Growth Hormone Is Necessary for the p53-Mediated, Obesity-Induced Insulin Resistance in Male C57BL/6J × CBA Mice

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Insulin resistance is a key marker of both obesity and GH excess. The purpose of the study was to assess the role of GH on p53-mediated insulin resistance of male mice with obesity due to a high-fat diet. C57BL/6J × CBA male mice fed on a high-fat diet (Obe) were studied; male mice fed a normal diet (Lean) or transgenic mice for bovine GH under the same genetic background (Acro) served as controls. The convergence of p53 and GH pathways was evaluated by Western blot. Obe mice had insulin resistance, which was sustained by a selective increased expression of p53 in adipose tissue. Normal insulin sensitivity was restored, and adipose p53 expression normalized when the GH pathway was blocked. Only the adipose p53 expression was sensitive to the GH blockage, which occurred through the p38 pathway. Adipose tissue of Obe mice had a coordinate overexpression of suppressors of cytokine signal 1–3 and signal transducers and activators of transcription-1, -3, and -5b, not different from that of Acro mice, suggesting an increased sensitivity of adipose tissue to GH. On the contrary, Lean mice were unaffected by changes of GH action. GH seems to be necessary for the increased adipose p53 expression and for insulin resistance of obese mice. **(*Endocrinology* 154: 4226–4236, 2013)**

Insulin resistance is a common feature of obesity and a leading event in the development of type 2 diabetes and the metabolic syndrome (1). Inability of insulin target tissues to respond adequately to insulin disrupts mechanisms responsible for the maintenance of glucose homeostasis (2). Although not completely understood, mechanisms of insulin resistance include an impaired activation of insulin-dependent phosphatidylinositol 3-kinase (PI3k) and downstream signaling (3–5), including a down-regulation of glucose transporter 4 (GLUT4), which leads to a reduced glucose transport into the cells (6, 7). Frequently insulin resistance develops in the hyperlipidemic and chronic proinflammatory conditions, which are both hallmarks of obesity. It has been suggested that hypoxia, deriving from a noncoordinate adipose tissue expansion and metabolic activity exceeding local oxygen supply, may lead to the activation of a local stress response, driving an autonomous inflammatory cytokine production (8).

Recently a crucial role of p53, expressed in the adipose tissue, in the regulation of insulin resistance, has been proposed (9). Increased expression of adipose p53 was a central finding of *Ay* animals (mice with dominant *agouti* mutations) with insulin resistance, whereas its inhibition ameliorated glucose metabolism and the inflammatory condition of adipose tissue (9).

GH has a contrainsular action, and the condition of GH excess, ie, acromegaly, is frequently associated with insulin resistance or type 2 diabetes (10). Patients with, or animal models of, acromegaly have increased GH and IGF-I concentrations, which have opposite effects on glu-

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Abbreviations: GHR, GH receptor; GLUT4, glucose transporter 4; IPGTT, ip glucose tolerance test; ITT, insulin tolerance test; MDM2, mouse double-minute 2 homolog; PI3k, phosphatidylinositol 3-kinase; SOCS, suppressors of cytokine signal; SREBP, sterol regulatory binding element; STAT, signal transducers and activators of transcription; TIGAR, Tp-53-induced glycolysis and apoptosis regulator; WAT, white adipose tissue.

cose metabolism. Whereas GH antagonizes, IGF-I promotes insulin action (11). In addition, cure of acromegaly, normalizing serum IGF-I, and GH concentrations, may ameliorate diabetes mellitus and insulin resistance, reducing the metabolic and cardiovascular risk (12).

Those evidences suggest a link between GH, insulin resistance, and obesity. Therefore, we hypothesized that GH may have a role in the insulin resistance of obesity. To test our hypothesis, we used mice that developed obesity under a high-fat diet, which is the most common cause of insulin resistance in humans; as controls, we used the same strain fed under normal caloric diet and transgenic mice overexpressing bovine GH under the same genetic background.

We uncover that GH is crucial for the development of insulin resistance of obese mice fed on a high-fat diet, affecting adipose p53 expression and function.

Materials and Methods

Animals

All procedures on animals were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals [Institute of Laboratory Animal Resources (13)]. The study protocol was approved by the local Board for Animals Experimentation at the University of Pisa. The environment of the animal rooms was controlled with a 12-hour light, 12-hour dark cycle, a relative humidity of 45%–55%, and a temperature of 20°C. Animals had free access to tap water and standard pellet chow (transgenic and lean animals) or high-fat diet pellet chow (Obe mice). For each experimental procedure, several groups of animals were used; each group consisted of 5–8 animals. Main features of the animals are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Transgenic animals

Transgenic mice overexpressing a coding sequence of the *bGH* gene under the control of the metallothionein promoter in the C57BL/6J × CBA genetic background have been described (14). The identity of bGH transgenic (Acro) mice was confirmed by PCR analysis as previously reported (15).

Lean animals

Wild-type animals with a C57BL/6J × CBA genetic background were used as controls and purchased from Harlan Italy and fed with normocaloric diet (18.5% protein, 5.5% fat, 4.5% fiber, and 6.0% ash; Harlan Italy (Lean mice).

Obe animals

A group of wild-type animals with a C57BL/6J × CBA genetic background were fed with a high-fat diet (23% protein, 34% fat, 5.0% fiber, and 5.5% ash; Mucedola) for 2 months, as previously reported (16) (Obe mice).

Treatments

Groups of Lean, Obe, or Acro animals were treated with pegvisomant (Pfizer), a specific antagonist of GH receptor (0.1 mg/daily, sc, for 15 d) as previously reported (15) or with pifithrin (Sigma-Aldrich), an inhibitor of p53 function (0.1 mg daily, ip, for 10 d) (17). Effectiveness of pegvisomant was evaluated by measuring serum IGF-I concentrations before and at the end of treatment; effectiveness of pifithrin was evaluated by Western blot, measuring the expression of p21 protein, which is regulated downstream by p53 (17).

Assays

Serum IGF-I concentrations were measured using a RIA commercial kit (Diagnostic System Laboratories), containing a specific goat antimouse/rat IGF-I serum, as previously reported (15); sensitivity was 21 ng/mL; intra- and interassay coefficients of variation were 12% and 9%, respectively. Plasma insulin levels were measured using an ELISA-based immunoassay kit (Mercodia AB); sensitivity was 0.2 ng/mL; intra- and interassay coefficients of variations were 3.2% and 6%, respectively.

The ip glucose tolerance test (IPGTT) and the insulin tolerance test (ITT) were performed as previously reported (18). For the IPGTT, fasted mice for 6 hours, were given glucose at a dose of 1 g/kg^{-1} (body weight); for the ITT, mice fasted for 2 hours were given human insulin ip (0.75 U/kg^{-1} body weight). Tail vein blood was collected at 0, 15, 30, 60, and 120 minutes, and blood glucose concentrations were measured with a glucose analyzer (Abbott), as reported (9).

Tissue samples

Animals were anesthetized with sodium pentobarbital overdose; organs, including white adipose tissue (WAT), skeletal muscles, liver, and kidney were separated and then immediately frozen in liquid nitrogen until further examination; for ex vivo experiments, adipose tissue was immediately used.

Tissue extracts and RNA preparation

Total tissue extracts were obtained by homogenizing each organ sample in lysis buffer [Tris HCl (pH 7.5), 50 mM; NaCl, 150 mM; sodium deoxycholate, 0.25%; Nonidet P-40 1%; protease inhibitor] as previously reported (15). After incubation on ice for 30 minutes and subsequent centrifugation, supernatants were stored at −80°C. Protein concentration was measured by a Bradford assay using the Bio-Rad reagent (Bio-Rad Laboratories). Total RNA was isolated by the standard TRIzol (Invitrogen) method, according to the manufacturer's protocols, and quality was assessed using an Agilent 2100 bioanalyzer instrument and the RNA 6000 Nano LabChip assay (Agilent Technologies).

Glucose uptake in WAT explants

Glucose uptake in WAT explants was evaluated as reported (19). Briefly, fat pad explants (20 mg), after exclusion of visible connective tissue and vessels, were placed in 0.5 mL incubation buffer (Krebs-Ringer HEPES, pH 7.4; 1.2 mM $CaCl_2$; 2% BSA). 2-Deoxyglucose (Sigma-Aldrich) (0.2 mM) and [^{3}H]-2-deoxyglucose (2 μCi/mL) (PerkinElmer) were added to the explants for 10 minutes at 37°C.

Explants were dissolved in NaOH 1 N and sodium dodecyl sulfate 0.1% at 50°C for 2 hours, and radioactivity was counted

in a Beckman scintillation β-counter. To evaluate the effect of p53 or GH on deoxyglucose uptake, WAT explants were preincubated in the presence or absence of pifithrin 1 μM or pegvisomant 100 nM, respectively, for 4 hours in 0.5 mL incubation buffer at 37°C. Data were expressed as picomoles of 2-deoxyglucose per milligram of wet weight.

Antibodies

The following antibodies were used: rabbit polyclonal anti-p53 (sc-6243), anti-p44/42 (sc-94), anti-GLUT4 (sc-7938), anti-pAKT1 (sc-7985-R), anti-sterol regulatory binding element (SREBP)-1 (sc-366), anti-suppressors of cytokine signal (SOCS)-3 (sc-9023), anti-signal transducers and activators of transcription (STAT)-1 (sc-464), anti-Tp-53-induced glycolysis and apoptosis regulator (TIGAR; sc-67273), anti-p21 (sc-397); goat polyclonal antihexokinase II (sc-6521); mouse monoclonal antiactin (sc-8432), anti-AKT1 (sc-5298), antimouse double-minute 2 homolog (MDM2; sc-965), anti-p38 (sc-7972), antiphospho-p38 (sc-7973), anti-STAT5b (sc-1656), and anti-STAT3 (sc-8019) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antiphospho-p53 (number 9284S), anti-SOCS1 (number 3950S), and anti-SOCS2 (number 2779S) were obtained from Cell Signaling Technology; rabbit antigoat, goat antirabbit, and antimouse IgG horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad Laboratories.

Western blotting

Total (50 μg) protein extracts (and protein extracts from immunoprecipitation, as appropriate) were resolved on a 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and stained with Ponceau red to verify the amount of proteins per lane. Transferred proteins were incubated overnight at 4°C in 50% Tris-buffered saline [200 mM Tris HCl (pH 7.6) and 1.4 M NaCl] and 50% Tris-buffered saline with 0.05% Tween 20 containing 5% nonfat dry milk and subsequently incubated with the appropriate primary antibody for 1 hour at room temperature. After washing with Tris-buffered saline with 0.05% Tween 20, an IgG horseradish peroxidase-conjugated secondary antibody was added for 1 hour at room temperature; positive proteins were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Membranes were incubated at 70°C for 10 minutes in stripping buffer [5 mM Tris HCl (pH 6.8), 2% sodium dodecyl sulfate, 67.5 mL ultrapure water, 0.8% β-mercaptoethanol] and reprobed for β-actin for loading normalization.

Films were scanned on densitometry (Bio-Rad Life Science), and band intensity was evaluated using GmbH software (Interfocus GmbH). Each sample value was normalized for loading errors (dividing by intensity of β-actin); data were expressed as arbitrary units that represent the ratio between the intensity of the band of interest and the intensity of the band corresponding to the control protein.

Immunoprecipitation

Five micrograms of p53 antibody were incubated with 500 μg of total tissue extracts overnight at 4°C. The mixture was then incubated with 50 μL of a 1:1 suspension of n-protein A-Sepharose beads (GE Healthcare) for 1 hour at 4°C with gentle rotation. The beads were pelleted and washed 3 times with radioimmunoprecipitation assay (Tris HCl, 50 mM, pH 8; NaCl, 150 mM; Nonidet P-40, 1%; sodium deoxycholate, 0.5%) buffer. The immunocomplexes were dissociated by boiling the mixture in SDS-PAGE sample buffer protein and analyzed by Western blot, with an MDM2 antibody.

Immunohistochemistry

Immunohistochemistry, using adipose tissue, was performed as previously reported (20). Five-micrometer sections were deparaffinized with xylene and rehydrated by ethanol treatment. Sections were pretreated with 1% hydrogen peroxide in methanol for 10 minutes at room temperature to inactivate endogenous peroxide activity. To unmask the antigens, slides were microwaved in 10 mM citrate buffer (pH 6) for 10 minutes. Nonspecific binding sites within the sections were blocked by incubating the sections with 5% BSA. Sections were incubated with the 1:100 diluted primary antibody (rabbit polyclonal antibody anti-p53, FL-393; Santa Cruz Biotechnology) at 4°C for 12 hours and then with the 1:500 diluted biotin-labeled secondary antibody at room temperature for 30 minutes, followed by incubation with avidin-biotin complex (Vector Laboratories, Inc) for an additional 30 minutes. The resultant immune peroxidase activity was developed in 0.5% 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp). Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were obtained by omitting the anti-p53-specific primary antibodies, which were replaced by rabbit immunoglobulins that did not react with the p53.

Quantitative real-time PCR

A total of 1 μg WAT RNA was transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) and pd(N)6 random hexamer (GE Healthcare) in a final volume of 20 μL. Five microliters of cDNA reaction were mixed with TaqMan universal PCR 1× master mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan gene expression assays; Applied Biosystems) in a final volume of 25 μL and amplified for 40 cycles as previously reported (21). The assays used were: Mm 01731287_m1 for p53 and Mm 00446973_m1 for TATA-box binding protein. Relative gene expression levels were quantified through the ABI 7700 sequence detection system instrument and software (Applied Biosystems), using a 5-point serially diluted standard curve. The gene expression was reported in arbitrary units and normalized relative to the housekeeping gene *TBP* to compensate for differences in cDNA loading.

Statistics

Results were expressed as mean ± SD. Differences among 3 or more groups were evaluated using the ANOVA; differences among curves of insulin or glucose concentrations during the IPGTT or ITT were evaluated using ANOVA for repeated measures; differences between 2 groups were evaluated using a post hoc test (Tukey). A $P < .05$ was considered statistically significant.

Results

Adipose p53 overexpression and abnormalities of glucose metabolism in obese mice

Obese and Acro animals had abnormalities of glucose metabolism (Figure 1, A–F). As expected, Obe animals had an impaired glucose tolerance (Figure 1A), whereas

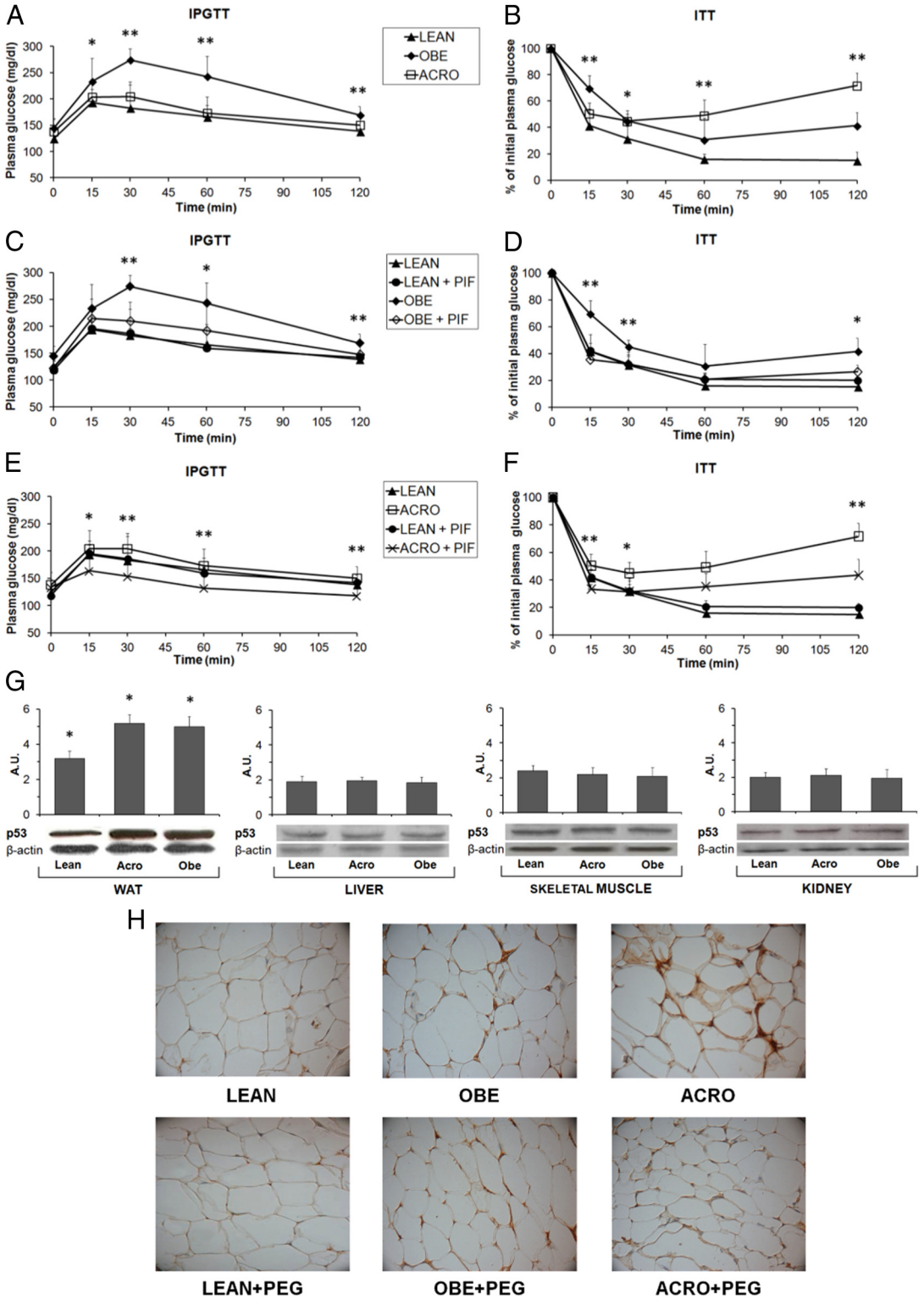

Figure 1. The abnormalities of glucose metabolism of obese mice are reverted by inhibition of p53. C57BL/6J × CBA mice were fed on normal diet (Lean) or high-fat diet (Obe); Acro animals were transgenic mice for bovine GH on the same genetic background. Pifithrin was given at the dose of 0.1 mg/d, ip, for 10 days. An IPGTT (n = 8) and an ITT (n = 5) were performed as reported in *Materials and Methods*. A, IPGTT in Lean, Obe, and Acro mice ($P < .02$, ANOVA for repeated measures) and after pifithrin (pif) ($P < .05$, ANOVA for repeated measures) (C and E). B, ITT in Lean, Obe, and Acro mice ($P < .0001$, ANOVA for repeated measures) and after pifithrin (pif) ($P < .0001$, ANOVA for repeated measures) (D and F). Expression of p53 in the whole adipose tissue was evaluated by Western blot (mean values of 3 replicates) and was higher in Obe and Acro mice than in Lean animals (G). In addition, immunohistochemistry confirmed changes in the expression of p53, mainly in adipocytes; Peg, pegvisomant (H). Panels A and B, Obe vs Lean; panel B, Acro vs Lean; panel C, Acro and Obe vs Lean; panels D and E, Obe + Pif vs Obe; panels F and G, Acro + Pif vs Acro. *, $P < .05$; **, $P < .005$, post hoc Tukey test. A.U., arbitrary units.

Acro mice had reduced insulin sensitivity (Figure 1B), revealed by IPGTT and ITT, respectively.

Insulin resistance was linked to a selective overexpression of adipose p53; in fact, the level of expression of p53 in liver, skeletal muscle, and kidney did not differ from that of lean mice (Figure 1, G and H). Notably, inhibition of p53, using pifithrin, reverted insulin resistance and normalized insulin sensitivity, confirming that adipose p53 is a key regulator of insulin resistance (Figure 1, C–F); treatment with pifithrin was associated with a reduced expression of p53 and its downstream effector, p21 (Supplemental Figure 1, A and B). The link between adipose tissue, GH, and p53 was further suggested by ex vivo experiments using adipose tissue; the uptake of ^{3}H-deoxyglucose was lower in adipose tissue from Obe than from Lean animals and partly restored when either GH or p53 action was blocked by pegvisomant or pifithrin, respectively (Figure 2).

The role of GH in insulin resistance of Obe mice

The observation that Acro mice had insulin resistance and reduced insulin sensitivity, both associated with overexpression of adipose p53 as in Obe mice (Figure 1, A and B), suggested a link between GH and p53. As in Obe mice, pifithrin, blocking p53 action, reverted the abnormalities of glucose metabolism of the Acro mice (Figure 1, E and F).

To explore the convergence of the GH signal on p53 and glucose metabolism, Obe mice were treated with a selective antagonist of GH receptor (GHR). When GHR was blocked, insulin resistance and sensitivity of Obe mice improved, whereas they did not change in lean animals (Figure 3, A–D). The block of GHR followed by IGF-I therapy revealed that insulin resistance was due to GH and not to IGF-I (Supplemental Figure 2, A–D).

In keeping with the biochemical improvement of insulin resistance, treatment with a GHR inhibitor was associated with a coordinate normalization of either total or phosphorylated adipose p53 of Obe mice (Figure 3E).

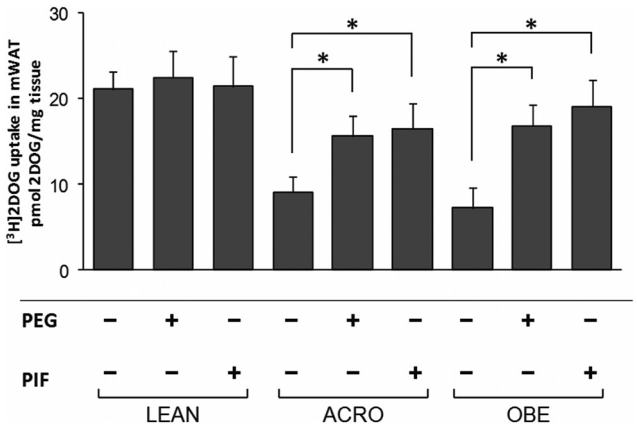

Figure 2. Glucose uptake in adipose tissue explants of obese mice. Adipose tissue (WAT) explants from Lean, Acro, and Obe mice, preincubated without or with pegvisomant (PEG; 100 nM) or pifithrin (PIF; 1 μM) for 4 hours were used for [^{3}H]-2-deoxyglucose (2DOG) uptake measurements. No significant differences were found in glucose uptake of WAT explants from Lean mice preincubated with pegvisomant or pifithrin, respectively. However, there was a 45% significant increase in the glucose transport in WAT explants from Acro and Obe mice preincubated with pegvisomant or pifithrin, respectively, compared with untreated animals. Results are expressed as mean ± SD of 3 uptake experiments, made in triplicate. *, $P < .05$, post hoc Tukey test.

Molecular findings of adipose insulin resistance of Obe mice

In keeping with the biochemical findings, Obe mice had a molecular phenotype of insulin resistance, including either a reduced expression of GLUT4, SREBP-1, and hexokinase II or an increased expression of TIGAR (Figure 3, F–I). Treatment with a GHR antagonist was associated with an increased expression of adipose GLUT4 and SREBP-1, whereas that of hexokinase II and TIGAR were unaffected, suggesting that GH may participate in insulin resistance, affecting both glucose and lipid metabolism.

GH affects adipose p53 expression through different mechanisms in Obe or Acro mice

Transcription of adipose p53 was significantly higher in Obe mice and, although to a lower degree, in Acro mice, than in lean animals (Figure 4A); the increased phospho-p38 expression suggested that GH affected p53 expression through the p38 pathway (Figure 4, B and C), whereas the PI3k-Akt and the p44/42 pathways appeared to be silent in Obe mice (Figure 4D). A posttranscriptional regulation of p53 was active in Acro but not in Obe mice; in fact, adipose MDM2, a key protein for the initiation of ubiquitin-dependent degradation of p53, was reduced in Acro but not in Obe mice; changes of MDM2 expression reverted after treatment with a GHR antagonist (Figure 4E).

Adipose tissue of obese mice resembles that of acromegalic animals

Obe mice had an increased expression of adipose SOCS1, -2, and -3 and a coordinate overexpression of STAT1, -5b, and -3, indistinguishable from that found in Acro mice (Figure 5, A–F). The findings that the intracellular regulators of GH actions, SOCSs, were increased both in Acro and Obe mice, leading to similar increased levels of their effectors, STATs, suggested that adipose tissue of Obe and that of Acro were similar, in term of GH responsiveness. The block of GHR greatly reduced the expression of all SOCS and STAT proteins in Acro mice, confirming that those changes were all secondary to GH excess; on the contrary, only the expression of SOCS2-STAT5b was reduced, following the GHR block, in Obe mice, suggesting that SOCS1-STAT1 and SOCS3-

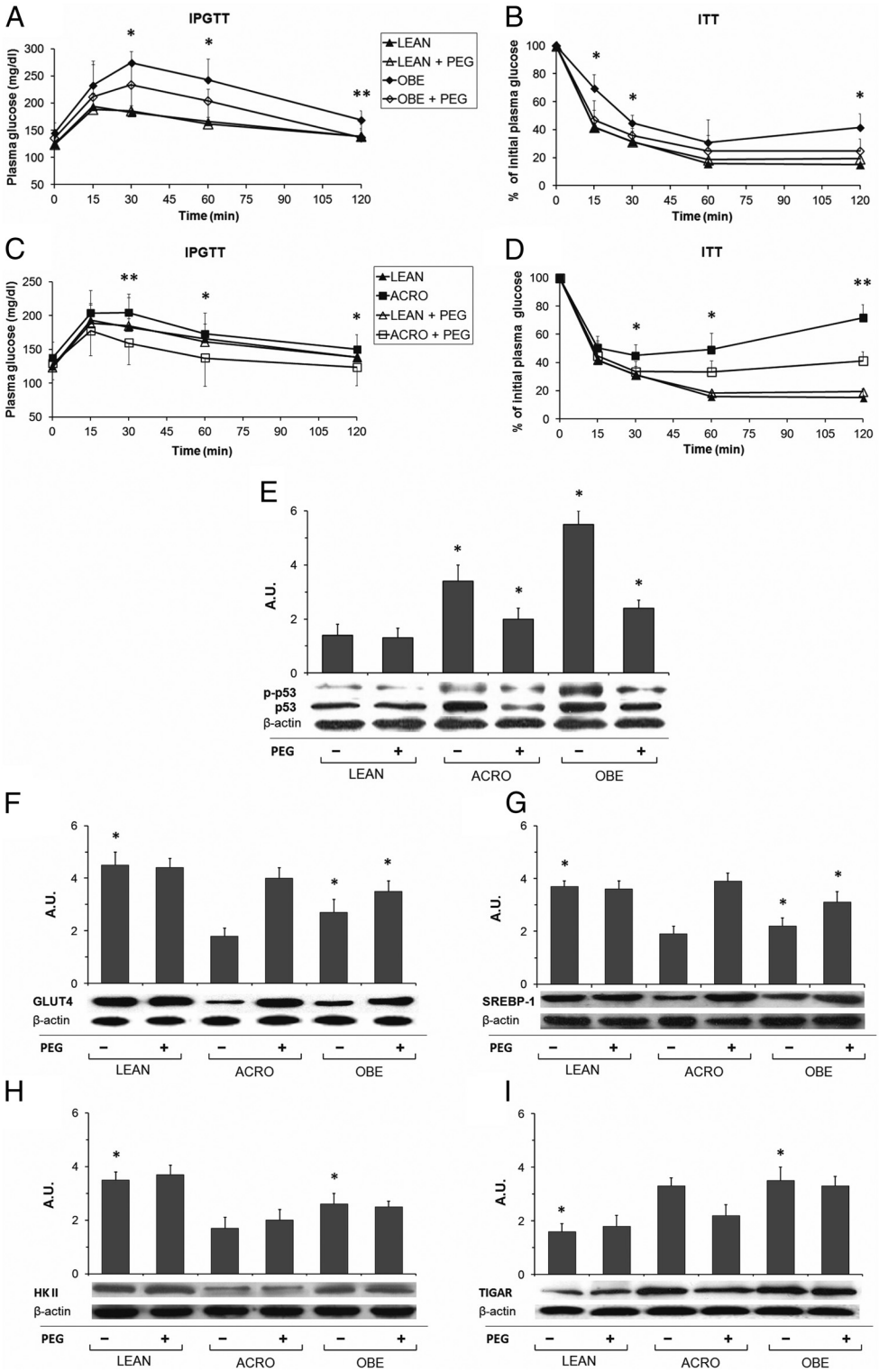

Figure 3. The inhibition of GHR signaling reverts biochemical and molecular changes associated with insulin resistance. GHR signaling was blocked giving a GHR inhibitor [pegvisomant (PEG) for 15 d] to animals, as reported (40). An IPGTT (n = 8) and an ITT (n = 5) were performed as reported in *Materials and Methods*. The IPGTT (panels A and C, $P = .006$, ANOVA for repeated measures) and ITT (panels B and D, $P < .0001$, ANOVA for repeated measures) improved in either Obese or Acro mice after treatment with PEG. Amelioration of the biochemical features of Obe (and Acro) mice was associated with a reduced expression of either total or phosphorylated adipose p53 after PEG treatment (panel E). The expression of GLUT4 (panel F); SREBP-1 (panel G); hexokinase II (HXII; panel H); TIGAR (panel I) was evaluated by Western blot (mean values of 3 replicates). Adipose GLUT4 and SREBP-1 expression was lower in obese than in lean mice and reverted after blocking GH receptor with a selective inhibitor (PEG). Panels A and B, Obe + PEG vs Obe; panes C and D, Acro + PEG vs Acro. *, $P < .05$; **, $P < .005$, post hoc Tukey test. A.U., arbitrary units.

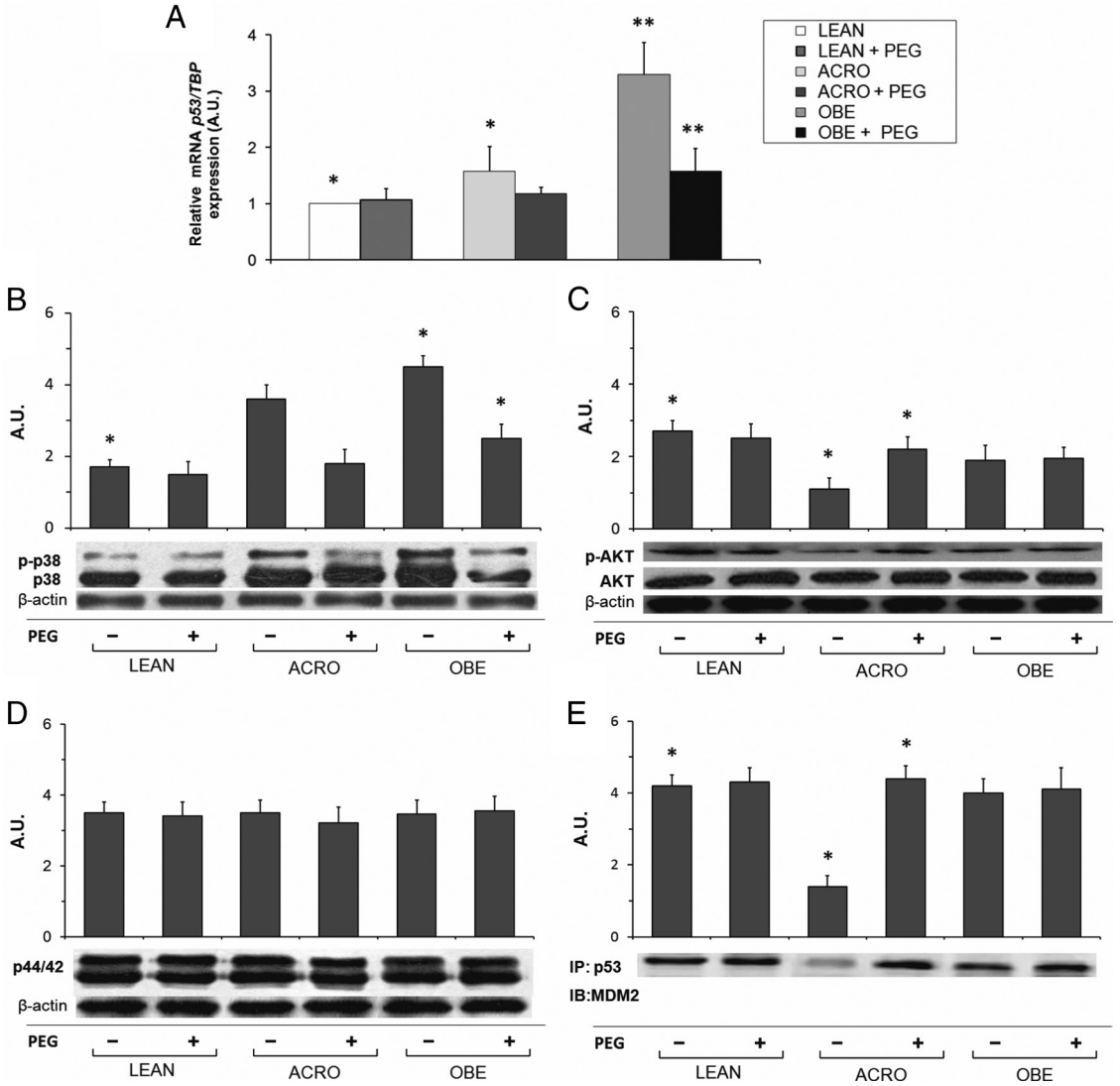

Figure 4. Mechanisms underlying the increased expression of adipose p53. Quantitative PCR (mean values of 4 replicates) was used for evaluating the transcriptional regulation of adipose p53; p53 transcripts increased mainly in obese mice and, although to lesser extent, in Acro mice than in Lean animals (A). Whole extracts from adipose tissue of Obe, Lean, or Acro mice were used for Western blotting (B–D) or coimmunoprecipitation (E). The expression of total and phosphorylated p38 (B), that of Akt (C), and that of p44/42 (D) was evaluated by Western blot. For coimmunoprecipitation experiments, protein extracts were first immunoprecipitated with a p53 antibody, and after dissociation of immunocomplexes and protein resolution on SDS-PAGE, the level of expression of MDM2 protein associated with p53 was evaluated by a specific MDM2 antibody by Western blot (E). *, $P < .05$; **, $P < .005$, post hoc Tukey test. A.U., arbitrary units.

STAT3 overexpressions were not due to the normal circulating GH concentrations found in obese animals. Instead, these findings suggested that the increased expression of adipose SOCS1 and SOCS3 were GH independent and likely contributed to the increased sensitivity of adipose tissue to GH, leading to an intracellular acromegalic-like condition, as shown by the increased SOCS2-STAT5b expression.

Discussion

In the present study, we have identified a convergence of GH signaling on the pathways mediated by p53, which led to insulin resistance. First of all, we confirmed that p53 was increased in adipose tissue of obese mice; more importantly, we showed that GH is necessary for the selective increased expression of adipose p53 and for the p53-mediated insulin resistance. In fact, abrogation of GH activity led to the normalization of adipose p53 expression and to a significant improvement of insulin resistance.

Adipose p53 has been reported to exert a crucial role in the development of insulin resistance in A*y* mice, which carried mutations in the *Agouti* gene (9). Excessive calorie intake led to a condition of oxidative stress, which, in turn, increased adipose tissue levels of p53 and proinflammatory cytokines; that condition triggered insulin resistance, which improved when p53 expression was selectively

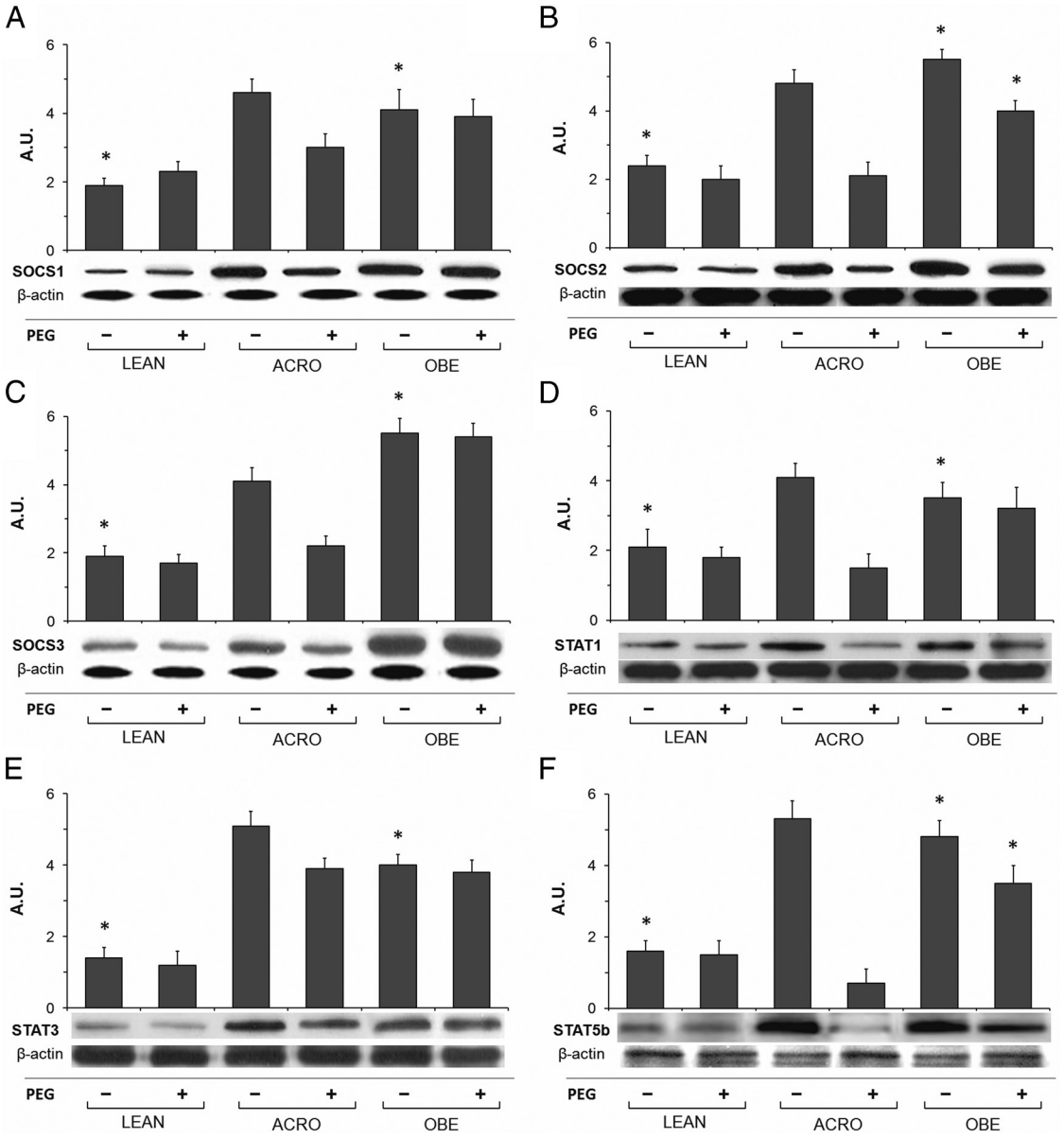

Figure 5. Adipose tissue of obese mice features a condition of GH excess. Adipose tissue of obese mice was indistinguishable from that of Acro mice in terms of increased expression of SOCS 1–3 (A–C) and STAT1, -3, and -5b (D–F). However, only SOCS2-STAT5b was reduced during pegvisomant treatment (B and F), suggesting that those changes were secondary to GH. On the contrary, the GH insensitivity of SOCS1-STAT1 and SOCS3-STAT3 suggests that their increased expression was mediated by other mechanisms. *, $P < .05$, post hoc Tukey test. A.U., arbitrary units.

abolished in adipose tissue (9), as shown in the present study.

An increasing body of evidence suggests that p53 is crucial for regulation of glucose metabolism, particularly in cancer cells (22). p53 may intervene in the regulation of several steps of glucose metabolism, for example, reducing glycolysis, down-regulating GLUT4 and glucose transport into the cells, increasing the expression of TIGAR and hexokinase II, and diverting glucose metabolism toward the pentose phosphate pathway (23) and promoting oxidative phosphorylation (23). However, depending on stress duration and the level of p53 expression, p53 may exert divergent effects, either promoting glycolysis or oxidative phosphorylation as well as inhibiting glycolysis, (24). A proposed model of p53 in the stress response machinery suggests that under conditions of basal stress, p53 may contribute to repairing damaged cells, whereas under conditions of persistent stressful events, p53 may promote the involution of the damaged cell (25, 26).

Data from the present study confirm that p53 reduces glucose uptake and glycolysis, affecting several regulatory steps (for example, increasing TIGAR and reducing GLUT4 expression), and maintains oxidative phosphorylation. However, increased p53 expression may drive detrimental metabolic consequences, leading to insulin resistance; a similar paradoxical behavior of p53 has been

reported in the regulation of reactive oxygen species; under nonstressful conditions, p53, regulating the pentose phosphate pathway, exerts an antioxidative function (27); however, under condition of stress, p53 can drive prooxidant functions (23). The present study suggests that constitutive levels of adipose p53 may help to maintain a normal glucose metabolism; on the contrary, in conditions of stress, such as those deriving from an inflammatory state triggered by obesity, p53 may react abnormally, as it occurs in other stressful situations (22). However, this response may contribute to the development of other diseases, such as diabetes. In fact, it has been reported that, on the one hand, p53 may protect from cancer development preventing cells damage and favoring removing damaged cells, and that, on the other hand, inhibition of p53 may have beneficial effects on tumor therapy making tumor cells more sensitive to drugs that affect metabolism (28, 29).

In our study, p53-dependent insulin resistance required GH either in Acro or Obe mice: in the former it was due to GH excess, whereas in the latter to the increased adipose sensitivity to GH. In fact, the expression of SOCS1, SOCS2, and SOCS3, which are the main intracellular regulators of GH action, was increased in Obe and indistinguishable from that of Acro. That the observed SOCSs pattern allowed an increased GH signal was sustained by the level of the expression of STAT1, -2, and -5b, which was higher than that of lean animals. It is interesting to note that the block of GHR with pegvisomant led to a down-regulation of all SOCS and STAT proteins in Acro mice, in keeping with the notion that those changes were all due to the GH excess. On the contrary, only SOCS2 and the corresponding STAT5b were reduced after abrogation of the GH signal in Obe mice.

Some considerations can be drawn from this observation: 1) the increased STAT1 and STAT3 level of expression was not due to GH but contributed instead to the increased sensitivity of adipose tissue of Obe mice to GH; 2) the increased SOCS2-STAT5b levels of Obe mice were due the increased adipose sensitivity to normal GH concentrations: in fact, the SOCS2-STAT5b levels in Obe were similar to those of Acro and were reduced with the block of GHR; and 3) it is likely that the increased SOCS2-STAT5b contributed to the GH-dependent, p53-mediated insulin resistance of Obe mice (Figure 6). It is worth noting that GHR inhibition did not affect glucose metabolism in Lean animals, in keeping with a very recent report, showing that adipose-specific GH receptor gene disrupted mice did not alter glucose metabolism (30).

It has been reported that several adipose cytokines play a role in promoting insulin resistance, linking inflammation to obesity and metabolic disease (31–36); for example, IL-6 and IL-8 (37, 38) and TNFα (39) have been shown to be increased in adipose tissue and may contribute to decrease insulin signaling (40). This may occur through increasing the expression of SOCS proteins (32), which, in turns, may impair insulin signal by binding directly the insulin receptor (32) or by promoting insulin

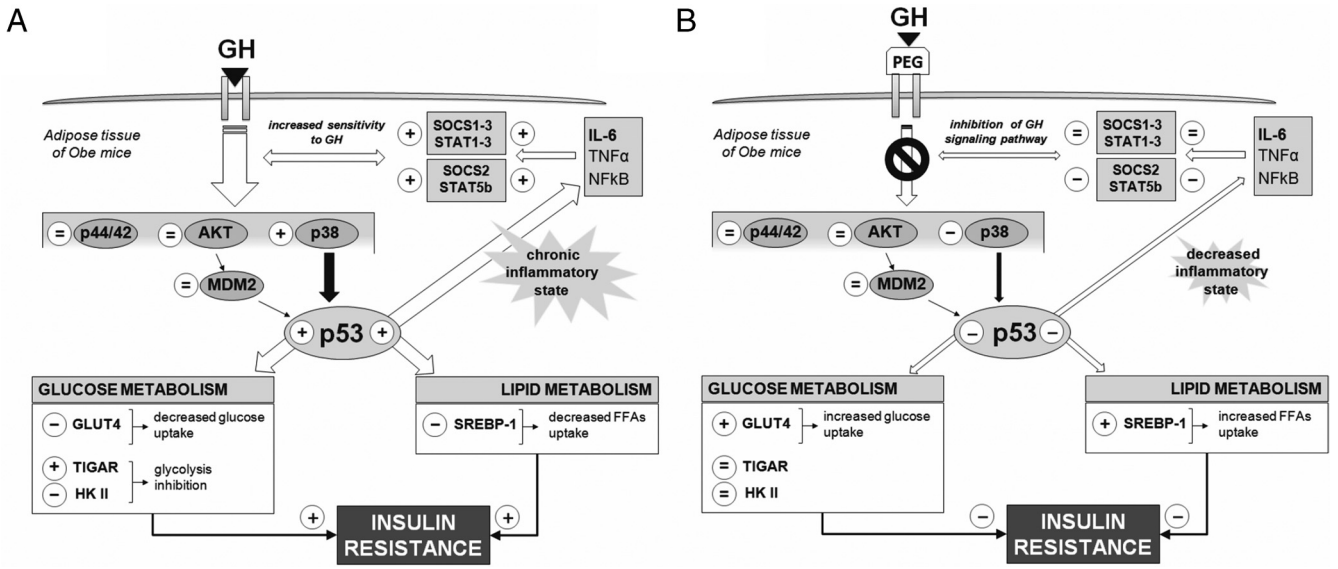

Figure 6. Proposed mechanisms of GH in the regulation of insulin resistance. In conditions of inflammation of adipose tissue, for example, obesity (A), inflammatory cytokines, including IL-6, increase the expression of adipose SOCS1-STAT1- and SOCS3-STAT3-coupled proteins, leading to a higher sensitivity to normal circulating GH concentrations, which, in turns, increase the expression of the SOCS2-STAT5b system. The high intracellular levels of SOCS proteins allow an increased GHR signal through the p38 (and PI3k-Akt in Acro mice) pathways toward p53, increasing its expression and activity. Higher adipose levels of p53 trigger insulin resistance, affecting glucose and lipid metabolism, through regulation of key steps. Blocking GH receptor with a specific inhibitor (B) reduces the abnormal GH action in adipose tissue, ameliorating insulin resistance.

receptor degradation (41). In fact, SOCS1 and SOCS3 have been reported to be increased in insulin-resistant states, such as endotoxemia and obesity (32), and their binding to insulin receptor or the inhibition of insulin receptor substrate-1 and insulin receptor substrate-2 led to a reduction of the glycogen synthesis and glucose transport in cell culture (32). However, whether cytokines promote a GH-dependent insulin resistance of Obe mice remains, at present, speculative.

Recently a central role of GH signaling in high-fat diet-induced glucose metabolism perturbation was revealed using Cre/loxP system inactivating GHR in postnatal skeletal muscle (42). Selective inactivation of GHR reduced obesity, improved insulin sensitivity, and lowered systemic inflammation; it is worth noting that SOCS2 expression was decreased in obese mouse GHR knockout mice. In fact, disruption of the *GHR* gene in mice, featuring the Laron's syndrome (43), is associated with increased insulin sensitivity (44–46), and patients with GH deficit have fasting hypoglycemia, increased insulin sensitivity, and diminished insulin secretion (47).

In conclusion, we have identified a triangulation between insulin resistance, adipose tissue, p53, and GH, which revealed an essential role of GH for impaired insulin action in diet-induced obesity.

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