Attenuated \textit{Pik3r1} Expression Prevents Insulin Resistance and Adipose Tissue Macrophage Accumulation in Diet-Induced Obese Mice

Carrie E. McCurdy,1,2,3 Simon Schenk,4 Michael J. Holliday,1,2,3 Andrew Philp,5 Julie A. Houck,1,2,3 David Patsouris,6 Paul S. MacLean,2 Susan M. Majka,2,3 Dwight J. Klemm,2,3 and Jacob E. Friedman1,7

Obese white adipose tissue (AT) is characterized by large-scale infiltration of proinflammatory macrophages, in parallel with systemic insulin resistance; however, the cellular stimulus that initiates this signaling cascade and chemokine release is still unknown. The objective of this study was to determine the role of the phosphoinositide 3-kinase (PI3K) regulatory subunits on AT macrophage (ATM) infiltration in obesity. Here, we find that the Pik3r1 regulatory subunits (i.e., p85α/p55α/p50α) are highly induced in AT from high-fat diet-fed obese mice, concurrent with insulin resistance. Global heterozygous deletion of the Pik3r1 regulatory subunits (αHZ), but not knockout of Pik3r3 (p85β), preserves whole-body, AT, and skeletal muscle insulin sensitivity, despite severe obesity. Moreover, ATM accumulation, proinflammatory gene expression, and ex vivo chemokine secretion in obese αHZ mice are markedly reduced despite endoplasmic reticulum (ER) stress, hypoxia, adipocyte hypertrophy, and Jun NH2-terminal kinase activation. Furthermore, bone marrow transplant studies reveal that these improvements in obese αHZ mice are independent of reduced Pik3r1 expression in the hematopoietic compartment. Taken together, these studies demonstrate that Pik3r1 expression plays a critical role in mediating AT insulin sensitivity and, more so, suggest that reduced PI3K activity is a key step in the initiation and propagation of the inflammatory response in obese AT. \textit{Diabetes} 61:2495–2505, 2012

Although the pathogenesis of insulin resistance in obesity is multifactorial, it is clear that chronic, low-grade inflammation is a major contributor, with the proinflammatory macrophage identified as the primary stimulus (1). Mechanistically, the current model for insulin resistance in obesity suggests that adipose tissue macrophage (ATM) infiltration and proinflammatory cytokine release activates inflammatory pathways such as inhibitor of kappa B kinase β (IKKβ) and Jun NH2-terminal kinase (JNK), which impinge upon the insulin signaling cascade by inhibiting tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), leading to impaired insulin activation of phosphoinositide 3-kinase (PI3K) and Akt (1).

The chemoattractant stimulus and the molecular details underlying cross-talk between infiltrating macrophage and the adipocyte is an area of intense investigation. Environmental cues including adipose tissue (AT) hypoxia, cell death, physical stress on adipocyte extracellular matrix, and increased lipolysis have all been identified as mechanisms that initiate ATM recruitment, primarily through their ability to either stimulate chemokine secretion (2) or increase free fatty acid (FFA) release (3). In turn, these factors activate proinflammatory signaling cascades within the monocyte to initiate migration (3–5). A wealth of data exists linking ATM accumulation with subsequent insulin resistance in obesity (6–8). Similarly, a recent study by Lee et al. (9) found that as few as 3 days of a high fat diet (HFD) feeding in mice led to significant reduction in insulin sensitivity, which was accompanied by an increase in ATM accumulation. Interestingly, however, the decrease in insulin sensitivity after 3 days of HFD occurred independently of ATM accumulation (9).

The class 1A PI3K regulates many cellular processes, including insulin-mediated glucose transport, cell growth, apoptosis, and immune cell motility. PI3K is a heterodimeric enzyme composed of a regulatory subunit (p85α, p55α, p50α, or p85β) and a catalytic subunit (p110α or p110β) (10). Studies in cell culture and transgenic mouse models demonstrate that complete deletion of the regulatory isoforms (p85α only, p85β only, and p55α/p50α double knockout) or heterozygous Pik3r1 deletion enhances PI3K activity and subsequent insulin sensitivity (11–14). Furthermore, inhibiting Pik3r1 expression improves insulin signaling and glucose homeostasis in HFD-fed, obese mice (15), and mice with genetically induced insulin resistance through heterozygous loss of the IR and IRS1 (16). Several studies have identified PI3K-independent roles for the p85α subunit that may explain the inverse relationship between p85α abundance and insulin sensitivity, including activation of phosphatase and tensin homolog (PTEN) (17), nuclear translocation of X-box binding protein 1 (18,19), and activation of JNK (20).

Here, we investigated the hypothesis that maintaining insulin action can prevent ATM recruitment, even with marked obesity. To do this, we studied ATM infiltration and cytokine profiles and systemic and tissue-specific insulin sensitivity in two well-characterized PI3K regulatory subunit mouse models, the Pik3r1 heterozygous (αHZ) mouse and

From the 1Department of Pediatrics, University of Colorado School of Medicine, Aurora, Colorado; the 2Department of Medicine, University of Colorado School of Medicine, Aurora, Colorado; the 3Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology, University of Colorado School of Medicine, Aurora, Colorado; the 4Department of Orthopaedic Surgery, University of California, San Diego, La Jolla, California; the 5Department of Neurobiology, Physiology, and Behavior, University of California Davis, Davis, California; the 6Faculté de Médecine Lyon-Sud, INSERM U1060, Université Lyon 1, Oullins, France; and the 7Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, Colorado.

Corresponding author: Carrie E. McCurdy, carrie.mccurdy@ucdenver.edu. Received 25 October 2011 and accepted 29 January 2012. DOI: 10.2337/db11-1433

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PI3K AND ADIPOSE TISSUE INFLAMMATION

PI3K and adipose tissue inflammation

In our study, we investigated the role of PI3K in adipose tissue inflammation and its contribution to obesity-induced insulin resistance. We used a mouse model of PI3K knockdown (bKO) to explore the effects on adipose tissue and metabolic parameters. Our results showed that PI3K knockdown in adipose tissue (eAT) reduces adipose tissue inflammation and improves insulin sensitivity in obese mice. These findings support the hypothesis that PI3K is a critical component of adipose tissue inflammation and insulin resistance.

### RESEARCH DESIGN AND METHODS

#### Animal experiments and tissue collection

All studies were approved by the Institutional Animal Care and Use Committee at the University of Colorado School of Medicine. Six-week-old, male Pik3r1+/− (eHZ) and Pik3r2−/− (bKO) and their wild-type (WT) littermates were placed on an HFD (40% of calories from fat) and control diet (CON; 10% of calories from fat) from Research Diets (New Brunswick, NJ) for 16 weeks. Basal and insulin-stimulated tissue was collected as previously described (21). Blood samples were collected after a 4-h fast through the retro-orbital sinus, and plasma cytokines/adipokines were measured by multiplex assay (Bio-Rad Laboratories, Hercules, CA).

#### BM Ts.

Male C57BL/6j (CD45.1) WT recipient mice (Jackson Laboratory, Bar Harbor, ME) and male C57BL/6sj Pik3r1+/− (CD45.2; aHZ) recipient mice received 1,000 rads of whole-body irradiation. BM was extracted from the tibia and femur of WT Pik3r1−/− (CD45.2), WT (CD45.1), and aHZ (CD45.2) donor mice, and 2.5 × 10⁶ cells were injected into the retro-orbital sinus cavity of irradiated mice. At 6 weeks, engraftment of donor BM was tested by flow cytometry for CD45.1 or CD45.2 antigens (anti-CD45.1-PE, anti-CD45.2-APC, and ter119-FTTC; BD Pharmingen) in peripheral blood (see Supplementary Fig. 3).

### RESULTS

#### Hypersulinemic-euglycemic clamp.

The hypersulinemic-euglycemic (HE) clamp was conducted using two jugular vein cannulas as previously described (22), except that clamps were conducted on overnight-fasted mice that were anesthetized with a drug cocktail (acepromazine, 10 mg/mL; midazolam, 5 mg/mL; and fentanyl, 0.05 mg/mL) as previously outlined (23). This anesthesia protocol does not alter glucose metabolism during an HE clamp (23).

### Immunoblotting.

Epidermal adipose tissue (eAT) was homogenized (3 g/mL) in ice-cold lysis buffer as previously described (22). Samples (50 μg) were run on a 7% acrylamide Bis-Tris gel and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Membranes were then exposed to the appropriate primary and secondary antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin was used as a loading control.

Phosphorytose-associated P38 activity.

PI3K was immunoprecipitated from 500 μg AT or skeletal muscle (SKM) homogenates by incubating overnight with a mouse anti-pY100 antibody (Cell Signaling, Danvers, MA). The kinase reaction was initiated with the addition of 2 μg phosphatidylinositol (PI) and 20 μCi [γ-32P]-ATP, incubated for 30 min, and then terminated by addition of 20 μL of 8 N HCl as previously described (22). The lipid products were run on a thin-layer chromatography plate, and phosphoinositide-l-phosphate [PI(3)P] was visualized on film.

### Immunohistochemistry.

eAT was immunohistochemically fixed in 10% neutral, phosphate-buffered formalin for 24–48 h and paraffin embedded, and 4-μm sections were stained with DAPI and a rat anti-mouse F4/80 monoclonal antibody (1:250; Abcam, Cambridge, MA).

### RNA isolation and quantitative real-time PCR.

RNA was isolated from eAT and SkM using a Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA was quantified and reverse transcribed (Invitrogen reverse transcriptase; Promega). Quantitative real-time PCR was performed using a Roche LightCycler 480 instrument. mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S. Primer sequences are presented in Supplementary Table 3.

### Lipid analysis.

SKM tibialis anterior (30 mg) and liver (70 mg) were lyophilized, weighed, and homogenized in MeOH along with an internal standard of tri- penta-decan-1-yl phosphocholine (Omni International, Marietta, GA). SKM lipid extraction, isolation, and analysis were performed as previously described (24).

### Flow cytometry.

eAT was digested in Dulbecco’s modified Eagle’s medium (DMEM) + 0.4% collagenase I, and cells were filtered and centrifuged to collect stromal vascular cells (SVCs). Cells were incubated for 10 min with Fix, followed by a 30-min incubation with anti-F4/80-PE and anti–CD11c-APC (eBioscience, San Diego, CA). Cells were then fixed with 1% paraformaldehyde in PBS. Analysis was performed using the FACS Calibur system with Cell Quest software.

### Cell secretion assay.

eAT was exposed to two conditions: one with serum-free DMEM and one with serum-free DMEM that also contained 1% FCS and 1 μM L-glutamate. Cells were differentiated into adipocytes by the addition of DMEM containing 10% FCS, 1 μM L-glutamate, 100 μM isobutylmethylxanthine, 1 μM dexamethasone, and 1 μg/mL insulin for 3 days. After differentiation, cells were maintained as previously described (25). For time course experiments, cells were serum starved overnight and then exposed to interleukin-6 (IL-6; 20 ng/mL) (Sigma–Aldrich) for indicated times.

### Statistical analysis.

Data were analyzed by two-way ANOVA for main effects of diet and genotype with Tukey post hoc analysis. Significant differences within genotype are indicated with ∗, and significant differences within diet group are indicated with #. Data from BMT studies were analyzed only within the HFD groups by two-way ANOVA for main effects of BM genotype and body genotype with Tukey post hoc analysis. The control group (WT/WT-CON) was used as reference to show that HFD induced a change independent of transplant. For BMT studies, ∗ indicates significant differences within BM genotype and # indicates significant differences within body genotype. Pearson χ² test of independence was used to compare cell size frequency distribution profiles. 3T3-L1 adipocyte studies were analyzed by one-way ANOVA with Bonferroni post hoc test, and ∗ indicates significant differences compared with t = 0.

### RESULTS

#### Limiting Pik3r1 expression, but not Pik3r2, ameliorates obesity-induced insulin resistance.

After 16 weeks on HFD, eHZ and bKO, and their respective WT littermates (i.e., Pik3r1+/− and Pik3r2−/−), gained significant weight compared with mice on CON, with the majority of weight gain due to an increase in body fat (Fig. 1A and B). Despite comparable obesity, whole-body insulin sensitivity was significantly greater in eHZ-HFD compared with WT-HFD mice as measured by the steady-state glucose infusion rate (GINF) during the H-E clamp (Fig. 1C and Supplementary Fig. 1A). The enhanced GINF in eHZ-HFD was primarily due to an increase in insulin-stimulated glucose disposal rate (IS-GDR), as suppression of hepatic glucose production by insulin was not different between eHZ-HFD and WT-HFD (Fig. 1D and E and Supplementary Fig. 1B). AT insulin sensitivity, measured by the ability of insulin to suppress FFA and glycerol levels during the clamp, was significantly impaired in WT-HFD mice, but was similar to CON in eHZ-HFD mice (Fig. 1F and G and Supplementary Fig. 1C and D). Surprisingly, the metabolic improvements did not manifest in bKO-HFD mice, which were indistinguishable from WT-HFD mice (Fig. 1C–G and Supplementary Fig. LA–D). Supporting the clamp data,
lipid analysis of SkM showed a significant twofold increase in triglyceride and diacylglyceride concentrations in WT-HFD, but not αHZ-HFD, compared with CON-fed mice, consistent with improved IS-GDR in αHZ-HFD (Fig. 1H). Hepatic triglycerides and diacylglycerides were elevated twofold in both WT and αHZ on HFD (Fig. 1I). In isolated adipocytes (Fig. 1J) and SkM (Fig. 1K), again, WT-HFD, but not αHZ-HFD, had reduced insulin-stimulated 2DG uptake. Because AT inflammatory cytokine secretion is thought to drive systemic insulin resistance, we measured insulin sensitivity in isolated EDL muscles after exposure to TNF-α from WT and αHZ mice after diet exposure (n = 3–5 per group). All data are mean ± SEM. Data were analyzed by two-way ANOVA for main effects of diet and genotype with a Tukey post hoc test. *P < 0.05 within same genotype and #P < 0.05 within same diet group compared with WT.
Obese Pik3r1\textsuperscript{−/−} mice have reduced AT inflammation and macropage accumulation. Inflammatory and macrophage markers were significantly elevated in AT and SkM from all groups compared with their respective CON groups, but were greatly reduced in aHZ-HFD vs. WT-HFD (Fig. 2A and Supplementary Fig. 2). Paralleling these changes, more F4/80\textsuperscript{+} cells were present in characteristic crown-like structures in WT-HFD and βKO-HFD, but not aHZ-HFD, compared with WT-CON (Fig. 2B). By flow cytometry, we found significant, two- and sevenfold increases in F4/80\textsuperscript{+} and F4/80\textsuperscript{+}/CD11c\textsuperscript{+} cells, respectively, in the SVF fraction of WT-HFD and βKO-HFD versus WT-CON (Fig. 2C). In contrast, there were significantly less F4/80\textsuperscript{+} and F4/80\textsuperscript{+}/CD11c\textsuperscript{+} cells in AT from aHZ-HFD compared to WT-HFD (Fig. 2C). Representative gated flow cytometry scatter plots with average percent of cells per quadrant for each group are presented in Supplementary Fig. 3.

Plasma insulin and some proinflammatory cytokine levels are reduced in obese Pik3r1\textsuperscript{−/−} mice. Plasma insulin, resistin, and plasminogen activator inhibitor-1 were significantly elevated in WT-HFD versus WT-CON mice, but not in aHZ-HFD mice. Additionally, plasma adiponectin levels were significantly decreased in WT-HFD, but not aHZ-HFD, mice as compared with CON (Table 1). Chemokine (C-C motif) ligand (CCL) 2 (CCL2)/monocyte chemotactic protein-1 (MCP-1) was not increased in the plasma of HFD mice; however, both IL-6 and leptin were significantly increased in HFD, regardless of genotype (Table 1).

**TABLE 1**

|                      | WT-CON            | WT-HFD          | aHZ-CON          | aHZ-HFD         |
|----------------------|-------------------|-----------------|------------------|-----------------|
| Insulin (ng/mL)      | 0.35 ± 0.1        | 1.0 ± 0.1\*,†   | 0.49 ± 0.17      | 0.73 ± 0.12     |
| Glucose (mg/dL)      | 76.1 ± 6.1        | 81.9 ± 7.3      | 69.3 ± 2.4       | 72.8 ± 7.2      |
| Leptin (pg/mL)       | 2,891.0 ± 736.5   | 7,878.2 ± 321.2\* | 2,053.1 ± 446.6 | 7,737.0 ± 758\* |
| MCP-1 (pg/mL)        | 64.9 ± 1.6        | 63.9 ± 2.0      | 61.9 ± 3.6       | 62.1 ± 2.5      |
| Resistin (pg/mL)     | 3,436.2 ± 451.2   | 5,672.8 ± 501.6\* | 4,428.8 ± 586.6 | 5,370.2 ± 587.7 |
| IL-6 (pg/mL)         | 163.3 ± 0.7      | 28.8 ± 3.8\*   | 18.7 ± 2.2       | 25.7 ± 4.3\*   |
| PAI-1 (pg/mL)        | 403.6 ± 81.8     | 1,181.0 ± 57.3\*,† | 553.6 ± 89.6    | 413.6 ± 40.8   |
| Adiponectin (mg/mL)  | 34.27 ± 1.5      | 25.74 ± 1.9\*,† | 35.11 ± 2.6      | 32.63 ± 1.3     |

Plasma factors were measured using Multiplex ELISA or a standard ELISA for insulin. Data presented as mean ± SEM. MCP-1, monocyte chemotactic protein-1. PAI-1, plasminogen activator inhibitor-1. *P < 0.05 within same genotype. †P < 0.05 within same diet group.
Secretion of chemotactic factors from AT is significantly reduced in αHZ-HFD mice. We profiled media collected from αHZ-HFD, WT-HFD, and WT-CON AT explants. Of the 62 cytokines measured, 10 inflammatory chemokines were differentially secreted in AT from WT-HFD versus αHZ-HFD mice (Table 2). Six of these secreted factors, chemokine (C-X-C motif) ligand (CXCL) 13 (CXCL13), CXCL12, CXCL4, CXCL16, CCL1, and L-selectin are chemoattractants for immune cells (2,29,30) and were elevated in WT-HFD but not αHZ-HFD. Macrophage colony stimulating factor (M-CSF), a cytokine that stimulates macrophage differentiation (31), and CXCL10, a proinflammatory cytokine released by macrophages (32), were also increased in conditioned media from WT-HFD only. IL-1α, IL-3, and tissue inhibitor of metalloproteinases 1 (TIMP-1) were differentially secreted in αHZ-HFD as compared with WT mice in a pattern that would positively influence extracellular matrix (ECM) remodeling and collagen synthesis (33,34). The full list of secreted factors can be found in Supplementary Table 1. Paralleling the changes in cytokine secretion, phosphorylation of IKKα/β (S180/181) in AT was increased twofold in WT-HFD versus WT-CON, but was not increased in αHZ-HFD (Supplementary Fig. 7A).

Greater adipocyte size in βKO and αHZ versus WT mice with HFD feeding. Adipocytes from HFD versus CON were larger in size but fewer in number (Supplementary Fig. 4A–D). In CON groups, a greater percentage of αHZ adipocytes were larger than WT, whereas βKO had a greater percentage of cells that were smaller (Supplementary Fig. 4A and B). In HFD groups, αHZ and βKO had a greater percentage of larger adipocytes compared with WT (Supplementary Fig. 4A and B). Notably, despite the larger cell size in both αHZ-HFD and βKO-HFD, only the αHZ-HFD mice were protected against ATM infiltration and adipocyte insulin resistance.

The protective effect of Pik3r1 knockdown on insulin sensitivity and ATM infiltration is independent of the hematopoietic compartment. BMT studies were performed in lethally irradiated Pik3r1-/- and WT mice. We created four chimeras: WT BM into WT mice (WT→WT; donor BM genotype−recipient mouse genotype), Pik3r1-/- BM into WT mice (αHZ→WT), Pik3r1-/- BM into WT mice (αHZ→αHZ), and Pik3r1-/- BM into Pik3r1-/- mice (WT→αHZ) (Fig. 3A). Flow cytometry on peripheral blood monocytes shows that engraftment was complete within 6 weeks of transplant (Supplementary Fig. 5A–D). Mice with >80% engraftment were placed on an HFD for 12 weeks and insulin sensitivity was measured by clamp. Body weight and percent body fat were significantly increased in all BMT-HFD mice compared with WT→WT-CON mice (Fig. 3B–C). Insulin sensitivity (GINF) was not impaired in obese αHZ→αHZ-HFD, but was significantly reduced in WT→WT-HFD mice versus WT→WT-CON (Fig. 3D). Replacing WT BM with Pik3r1-/- BM did not improve insulin sensitivity in obese WT mice (αHZ→WT), and GINF was similar to WT→WT-HFD mice (Fig. 3D). Replacing Pik3r1-/- BM with WT BM did not impair insulin sensitivity as GINF in WT→αHZ-HFD was similar to αHZ→αHZ-HFD mice (Fig. 3D).

Complementing the insulin sensitivity data, gene expression of inflammatory and macrophage markers in AT was significantly increased in WT→WT-HFD and αHZ→WT-HFD mice compared with WT→WT-CON, but were attenuated in αHZ→αHZ-HFD and WT→αHZ-HFD mice (Fig. 4A). In addition, only AT from WT→WT-HFD and αHZ→WT-HFD mice had a significant increase in F4/80 + cells forming crown-like structures by immunohistochemistry and more F4/80+/CD11c+ (approximately twofold) and F4/80+/CD11c+ (approximately eightfold) cells counted in the SVCs compared with WT→WT-CON mice (Fig. 4B and C). Macrophage infiltration was markedly reduced and essentially absent in WT→αHZ-HFD and αHZ→αHZ-HFD mice (Fig. 4B and C).

Proximal insulin signaling, but not PI3K-Akt signaling, is impaired in AT from obese αHZ mice. Insulin-stimulated tyrosine phosphorylation of IRS1 (pIRS1[Y612]) was significantly and equally impaired (approximately threefold) in

### Table 2: Cytokine and chemokine levels in conditioned media from AT explants

|         | WT-CON | WT-HFD | αHZ-HFD |
|---------|--------|--------|---------|
| AXL     | 36 ± 1 | 61 ± 8* | 33 ± 5† |
| CXCL13  | 41 ± 4 | 51.0 ± 5 | 26 ± 5† |
| CD40    | 35 ± 1 | 50 ± 8  | 24 ± 3  |
| CRG-2   | 35 ± 2 | 48 ± 7  | 23 ± 4† |
| CTACK   | 49 ± 5.4 | 61 ± 3.1 | 39 ± 6  |
| CXCL16  | 80 ± 2 | 183 ± 14* | 129 ± 12† |
| FAS ligand | 25 ± 1 | 32 ± 6  | 23 ± 5  |
| G-CSF   | 134 ± 35 | 261 ± 37 | 215 ± 20 |
| GM-CSF  | 51 ± 11 | 50 ± 10 | 27 ± 20 |
| IFN-γ   | 39 ± 9  | 34 ± 14 | 29 ± 7  |
| sGF-BP6 | 71 ± 3  | 139 ± 14* | 59 ± 7  |
| IL-1α   | 69 ± 7  | 70 ± 6  | 43 ± 5†  |
| IL-1β   | 41 ± 1  | 52 ± 10 | 26 ± 2†  |
| IL-2    | 39 ± 3  | 51 ± 9  | 25 ± 2  |
| IL-3    | 40 ± 1  | 39 ± 3  | 23 ± 2†  |
| L-selectin | 72 ± 4 | 65 ± 11* | 58 ± 1†  |
| MCP-1   | 381 ± 62 | 399 ± 25 | 285 ± 55 |
| MCP-5   | 39 ± 3  | 32 ± 5  | 19 ± 10 |
| M-CSF   | 107 ± 38 | 188 ± 25* | 65 ± 19† |
| MIG/CXCL9 | 37 ± 21 | 47 ± 11 | 301 ± 5 |
| MIP-1α/CCL3 | 41 ± 17 | 27 ± 7  | 28 ± 13 |
| CXCL4   | 61 ± 14 | 118 ± 10* | 65 ± 8† |
| P-selectin | 86 ± 18 | 94 ± 12 | 94 ± 7 |
| RANTES/CCL5 | 77 ± 22 | 87 ± 54 | 35 ± 7 |
| CXCL12α | 26 ± 4 | 58 ± 9† | 26 ± 5† |
| TARC/CCL7 | 28 ± 1 | 39 ± 4  | 26 ± 5  |
| CCL1    | 48 ± 12 | 88 ± 3* | 48 ± 5† |
| TECK/CCL25 | 30 ± 1 | 47 ± 13 | 25 ± 4  |
| TIMP-1  | 75 ± 7  | 92 ± 3  | 51 ± 4† |
| TNF-α   | 30 ± 9  | 17 ± 0.3 | 29 ± 4.6 |
| sTNF RI | 241 ± 47 | 324 ± 24 | 332 ± 19 |
| sTNF RII | 76 ± 3  | 146 ± 21* | 189 ± 14* |
| VCAM-1  | 180 ± 3 | 304 ± 16* | 323 ± 24* |

Media from AT explants was analyzed by immunoblot assay. Data (presented as mean ± SEM) are expressed relative to an internal positive control. Values in bold indicate a statistical difference. *P < 0.05 vs. WT-CON. †P < 0.05 vs. WT-HFD. CD40, cluster of differentiation (CD) 40; CRG-2, cytotoxic responsive gene-2; CTACK, cutaneous T-cell attracting chemokine; Fas, also known as CD95; G- and GM-CSF, granulocyte- and granulocyte-macrophage colony stimulating factor (CSF); IFN, interferon; IFGBP6, insulin like growth factor binding protein 6; MCP-1 and -5, monocyte chemotactic protein-1 and -5; M-CSF, macrophage-CSF; MIG/CXCL9, monokine-induced by interferon/chemokine (C-X-C motif) ligand (CXCL) 9; MIP-1α/CCL3, macrophage inflammatory protein-1α/chemokine (C-C motif) ligand (CCL) 3; RANTES, regulated upon activation, normal T-cell expressed and secreted; TARC, thymus and activated regulated chemokine; TECK, thymus expressed chemokine; TIMP-1, tissue inhibitor of metalloproteinases 1; sTNF RI and RII, soluble tumor necrosis factor receptor I and receptor II; VCAM-1, vascular cell adhesion molecule-1.
AT of all HFD versus CON mice (Fig. 5A and B and Supplementary Fig. 6A). The decrease in IRS1 activation was accompanied by a significant reduction (~50%) in IRS1 abundance (Fig. 5A and Supplementary Fig. 6A). This reduction was likely due to increased IRS1 serine phosphorylation as JNK1(T183/Y185) phosphorylation was equally upregulated in AT from WT-HFD and αHZ-HFD mice (Supplementary Fig. 7B). X-box binding protein 1 splicing and gene expression of the ER stress markers growth arrest and DNA damage-inducible gene-153 (GADD153) and glucose-regulated protein, 78kDa (GRP78) and hypoxia markers, DNA damage-inducible gene-153 (GADD153) and glucose-regulated protein, 78kDa (GRP78) and hypoxia markers, vascular endothelial growth factor (VEGF), GLUT1, and hypoxia-inducible factor (HIF) 1α were elevated in AT with hypoxia occurrence without profound ATM accumulation in αHZ-HFD. Despite reduced insulin-stimulated IRS1 activation, insulin-stimulated Akt(S473) and Akt(T308) phosphorylation was equally upregulated in AT from WT-HFD and αHZ-HFD mice (Supplementary Fig. 7B). These findings are important in that the decrease in IRS1 activation and upregulation of pJNK1(T183/Y185), ER stress, and hypoxia occurred without profound ATM accumulation in αHZ-HFD. Despite reduced insulin-stimulated IRS1 activation, insulin-stimulated Akt(S473) and Akt(T308) phosphorylation was not impaired in αHZ-HFD, but was impaired in WT-HFD versus CON mice (Fig. 5A, D, and E). Enhanced insulin-stimulated Akt activation did not occur in βKO-HFD mice (Supplementary Fig. 6A). Insulin-stimulated, pY-associated PI3K activity in AT was also reduced twofold in WT-HFD versus WT-CON mice, but was not reduced in αHZ-HFD mice (Fig. 5F). There was no difference in basal pY-associated PI3K activity (Fig. 5F).

Pik3r1 abundance is increased in AT from WT-HFD but not αHZ-HFD mice. In parallel with the insulin-stimulated PI3K activity, there was a significant increase in the abundance of the shorter isoforms of Pik3r1 (p55α and p50α; four- and twofold, respectively) and a smaller (40%) increase in p85α in AT from WT-HFD and βKO-HFD versus WT-CON, which did not occur in αHZ-HFD mice (Fig. 5A and C and Supplementary Fig. 6A). Changes in p55α/p50α abundance in WT-HFD were paralleled by an increase in mRNA levels of p55α and p50α, but not p85α (Supplementary Fig. 7F). P110α abundance was not different across groups (Fig. 5A). Interestingly, we observed only a modest, nonsignificant decrease in p85α abundance in AT from Pik3r1−/− mice despite a significant, 50% decrease in mRNA expression, suggesting that Pik3r1 protein stability is likely increased. We speculate that increased protein stability may be due to a greater number of the Pik3r1 regulatory subunits binding to the catalytic subunit to form heterodimers or to other p85α binding partners. Indeed, association with the catalytic subunit has been previously shown to increase heterodimer stability (35). Thus, these data suggest that HFD appears to reduce insulin-stimulated PI3K activity and downstream signaling to Akt, at least in part through upregulation of p85α, p55α, and p50α expression and protein abundance in AT.

SkM insulin signaling is not impaired in αHZ-HFD mice. Despite severe obesity, SkM insulin signaling was not impaired in αHZ-HFD mice compared with αHZ-CON, whereas WT-HFD mice had significant impairments in insulin-stimulated tyrosine phosphorylation of pIRS1(Y612) (~50%), insulin-stimulated p-associated PI3K activity, and Akt(S473) phosphorylation compared with WT-CON (Supplementary Fig. 8A, B, E, and F). Similar to AT, p85α...
abundance was increased in WT-HFD SkM, with no change in p110 abundance (Supplementary Fig. C–E). Although there was no significant decrease in p85α abundance in αHZ SkM, we did find an ~50% decrease in p110 catalytic subunit (Supplementary Fig. C–E). This decrease in p110 abundance was reflected in a 50% decrease in insulin-stimulated, pY-associated PI3K activity in both αHZ SkM. It is notable that catalytic subunit abundance in all groups, insulin-stimulated, pY-

associated PI3K activity/p110 was significantly higher in αHZ groups compared with their diet-matched WT group (Supplementary Fig. 6F). The adjusted insulin-stimulated PI3K activity matches the increase in Akt phosphorylation seen in αHZ mice compared with WT groups (Supplementary Fig. 8B and F).

**STAT3 activation in AT parallels increased p55α/p50α expression.** Nuclear localization of signal transducer and activator of transcription 3 (STAT3) (Fig. 6A) was increased in AT of WT-HFD and αHZ-HFD versus WT-CON. Because IL-6 was elevated in both WT-HFD and αHZ-HFD and is known to activate STAT3 (36), we studied the effects of IL-6 treatment on p85α, p55α, and p50α expression in differentiated 3T3-L1 adipocytes. IL-6 rapidly activated STAT3(Y705) phosphorylation within 15 min (Fig. 6B), corresponding to an acute rise in STAT3 genes IL-6 and SOCS3 (Fig. 6C). Interestingly, STAT3 activation by IL-6 appeared biphasic, with peak phosphorylation at 1 and 24 h (Fig. 6B). This pattern of activation suggests the possibility that acute IL-6 stimulation of STAT3 may lead to secretion of a second factor that then reactivates STAT3 signaling. Only chronic IL-6 stimulation led to a significant increase in p55α and p50α expression (Fig. 6D). No increase in p85α expression was found in response to IL-6 stimulation (Fig. 6D).

**DISCUSSION**

Despite strong evidence for a link between macrophage accumulation in AT and insulin resistance in obesity, the factors that initiate chemokine secretion for macrophage recruitment remain elusive. In this study, we introduce the idea that AT insulin resistance is an important factor that links cellular metabolism, particularly insulin action, to chemokine secretion and initiation of macrophage recruitment.

Elegant transgenic mouse studies have demonstrated that AT inflammation and macrophage chemotaxis are reduced when signaling pathways, such as JNK, toll-like receptor 4, and IKK, are manipulated in the hematopoietic compartment (6–8). The underlying conclusion from these and comparable studies (1) is that AT inflammation is driven by macrophage infiltration, and secondary to this infiltration, AT insulin resistance manifests. In contrast, however, several studies have found dissociation between ATM infiltration and the development of insulin resistance. For example, in lean mice null for transforming growth factor-β receptor 4 and apolipoprotein E, there is massive T-cell activation, hyperlipidemia, ATM infiltration, and up-regulation of inflammatory cytokines but no evidence of adipocyte or systemic insulin resistance (37). In our study, AT from WT-HFD had significant increases in F4/80+ and F4/80+/CD11c+ cells, whereas accumulation of these cells in AT was attenuated in αHZ-HFD mice. Our BMT experiments demonstrate that the metabolic improvements in αHZ-HFD mice are independent of changes in Pik3r1 expression in myeloid cells. In fact, even when αHZ mice were transplanted with WT BM (WT→αHZ), which would be expected to facilitate F4/80+/CD11c+ infiltration and AT inflammation in obese mice, F4/80+/CD11c+ cells were significantly reduced compared with obese WT→WT mice. Taken together, these experiments demonstrate that the impact of reduced P3K regulatory subunits on improved insulin sensitivity and macrophage recruitment in obese Pik3r1<sup>−/−</sup> mice is driven by non-hematopoietic–derived cells.
Local hypoxia, upregulation of proinflammatory JNK1 and IKKβ pathways, and induction of ER stress have all been shown to be key signaling pathways that contribute to AT insulin resistance, primarily through inhibition of insulin signaling at the level of IRS1 (1). We found that activation of JNK, ER stress, and hypoxia were induced similarly in AT from WT-HFD, bKO-HFD, and αHZ-HFD mice, in parallel with reduced insulin-stimulated activation of IRS1. Despite these changes in IRS1, insulin-stimulated PI3K activity was not reduced in AT from αHZ-HFD mice but was impaired in WT-HFD. Thus, these data demonstrate that partial deletion of Pik3r1, and subsequent maintenance of PI3K function, bypasses the obesity- and inflammation-mediated reductions in tyrosine activation of IRS1. Moreover, our results clearly demonstrate that PI3K-dependent signaling is critical for ATM recruitment in obesity. A separate study found that increased AT lipolysis leads to a rapid increase in ATM accumulation in lean mice.

**FIG. 5.** Proximal insulin signaling but not PI3K/Akt signaling is impaired in AT from obese αHZ-HFD mice. Insulin signaling protein abundance and activation were measured in eAT before and after maximal insulin stimulation in WT and αHZ mice. A: Representative immunoblots are shown. Quantitation of signaling proteins from immunoblots for IRS1(Y612) phosphorylation (B), p85/p55/p50α abundance (C), Akt(S473) phosphorylation (D), Akt(T308) phosphorylation (E), and PY-associated PI3K activity (F) was assayed in basal and insulin-stimulated eAT. Data shown as the mean ± SEM. n = 6–10 per group. Data were analyzed by two-way ANOVA for main effects of diet and genotype with a Tukey post hoc test. *P < 0.05 within same genotype and #P < 0.05 within same diet group.
after fasting or after initial weight loss in HFD-fed mice, suggesting that increased FFA may be an important cue for macrophage recruitment (38). Like the findings of Kosteli et al. (38), aHZ-HFD mice had no impairments in insulin suppression of FFAs or glycerol during a clamp and significantly less ATM accumulation as compared with WT-HFD mice, which failed to suppress lipolysis during the clamp. Mechanistically, we believe that the local tissue environment plays a major role in reducing ATM recruitment in Pik3r1+/− mice. To this end, AT explants from aHZ-HFD mice secreted less proinflammatory cytokines compared with WT-HFD mice. This reduction in chemokine and cytokine secretion may be linked to the reduction in IKK (S180/S181) phosphorylation in AT from aHZ-HFD compared with WT-HFD mice. Activation of IKKβ is thought to be a key regulator of inflammatory gene expression in obesity through regulation of nuclear factor-κB activity (7). Alternatively, reduced inflammation in aHZ-HFD compared with WT-HFD mice may be linked to the increased circulating adiponectin, which has potent anti-inflammatory effects (39).

Although PI3K is a key regulator of insulin signaling, surprisingly, little is known about the signaling mechanisms that modulate the expression of the regulatory subunits of PI3K in response to nutrient flux. Increased expression of p85α in peripheral tissues occurs in response to increased insulin (40), growth hormone (21), and dexamethasone (41). Additionally, rosiglitazone increases p85α expression in human adipocytes through peroxisome proliferator-activated receptor γ activation (42), and SkM p85α expression is strongly induced by peroxisome proliferator-activated receptor α activation (43). We speculate that the increase in p85α in WT-HFD AT and SkM may be due to increased fasting insulin in WT-HFD mice. Regarding p55α/p50α transcription, Abell et al. (44) reported that STAT3 activation increases p55α/p50α transcription during mammary gland differentiation, stimulating apoptosis through inhibition of PI3K activity. A similar pattern of PI3K regulation has recently been proposed in SkM in response to caloric restriction (22). In agreement, we found that AT p55α/p50α abundance was increased in parallel with increased STAT3 nuclear localization in WT-HFD mice and in 3T3-L1 cells in response to chronic IL-6 exposure. Limiting STAT3 transcription of Pik3r1 in AT could be an attractive target for preventing obesity-induced insulin resistance. In support of this, mice with AT-specific knockout of STAT3 have increased body weight and adiposity with no impairment in insulin sensitivity (45).

In summary, our results suggest that impaired AT insulin sensitivity in obesity at the level of PI3K is a critical step necessary for ATM infiltration and inflammation. Moreover, they suggest a model in which reduced PI3K function and
AT insulin resistance manifests due to increased adipocyte activation of STAT3 and p55α/p50α transcription. These data also raise new questions as to whether the reduced inflammatory response in AT of Pik3r1−/− mice is due directly to improvements in insulin action or, alternatively, related to improved P3K activity independent of the insulin signaling pathway. Thus, the molecular mechanism of how P3K signaling affects macrophage function remains to be further defined; however, the present results suggest that modulating AT Pik3r1 expression and P3K function is a potentially attractive avenue for the development of novel therapies to treat AT insulin resistance and inflammation in obesity.

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C.E.M., S.S., and A.P. researched data, contributed to the discussion, and wrote and edited the manuscript. M.J.H. and J.A.H. researched data and contributed to the discussion. D.P., P.S.M., S.M.M., and D.J.K. researched data, contributed to the discussion, and edited the manuscript. J.E.F. contributed to the discussion and edited the manuscript. C.E.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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