Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone that also plays a regulatory role in fat metabolism. In 3T3-L1 cells, resistin was demonstrated to be a key mediator of GIP stimulation of lipoprotein lipase (LPL) activity, involving activation of protein kinase B (PKB) and reduced phosphorylation of liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK). The current study was initiated to determine whether resistin has additional roles in GIP-regulated adipocyte functions. Analysis of primary adipocytes isolated from Retn+/−, Retn−/−, and Retn+/− mice found that GIP stimulated the PKB/LKB1/AMPK/LPL pathway and fatty acid uptake only in Retn+/− adipocytes, suggesting that GIP signaling and/or GIP responsiveness were compromised in Retn−/− and Retn−/− adipocytes. GIP receptor (GIPR) protein and mRNA were decreased in Retn−/− and Retn−/− adipocytes, but resistin treatment rescued LPL responsiveness to GIP. In addition, genes encoding tumor necrosis factor (TNF), adiponectin, and resistin were measured in Retn−/− adipocytes by microarray analysis and found to be increased in Retn−/− adipocytes but unaffected in Retn+/− adipocytes.

RESULTS

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone that also plays a regulatory role in fat metabolism. In 3T3-L1 cells, resistin was demonstrated to be a key mediator of GIP stimulation of lipoprotein lipase (LPL) activity, involving activation of protein kinase B (PKB) and reduced phosphorylation of liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK). The current study was initiated to determine whether resistin has additional roles in GIP-regulated adipocyte functions. Analysis of primary adipocytes isolated from Retn+/−, Retn−/−, and Retn+/− mice found that GIP stimulated the PKB/LKB1/AMPK/LPL pathway and fatty acid uptake only in Retn+/− adipocytes, suggesting that GIP signaling and/or GIP responsiveness were compromised in Retn−/− and Retn−/− adipocytes. GIP receptor (GIPR) protein and mRNA were decreased in Retn−/− and Retn−/− adipocytes, but resistin treatment rescued LPL responsiveness to GIP. In addition, genes encoding tumor necrosis factor (TNF), adiponectin, and resistin were measured in Retn−/− adipocytes by microarray analysis and found to be increased in Retn−/− adipocytes but unaffected in Retn+/− adipocytes.

RESEARCH DESIGN AND METHODS

Animals. Retn+/− mice were provided by Dr. Mitchell Lazar, University of Pennsylvania School of Medicine. Breeding and genotyping were performed at the University of British Columbia (UBC). Animal experiments were conducted in accordance with guidelines of the UBC Committee on Animal Care.

LPL enzyme activity and fatty acid uptake assay. Primary adipocytes were isolated (18) from male Retn+/−, Retn−/−, and Retn+/− mice (10–12 weeks old) and plated using Matrigel (BD Biosciences). After GIP plus insulin treatment, adipocytes were recovered and LPL activity was measured, as described (6). For the fatty acid uptake assay (19), adipocytes were incubated with 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-9-dodecanolic acid (C12-BODIPY500/510-C15, Molecular Probes) and sorted on a BD LSR II flow cytometer.

Quantitative PCR and RT² Profiler Arrays. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) and Gipr expression measured, as described previously (10). RT² Profiler PCR Arrays were performed to quantify mRNAs of multiple genes, including housekeeping genes, according to the manufacturer’s protocol.

Chromatin immunoprecipitation and Western blot analysis. Protein samples were immunoprecipitated using Dynabead protein A or protein G (Invitrogen) and c-Jun or phospho-serine/threonine antibody (Cell Signaling; ECM Biosciences). Precipitated products or protein samples were resolved by SDS-PAGE and probed with the antibodies indicated in the Figure legends.

RESULTS

GIP regulates a PKB/LKB1/AMPK/LPL pathway and resistin secretion in Retn+/− adipocytes but not in Retn−/− or Retn−/− adipocytes. Mating of heterozygous Retn+/− mice resulted in expected Retn+/+, Retn+/−, and
Retn<sup>−/−</sup> genotypes (14). The mice did not differ significantly in body weight, glucose tolerance, or epididymal white adipose tissue (eWAT) adipocyte size (Supplementary Fig. 1A–E). Immunoreactive resistin was undetectable in Retn<sup>−/−</sup> mice plasma and eWAT extracts, whereas plasma and fat levels in Retn<sup>−/−</sup> mice were <25% of Retn<sup>+/+</sup> levels (Supplementary Fig. 1F and G).

To investigate the role of resistin in GIP-mediated increases in LPL activity, responses of Retn<sup>+/+</sup>, Retn<sup>−/−</sup>, and Retn<sup>+/−</sup> adipocytes to GIP were examined. Incubation of Retn<sup>+/+</sup> adipocytes with GIP plus insulin (1 nmol/L) resulted in concentration-dependent stimulation of LPL activity (Fig. 1A), increased levels of phospho-PKB (Ser473), and decreased levels of phospho-LKB1 (Ser428) and phospho-AMPK (Thr172), but was without effect on Retn<sup>−/−</sup> or Retn<sup>+/−</sup> adipocytes (Supplementary Fig. 2A–C). In addition, treatment of Retn<sup>+/+</sup> adipocytes with GIP plus insulin (1 nmol/L) resulted in significantly increased fatty acid uptake, but was without effect on Retn<sup>−/−</sup> or Retn<sup>+/−</sup> adipocytes (Fig. 1B). The complete lack of Retn<sup>−/−</sup> adipocyte responses was surprising and suggested a critical resistin secretion level was required for GIP-mediated stimulation of LPL activity and/or adipocyte responsiveness to GIP was compromised.

The possibility that resistin secretion from Retn<sup>−/−</sup> adipocytes was reduced to ineffective levels for activating the PKB/LKB1/AMPK pathway was first tested. Incubation of Retn<sup>−/−</sup> adipocytes with GIP plus insulin (1 nmol/L) for 2 h resulted in concentration-dependent stimulation of resistin secretion (Supplementary Fig. 3A). Basal secretion of resistin by Retn<sup>−/−</sup> adipocytes was significantly reduced, and secretion by Retn<sup>+/−</sup> adipocytes was undetectable. Resistin secretion by Retn<sup>−/−</sup> adipocytes was unresponsive to GIP plus insulin (1 nmol/L). In addition, insulin alone had no effect on resistin secretion by Retn<sup>−/−</sup> adipocytes (Supplementary Fig. 3B). These results indicated that the inability of GIP to activate the PKB/LKB1/AMPK/LPL pathway in Retn<sup>−/−</sup> adipocytes involved severely blunted resistin secretion.

The GIP receptor (GIPR) is downregulated in Retn<sup>−/−</sup> and Retn<sup>+/−</sup> adipocytes. To examine the involvement of altered receptor expression, GIPR protein and mRNA levels were determined, and Retn<sup>−/−</sup> and Retn<sup>+/−</sup> adipocytes demonstrated severe downregulation (Fig. 1C and D). We next examined whether resistin acted as a direct regulator of GIPR expression. Treatment of Retn<sup>−/−</sup> adipocytes with resistin (100 nmol/L) resulted in a delayed increase in GIPR expression, after 72-h incubation (Fig. 2A), indicating chronic effects on expression, and 72-h treatment with resistin rescued GIP-induced LPL activity in Retn<sup>−/−</sup> and Retn<sup>+/−</sup> adipocytes (Fig. 2B).

Intriguingly, GIPR protein was also downregulated in brown adipose tissue and the stromal vascular fraction of Retn<sup>−/−</sup> and Retn<sup>+/−</sup> mice (Supplementary Fig. 4A and B), whereas no significant differences were noted in skeletal muscle or pancreatic islet GIPR protein expression among the groups (Supplementary Fig. 4C and D). In addition, treatment of Retn<sup>+/−</sup> islets with resistin (100 nmol/L) had no effect on GIPR expression (Supplementary Fig. 4E), implying that regulation was tissue-specific.

Adipocyte Gipr expression is regulated via a c-Jun-mediated pathway. With a view to identifying proteins involved in regulating the adipocyte Gipr, a PCR array was used to screen a panel of 84 genes associated with obesity, insulin resistance, and early onset of diabetes (RT-Profiler) in eWAT from Retn<sup>+/+</sup> and Retn<sup>−/−</sup> mice. This
revealed large reductions in Retn−/− fat expression of genes coding for proteins involved in signaling (ectonucleotide pyrophosphatase/phosphodiesterase 1 [Enpp1]), intercellular adhesion (intracellular adhesion molecule [Icam1]), GLUT-4 trafficking (syntaxin 4a [Stx4a]), and glyceroneogenesis (soluble phosphoenolpyruvate carboxykinase 1 [Pck1]) (Fig. 3A). Of particular interest, genes encoding tumor necrosis factor (TNF [Tnf]), tumor necrosis factor receptor 2 (TNFR2 [Tnfrsf1b]), and the downstream signaling protein(s) stress-activated protein kinase (SAPK)/Jun NH₂-terminal kinase (JNK) (Mapk8), were decreased in Retn−/− fat tissue. In contrast, Gsk3b (glycogen synthase kinase 3 [GSK3]-β) expression, a negative regulator of c-Jun (20) was increased in Retn−/− fat (Fig. 3B). Levels of phospho-SAPK/JNK (Thr183/Tyr185) and phospho-c-Jun, were also decreased (Figs. 3C and D). Together, these results suggested that decreased adipocyte GIPR expression involved reduced signaling via TNF activation of SAPK/JNK and c-Jun.

FIG. 2. Resistin increases GIPR expression in Retn+/− adipocytes and rescues GIP responsiveness of Retn−/− and Retn−/− adipocytes. A: Effect of resistin on GIPR expression in Retn+/− adipocytes. Primary adipocytes were isolated from Retn+/+ mice, plated using Matrigel, and treated with resistin for the indicated periods of time. Protein extracts were isolated and Western blot analyses performed with antibodies against GIPR and β-actin. B: Effect of resistin on GIP-mediated LPL activation in Retn−/− and Retn−/− adipocytes. Primary adipocytes were isolated from Retn+/+ and Retn−/− mice and plated using Matrigel. Adipocytes were treated with resistin for 72 h, further treated with GIP in the presence of insulin (1 nmol/L) for 24 h, and LPL activity was determined. Significance was tested using ANOVA with Newman-Keuls post hoc test. **P < 0.05 vs. control or indicated group. Data represent the mean ± SEM.
FIG. 3. JNK/c-Jun expression is downregulated in Retn<sup>−/−</sup> adipocytes. A and B: Gene expression profiles. Total RNA was extracted from eWAT of Retn<sup>+/+</sup> and Retn<sup>−/−</sup> mice, and RT<sup>2</sup> Profiler PCR Arrays were performed to simultaneously quantify mRNA expression levels of multiple genes. Genes changed in Retn<sup>−/−</sup> adipocytes: Enpp1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), Icam1 (intercellular adhesion molecule 1), Pck1 (phosphoenolpyruvate carboxykinase 1 [soluble]), Stx4a (syntaxin 4a), Mapk8 (SAPK/JNK), Tnf (TNF), Tnfrsf1b (TNFR2) (A) and Gsk3b (glycogen synthase kinase 3 [GSK3]-β) (B). C and D: JNK/c-Jun expression in Retn<sup>+/+</sup> and Retn<sup>−/−</sup> adipocytes. Primary adipocytes were isolated from eWAT of Retn<sup>+/+</sup> and Retn<sup>−/−</sup> mice and protein extracts prepared. Western blot analyses were performed with antibodies against phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, and β-actin. Protein extracts were immunoprecipitated (IP) with phospho-serine (Ser)/threonine (Thr) or c-Jun, followed by immunoblotting (IB) for c-Jun or phospho-Ser/Thr. Input represents one-tenth of protein extracts used in assay. Western blots are representative of n = 3. All data represent the mean ± SEM. Significance was tested using Student t test. **P < 0.05 vs Retn<sup>+/+</sup> adipocytes.
FIG. 4. c-Jun mediated adipocyte Gipr expression. A and B: c-Jun binds to the TRE-III of Gipr promoter in Retn+/+ adipocytes. A: Identification of a putative TRE sequence in the promoter of the mouse Gipr gene. The TRE location and the consensus sequence (bold) are presented. B: Quantitative ChIP in Retn+/+ and Retn+/− adipocytes. Primary adipocytes were isolated from eWAT of Retn+/+ and Retn+/− mice. c-Jun was immunoprecipitated from intact chromatin isolated from Retn+/+ and Retn+/− adipocytes using anti-c-Jun antibody. Precipitated DNA fragments were analyzed by SYBR green real-time PCR using primers flanking the TRE site in the Gipr promoter. C: Quantitative ChIP in resistin-treated Retn+/+ adipocytes. Primary adipocytes were isolated from Retn+/+ mice and treated with resistin (100 nmol/L) for the indicated intervals in the presence or absence of SAPK/JNK inhibitor, JNKi (SP600125, 50 μmol/L). c-Jun was immunoprecipitated as described above, and precipitated DNA fragments were analyzed by SYBR green real-time PCR using primers flanking the TRE-III site. D: GIPR mRNA levels in resistin-treated Retn+/+ adipocytes. Retn+/+ adipocytes were treated as described in C, and real-time RT-PCR was performed to quantify Gipr mRNA levels, which are shown as the fold difference vs control normalized to 18S rRNA expression levels. E: Effect of SAPK/JNK inhibitor on GIPR protein levels. Retn+/+ adipocytes were treated with resistin (100 nmol/L) for 72 h in the presence or absence of JNKi. Protein extracts were isolated and Western blot analyses...
In view of the decreased Gipr expression and c-Jun phosphorylation in Retn−/− fat tissue, we focused on establishing a functional link. Analysis of mouse Gipr promoter sequences using MatInspector (Abteling Genetik, Germany) revealed three potential TREs for c-Jun binding, residing between -3583 and -3577, -2936 and -2930, and -298 and -292, that we termed TRE-I, -II, and -III, respectively. TRE-I (TGATTCA), TRE-II (TGAGCCCA), and the inverted TRE-III (TGAGTAA) exhibit 85.7% homology to the consensus TRE (TGAG/CTCA) (Fig. 4A). To identify functional TRE(s), c-Jun binding to the Gipr promoter was determined by measuring TRE occupancy using ChIP assays. Significantly increased c-Jun occupancy in Retn−/+ over Retn−/− adipocytes was detectable only in TRE-III (Fig. 4B), indicating it is the functional cis-acting element for c-Jun–mediated regulation of the mouse Gipr promoter. Resistin (100 nmol/L) treatment of Retn−/+ adipocytes significantly increased c-Jun occupancy in Gipr promoter TRE-III (Fig. 4C) and Gipr mRNA levels (Fig. 4D) by 24 h, with a further increase up to 72 h. c-Jun occupancy and Gipr mRNA levels at 72 h after resistin treatment were both greatly reduced by the SAPK/JNK inhibitor, JNKi. Resistin-mediated Gipr protein expression was also greatly attenuated by JNKi (Fig. 4E), strongly supporting the involvement of a SAPK/JNK/c-Jun–mediated pathway in the regulation of GIPR expression.

**DISCUSSION**

We previously demonstrated that GIP stimulated LPL activity in 3T3-L1 cells and human adipocytes through a pathway involving resistin-mediated increases in phosphorylation of PKB and decreases in LKB1 and AMPK phosphorylation (6–8); a similar role was confirmed in Retn−/+ adipocytes (Fig. 1A and Supplementary Fig. 2). However, although resistin content of Retn−/+ adipocytes was ~25% of that in Retn−/+ mice, GIP responses were almost completely ablated, suggesting that a critical level of resistin signaling was essential for GIP action and/or that GIP responsiveness was greatly reduced. Subsequently, GIPR protein and mRNA were both found to be downregulated in Retn−/+ and Retn−/− adipocytes, indicating that attenuated adipocyte GIPR expression was the major contributor to blunted GIP responsiveness (Figs. 1B and C). In addition, resistin treatment of Retn−/+ adipocytes for 72 h increased GIPR expression in Retn−/+ adipocytes (Fig. 2A) and rescued GIP responsiveness of LPL activity in Retn−/+ and Retn−/− adipocytes (Fig. 2B), supporting a role in its long-term regulation. Although resistin treatment of Retn−/+ adipocytes resulted in a delayed increase in GIPR protein expression after 72-h incubation (Fig. 2A), increases in Gipr mRNA (Fig. 4D) and c-Jun Gipr promoter occupancy (Fig. 4C) were detectable after 24-h incubation. These results suggest involvement of complex post-translational regulation in resistin-mediated GIPR protein expression. The receptor is glycosylated, and the islet GIPR was downregulated in response to elevated glucose by a process involving ubiquitination (21), although whether these processes are involved in the delayed expression is unclear.

The PCR array study revealed Retn−/+ adipose tissue downregulation of genes encoding TNF (Tnf), TNFR2 (Tnfrsf1b), and the downstream signaling protein SAPK/JNK (Mapk8), whereas expression of Gsk3b was greatly increased (Figs. 3A and B). Resistin stimulates TNF-α gene expression in human and mouse macrophages (22), and TNF-α increases resistin protein and mRNA expression via a JNK pathway in human macrophages (23). Because activation of SAPK/JNK/c-Jun by TNF-α is an established pathway for regulating gene transcription (24), we considered it possible that its downregulation may contribute to the reduced GIP expression in Retn−/+ eWAT. Supporting this, phosphorylated levels of c-Jun and SAPK/JNK (Thr183/Tyr185) were decreased in Retn−/+ eWAT (Fig. 3C and D). In addition, using ChIP assays, we established that TRE-III in the mouse Gipr promoter was functional and responsible for c-Jun–mediated transcriptional activation, which was downregulated in Retn−/+ adipocytes (Fig. 4F).

Treatment of Retn−/+ adipocytes with a SAPK/JNK inhibitor greatly reduced resistin-induced increases in c-Jun occupancy in TRE-III of the Gipr promoter (Fig. 4C) and Gipr mRNA and protein expression (Figs. 4D and E), strongly supporting functional involvement of a SAPK/JNK/c-Jun–mediated pathway in GIPR expression regulation. The elevation in Gsk3b would also be expected to result in phosphorylation-induced reduction in transcriptional activity of c-Jun (20). These results strongly support involvement of c-Jun in regulating Gipr expression; however, other factors could clearly be involved.

Although a strong link between resistin and TNF-α has been identified in humans (12), the context in which resistin normally stimulates the TNF/TNFR2 pathway in adipocytes is unclear. TNFR1 and TNFR2 are the primary receptors for soluble and membrane-bound TNF, respectively (24). Most cell types constitutively express TNFR1, whereas TNFR2 expression is regulated by multiple factors. These receptors activate several cellular signaling modules, including SAPK/JNK, and GIP stimulates resistin secretion via activation of SAPK/JNK- and p38 MAPK-mediated pathways (8). Therefore, a complex interplay exists among GIP, resistin, and TNF-α.

In summary, effects of GIP on PKB/LKB1/AMPK/LPL in Retn−/+ adipocytes were almost completely abolished in Retn−/+ and Retn−/− adipocytes largely due to greatly reduced GIPR expression, associated with downregulated TNFα signaling and c-Jun–mediated transcriptional activation of the Gipr. In view of the use of incretin-related compounds for diabetes treatment, it will be important to establish the physiological and pathophysiological significance of the strong interaction between GIP and resistin actions on the adipocyte, especially in humans.

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

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