Resistin is a key mediator of glucose-dependent insulinotropic polypeptide (GIP) stimulation of lipoprotein lipase (LPL) activity in adipocytes

Su-Jin Kim, Cuilan Nian and Christopher H.S. McIntosh

From the Department of Cellular & Physiological Sciences and the Diabetes Research Group, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, Vancouver, B.C., V6T 1Z3, Canada

Running Title: Resistin-mediated action of GIP on LPL

Address correspondence to: Dr. C.H.S. McIntosh, Department of Cellular & Physiological Sciences, Faculty of Medicine, University of British Columbia, 2350 Health Sciences Mall, Vancouver, B.C., V6T 1Z3, Phone: 604-822-3088; Fax: 604-822-6048, Email: mcintosh@interchange.ubc.ca

Abbreviations list
GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; GPCRs, G protein-coupled receptors; GIPR, GIP receptor; GLP-1R, GLP-1 receptor; LPL, lipoprotein lipase; PKB, protein kinase B; AMPK, AMP-activated protein kinase; VDF, Vancouver Diabetic Fatty; GSIS, glucose-stimulated insulin secretion; PI3-K, phosphatidylinositol 3-kinase; MAPK, mitogen activated protein kinase; p38 MAPK, p38 mitogen activated protein kinase; SAPK/JNK, stress-activated protein kinase/Jun-amino-terminal kinase; TG, triglyceride(s); RNAi, RNA interference
Abstract

Studies on the physiological roles of the incretin hormone, glucose-dependent insulino tropic polypeptide (GIP) have largely focused on its insulino tropic action and ability to regulate β-cell mass. In previous studies on the stimulatory effect of GIP on adipocyte lipoprotein lipase (LPL), a pathway was identified involving increased phosphorylation of protein kinase B (PKB) and reduced phosphorylation of LKB1 and AMP-activated protein kinase (AMPK). The slow time of onset of the responses suggested that GIP have induced release of an intermediary molecule, and the current studies focused on the possible contribution of the adipokine resistin.

In differentiated 3T3-L1 adipocytes, GIP, in the presence of insulin, increased resistin secretion through a pathway involving p38 mitogen activated protein kinase (p38 MAPK) and the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK). The other major incretin hormone, glucagon-like peptide-1 (GLP-1), exhibited no significant effects. Chronic elevation of circulating GIP levels in the Vancouver Diabetic Fatty (VDF) Zucker rat resulted in increases in circulating resistin levels and activation of p38 MAPK or SAPK/JNK in epididymal fat tissue, suggesting the existence of identical pathways in vivo as well as in vitro. Administration of resistin to 3T3-L1 adipocytes mimicked the effects of GIP on the PKB/LKB1/AMPK/LPL pathway: increasing phosphorylation of PKB, reducing levels of phosphorylated LKB1 and AMPK, and increasing LPL activity. Knockdown of resistin using RNA interference (RNAi) attenuated the effect of GIP on the PKB/LKB1/AMPK/LPL pathway in 3T3-L1 adipocytes, supporting a role for resistin as a mediator.

Introduction

Glucose-dependent insulino tropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the two major incretin hormones that potentiate glucose-stimulated insulin secretion (GSIS) during a meal (1-4). There is an extensive literature on the insulino tropic effects of the incretins and their ability to promote β-cell proliferation and survival (1-6). Receptors for both GIP (GIPR) and GLP-1 (GLP-1R) have been shown to be expressed in tissues other than the pancreas, including adipose tissue (7-9), the gastrointestinal tract (10,11) and the brain (12,13). As a result of the recent introduction of the GLP-1 agonist Exenatide and inhibitors of the incretin-degrading enzyme dipeptidyl peptidase IV as therapeutics for type 2 diabetes (14), considerable interest has developed in the non-β-cell effects of the incretins.

There is strong evidence that GIP plays an important regulatory role in fat metabolism (7,8,15). During a meal, GIP is secreted in response to long chain fatty acids (FAs) released from triglycerides (TGs) (16,17). GIP promotes the clearance of chylomicron-associated TG from blood (18) and infusion of GIP lowers rat plasma TG responses to intraduodenal fat (19). A direct adipogenic role was suggested by the demonstrations of GIP enhancement of adipose tissue FA synthesis from acetate (20) and its incorporation into triglyceride (21). The physiological importance of these effects was
emphasized by the finding that GIP receptor (GIPR) knockout mice demonstrated reduced adipose tissue accretion on feeding a high fat diet (22).

In an earlier study we demonstrated that GIP increased lipoprotein lipase (LPL) activity and triglyceride (TG) accumulation in differentiated 3T3-L1 cells and human subcutaneous adipocytes through a pathway involving increased phosphorylation of protein kinase B (PKB) and decreases in LKB1 and AMP-activated protein kinase (AMPK) phosphorylation (23). An anomaly of these studies was the slow time of onset of the responses, suggesting that GIP may have acted through release of an intermediary molecule. Recently, Hansotia and coworkers administered the long-acting GIPR agonist, [D-Ala²]GIP, to mice and observed an increase in plasma resistin that was absent in GIPR null mice and was not mimicked by the long-acting GLP-1R agonist, exendin-4 (24). We have now examined the possibility that specific actions of GIP are mediated through the direct regulation of resistin release at the level of the adipocyte, resulting in activation of PKB and increased LPL activity.

**Experimental Procedures**

**Cell Culture**—3T3-L1 cells (American Type Culture Collection; ATCC) were cultured in 25 mM glucose DMEM (Invitrogen, Burlington, Ontario, Canada) supplemented with 5% newborn calf serum, 100 unit/ml penicillin G-sodium and 100 µg/ml streptomycin sulfate. INS-1 cells (clone 832/13) were kindly provided by Dr. C. B. Newgard (Duke University Medical Center, Durham, North Carolina). INS-1 (832/13) cells were cultured in 11 mM glucose RPMI 1640 (Sigma Laboratories, Natick, MA) supplemented with 2 mM glutamine, 50 µM β-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 10 % fetal bovine serum, 100 unit/ml penicillin G-sodium and 100 µg/ml streptomycin sulfate.

**Differentiation of 3T3-L1 Adipocytes**—3T3-L1 cells were differentiated into the adipocyte phenotype as described previously (8). Briefly, 2 days after cells were confluent, medium was supplemented with dexamethasone (0.6 µM), 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM) and insulin (16 µM) for 72 h, after which cells were cultured in 25 mM glucose DMEM + 10 % FCS for 7 further days. Differentiation of cells was confirmed by Oil-Red-O staining and fully differentiated cells (> 85 % adipose cells) from passages 2–6 were used in all experiments.

**Resistin ELISA (Enzyme-Linked ImmunoSorbent Assay)**—For studies on the effect of GIP or GLP-1 on the secretion of resistin, INS-1 β-cells or 3T3-L1 adipocytes were treated with GIP or GLP-1 as indicated in the figure legend. The levels of resistin in the media were determined using a Resistin ELISA kit (Cayman Chemical, Ann Arbor, MI).

**Quantitative Real-time RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)**—Total RNA was extracted and cDNAs generated by reverse transcription. 100 ng cDNA were used in the real-time polymerase chain reaction (PCR) to measure resistin expression, whereas 10 ng cDNA were used for the β-actin internal control. The primer and probe sequences used for the
amplification of resistin cDNA are as follows: forward primer, 5’-AAC CTT TCA TTT CCC CTC CTT TTC-3’; reverse primer, 5’-GGA AGC GAC CTG CAG CTT ACA GCA-3’; probe, 5’FAM-AGT CTC CTC CAG AGG GAA GTT GG-3’TAMRA. All reactions followed the typical sigmoidal reaction profile, and cycle threshold was used as a measurement of amplicon abundance.

Western Blot Analysis— For studies on the effect of GIP or GLP-1 on intracellular resistin and protein kinase levels, INS-1 β-cells or 3T3-L1 adipocytes were incubated with GIP or GLP-1, as indicated in the figure legends. Total cellular extracts from each sample were separated on a 13 % sodium dodecyl sulfate (SDS)/polyacrylamide gel (PAGE) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON). Probing of the membranes was performed with resistin (ProSci Inc, Poway, CA), phospho-p38 MAP Kinase (Thr/180/Tyr-182), p38 MAP Kinase, phospho-p42/44 MAP Kinase (Thr/202/Tyr-204), p42/44 MAP Kinase, phospho-SAPK/JNK (Thr/183/Tyr-185), phospho-PKB (Ser-473), PKB, phospho-LKB1 (Ser-428) and phospho-AMPK (Thr-172), AMPK and β-actin antibodies (Cell Signaling Technology, Beverly, MA). Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) using horseradish peroxidase-conjugated IgG secondary antibodies.

Lipoprotein lipase (LPL) Enzyme Activity Assays— The LPL activity assay kit (Roar Biomedical Inc. NY) was used to measure enzyme activity, according to the manufacturer’s protocol. Enzyme activity is presented as relative activity normalized to protein concentration.

Knockdown of Resistin by RNA Interference— To reduce the synthesis and secretion of resistin, 3T3-L1 adipocytes were transfected with a pool of 3 siRNAs for resistin (sc-39723, Santa Cruz) using LIPOFECTAMINE 2000™ transfection reagent, and incubated for 72 hr. The down-regulation of resistin protein by RNA interference was confirmed by Western blot hybridization using antibody against resistin.

In Vivo GIP Infusion and Measurements of Circulating Resistin Levels— Obese VDF rats and their lean littermate (12 weeks old) were subjected to a 2 week continuous infusion of GIP (10 pmol/kg·min). The infusion was performed using an Alzet miniosmotic pump (Alzet Corp., Minneapolis, MN) implanted in the intraperitoneal region under pentobarbital (40 mg/kg) anesthesia. For the controls, PBS vehicle-containing minipumps were implanted. Blood samples were taken at the indicated time points, and circulating plasma resistin levels were determined. Experiments were conducted in accordance with guidelines of the UBC Animal Care Committee and Canadian Council on Animal Care.

Immunohistochemistry— Paraffin sections containing epididymal fat tissue were prepared from GIP-treated and control rats and subjected to immunostaining for resistin. The sections were visualized with Alexa fluor® 488 conjugated anti-mouse secondary antibody, and imaged using a
Zeiss laser scanning confocal microscope (Axioskop2).

Statistical Analysis— Data are expressed as means ± Standard Errors of the Mean (SEM) with the number of individual experiments presented in the figure legend. Data were analyzed using the non-linear regression analysis program PRISM (GraphPad, San Diego, CA) and significance was tested using analysis of variance (ANOVA) with Newman-Keuls hoc test ($P < 0.05$) as indicated in figure legends.

Results

GIP, but not GLP-1 increases the secretion of resistin by 3T3-L1 adipocytes. The effect of GIP and GLP-1 on the secretion of resistin in vitro was first examined in differentiated 3T3-L1 adipocytes. Incubation of 3T3-L1 adipocytes with GIP (100 nM) in the presence of insulin (1 nM) for 2 h resulted in ∼2.3-fold increases in resistin secretion, compared to control cells (Fig. 1A). Concentration dependent effects of GIP on resistin secretion were observed with EC$_{50}$ values of 9.6 ± 0.2 nM (Fig. 1B). In contrast, treatment of 3T3-L1 adipocytes with GLP-1 (100 nM) had no effect on resistin secretion. Additionally, although resistin was detectable in extracts from INS-1 (832/13) β-cells, neither GIP nor GLP-1 affected secretion (Fig. 1A and B).

GIP increases resistin (Retn) mRNA expression in 3T3-L1 adipocytes. Treatment of 3T3-L1 adipocytes (Supplementary Figure 1) or INS-1 β-cells (Supplementary Figure 2) with GIP or GLP-1 (100 nM) was not found to alter resistin protein levels. One possible explanation for the lack of change in cell protein levels is that increases in expression only maintain sufficient newly synthesized resistin to support the increased secretion rate, without intracellular accumulation. To investigate this possibility, Retn mRNA levels were determined in 3T3-L1 adipocytes under basal and stimulated conditions. As shown in Figure 1C, GIP in the presence of insulin, increased Retn mRNA levels whereas GLP-1 had no effect. It is therefore likely that GIP-potentiated resistin secretion resulted from increased expression.

GIP increases phosphorylation of p38 mitogen activated protein kinase (p38 MAPK (Thr180/Tyr182)) and stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK (Thr183/Tyr185)) in 3T3-L1 adipocytes. Signaling modules potentially involved in GIP-mediated resistin secretion were next studied. Because resistin secretion was evident within 1 h following GIP treatment, we first examined faster-acting MAPK signaling modules, including p42/44 MAPK, p38 MAPK and SAPK/JNK. There was no significant effect of GIP or GLP-1 on the levels of phosphorylated p42/44 MAPK (Thr202/Tyr204) in 3T3-L1 adipocytes during the time-course of the study (Figure 2A and 2B). On the other hand, GIP, in the presence of insulin, was found to increase phosphorylation of p38MAPK (Thr180/Tyr182) transiently, peaking at 5-10 minutes (Figures 2C and 2G), and SAPK/JNK (Thr183/Tyr185) in a more sustained manner (Figures 2E and 2H), whereas GLP-1 was without effect on either kinase (Figures 2D, 2F, 2G and 2H). There were no significant changes in the phosphorylation of p42/44 MAPK (Thr202/Tyr204), p38MAPK.
(Thr180/Tyr182) or SAPK/JNK (Thr183/Tyr185) with either treatment of insulin (1 nM) or GIP (100 nM) alone (Supplementary Figure 3). These results indicate that synergistic action between insulin and GIP is required for phosphorylation.

p38 MAPK and SAPK/JNK are involved in GIP-mediated resistin secretion. In studies designed to examine the involvement of p38 MAPK and SAPK/JNK in GIP-mediated resistin secretion, 3T3-L1 adipocytes were incubated in the presence or absence of selective inhibitors of MAPK pathways. There was no significant effect on GIP-mediated resistin secretion by treatment with the MAP Kinase Kinase (MEK) 1/2 inhibitors, PD098059 (75 µM) or U0126 (25 µM). However, resistin secretion in response to GIP, was greatly reduced by the p38 MAPK inhibitor, SB203580, and the SAPK/JNK inhibitor, SP600125 (50 µM) (Figure 3A). There were no significant differences in cellular resistin levels following treatment with these inhibitors (Figure 3B). These results strongly suggest that p38 MAPK and SAPK/JNK, but not ERK1/2, are involved in GIP-mediated resistin secretion in 3T3-L1 adipocytes and that changes in protein expression are not involved.

The effect of GIP on epididymal fat in vivo. To determine whether GIP can regulate resistin secretion in vivo, peptide was systemically administrated to lean (Fa/?) or obese (fa/fa) VDF Zucker rats, using osmotic minipumps and conditions previously shown to increase adipose tissue LPL levels (23). Resistin was shown to be expressed in epididymal fat tissue of lean and obese VDF Zucker rats (Figure 4A), and 2 weeks continuous infusion of low concentrations of GIP (10 pmol/kg-min) resulted in increased circulating resistin levels (Figure 4B), but no detectable change in resistin protein levels in epididymal fat tissue (Figure 4C). However, GIP infusion did increase tissue levels of phosphorylated p38MAPK (Thr180/Tyr182) and SAPK/JNK (Thr183/Tyr185) in both lean and obese animals, when compared to controls (Figures 4D and 4E). These results correlate well with the in vitro results in 3T3-L1 adipocytes, and support a role for GIP in the regulation of resistin secretion in vivo, acting through a p38MAPK and JNK/SAPK signaling module.

Resistin modulates PKB/LKB1/AMPK phosphorylation and LPL activity. Next, we examined the functional implications of GIP-mediated resistin secretion. In the earlier study, we demonstrated that GIP increased LPL activity and TG accumulation through a pathway involving increased phosphorylation of PKB and reductions in phosphorylated LKB1 and AMPK (23). Responses to GIP under these conditions were relatively slow and the possible involvement of an intermediary could not be excluded. We therefore tested whether resistin could act as a mediator of GIP’s action on adipocytes. Treatment of 3T3-L1 adipocytes with resistin (10 nM) mimicked the effects of GIP, resulting in increased levels of phosphorylated PKB (Ser473), and decreased levels of LKB1 (Ser428) and AMPK (Thr172) (Figures 5A, 5B and 5C). In control experiments, there were no significant changes in levels of phosphorylated PKB (Ser473), LKB1 (Ser428) or AMPK (Thr172) during the
time course of the study, confirming that these effects were GIP-dependent (Supplementary Figure 4). Treatment of 3T3-L1 adipocytes with resistin (10 nM) for 12 h resulted in a 3.4-fold increase in LPL activity, compared to control (Figure 5D) and concentration-dependent effects of resistin on LPL activity were observed with EC_{50} values of 15.8 ± 0.2 nM (Fig. 5E). Taken together, these results suggest that resistin could be an important mediator of GIP stimulation of LPL activity.

**Resistin knockdown greatly reduces the effect of GIP on the PKB/LKB1/AMPK/LPL cascade.** To evaluate further the functional contribution of resistin secretion to GIP effects on the PKB/LKB1/AMPK/LPL cascade, resistin was knocked down by RNAi treatment. As shown in Figures 6A and 6B, 3T3-L1 adipocytes treated with 100 nM of a pool of 3 resistin siRNAs resulted in greatly reduced resistin protein levels and an approximate 65% and 75% reduction in basal- and GIP-stimulated resistin secretion, respectively. The reduction in GIP-mediated responses was associated with attenuation of the effects of GIP on phosphorylation of PKB, LKB1 and AMPK (Figure 6C, 6D and 6E). These results complement the finding that resistin treatment resulted in decreased levels of phosphorylated LKB1 and AMPK (Figure 5B and 5C). Furthermore, GIP-mediated LPL activation was also greatly reduced in resistin RNAi-treated cells (Figure 6F), supporting a role for resistin as a mediator of GIP-mediated increases in LPL activity.

Basal phospho-LKB1 and AMPK levels appeared to be slightly increased in RNAi-treated cells in the absence of GIP (Figure 6D and E). Basal phospho-PKB levels were undetectable. However resistin may normally maintain a pool of PKB in the phosphorylated state, thus suppressing levels of phospho-LKB1 and AMPK, with loss of resistin removing this constraint. Since GIP-treated cells showed both a reduction in phospho-LKB1/AMPK and a small increase in LPL, there is likely an alternative pathway by which it can act.

**Discussion**

Since its identification as an adipocyte-secreted protein (25,26), there has been controversy over the physiological roles played by resistin (Adipocyte Secreted Factor; ADSF). Since resistin administration to C57BL/6J mice resulted in glucose intolerance and reduced insulin sensitivity, while anti-resistin serum improved insulin resistance, it was suggested that resistin was the long sought after link between obesity and type 2 diabetes (25). Resistin levels were found to be markedly elevated in serum from insulin resistant mice placed on a high-fat diet and in obese rats and mice (25). Subsequently a number of groups reported a reduction in resistin expression in white adipose tissue from a number of obese rodent models including ob/ob, db/db, tub/tub and KKA (y) mice (27-29). Despite this, elevated serum resistin levels have found in high fat fed or monogenic obese mice (28), similar to the elevated levels observed in the obese VDF Zucker rats.

A number of studies have shown that resistin can act in an autocrine/paracrine manner in white adipocytes, although its actual role is obscure. Resistin mRNA is undetectable in 3T3-L1 cell preadipocytes, but its expression increases during
differentiation to the adipocyte phenotype (30). Addition of recombinant resistin, or increasing resistin expression via gene transfection promoted differentiation of preadipocytes to adipocytes. Conflicting results have been reported for resistin effects on mature adipocytes. Fat specific overexpression of resistin in a spontaneously hypertensive rat model was found to impair reesterification of FFAs to triglycerides (31), whereas treatment of mouse adipose explants with recombinant FIZZ3, the human resistin equivalent, resulted in increases in both lipolysis and reesterification (32). Resistin can also inhibit insulin-induced glucose uptake in 3T3-L1 adipocytes (25). Resistin is additionally expressed in both human and mouse pancreatic islets (33), and exogenous resistin impaired glucose-stimulated insulin secretion in isolated islets (34), although its physiological islet function is unknown.

We showed previously that GIP, in the presence of insulin, increased LPL enzyme activity and triglyceride accumulation, through a pathway involving increased phosphorylation of PKB and reductions in the phosphorylated forms of LKB1 and AMPK (23). Unlike GIP action on PKB in INS-1 beta cells (5), responses in 3T3-L1 adipocytes demonstrated a slow time of onset, suggesting responses involved release of an intermediary molecule that acted upstream of PI3-K/PKB. The present results suggest that resistin is a major contributor to the lipogenic effects of GIP in 3T3-L1 adipocytes since it induced identical changes in PKB, LKB1 and AMPK (Figure 5). Knockdown of resistin using RNA interference resulted in attenuation of GIP’s effects (Figure 6). The incomplete abolition of GIP’s effects is likely due to the involvement of other growth factors and/or hormones secreted by adipocytes.

The current studies therefore provide a plausible explanation for the delayed responses to GIP: increased secretion and actions of resistin. There are several ways by which GIP could have produced an increase in resistin secretion. It is unlikely to be through a major effect on resistin biosynthesis, since neither resistin levels in 3T3-L1 adipocytes nor those in INS-1 832/13 β-cells, were influenced by GIP treatment (Figures 2 and 3), even with incubation periods of up to 6 hours. There have been only few previous studies on in vitro adipocyte resistin secretion. In 3T3-L1 cells, extended (24h) incubation with insulin stimulated resistin secretion (35), whereas IGF-I (36) and endothelin-1 (35) reduced secretion. GIP-induced resistin secretion peaked within approximately 2 hours. Wide variability in the patterns of adipokine secretion have been described in the literature, with a slow linear release of leptin over 24h under unstimulated conditions (37), but acute secretory responses of leptin (38,39) and adiponectin (40) to insulin, and of proinflammatory adipokines to TNFα (41). The cellular mechanisms underlying secretion of the adipokines are still largely undefined. Following biosynthesis, leptin undergoes trafficking through the endoplasmic reticulum and golgi network to reside in the limited cytoplasmic space surrounding the fat droplets (39,42). A fraction of adipocyte leptin appears to be localized in small vesicles (39,42,43). It is therefore possible that resistin also resides in intracellular vesicles and that GIP stimulates their production and
secretion, as proposed for leptin (44). In the present study, although GIP was shown to stimulate resistin secretion, there was no detectable effect on the levels of resistin protein in either acute experiments on 3T3-L1 adipocytes or in vivo in VDF rats. A potential explanation for this observation is that GIP increases resistin expression so as to maintain sufficient newly synthesized protein to support the increased secretion rate, without intracellular accumulation. This possibility was supported by the finding that GIP treatment resulted in increased Retn mRNA expression (Figure 1C). In studies designed to identify signaling pathways responsible for GIP-induced resistin secretion, we identified p38 MAPK and SAPK/JNK as potential targets (Figures 2G and 2H). Interestingly, increased phosphorylation of p38 MAPK was rapid, but transient, whereas increases in SAPK/Jnk did not reach peak levels until 1 h. This may explain why GIP-induced resistin secretion is maximal at 1-2 h following initiation of stimulation. As discussed previously (23), low concentrations of insulin were included in all the studies since it is essential for the stimulatory effect of GIP on LPL activity and triglyceride synthesis. Under these conditions the ability of GIP to stimulate adenylyl cyclase and activate protein kinase A is blocked. It is therefore unlikely that cyclic AMP-mediated pathways contributed to the stimulation of secretion. A pharmacological approach was taken to confirm the kinase pathways involved. Application of MEK inhibitors did not influence resistin secretion, whereas inhibition of either SAPK/JNK or p38 MAP kinase greatly reduced secretion. Exactly where in the secretory pathway these kinases act is currently unknown. An additional uncertainty is the exact mechanism by which resistin acts in the adipocyte. As previously discussed (23), application of the pharmacological inhibitors of PI3-K, LY294002 and wortmannin, resulted in reduced phosphorylated LKB1/AMPK strongly suggesting that PKB at least partially mediated these effects of GIP (23). The responses to resistin shown in the current studies did not reveal a definitive temporal relationship between PKB phosphorylation and reduced LKB1/AMPK, possibly due to close intracellular interaction between these enzymes. It has been shown that resistin increases phosphorylation of ERK1/2 in cultured human endothelial cells (45) and aortic smooth muscle (46), whereas resistin’s ability to impair insulin-receptor phosphorylation has been attributed to increasing gene expression of Suppressor of Cytokine Signaling 3 (SOCS3) (47). Resistin also induced a transient increase in phospho-PKB in endothelial cells (48), although studies on resistin null ob/ob mice indicated that resistin normally suppressed PKB phosphorylation in skeletal muscle and liver. Importantly, as found in 3T3-L1 adipocytes, resistin treatment reduced AMPK phosphorylation in both the liver (49,50) and skeletal muscle (51), and this may be a key intermediate molecule in resistin action. There are therefore clearly cell-type specific responses to resistin and the mechanistic link between PKB activation and reduced LKB1/AMPK phosphorylation remains to be clarified.

Finally, what are the implications of these studies for the regulation of human resistin. There is only moderate sequence homology (~53 %) in coding and noncoding regions between mouse and human resistin (FIZZ3) (52).
Additionally, although recent studies have confirmed that FIZZ3 is expressed in human adipocytes (53), monocytes/macrophages within adipose tissue express much higher levels, raising doubt as to its role in human adipose tissue (54). However, resistin/FIZZ3 originating from immune cells could still play an important autocrine function in the regulation of adipocyte development and function. In view of the increasing evidence linking chronic inflammation in fat with the development of insulin resistance and type II diabetes (55,56) it will be important to establish whether there is a similar entero-adipocyte axis involving GIP and resistin/FIZZ3 in human fat.

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Figure Legends

Figure 1. The effect of GIP and GLP-1 on the secretion of resistin. A. Time course of GIP and GLP-1 action on resistin secretion. Differentiated 3T3-L1 adipocytes were serum starved in 3mM glucose DMEM containing 0.1 % BSA overnight and INS-1 (832/13) cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight. 3T3-L1 adipocytes and INS-1 cells were treated for the indicated periods of time with GIP or GLP-1 (100 nM) in the presence of insulin (1 nM). B. Concentration-dependence of GIP and GLP-1 effects on resistin secretion. 3T3-L1 adipocytes and INS-1 (832/13) cells were treated for 2 h with the indicated concentrations of GIP or GLP-1 in the presence of insulin (1 nM). Resistin levels in culture media were determined using resistin ELIZA kit. C. The effect of GIP and GLP-1 on resistin mRNA expression. 3T3-L1 adipocytes were treated as described above and incubated with GIP or GLP-1 (100 nM) for 6 h in the presence of insulin (1 nM). Total RNA was isolated from each sample and real-time RT-PCR was performed to quantify resistin mRNA (Retn) levels; shown as the fold difference vs control normalized to β-actin expression levels. All data represent three independent experiments, each carried out in duplicate. Significance was tested using ANOVA with Newman-Keuls post hoc test, where ** represents p<0.05 vs Control.

Figure 2. GIP increases phosphorylation of p38MAPK (Thr180/Tyr182) and SAPK/JNK (Thr183/Tyr185) in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and stimulated for the indicated periods of time with GIP or GLP-1 (100 nM) in the presence of insulin (1 nM). Effect of treatment with GIP (A, C, E) or GLP-1 (B, D, F) on phosphorylation of p42/44 MAPK Thr202/Tyr204 (A, B), p38MAPK (Thr180/Tyr182) (C, D) and SAPK/JNK (Thr183/Tyr185) (E, F). Densitomeric analysis of Thr180/Tyr182 p38MAPK (G) and Thr183/Tyr185 SAPK/JNK (H) phosphorylation in response to GIP or GLP-1. Western blots are representative of n = 3 and significance was tested using ANOVA with Newman-Keuls post hoc test, where * represents p<0.05 vs Basal.

Figure 3. p38MAPK and SAPK/JNK are involved in GIP-mediated resistin secretion. A. Effect of inhibiting p38MAPK or SAPK/JNK on resistin secretion. Differentiated 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and stimulated for 2 h with 100 nM GIP in the presence or absence of inhibitors of MAPK pathways. PD98059 (75 µM), U0126 (25 µM), SB203580 (10 µM) and SP600125 (50 µM) were added to cells during 1 hr preincubation as well as during GIP stimulation. Resistin levels in the culture media were determined using a resistin ELISA. B. Effect of p38MAPK or SAPK/JNK inhibition on resistin protein levels. 3T3-L1 adipocytes were treated as described above, total cellular extracts were isolated from each sample and resistin determined by Western blotting. All data represent three independent experiments, each carried out in duplicate and Western blots are representative of n = 3. Significance was tested using ANOVA with Newman-Keuls post hoc test, where ** represents p<0.05 vs Control, ## represents p<0.05 vs Control + GIP.
Figure 4. Effect of GIP on resistin levels in vivo. VDF rats and their lean littermate controls received a 2 week continuous infusion of GIP (10 pmol/kg-min) or PBS vehicle. A. Resistin expression in epididymal fat tissue. Immunohistochemical staining was performed using resistin antibody and imaging performed using a Zeiss laser scanning confocal microscope (Axioskop2). B. The effect of GIP infusion on circulating plasma resistin levels. Following a 2 week GIP infusion, plasma samples were taken from each group of animals, and plasma resistin levels determined as described in Materials and Methods. C. The effect of GIP infusion on resistin expression in epididymal fat tissue. Total cellular extracts were prepared from epididymal fat tissues and Western blot analyses performed using antibodies against resistin and β-actin. D-E. The effect of GIP on kinase phosphorylation. Western blot analyses were performed using antibodies against p38MAPK (Thr180/Tyr182) (D), SAPK/JNK (Thr183/Tyr185) (E) and β-actin. All imaging data were analyzed using the Northern Eclipse program (ver.6) and scale bar indicates 50 µm. Data represent three independent experiments, each carried out in duplicate and Western blots are representative of n = 3. Significance was tested using ANOVA with Newman-Keuls post hoc test, where ** represents p<0.05 vs Control, ## represents p<0.05 vs Fatty.

Figure 5. Effect of resistin on phosphorylation of PKB, LKB1 and AMPK, and LPL activity. Time course of phosphorylation of PKB (A), LKB1 (B) and AMPK (C) in the presence of resistin. 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and stimulated for the indicated periods of time with resistin (10 nM). Total cellular extracts were isolated and Western blot analyses performed with antibodies against phosphorylated Thr172-AMPK, AMPK, Ser428-LKB1, Ser473-PKB or PKB, and β-actin. Time course (D) and concentration-response effect (E) of resistin on LPL activity. 3T3-L1 adipocytes were treated for the indicated periods of time with resistin (10 nM) (A-D) or for 24 h with the indicated concentrations of resistin (E). Western blots are representative of n = 3 and significance was tested using ANOVA with Newman-Keuls post hoc test, where * represents p<0.05 vs Basal.

Figure 6. The effects of resistin knockdown using siRNA on GIP-mediated changes in phosphorylation of PKB, LKB1, and AMPK, and LPL activity. Concentration-dependent effects of RNAi-mediated resistin knock-down on resistin protein levels (A) and secretion (B). 3T3-L1 adipocytes were transfected with resistin or control scrambled siRNAs (100 nM) and treated with GIP (100 nM) plus insulin (1 nM). Western blot analyses were performed using antibodies against resistin (A) and resistin levels in culture media were determined using resistin ELISA kit (B). Effect of resistin knockdown using siRNA on kinase phosphorylation. Western blot analyses were performed using antibodies against phospho Ser473-PKB (C), phospho Ser428-LKB1 (D) or phospho Thr172-AMPK (E) and β-actin. F. Effect of resistin siRNA treatment on LPL activity. All data represent three independent experiments, each carried out in duplicate. Significance was tested using ANOVA with Newman-Keuls post hoc test, where ** represents p<0.05 vs Control, ## represents p<0.05 vs Control + GIP.
**Figure 7. Proposed pathway by which GIP increases resistin secretion and LPL activity in adipocytes.** GIP receptor interaction results in activation of p38 MAPK and SAPK/JNK, leading to the secretion of resistin. Secreted resistin acts in either an autocrine or paracrine manner to increase PKB phosphorylation and decrease LKB1 and AMPK phosphorylation, resulting in increased LPL activity. There is the potential for involvement of other growth factors/hormones in the regulatory pathway.

**Supplementary Figure 1. Levels of resistin in 3T3-L1 adipocytes are unaffected by GIP or GLP-1 treatment.** Differentiated 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and treated for up to 6 hours with (A) GIP or (B) GLP-1 (100 nM) or with the concentrations of GIP (B) or GLP-1 (D) indicated, in the presence of insulin (1 nM). Total cellular extracts were isolated from each sample and Western blot hybridizations were performed. Western blots are representative of \( n = 3 \).

**Supplementary Figure 2. Levels of resistin in rat insulinoma INS-1 (832/13) cells are unaffected by GIP or GLP-1 treatment.** INS-1 (832/13) cells were serum starved in 3 mM glucose RPMI containing 0.1 % BSA overnight and treated for up to six hours with (A) GIP or (B) GLP-1 (100 nM) or with the concentrations of GIP (B) or GLP-1 (D) indicated, in the presence of insulin (1 nM). Western blot hybridizations were performed from total cellular extracts and are representative of \( n = 3 \).

**Supplementary Figure 3. Effect of treatment with insulin (A, B, C) or GIP (D, E, F) alone on phosphorylation of p42/44 MAPK Thr202/Tyr204 (A, D), p38MAPK (Thr180/Tyr182) (B, E) and SAPK/JNK (Thr183/Tyr185) (C, F).** Differentiated 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and stimulated for the indicated periods of time with GIP (100 nM) or insulin (1 nM) alone.

**Supplementary Figure 4. Control levels of phosphorylated PKB, LKB1 and AMPK during the 24h-time course.** 3T3-L1 adipocytes were serum starved in 3 mM glucose DMEM containing 0.1 % BSA overnight and total cellular extracts were isolated at the indicated time point. Western blot analyses were performed with antibodies against phosphorylated Thr172-AMPK, AMPK, Ser428-LKB1, Ser473-PKB or PKB, and \( \beta \)-actin.
Figure 1.

A. Resistin secretion in 3T3-L1 adipocytes and INS-1 (832/13) β-cells treated with GIP and GLP-1. Bars indicate mean ± SEM.

B. Resistin secretion as a function of Log10 GIP or GLP-1 concentration in 3T3-L1 adipocytes and INS-1 (832/13) β-cells.

C. Retn mRNA expression levels in control, GIP, and GLP-1 treated groups. Bars indicate mean ± SEM.
Figure 2.

A.

\[ \text{p-p42/44 MAP Kinase (Thr202/Tyr204) } \rightarrow \]

\[ \text{p42/44 MAP Kinase } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

B.

\[ \text{p-p42/44 MAP Kinase (Thr202/Tyr204) } \rightarrow \]

\[ \text{p42/44 MAP Kinase } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

C.

\[ \text{p-p38 MAP Kinase (Thr180/Tyr182) } \rightarrow \]

\[ \text{p38 MAP Kinase } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

D.

\[ \text{p-p38 MAP Kinase (Thr180/Tyr182) } \rightarrow \]

\[ \text{p38 MAP Kinase } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360
Figure 2.

E.

\[ \text{p-SAPK/JNK (Thr183/Tyr185)} \rightarrow \beta\text{-actin} \rightarrow \text{p-SAPK/JNK (Thr183/Tyr185)} \rightarrow \beta\text{-actin} \rightarrow \text{Time (mins): } 0 \ 5 \ 10 \ 15 \ 30 \ 60 \ 120 \ 240 \ 360 \]

F.

\[ \text{p-SAPK/JNK (Thr183/Tyr185)} \rightarrow \beta\text{-actin} \rightarrow \text{p-SAPK/JNK (Thr183/Tyr185)} \rightarrow \beta\text{-actin} \rightarrow \text{Time (mins): } 0 \ 5 \ 10 \ 15 \ 30 \ 60 \ 120 \ 240 \ 360 \]

G.

\[ \text{GIP} \quad \text{GLP-1} \]

H.

\[ \text{p46 SAPK/JNK} \quad \text{p54 SAPK/JNK} \]
Figure 3.

A. Resistin Secretion (ng/ml)

GIP: - + - + - +

PB098059 U0126 SB203580 SP600125

B. Resistin → β-actin

GIP: - + - + - +

PB098059 U0126 SB203580 SP600125
Figure 4.

A.

Lean: 

Lean + GIP:

Fatty: 

Fatty + GIP:

B.

Circulating Resistin Levels (ng/ml)

Lean Lean + GIP Fatty Fatty + GIP

C.

Resistin → β-actin

D.

p-p38 MAP Kinase (Thr180/Tyr182) → p38 MAP Kinase

E.

p-SAPK/JNK (Thr183/Tyr185) → β-actin
Figure 5.

A. PKB → p-AMPK (Thr172) → p-PKB (Ser473)

B. p-LKB1 (Ser428) → β-actin

C. p-AMPK (Thr172) → AMPK

D. E. Relative LPL activity (fold difference vs. basal)
Figure 6.

A.

Resistin →

β-actin →

GIP (100 nM): - + - + - + - + - +
Control siRNA (nM): 100 - - - - - -
Resistin siRNA (nM): - 0.01 0.1 10 100

B.

Resistin Secretion (ng/ml)

GIP (100 nM): - + - + - + - + - +
Control siRNA (nM): 100 - - - - - -
Resistin siRNA (nM): - 0.01 0.1 10 100

C.

p-PKB (Ser473) →

PKB →

GIP (100 nM): - + - + - + - + - +
Control siRNA (nM): 100 - - - - - -
Resistin siRNA (nM): - 0.01 0.1 10 100

D.

p-LKB1 (Ser428) →

β-actin →

GIP (100 nM): - + - + - + - + - +
Control siRNA (nM): 100 - - - - - -
Resistin siRNA (nM): - 0.01 0.1 10 100
**Figure 6.**

E.  
![Image](image1.png)

Relative LPL activity (fold difference vs control)  

| GIP (100 nM): | Control siRNA (nM): 100 | Resistin siRNA (nM): 0.01 0.1 10 100 |
|--------------|-------------------------|-------------------------------|
| Control siRNA (nM): | 100 | - | - | - | - | - | - | - |
| Resistin siRNA (nM): | - | 0.01 | 0.1 | 10 | 100 |

F.  
![Image](image2.png)

Relative LPL activity (fold difference vs Control)  

| GIP (100 nM): | Control siRNA (nM): 100 | Resistin siRNA (nM): 0.01 0.1 10 100 |
|--------------|-------------------------|-------------------------------|
| Control siRNA (nM): | 100 | - | - | - | - | - | - | - |
| Resistin siRNA (nM): | - | 0.01 | 0.1 | 10 | 100 |
Figure 7.

- GIP
- GIPR
- p38 MAPK
  - Thr180/Tyr182
- SAPK/JNK
  - Thr183/Tyr185
- Resistin secreton
- Resistin Receptor?
- Resistant secreton
- PI3-K
  - ↑
- PKB Ser473
  - ↑
- LKB1 Ser428
  - ↓
- AMPK Thr172
  - ↓
- LPL
  - ↑
- Adipocytes
Supplementary Figure 1.

A. Resistin → β-actin
   Time (hours): 0' 1' 2' 4' 6'

B. Resistin → β-actin
   Log10 [GIP]: -11 -10 -9 -8 -7 -6

C. Resistin → β-actin
   Time (hours): 0' 1' 2' 4' 6'

D. Resistin → β-actin
   Log10 [GLP-1]: -11 -10 -9 -8 -7 -6
Supplementary Figure 2.

A. Resistin → β-actin
   Time (hours): 0' 1' 2' 4' 6'

B. Resistin → β-actin
   Log₁₀ [GIP]: 0 -11 -10 -9 -8 -7 -6

C. Resistin → β-actin
   Time (hours): 0' 1' 2' 4' 6'

D. Resistin → β-actin
   Log₁₀ [GLP-1]: 0 -11 -10 -9 -8 -7 -6
Supplementary Figure 3.

A.

\[ \text{p-p42/44 MAP Kinase (Thr202/Tyr204)} \rightarrow \]

\[ \text{p42/44 MAP Kinase} \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

B.

\[ \text{p-p38 MAP Kinase (Thr180/Tyr182)} \rightarrow \]

\[ \text{p38 MAP Kinase} \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

C.

\[ \text{p-SAPK/JNK (Thr183/Tyr185)} \rightarrow \]

\[ \text{β-actin} \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

D.

\[ \text{p-p42/44 MAP Kinase (Thr202/Tyr204)} \rightarrow \]

\[ \text{p42/44 MAP Kinase} \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360
Supplementary Figure 3.

E.

\[
p\text{-p38 MAP Kinase (Thr180/Tyr182) } \rightarrow \]

\[
p38 \text{ MAP Kinase } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

F.

\[
p\text{-SAPK/JNK (Thr183/Tyr185) } \rightarrow \]

\[
\beta\text{-actin } \rightarrow \]

Time (mins): 0 5 10 15 30 60 120 240 360

\[\text{← p54 SAPK/JNK} \text{ ← p46 SAPK/JNK}\]
Supplementary Figure 4.

A.  
\[ \text{p-PKB (Ser473)} \rightarrow \text{PKB} \rightarrow \]
\[ \text{p-AMPK (Thr172)} \rightarrow \]
\[ \text{Time (hours): 0' 1' 2' 4' 6' 12' 24'} \]

B.  
\[ \text{p-LKB1 (Ser428)} \rightarrow \text{\beta-actin} \rightarrow \]
\[ \text{Time (hours): 0' 1' 2' 4' 6' 12' 24'} \]

C.  
\[ \text{p-AMPK (Thr172)} \rightarrow \text{AMPK} \rightarrow \]
\[ \text{Time (hours): 0' 1' 2' 4' 6' 12' 24'} \]
Resistin is a key mediator of glucose-dependent insulinoitropic polypeptide (GIP) stimulation of lipoprotein lipase (LPL) activity in adipocytes

Su-Jin Kim, Cuilan Nian and Christopher H.S. McIntosh

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