Cell Metabolism
Short Article

Pigment Epithelium-Derived Factor Contributes to Insulin Resistance in Obesity

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SUMMARY

Obesity is a major risk factor for insulin resistance; however, the factors linking these disorders are not well defined. Herein, we show that the noninhibitory serine protease inhibitor, pigment epithelium-derived factor (PEDF), plays a causal role in insulin resistance. Adipocyte PEDF expression and serum levels are elevated in several rodent models of obesity and reduced upon weight loss and insulin sensitization. Lean mice injected with recombinant PEDF exhibited reduced insulin sensitivity during hyperinsulinemic-euglycemic clamps. Acute PEDF administration activated the proinflammatory serine/threonine kinases c-Jun terminal kinase and extracellular regulated kinase in both muscle and liver, which corresponded with reduced insulin signal transduction. Prolonged PEDF administration stimulated adipose tissue lipolysis, resulted in ectopic lipid deposition, and reduced insulin sensitivity, while neutralizing PEDF in obese mice enhanced insulin sensitivity. Overall, these results identify a causal role for PEDF in obesity-induced insulin resistance.

INTRODUCTION

The increased prevalence of obesity in industrialized countries is closely associated with the development of chronic diseases including atherosclerosis, nonalcoholic fatty liver disease, dyslipidemia, and type 2 diabetes (Wellen and Hotamisligil, 2005). Insulin resistance is a central feature of the pathophysiology of most obesity-related disorders including type 2 diabetes, and is defined as a subnormal response of tissues to the actions of insulin. Several possible mediators of insulin resistance in obesity, including dysregulation of lipid metabolism (Savage et al., 2007) and low-grade inflammation (Wellen and Hotamisligil, 2005), have been identified. However, the mechanistic link between these parameters is not understood.

Adipocytes are known to regulate whole-body metabolism, at least in part via the release of secretory or endocrine factors such as leptin (Halaas et al., 1995) and adiponectin (Maeda et al., 2002). Modifications in the secretion of several adipocyte secreted factors contributes to dysregulation of metabolism either via central or peripheral effects (Rosen and Spiegelman, 2006). This has led to major investigation of adipose secretory factors in the hope of identifying other regulatory molecules that might play other as-yet-unidentified roles in whole-body metabolism. One of the limitations with many studies in this area is that the contribution of adipose tissue secretion to circulating levels of such factors is not clear, and in some cases it remains controversial if adipocytes per se are the source of the secretory factor, as opposed to nonparenchymal cells such as endothelial cells.

In the present studies we identify the serine protease inhibitor (serpin), pigment epithelium-derived factor (PEDF, SerpinF1) as a bona fide adipocyte secretory factor. This is an exciting observation as circulating PEDF was recently found to be upregulated in individuals with the metabolic syndrome (Yamagishi et al., 2006) and patients with type 2 diabetes mellitus (Jenkins et al., 2008; Ogata et al., 2007). PEDF is a multifunctional protein that promotes neuronal survival and differentiation and possesses anti-angiogenic activities (Tombran-Tink and Barnstable, 2003). It is a unique serpin because its c-reactive loop is inactive and is thereby noninhibitory, meaning that it does not directly inhibit serine proteases or other serpin targets such as caspases. In this way, PEDF may provide a link between obesity and insulin resistance. Using a range of physiological methods, we provide causal evidence in favor of this claim.

RESULTS

Identification of the Adipocyte Secretome by Mass Spectrometry

Previous efforts to identify the secretome of adipocytes have included the use of a signal sequence trap cloning procedure and proteomics. In the case of proteomics, media is usually collected from cells grown in culture; proteins are concentrated and then sequenced using mass spectrometry. A major

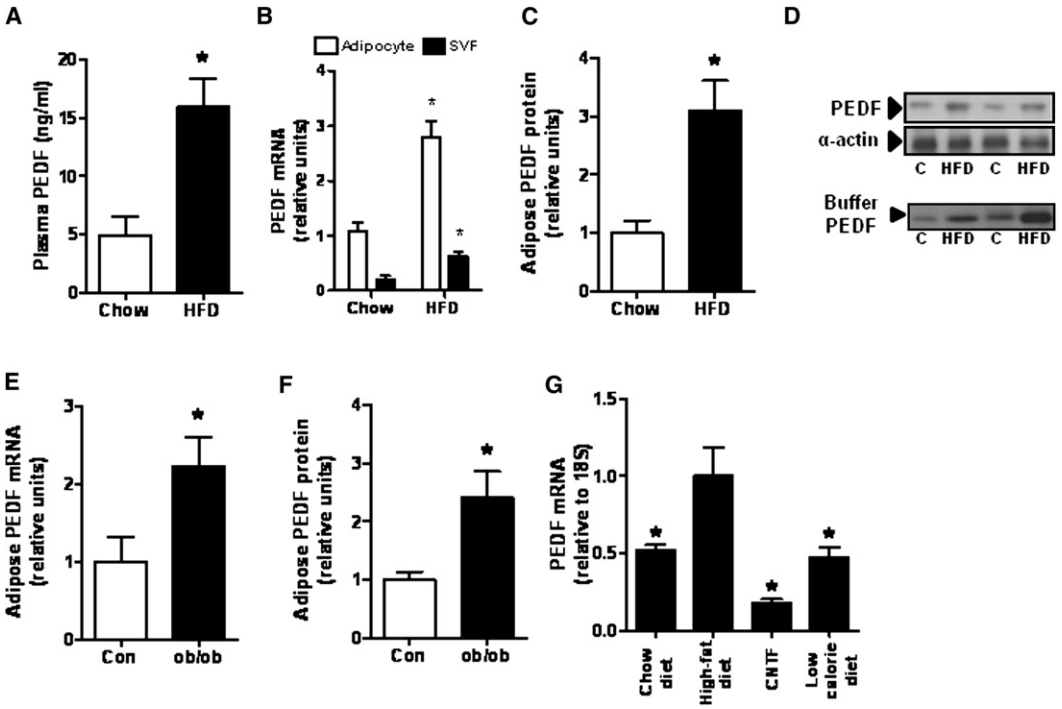

Figure 1. PEDF Is Elevated in Obesity and Reduced by Weight Loss

(A) Plasma PEDF concentrations in mice fed a control (chow) and high-fat diet (HFD) for 12 weeks.

(B–D) PEDF mRNA in the adipocyte and stomal vascular fraction (SVF), (C) PEDF protein expression, and (D) PEDF release from epididymal adipose tissue of mice after chow and HFD.

(E and F) PEDF mRNA and (F) protein in obese *ob/ob* mice and lean littermates.

(G) PEDF mRNA content in mice fed a HFD or mice that lost ~15% of their body mass by 7 day ciliary neurotrophic factor (CNTF) administration or caloric restriction (low-calorie diet). n = 8 mice for all groups; error bars are ± SEM; *p < 0.05 versus group with open bars.

limitation is the presence of contaminants in the media due to cell surface shedding or cellular lysis and release of intracellular content. To circumvent these limitations, we have implemented a lectin affinity chromatography step to enrich for glycoproteins prior to mass spectrometry on the assumption that many, but not all, secretory proteins undergo complex N-linked glycosylation prior to release. Using this method, we screened conditioned media from 3T3-L1 adipocytes. One of the most abundant proteins identified in this screen based upon peptide coverage in the mass spectrum (20.6% of the full-length protein detected) was PEDF. While PEDF has been implicated as an important metabolic regulatory secretory factor, little is known of its mechanism of action.

Adipose Tissue and Plasma PEDF Levels Are Elevated in Obesity

We initially examined plasma PEDF levels in obesity. Mice were fed a low-fat (chow, 4% fat) or high-fat (HFD, 60% fat) diet for 12 weeks. The high-fat diet resulted in elevated body mass (chow, 28.9 ± 0.6 g versus HFD, 35.8 ± 1.2 g) and adipose tissue mass (epididymal fat pad, chow: 335 ± 29 mg versus HFD: 1439 ± 192 mg). Mice fed a HFD were mildly hyperglycaemic (blood glucose: chow, 8.8 ± 0.4 mM versus HFD, 10.2 ± 0.9 mM) and hyperinsulinemic (plasma insulin: chow, 49 ± 6 pM versus HFD, 107 ± 21 pM), suggestive of insulin resistance. The plasma PEDF concentration averaged 4.9 ± 1.6 ng/ml for chow and was increased 3.2-fold by HFD (Figure 1A). A similar increase in the PEDF mRNA (Figure 1B) and protein-expression levels (Figure 1C) in adipose tissue was also observed in HFD mice. PEDF expression was increased in both the adipocyte and stromal vascular fraction of adipose tissue (Figure 1B), and most of the whole-tissue increase was attributable to enhanced adipocyte expression. Ex vivo analysis revealed that adipose tissue PEDF secretion was greater in obese versus lean mice (Figure 1D), supporting a link between adipose tissue PEDF production and plasma levels. While PEDF was readily detected as an adipose secretory factor, only modest PEDF secretion was detected in cultured hepatocytes and myocytes and in whole mouse liver and skeletal muscle incubated ex vivo for 5 hr in oxygenated Krebs buffer (data not shown). This is intriguing because this pattern does not correspond to the relative tissue-specific expression profile of PEDF (Figure S1). This suggests that adipocytes likely make an important contribution to circulating levels of this factor. The level of the PEDF mRNA and protein was also increased in the adipose tissue of obese, leptin-deficient *ob/ob* mice compared with lean littermate controls (Figures 1E and 1F). There was no striking effect of obesity on skeletal muscle or liver PEDF expression (Figure S2). We then assessed the effects of weight loss on adipose PEDF expression in obese mice using either caloric restriction or ciliary neurotrophic factor administration as described previously (Crowe et al., 2008). PEDF mRNA expression was decreased by 2.1- and

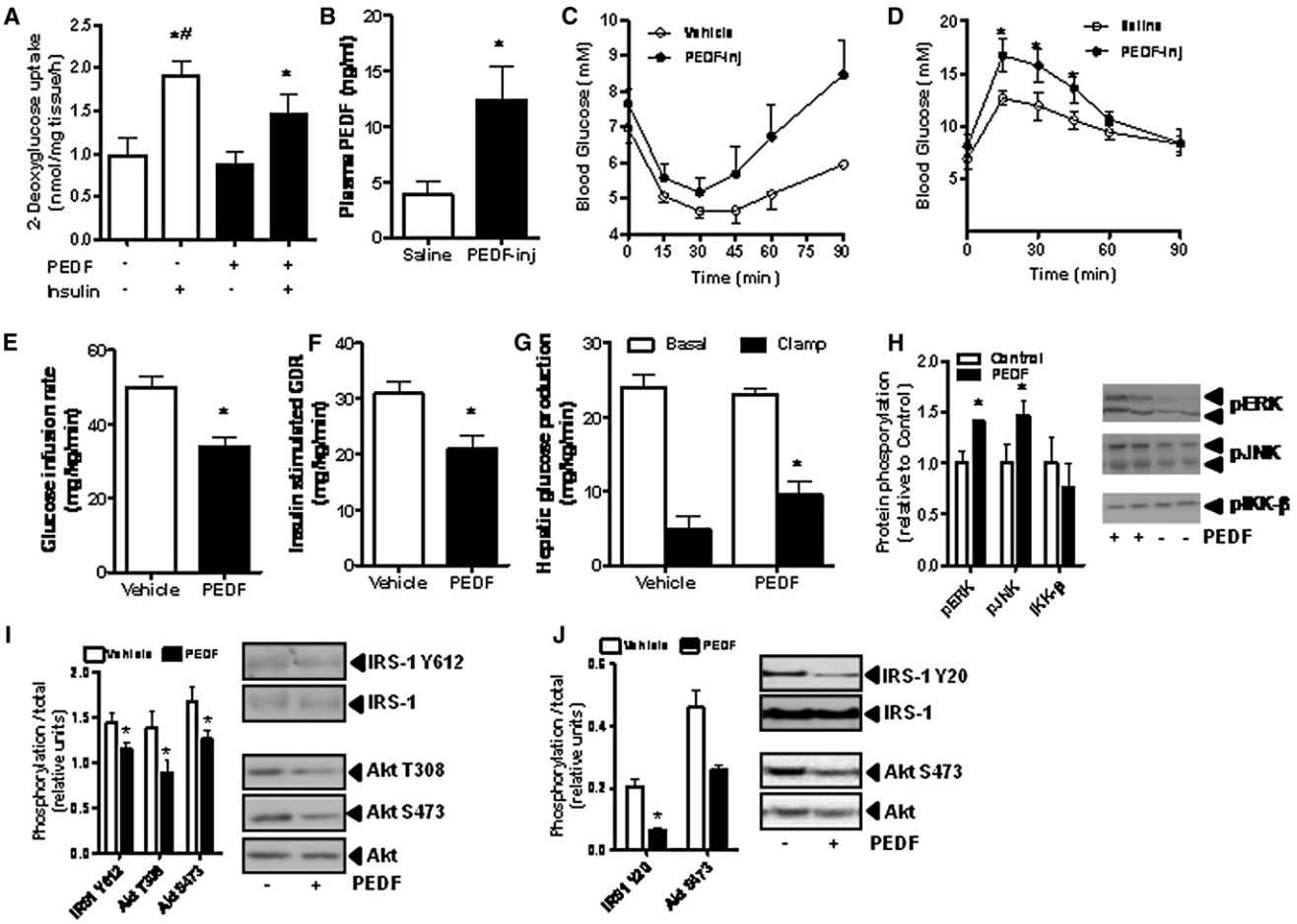

Figure 2. Acute PEDF Administration Causes Insulin Resistance in Skeletal Muscle and Liver

(A) 2-deoxyglucose uptake experiments in EDL muscles isolated from C57Bl/6 mice. Muscles were pretreated without (open bars) or with (closed bars) 100 nM PEDF for 2 hr. The media was removed and basal and insulin stimulated 2DG uptake determined. n = 8 EDL muscles from independent mice for each group. *p < 0.05 versus basal within the same treatment; #p < 0.05 versus PEDF insulin treatment. Values are means ± SEM.

(B) Plasma PEDF concentrations 2 hr after C57Bl/6 mice were injected with saline (open bars) or PEDF (closed bars) in the intraperotineal cavity. *p < 0.05 versus saline; n = 8 mice for each group.

(C and D) Insulin tolerance tests and (D) glucose tolerance tests 2 hr after C57Bl/6 mice were injected with saline (open bars) or PEDF (closed bars) in the intraperotineal cavity. n = 6 mice for each group; *p < 0.05 versus saline.

(E–G) Direct measures of insulin sensitivity by hyperinsulinemic-euglycemic clamp after PEDF injection. Lean C57Bl/6 mice aged 10 weeks were injected with PEDF 2.5 h prior to clamps where whole-body glucose infusion rate (E), glucose disposal rate (F), and hepatic glucose production (G) were determined. *p < 0.05 versus vehicle; n = 8 mice for each group.

(H) Phosphorylation of the serine/threonine kinases ERK, JNK, and IKK-β in skeletal muscle at the end of the hyperinsulinemic-euglycemic clamp. (n = 8 per group); *p < 0.05 versus vehicle.

(I and J) Insulin signaling in muscle (I) and liver (J) at the end of the hyperinsulinemic-euglycemic clamp. n = 8 per group; *p < 0.05 versus vehicle; error bars are ± SEM.

5.4-fold, respectively, compared with mice fed a HFD (Figure 2F). Together, these data show that circulating levels of PEDF as well as its expression in adipose tissue correlates very well with whole-body adiposity and insulin sensitivity.

PEDF Regulates Glucose Metabolism in Skeletal Muscle and Liver

To determine whether PEDF directly regulates skeletal muscle insulin sensitivity, we treated isolated EDL muscles from lean mice with recombinant PEDF for 2 hr and assessed 2-deoxy-D-glucose (2DG) uptake. PEDF did not affect basal 2DG uptake but reduced insulin-stimulated 2DG disposal by ~30% (Figure 2A). The specific effect of PEDF on skeletal muscle glucose uptake was also verified in L6 myotubes (Figure S3) and was maintained for 24 hr (data not shown). To test whether these effects could be recapitulated in vivo, lean mice were injected with 50 μg PEDF 2 hr prior to an insulin tolerance test (ITT), resulting in an increase in circulating PEDF levels from 3.8 ± 1.2 ng/ml to 12.4 ± 3.1 ng/ml (Figure 2B). These levels are similar to those observed in lean and obese mice, respectively (Figure 1A). There was no change in basal plasma glucose concentration following PEDF injection (saline: 6.9 ± 0.7 mM, PEDF: 7.6 ± 0.5 mM, p = 0.45) whereas whole-body insulin sensitivity was decreased (Figure 2C). Mice pretreated with PEDF also

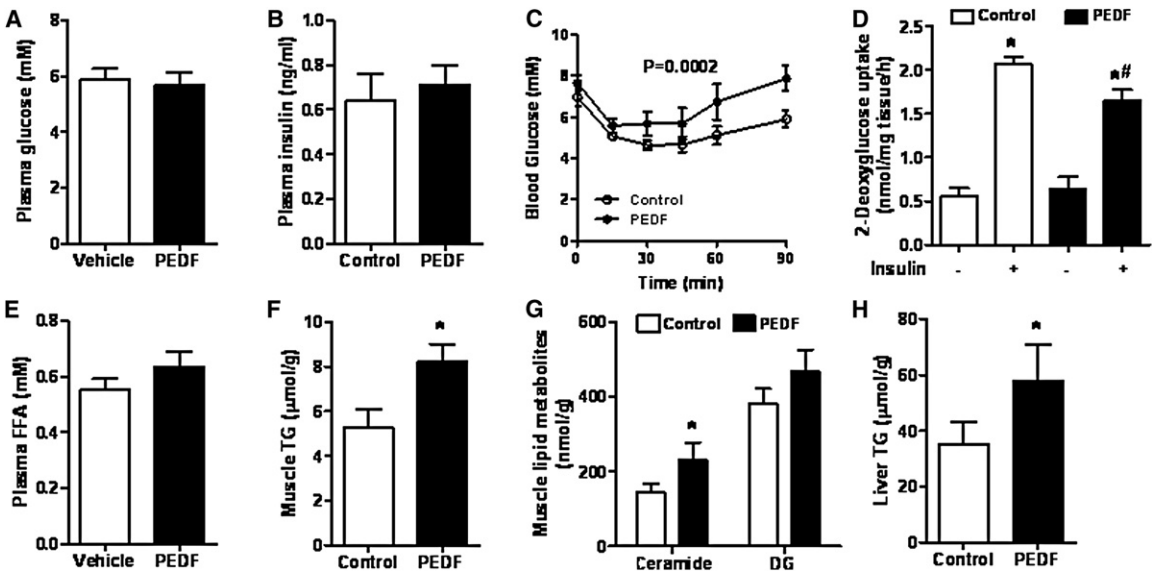

Figure 3. Prolonged PEDF Administration Mediates Insulin Resistance in Lean Mice

(A and B) Lean C57bl/6 mice were continuous infused with recombinant PEDF (closed bars) or sterile saline (open bars) for 5 days. Plasma glucose (A) and insulin (B) were assessed in 4 hr fasted mice.

(C) Whole-body insulin sensitivity was assessed by ITT.

(D) Skeletal muscle 2DG uptake was assessed in EDL muscle ex vivo with or without the addition of 10 nM insulin. *$p < 0.05$ versus basal; #$p < 0.05$ versus control insulin.

(E) Plasma FFA were assessed in 4 hr fasted mice.

(F and G) Skeletal muscle was removed and triglyceride (TG), diglyceride (DG) and ceramide assessed.

(H) Liver TG. *$p < 0.05$ versus control. For all experiments, n = 6 mice per group. Error bars are ± SEM.

displayed impaired glucose tolerance (Figure 2D), independent of changes in plasma insulin levels (Table S1). These effects of PEDF raise the possibility that obesity-associated elevations in PEDF might play a role in insulin resistance.

To investigate this further and to quantitatively examine the contribution of liver and skeletal muscle to PEDF-induced insulin resistance, we injected lean mice with PEDF 2.5 hr prior to hyperinsulinemic-euglycemic clamps. There were no differences in basal glucose turnover between vehicle (24 ± 1.6 mg/kg/min) or PEDF (23 ± 0.8 mg/kg/min) treated mice; however, the amount of exogenous glucose required to maintain euglycemia during insulin stimulation was significantly diminished with prior PEDF administration, indicating whole-body insulin resistance (Figure 2E). The insulin-stimulated glucose disposal rate, primarily reflecting skeletal muscle glucose disposal, was decreased with PEDF (Figure 2F). In addition, suppression of hepatic glucose production was blunted in PEDF treated animals (Figure 2G), as indicated by the increased rate of glucose production by the liver during the clamp. It is unlikely that these effects are due to changes in insulin secretion as plasma insulin levels were identical between PEDF treated and control mice during the hyperinsulinemic clamps. Moreover, basal plasma insulin levels were not different 2 hr after PEDF injection or following a glucose load (Table S1).

PEDF Induces Proinflammatory Signaling and Impairs Insulin Signaling

We next sought to identify the mechanism for PEDF-induced insulin resistance. PEDF induces proinflammatory signaling in several cell types (Tombran-Tink and Barnstable, 2003), which is of interest in view of the possible role of inflammation in insulin resistance (Aguirre et al., 2000; Bost et al., 2005; Gao et al., 2002). PEDF was associated with a decrease in phospho-ERK and phospho-JNK in skeletal muscle (Figure 2H) and liver (not shown), concomitant with a reduction in the insulin-dependent activation of insulin receptor substrate-1 and Akt, as indicated by reduced phosphorylation at their activating sites (Figures 2I and 2J) during the hyperinsulinemic-euglycemic clamp studies. Similar effects were observed in cultured myotubes (Figure S4). Importantly, serine/threonine kinase activation and impaired insulin signal transduction occurred independent of changes in other known mediators of insulin resistance including bioactive lipid metabolite accumulation and endoplasmic reticulum stress (data not shown).

Prolonged PEDF Administration in Lean Mice Causes Ectopic Lipid Deposition and Insulin Resistance

To explore the effect of prolonged PEDF administration in vivo, miniosmotic pumps were surgically implanted into lean C57Bl/6 mice. PEDF was continuously infused for 5 days as validated and described previously (Apte et al., 2004). Fasting plasma glucose and insulin were unaffected by PEDF (Figures 3A and 3B), whereas whole-body insulin sensitivity (Figure 3C) and insulin-stimulated glucose uptake into skeletal muscle were reduced (Figure 3D).

Obesity is characterized by excessive basal adipose tissue lipolysis and increased postabsorptive circulating fatty acid levels (Horowitz et al., 1999; Wolfe et al., 1987) that in turn

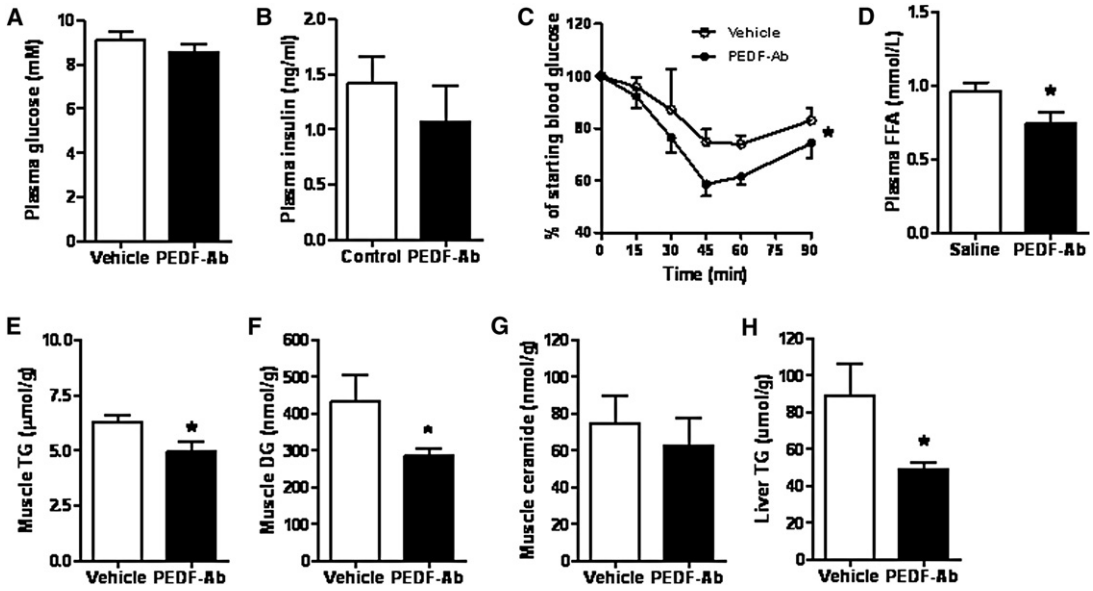

Figure 4. PEDF Neutralization in Obese, Insulin-Resistant Mice Improves Insulin Sensitivity

(A–D) Miniosmotic pumps were placed in obese mice and saline or PEDF-neutralizing antibody was infused for 5 days. Plasma glucose (A), insulin (B), and FFA (D) were assessed in 4 hr fasted mice. In (C), mice were fasted for 4 hr and insulin tolerance tests were performed. n = 6 mice per group; *$p < 0.05$ main effect for treatment.

(E–H) Skeletal muscle and liver lipids are decreased with PEDF neutralizing antibody treatment. Skeletal muscle triglyceride (E; TG), diglyceride (F; DG), and ceramide (G), and liver TG (H) were assessed. n = 5 mice per group; $p < 0.05$ versus vehicle; error bars are ± SEM.

contribute to the development of ectopic lipid deposition and insulin resistance (Boden and Chen, 1995). We next explored the role of PEDF in lipid metabolism by examining its effects on adipose tissue lipolysis. As shown in Figure S5A, PEDF acutely increased lipolysis in cultured 3T3-L1 adipocytes and in mice in vivo as assessed by plasma glycerol and FFA levels (byproducts of triacylglycerol lipolysis) (Figure S4b). Plasma FFA levels were increased following prolonged in vivo PEDF administration (15%, $p = 0.13$; Figure 3E) coupled with an increase in muscle lipid storage (Figures 3F and 3G). Liver TG was elevated by 66% in animals treated with PEDF (Figure 3H). Together, these data support two possible mechanisms for PEDF-induced insulin resistance, via stress signaling events in the short term (Figures 2A–2J) and via lipid deposition in skeletal muscle and liver in the longer term.

PEDF Neutralization in Obese, Insulin-Resistant Mice Improves Insulin Sensitivity

We next tested whether blocking PEDF action could restore insulin sensitivity in obese mice. Miniosmotic pumps were implanted into obese C57Bl/6 mice (mass: 36.1 ± 0.5 g) and neutralizing PEDF antibody or control solution was continuously infused for 5 days (Apte et al., 2004). The PEDF neutralizing antibody did not affect fasting blood glucose (Figure 4A) and insulin levels (Figure 4B) but did improve whole-body insulin sensitivity in these obese mice (Figure 4C), independent of changes in body mass and adiposity (Table S2). Consistent with this notion, parallel studies in cultured myotubes showed that adipocyte conditioned media containing PEDF caused insulin resistance, whereas coincubation with the PEDF-neutralizing antibody largely restored insulin action (Figure S6).

Sequestering fatty acids in adipocytes and/or reducing adipose tissue lipolysis reduces fatty acid delivery to nonadipose tissues such as skeletal muscle and liver, thereby limiting the progression of insulin resistance (Bajaj et al., 2005; Unger, 2003). It is believed that this effect is mediated, in part, by minimizing the accumulation of bioactive lipid metabolites known to cause insulin resistance (Holland et al., 2007; Yu et al., 2002). Plasma FFA was reduced with PEDF antibody administration (Figure 4D), suggesting an inhibition of adipose tissue lipolysis. Skeletal muscle triacylglycerol and diacylglycerol contents were reduced with PEDF antibody administration (Figures 4E and 4F). Furthermore, liver triacylglycerol (Figure 4I), diacylglycerol and ceramide (Figure S7) levels were also reduced with PEDF neutralizing antibody treatment. These data demonstrate that reducing PEDF action in vivo restores insulin action in the context of obesity. The reduction in circulating FFA and muscle and liver lipid accumulation may have been one mechanism underlying this insulin-sensitizing effect of PEDF neutralization obesity.

DISCUSSION

Changes in fat-cell size are accompanied by reprogramming of the fat-cell secretory profile, and this is thought to play an important role in the link between obesity and insulin resistance (Guilherme et al., 2008; Tilg and Moschen, 2006). In this study, we describe a role for a novel adipokine, PEDF, in whole-body metabolism. Expression of PEDF in adipose tissue and plasma levels of PEDF positively correlate with obesity and insulin resistance. This is consistent with studies reporting a significant correlation between plasma PEDF and adiposity in humans

(Jenkins et al., 2008; Yamagishi et al., 2006). To test the relationship between circulating PEDF and insulin resistance, we administered either PEDF or PEDF-neutralizing antibodies to mice and showed that this was accompanied by reduced or enhanced whole-body insulin sensitivity, respectively. These studies therefore provide novel insights into yet another factor secreted from adipose tissue that appears to play a key role in regulating whole-body metabolism.

The mechanism by which PEDF induces insulin resistance is not fully resolved. In addition to its role in neurogenesis and angiogenesis, PEDF induces inflammatory signaling in several cell types (Filleur et al., 2009) and circulating PEDF levels correlate with inflammation in individuals with type 1 diabetes (Jenkins et al., 2007). Obesity triggers a low-grade inflammatory state that results from the excessive release of proinflammatory cytokines and chemokines from adipocytes and activated macrophages that surround necrotic adipocytes (Cinti et al., 2005; Lumeng et al., 2007). This inflammatory stress activates several serine/threonine signaling molecules including IKKβ, JNK, and ERK that in turn inhibit downstream insulin signal transduction. The importance of both JNK and IKK to the etiology of insulin resistance is highlighted by the consistent observations that the global (Hirosumi et al., 2002; Yuan et al., 2001) or tissue-specific deletion of these stress kinases markedly improves insulin sensitivity and glucose homeostasis in genetically obese (*ob*/*ob*) and high-fat fed mice (Arkan et al., 2005; Sabio et al., 2008; Solinas et al., 2007). Consistent with this notion, we show that PEDF directly activates several proinflammatory signaling proteins in skeletal muscle and liver in vivo, which is associated with reduced insulin signal transduction. These effects occur within 30 min of PEDF administration, and prior to the upregulation of other known mediators of insulin resistance including lipid metabolite accumulation and ER stress induction.

The ''lipocentric'' view of insulin-resistance development suggests that obesity is characterized by elevated basal adipose tissue lipolysis (Langin et al., 2005) and greater systemic FFA availability (Horowitz et al., 1999) that in turn contribute to excessive ectopic lipid deposition and the development of insulin resistance (Holland et al., 2007; Schmitz-Peiffer et al., 1999; Yu et al., 2002). A striking observation in this study was that several of these metabolic events were recapitulated with PEDF treatment in lean mice. We show that PEDF can cause diabetogenic effects indirectly through stimulation of adipocyte lipolysis and subsequent ectopic diacylglycerol and ceramide deposition in insulin-sensitive tissues. These events were reversed with PEDF neutralization in obese, insulin-resistant mice. Taken together, our studies indicate that PEDF can induce insulin resistance through several mechanisms, including acute activation of serine/threonine kinases known to impair insulin signal transduction and by altering systemic lipid metabolism that results in ectopic tissue lipid deposition. This dual effect is reminiscent of other adipokines known to cause insulin resistance. For example, tumor necrosis factor (TNF) α, resistin, and interleukin-6 are all capable of inducing serine/threonine kinase activation and lipolysis (Ort et al., 2005; Ryden and Arner, 2007). Interestingly, PEDF appears to rapidly induce lipolytic stimulation by interacting directly with the key lipolytic protein adipose triglyceride lipase (Chung et al., 2008), while other adipokines such as TNFα act more slowly (Plomgaard et al., 2008), presumably by modifying translational control of lipid droplet associated proteins such as perilipin A (Ryden and Arner, 2007). Evidently, other factors are likely to contribute to PEDF-induced insulin resistance and will require further detailed analysis. More definitive elucidation of PEDF interactions with its putative receptors and high-affinity ligands (Filleur et al., 2009) will further assist in deciphering the biological roles of PEDF.

An important question raised by these experiments is, why do adipocytes secrete PEDF in obesity? In light of PEDFs powerful anti-angiogenic function, there is good reason to assume that PEDF is secreted to act as a brake on excessive vascularization during adipocyte expansion. Adipocytes and stromal vascular cells produce multiple angiogenic factors including leptin, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-β, and TNFα that contribute to adipose neovascularisation, which is undoubtedly an essential process for adipose tissue development (Cao, 2007). Precursor fibroblasts, which exist to expand adipose tissue depots, produce these proangiogenic factors, and their production is downregulated during adipogenesis (Figure S8A). Meanwhile, the anti-angiogenic PEDF is upregulated during adipogenesis and is mostly produced by mature adipocytes (Figure S8B). It is likely then that anti-angiogenic factors are secreted by mature adipocytes to restrict excessive vascularization and maintain homeostasis during adipose tissue growth. Indeed, a number of angiogenesis inhibitors including thrombospondin 1, soluble VEGFR2, and endostatin are produced in overweight or obese humans (Silha et al., 2005; Voros et al., 2005). Both the observations reported here and previously (Yamagishi et al., 2006) suggest that PEDF can be added to this list and supports the premise that the net balance between proangiogenic factors secreted from fibroblasts and anti-angiogenic factors secreted from mature adipocytes is important for appropriate vascularization during white adipose-tissue development (Cao, 2007).

In conclusion, we have identified a novel role for PEDF as a negative regulator of insulin action in obesity. Given the observation that PEDF is increased in obese type 2 diabetic humans (Jenkins et al., 2008; Yamagishi et al., 2006), therapeutic strategies to inhibit PEDF action in muscle and liver, or prevent adipocyte PEDF release, may prove a viable approach to ameliorate obesity-induced insulin resistance and its associated pathologies.

EXPERIMENTAL PROCEDURES

Production of Recombinant PEDF

Recombinant PEDF was purified from a HEK293 cell line stably transfected with human PEDF (Duh et al., 2002), and bioactivity was assessed by proliferation assay of SaOS-2 osteosarcoma cells.

Animal Maintenance and Experimental Protocols

Experimental procedures were approved by the St. Vincent's Hospital Animal Experimentation Ethics Committee, the School of Biomedical Sciences Animal Ethics Committee (Monash University), and the Committee on Animal Research at the University of California, Los Angeles. Eight-week-old male C57Bl/6J mice (Monash Animal Services; Victoria, Australia) were fed a standard chow diet or a high-fat diet (60% calories from fat) for 16 weeks. Mice were fasted for 4 hr prior to all experiments. See the Supplemental Experimental Procedures for details of ex vivo, GTT, and ITT experiments. Metabolic monitoring was performed in a Comprehensive Lab Animal Monitoring System (Columbus Instruments; Columbus, OH).

Hyperinsulinemic-Euglycemic Clamps

Glucose clamp studies were performed on 16-week-old normal chow-fed C57Bl/6J mice (Jackson Laboratory) 3 days after chronic cannulation as previously described (Hevener et al., 2003) and detailed in SI methods.

Glucose Uptake

2DG uptake was measured by adding 2-deoxy-D-[1,^{3}H] glucose (1 mM, 0.5 μCi / ml) and D-[1-^{14}C]mannitol (8 mM, 0.2 μCi /ml) (Amersham Biosciences) to Krebs buffer and is outlined in the Supplemental Experimental Procedures.

Plasma Hormone and Metabolite Analysis

PEDF was analyzed by ELISA for mouse PEDF (Chemicon, Temecula, CA). Plasma glucose was assessed by a glucose oxidase method (Sigma), FFA by an enzymatic colorimetric method (Wako), and insulin by RIA.

ConA Affinity Chromatography and Mass Spectrometry

3T3-L1 adipocytes were washed in PBS, once in serum-free DMEM, and left overnight serum-free DMEM supplemented with 100 nM insulin. Conditioned medium was collected, pooled, and dialyzed overnight with a 3000 MW cut-off membrane. Concanavalin A sepharose 4B (GE Healthcare 17-0440-03) was added and left to mix overnight. Glycoproteins were eluted and proteins precipitated, resuspended in Laemmle buffer, and separated on SDS-PAGE gels. Lanes were excised, dehydrated, and digested with trypsin. Peptides were separated by liquid chromatography and subjected to tandem mass spectrometry on a Waters Ultima quadruple time of flight (QTOF) mass spectrometer, as described previously (Larance et al., 2005).

qRT-PCR

Total RNA was extracted, reverse transcribed and quantitative PCR was performed as described (Steinberg et al., 2007). PEDF and 18S primers were purchased from Applied Biosystems.

Immunoblot Analysis

Immunoblotting was performed antibodies against IRS1, IRS-1 Y20, Akt, Akt S473, Akt T308, phosphor- and total ERK1/2, JNK1/2, and IKK-β from Cell Signaling (Danvers, MA), α-actin (Sigma), tubulin (Sigma T9026), IRS1 Y612 (Biosource; Carlsbad, CA) and PEDF (Duh et al., 2002). Immunofluorescence analysis is detailed in the Supplemental Experimental Procedures.

Muscle Lipids

Muscle lipids were extracted in chloroform: methanol. TG was assessed by measuring glycerol after saponification and DG and ceramide were measured by a radiometric method as described (Watt et al., 2006).

Statistical Analysis

Statistical analysis was performed using unpaired Student's t test. A two-way ANOVA with repeated measures was applied where appropriate and a Student Newman-Keuls post hoc analysis performed. Statistical significance was set at $p < 0.05$.

SUPPLEMENTAL DATA

The Supplemental Data include eight figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(09)00159-4.

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