Angiopoietin-like Protein 2 Promotes Chronic Adipose Tissue Inflammation and Obesity-Related Systemic Insulin Resistance

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SUMMARY

Recent studies of obesity have provided new insights into the mechanisms underlying insulin resistance and metabolic dysregulation. Numerous efforts have been made to identify key regulators of obesity-linked adipose tissue inflammation and insulin resistance. We found that angiopoietin-like protein 2 (Angptl2) was secreted by adipose tissue and that its circulating level was closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans. Angptl2 activated an inflammatory cascade in endothelial cells via integrin signaling and induced chemotaxis of monocytes/macrophages. Constitutive Angptl2 activation in vivo induced inflammation of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased permeability. Angptl2 deletion ameliorated adipose tissue inflammation and systemic insulin resistance in diet-induced obese mice. Conversely, Angptl2 overexpression in adipose tissue caused local inflammation and systemic insulin resistance in nonobese mice. Thus, Angptl2 is a key adipocyte-derived inflammatory mediator that links obesity to systemic insulin resistance.

INTRODUCTION

Obesity is a pandemic medical and social problem that is associated with several adverse health outcomes, including type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, and cancer (Eckel et al., 2005; Mokdad et al., 2003), all of which result in increased mortality. A major metabolic manifestation of obesity is systemic insulin resistance. Recently, the concept has emerged that chronic low-grade activation of proinflammatory pathways in adipose tissue directly promotes systemic insulin resistance (Apovian et al., 2008; Neels and Olefsky, 2006; Schenk et al., 2008). Adipocytes and macrophages could be a source of several proinflammatory cytokines that activate inflammatory pathways in resident and infiltrating cells within adipose tissue in a paracrine or autocrine fashion (Kanda et al., 2006; Weisberg et al., 2006). However, the molecular mechanisms underlying inflammation of adipose tissue in obesity have not fully clarified.

Fibrinogen promotes leukocyte adhesion and cytokine secretion at sites of inflammation through integrin-dependent inflammatory pathways (Herrick et al., 1999; Mosesson, 2005).
Fibrinogen-binding integrins are abundantly expressed by monocytes/macrophages and endothelial cells, and fibrinogen must undergo oligomerization or polymerization to display its activity. The presence of extravascular fibrinogen at sites of inflammation has been documented by pathologists for decades (Dvorak et al., 1985). These findings prompted us to ask whether an oligomeric protein derived from adipose tissue and containing a fibrinogen-like sequence might play a pathological role in inflammatory changes of adipose tissue associated with obesity. Recently, we and others identified seven angiopoietin-like proteins (Angptls), which possess a coiled-coil domain at the N terminus for oligomerization and a C-terminal fibrinogen-like domain (Kim et al., 1999; Kubota et al., 2005a; Oike et al., 2004). Angptls are structurally similar to Tie-2 receptor ligands (angiopoietins), but Angptls do not bind to either Tie2 or the homologous Tie1 protein, indicating that their role differs from that of angiopoietins.

Here we show that angiopoietin-like protein 2 (Angptl2) is primarily secreted by adipose tissue and that its expression is increased by obesity and obesity-related pathological conditions, including hypoxia and endoplasmic reticulum (ER) stress. We found that increased circulating Angptl2 levels were closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans. Angptl2 acted on endothelial cells and monocytes/macrophages via integrin signaling, resulting in the promotion of inflammation. Constitutive activation of Angptl2 in mouse skin tissue induced chronic inflammation, including inflammatory changes of the vasculature characterized by abundant attachment of leukocytes to the vessel walls and increased permeability. Deletion of Angptl2 led to reduced inflammation in adipose tissue and ameliorated systemic insulin resistance in mice with dietary obesity. Conversely, persistent overexpression of Angptl2 in adipose tissue caused local inflammation and systemic insulin resistance in nonobese mice. These findings establish Angptl2 as a key adipocyte-derived inflammatory mediator linking obesity to systemic insulin resistance and identify it as a new molecular target that could be used to improve the diagnosis and treatment of obesity and related metabolic diseases.

**RESULTS**

**Angptl2 Expression in White Adipose Tissue Is Increased by Obesity and Obesity-Related Stress**

Angptl2 mRNA was widely expressed in various organs of mice, but its level was particularly elevated in visceral white adipose tissues (Figure 1A). Differentiated 3T3-L1 adipocytes expressed Angptl2 mRNA (Figure 1B), and its expression was increased by hypoxia (Figure 1C), which occurs in obese adipose tissue (Hosogai et al., 2007; Nishimura et al., 2008; Schenk et al., 2008; Ye, 2009). We found significantly increased ER stress in adipocytes from obese mice compared with cells from nonobese mice (see Figure S1 available online). Serum levels of long-chain saturated fatty acids (LCSFAs) are elevated in obesity, and LCSFAs promote ER stress in adipocytes (Schenk et al., 2008). Our in vitro study of cultured 3T3-L1 cells revealed that ER stress was induced in adipocytes after treatment with palmitate, one of the LCSFA, or thapsigargin, an ER stress inducer. As
Angptl2 Causes Adipose Tissue Inflammation

Figure 2. Circulating Angptl2 Is Correlated with Adiposity, Insulin Resistance, and Inflammation in Humans

(A) Immunohistochemical staining for Angptl2 in human adipose tissue. Scale bar, 100 μm.

(B) Correlation of the serum Angptl2 level with the body mass index or serum insulin, glucose, and CRP levels in healthy volunteers (n = 98).

(C) Serum Angptl2 levels in healthy volunteers (Healthy, n = 98) and in patients with type 2 diabetes (DM, n = 89) or coronary artery disease (CAD, n = 109). Horizontal bars represent the 10%–90% percentile range, and boxes indicate the 25%–75% percentile range. The horizontal line in each box corresponds to the median.

(D) Correlation of the serum Angptl2 level with the visceral fat area, HOMA-R index, M value, and CRP level in diabetic patients.

(E and F) Changes of the plasma Angptl2 level in obese diabetic male patients after pioglitazone treatment (n = 27). Plasma Angptl2 levels (mean ± SEM) before and after treatment (E). Correlation of the change (%) of the plasma Angptl2 level with the change (%) of the visceral fat area, subcutaneous fat area, HOMA-R index, and CRP level. Correlation coefficient (R) and probability (P) values are shown (F). *p < 0.05 and **p < 0.01 compared with controls.

As a result, both the cellular Angptl2 mRNA level and its protein concentration in the culture medium were significantly increased (Figures 1D and 1E, Figure S2, and data not shown). Angptl2 mRNA in mesenteric white adipose tissue and serum Angptl2 protein levels were increased in obese mice fed a high-fat diet (Figures 1F and 1G), suggesting that Angptl2 is a bioactive adipocyte-derived factor that has a role in obesity and related metabolic diseases.

Circulating Angptl2 Level Is Correlated with Adiposity, Systemic Insulin Resistance, and Inflammation in Humans

Immunohistochemical analysis revealed that Angptl2 was expressed by the adipocytes of human adipose tissue (Figure 2A). We analyzed the circulating levels of Angptl2 in various human subjects by using an enzyme-linked immunosorbent assay (ELISA). In healthy normal-weight volunteers aged from 20 to 59 years, the serum Angptl2 concentration ranged from 1.36 to 4.98 ng/ml, and the distribution was normal after log transformation (Figure S3A). Plasma levels were comparable and strongly correlated with the corresponding serum levels (Figure S3B). There was no significant difference of serum Angptl2 concentration between genders (data not shown). Angptl2 level showed a positive correlation with body mass index, serum insulin level, and serum C-reactive protein (CRP) level. In contrast, the level of Angptl4, which has already been identified as an adipocyte-derived Angptl, showed no correlation with these factors in normal-weight healthy subjects (Figure 2B and Figure S4). An increase of the body mass index, serum insulin level, and CRP level is associated with the development of type 2 diabetes and atherosclerosis (Eckel et al., 2005; Mokdad et al., 2003). Indeed, serum Angptl2 was also significantly increased in patients with type 2 diabetes or coronary artery disease (Figure 2C). In 935 consecutive persons aged 27–84 years who underwent a medical checkup and gave informed consent for measurement of serum Angptl2 at the Japanese Red Cross Kumamoto Health Care Center, the Angptl2 level was positively correlated with the body mass index, abdominal circumference, and serum CRP level (Figure S5). In patients with type 2 diabetes, Angptl2 was positively correlated with the visceral fat area, homeostasis model assessment of insulin resistance (HOMA-R) index (Matthews et al., 1985), and serum CRP level, but not with the subcutaneous fat area. Angptl2 level was inversely correlated with the insulin sensitivity index (M value), as assessed...
by the hyperinsulinemic euglycemic clamp test (DeFronzo et al., 1979) (Figure 2D).

These observations led us to ask whether improvement of systemic insulin resistance or inflammation would influence the circulating level of Angptl2. We observed a significant decrease of the plasma Angptl2 level in 27 obese diabetic men following treatment with pioglitazone at 30 mg/day for 3 months (Figure 2E). The percent decrease of the plasma Angptl2 level was correlated with the percent decrease of the visceral fat area, HOMA-R index, and serum CRP level (Figure 2F). These results suggested that visceral fat was likely to be the main source of circulating Angptl2, the concentration of which was significantly correlated with systemic insulin resistance and inflammation.

**Angptl2 Activates Migration and Inflammatory Changes of Endothelial Cells and Monocytes/Macrophages via Integrins**

Since the vasculature has an important role in tissue inflammation (Jackson et al., 1997), we examined the effect of Angptl2 on endothelial cells. First, we found a dose-dependent increase of cell adhesion when human umbilical vein endothelial cells (HUVECs) and human arterial endothelial cells (HAECs), which express several integrins on their surfaces, were plated on Angptl2-coated plates (Figures 3A and S6). We next analyzed cell adhesion in the presence of a series of function-blocking antibodies for specific integrins. A neutralizing antibody for integrin α5β1 inhibited endothelial cell adhesion to Angptl2-coated plates, as did RGD peptide, which blocks RGD-dependent integrins (Figure 3B), suggesting that Angptl2-induced cell adhesion was an α5β1-dependent process, although the involvement of untested integrins could not be excluded. Integrin α5β1 activates NF-κB in endothelial cells (Klein et al., 2002). Consistently, there was increased translocation of NF-κB to the nucleus and degradation of IκB in HUVECs stimulated with recombinant human Angptl2 protein (Figures 3C and 3D).

Angptl2 also promoted the migration of HUVECs and HAECs through a microchemotaxis membrane (Figure 3E). Time-lapse imaging of HUVECs or HAECs cultures revealed that protrusion of lamellipodia and membrane ruffling were rapidly induced following the addition of Angptl2 (Movies S1 and S2). Since Rac1, a small Rho-GTPase, plays a pivotal role in the protrusion of lamellipodia, membrane ruffling, and cell migration (Bar-Sagi and Hall, 2000; Fryer and Field, 2005), we investigated whether Rac1 was activated in HAECs and HUVECs by performing a pull-down assay. Activation of Rac1 was detected in both Angptl2-stimulated HUVECs and HAECs (Figure 3F). In viable Angptl2-stimulated HUVECs, a single-molecule probe was used to determine Rac1 activity, showing that it was diffusely activated at the plasma membrane, with this activation being followed by protrusion of lamellipodia and membrane ruffling (Figure 3G and Movie S3). Moreover, Angptl2 no longer stimulated the protrusion of lamellipodia and membrane ruffling in HUVECs transfected with a dominant-negative Rac1 mutant expressing red fluorescent protein (RacN17-IRE5-RFP) (Figure 3H and Movie S4). These findings suggest that Angptl2-stimulated lamellipodia formation and membrane ruffling in endothelial cells were both mediated by activation of Rac1. Next, we investigated whether Angptl2 could induce in vivo chemotaxis of endothelial cells in a mouse cornea assay. Implanted pellets containing Angptl2 markedly induced neovascularization in the mouse cornea, whereas pellets containing PBS alone did not (Figure 3I). Monocytes/macrophages express several integrin receptors that are responsible for adhesion, migration, and extravasation into the peripheral tissues (Friedl and Weigelin, 2008; Rose et al., 2007). We found that the THP-1 human monocytic cell line expressed integrins α4, β1, β2, and α5β1 (Figure 3J). THP-1 cells adhered to Angptl2-coated plates in a dose-dependent manner (Figure 3K). FACs analysis revealed that Angptl2 bound to THP-1; this binding was completely inhibited by neutralizing antibodies for integrins α4 or β2 and was partially blocked by antibodies for integrin α5β1 or β1 (Figure 3L). Angptl2 also promoted transmigration by THP-1 cells and primary human monocytes (Figure 3M and Figure S7).

**Constitutive Angptl2 Activation Induces Local Inflammation in Mouse Skin Tissue**

To further investigate the role of Angptl2 in the inflammatory process, we generated transgenic mice expressing Angptl2 driven by the keratinocyte-specific promoter K14 (K14-Angptl2) and therefore constitutively expressing Angptl2 in the epidermis (Figures S8A and S8B). The ears, snouts, and eyelids of K14-Angptl2 mice were redder than those of controls. The tails of K14-Angptl2 mice were not only reddish but also swollen and showed loss at the tips (Figure 4A), indicating local inflammation. Lectin staining showed an increase of adherent leukocytes, a common feature of inflammatory vasculature (McDonald, 1994), in enlarged vessels of the skin tissue specimens from K14-Angptl2 mice (Figure 4B), while there was no difference of vessel length between the genotypes (Figure S8C). The vessels of K14-Angptl2 mice were significantly more permeable than the vessels of wild-type controls after inflammation was induced by topical application of mustard oil, a potent proinflammatory agent (Figure 4C). As expected, even before mustard oil application, lumens of CD31 ‘LYVE-1’ lymphatics were enlarged in the skin of K14-Angptl2 mice, while such changes were not observed in controls (Figure 4D), suggesting that increased drainage via lymphatics was compensating for the excessive leakiness of Angptl2-stimulated vessels in the dermis. These findings indicate that Angptl2 induces inflammatory vascular remodeling rather than angiogenesis.

**Reduction of Adiposity and Obesity-Related Adipose Tissue Inflammation in Angptl2<sup>−/−</sup> Mice**

Next, we investigated the pathophysiological role of Angptl2 by generating Angptl2 knockout (Angptl2<sup>−/−</sup>) mice (Figure S9). Angptl2<sup>−/−</sup> mice were born alive following Mendelian inheritance and appeared to be grossly normal. Interestingly, when fed normal chow, Angptl2<sup>−/−</sup> mice weighed slightly less (Figure 2H) and appeared to be grossly normal. Interestingly, when fed normal chow, Angptl2<sup>−/−</sup> mice weighed slightly less (Figure S10A) and had a lower body fat mass estimated by computed tomography (CT) (Figures S10B and S10C) than heterozygotes or wild-type mice fed the same normal diet, although there was no significant difference of daily food intake or energy expenditure between the groups (Figures S10D and S10E). In addition, Angptl2<sup>−/−</sup> mice showed slightly, but significantly, better glucose tolerance and insulin sensitivity (Figures S10F and S10G). Next, we fed 8-week-old mice a high-fat diet containing 32% (wt/wt) fat to stimulate weight gain. After
8 weeks of high-fat diet feeding, Angptl2−/− mice had a body weight 12% lower than that of wild-type mice (Figure 5A). The visceral and subcutaneous fat mass and total body fat percentage were moderately decreased in Angptl2−/− mice compared to wild-type mice (Figures 5B and 5C). Considerable accumulation of fat was seen in the liver and skeletal muscle of wild-type mice, whereas these changes were mild in Angptl2−/− mice (Figures 5D and 5E). Although there were no obvious differences of food intake or energy expenditure between the two groups, the respiratory quotient was significantly lower in the Angptl2−/− group (Figures S11A–S11C).

We next examined the expression of mRNAs for inflammatory cytokines (IL-6 and TNF-α), a chemokine (MCP-1), various macrophage markers (F4/80, CD68, CCR2, Mgl1, and Mgl2), Angptl2 Causes Adipose Tissue Inflammation

Figure 3. Angptl2 Activates Endothelial Cells and Monocytes

(A) Adhesion of HUVECs or HAECs to culture dishes coated with various concentrations of recombinant human Angptl2 (n = 3).

(B) HUVECs or HAECs were preincubated with or without 25 μg/ml of blocking antibodies (anti-α5β1, anti-αvβ3, or anti-αvβ5) or RGD or RGE peptides (300 μM), and cell adhesion was assessed (n = 3). As a negative control, cell adhesion was assayed in the presence of 10 μM EDTA, which inhibits integrin binding.

(C) Nuclear translocation of NF-κB subunit p65 in HUVECs at 2 hr after Angptl2 stimulation. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Scale bar, 20 μm.

(D) Representative western blots of κB and Hsc70 protein (internal control) in HUVECs at the indicated times after Angptl2 stimulation.

(E) Migration of HUVECs or HAECs in response to Angptl2 (n = 4).

(F) HUVECs or HAECs were cultured with Angptl2 for 30 min and then subjected to the pull-down assay using GST-PAK-CRIB followed by western blotting with an anti-Rac1 antibody. Representative images are shown.

(G) HUVECs expressing Raichu-Rac1 (a probe for active Rac1) at the indicated times (min) after Angptl2 stimulation. Arrows indicate nascent and retracting lamellipodia. Ratio ranges are shown on the right.

(H) HUVECs that were either untransfected or transfected with RacN17 (shown in red in the left panel and by red stars in the center and right panels) and stimulated with Angptl2 at 200 μg/ml and then subjected to the pull-down assay using GST-PAK-CRIB followed by western blotting with an anti-Rac1 antibody. Representative images are shown.

(I) Macroporous appearance of neovascularization in the mouse cornea. Pellets containing vehicle or Angptl2 (0.5 μg) were implanted into micropockets cut in the corneal stroma.

(J) Integrin expression by THP-1 cells. Typical profiles obtained by FACS analysis with the indicated anti-integrin antibodies (black line traces) or isotype-matched control IgG (filled gray traces).

(K) Adhesion of THP-1 cells to culture dishes coated with various concentrations of Angptl2 (n = 3).

(L) Inhibition of Angptl2 binding to THP-1 cells by integrin-neutralizing antibodies. THP-1 cells were preincubated with (red line traces) or without (blue line traces) the indicated anti-integrin neutralizing antibodies, and then incubated with FLAG-tagged Angptl2 followed by detection with FITC-conjugated anti-FLAG antibody. Negative controls (filled gray traces) had omission of Angptl2.

(M) Migration of THP-1 cells in response to Angptl2 (n = 7–9). Data are the mean ± SD, *p < 0.05 and **p < 0.01 compared with controls.
and insulin-sensitizing adipocytokines (adiponectin and leptin) in the adipose tissue of mice fed a high-fat diet. As shown in Figure 5F, adiponectin expression was increased, while TNF-α and general (F4/80, CD68) and inflammatory (CCR2) macrophage markers were all decreased in the adipose tissue of Angptl2−/− mice. However, the expression of residential macrophage markers (Mgl1 and Mgl2) remained unchanged. Furthermore, expression of F4/80 mRNA was positively correlated with the adipose tissue weight in controls, indicating that adiposity was significantly correlated with macrophage infiltration into adipose tissue. In contrast, there was no significant correlation between adipose tissue weight and macrophage infiltration in Angptl2−/− mice (Figure 5G), suggesting that this decrease of macrophage infiltration may be independent of reduced adiposity in Angptl2−/− mice. Furthermore, immunohistochemistry using the macrophage marker Mac2 revealed accumulation of Mac2-positive macrophages in crown-like structures within the adipose tissue of wild-type mice, while fewer Mac2-positive cells were observed in the adipose tissue of Angptl2−/− mice (Figure 5H). The high-fat diet caused impaired glucose tolerance and insulin resistance in controls, whereas Angptl2−/− mice showed better glucose tolerance and insulin sensitivity based on the results of intraperitoneal glucose and insulin tolerance tests (GTT and ITT, respectively) (Figures 5I and 5J). To explore which organ(s) contributed to the improved insulin sensitivity in Angptl2−/− mice, we next performed western blotting analysis of the insulin signaling pathway. Tyrosine phosphorylation of insulin receptor β and serine phosphorylation of Akt after insulin injection were significantly increased in both the liver and skeletal muscle of Angptl2−/− mice compared with wild-type mice (Figure 5K). To confirm these results, we performed hyperinsulinemic-euglycemic clamp experiments. Both glucose infusion rate and whole-body glucose disposal rate were significantly increased in Angptl2−/− mice, while clamp endogenous glucose production was significantly reduced. In addition, the percent decrease in endogenous glucose production from basal to clamp states was significantly higher in Angptl2−/− mice than in wild-type mice (Figure 5L). These results indicated that insulin sensitivity was improved in both the skeletal muscle and liver of Angptl2−/− mice fed a high-fat diet.

Angptl2 Promotes Local Inflammation in Adipose Tissue and Systemic Insulin Resistance

Finally, we determined whether sustained overexpression of Angptl2 in adipose tissue promoted systemic insulin resistance by generating transgenic mice that overexpressed Angptl2 in adipose tissue under the control of aP2, an adipose tissue-specific promoter (aP2-Angptl2) (Figure S12A). Based on the level of Angptl2 expression, we considered that line 5 was the most acceptable model for examining the pathological role of increased Angptl2 expression in obese mice (Figure S12B), so we performed subsequent analyses using line 5 and wild-type mice.
**Figure 5. Deletion of Angptl2 Reduces Adipose Tissue Inflammation and Systemic Insulin Resistance in Dietary Obese Mice**

Analyses of Angptl2−/− and wild-type mice fed a HFD for 8 weeks (A–L).

(A) Body weight of each genotype (n = 8–16 per group) at the indicated times (weeks) after initiation of a HFD.

(B and C) Representative CT findings (B) and quantitative comparison of the visceral (V) and subcutaneous (S) fat volume and total percent body fat (C) in Angptl2−/− and wild-type mice (n = 5–7 per group).

(D) HE-stained liver sections from Angptl2−/− and wild-type mice. Scale bar, 100 μm.

(E) Triglyceride (TG) content of liver and skeletal muscle from Angptl2−/− and wild-type mice (n = 6 per group).

(F) Quantitative RT-PCR of mRNAs encoding adipocytokines and macrophage markers in epididymal adipose tissue from Angptl2−/− and wild-type mice (n = 11–12 per group).

(G) Correlation between F4/80 mRNA expression and epididymal adipose tissue weight in Angptl2−/− mice and wild-type mice (n = 11–12 per group). Correlation coefficient (R) and probability (P) values are shown.

(H) Immunohistochemistry of adipose tissue using the macrophage marker MAC2 and adipocyte marker perilipin. Representative photographs and quantitative comparisons of MAC2-positive cells (n = 6 per group) are shown. Scale bar, 100 μm.

(I and J) Glucose (I) and insulin (J) tolerance tests in Angptl2−/− and wild-type (WT) mice. Data are mean ± SEM, *p < 0.05 and **p < 0.01 compared with controls.

There was no difference of weight gain between aP2-Angptl2 mice and control wild-type mice fed a normal chow diet (Figure 6A). However, immunohistochemistry using Mac2 revealed accumulation of macrophages in crown-like structures within the adipose tissue of aP2-Angptl2 mice, whereas fewer Mac2-positive cells were observed in wild-type mice (Figure 6B). RT-PCR analysis revealed that inflammatory cytokines (IL-6, TNF-α, and IL-1β) and general (CD68) and inflammatory (CCR2) macrophage markers were increased in the adipose tissue of aP2-Angptl2 mice, while adiponectin and leptin were unchanged (Figure 6C). Lectin staining showed an increase of adherent leukocytes in vessels within the adipose tissue of aP2-Angptl2 mice, while few leukocytes were detected in the vessels of wild-type mice (Figure 6D). There was no
Figure 6. Angptl2 in Adipose Tissue Induces Local Inflammation and Systemic Insulin Resistance
Analyses of aP2-Angptl2 and wild-type mice at 16 weeks of age (B–H).
(A) Body weight of aP2-Angptl2 and wild-type mice (n = 14–16 per group) at the indicated ages (months).
(B) Immunohistochemistry of adipose tissue using the macrophage marker MAC2 and adipocyte marker perilipin. Representative photographs and quantitative comparison of MAC2-positive cells are shown (n = 6 per group). Scale bar, 50 μm.
(C) Quantitative RT-PCR of mRNAs encoding adipocytokines and macrophage markers in epididymal adipose tissue from aP2-Angptl2 mice and wild-type mice (n = 6 per group).
(D) Blood vessels in epididymal adipose tissue from aP2-Angptl2 and wild-type mice. Arrows indicate adherent leukocytes on the walls of enlarged vessels in aP2-Angptl2 mice. Scale bar, 25 μm.
(E and F) Glucose (E) and insulin (F) tolerance tests in aP2-Angptl2 mice and wild-type mice (n = 10–12 per group).
(G) Insulin signaling in the liver and skeletal muscle of aP2-Angptl2 (TG) and wild-type (WT) mice. Representative western blots and quantitative data for the total and phosphorylated forms of insulin receptor β subunit (IRβ) and Akt are shown (n = 4 per group).
(H) Glucose infusion rate (GIR), glucose disposal rate, endogenous glucose production (EGP) during the basal and clamped states, and percent change in EGP between the states in aP2-Angptl2 (TG) and wild-type (WT) mice (n = 7 per group). Data are mean ± SEM, *p < 0.05 and **p < 0.01 compared with controls.

We demonstrated that Angptl2, a member of the Angptl family, is a key mediator of chronic adipose tissue inflammation and obesity-related systemic insulin resistance.

Here we showed that Angptl2 is an adipocyte-derived inflammatory mediator, with increased expression at both the mRNA and protein levels in obesity. Hypoxia and ER stress, which are enhanced in obese adipose tissue (Hosogai et al., 2007; Nishimura et al., 2008; Schenk et al., 2008; Ye, 2009), both increased Angptl2 expression or secretion in adipocytes. Various changes of the microenvironment observed in the adipose tissue of obese animals, such as inflammation and hypoxia, could also promote ER stress (Schenk et al., 2008). Therefore, Angptl2 production by adipocyte should be increased by hypoxia and ER stress in obesity.
It is noteworthy that the circulating Angptl2 level was positively correlated with obesity-related metabolic changes. The difference of circulating Angptl2 protein levels between Angptl2 Tg mice and wild-type mice was only 1.5-fold, but tissue Angptl2 levels showed a 3- to 5-fold difference (data not shown). Therefore, the modest difference of circulating Angptl2 levels in humans may reflect a larger alteration of adipose tissue Angptl2 expression, which could promote inflammation of adipose tissue, resulting in systemic insulin resistance. We also do not exclude the possibility that there is a direct inhibitory effect of circulating Angptl2 on insulin sensitivity in other peripheral tissues, such as skeletal muscle or the liver, because glucose clamp studies and western blotting analysis of insulin signaling revealed that both skeletal muscle and liver were target organs for Angptl2-related insulin resistance in mice. Other Angptl family molecules function in an endocrine manner to regulate lipids, glucose, and energy metabolism (Hato et al., 2008; Oike et al., 2005a, 2005b), so further studies are needed to clarify whether Angptl2 might also act in an endocrine manner.

Angptl2 contains an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. The coiled-coil domain is required for oligomerization, which is necessary for its maximum activity, while the fibrinogen-like domain shares high homology with the analogous domain of fibrinogen. Fibrinogen acts as a ligand of the receptors for integrins such as αvβ3, α5β1, and αMβ2 (Herrick et al., 1999; Mosesson, 2005), which are heterodimeric transmembrane glycoproteins that mediate cell-extracellular matrix and cell-cell adhesion (Hynes, 2002). Angptl3 was reported to promote angiogenesis through integrin αvβ3 (Camenisch et al., 2002). In this study, we found that Angptl2 acted on endothelial cells through integrin α5β1 and influenced macrophages/macrophages through integrins α4 or β2. Several reports have indicated that integrin α5β1 signaling activates Rac1 in endothelial cells (Dormond et al., 2001; Mettouchi et al., 2001), in agreement with our finding that Angptl2 promotes Rac1 activation in endothelial cells. We also found that Angptl2 induced the chemotaxis of endothelial cells by in vitro time-lapse imaging analysis and in an in vivo mouse cornea neovascularization assay. In contrast, constitutive overexpression of Angptl2 in mouse skin or adipose tissue induced pathological vascular inflammation but did not increase vascularization or ameliorate hypoxia in the adipose tissue of mice with dietary obesity (Figure S12D). The cornea is an avascular tissue and thus is isolated from circulating soluble bioactive mediators, whereas various angiogenesis-related factors exist in highly vascular tissues such as the skin and adipose tissue. Taken together, these findings indicate that Angptl2 may function differently in different tissues, but it promotes vascular inflammation rather than angiogenesis, at least in adipose tissue that develops in obese mice.

Potentially relevant to these findings, we observed that Angptl2 stimulated the nuclear translocation of NF-κB and degradation of IkBα in cultured vascular endothelial cells, findings consistent with a previous report that integrin α5β1 signaling activates NF-κB-dependent expression of genes that are important for inflammation (Klein et al., 2002). There have been several other reports that Rac1 activates NF-κB (Perona et al., 1997; Sulciner et al., 1996), which is also consistent with our findings. An important aspect of inflammation is the recruitment of immune cells to affected tissues (Luster et al., 2005). This process requires adhesion of the immune cells to endothelial cells, allowing extravasation into the interstitium, followed by adhesion of immune cells to the extracellular matrix that enables migration toward the site of inflammation. In this regard, Angptl2 not only activated NF-κB in endothelial cells, which could induce expression of adhesion molecules (such as ICAM, VCAM, and selectin) and thus facilitate adhesion of immune cells to endothelial cells, but also promoted the migration of monocytes. Immune cells express integrins α4 or β2, as well as α5β1, which mediate cell adhesion, migration, activation, and production of proinflammatory cytokines through activation of NF-κB (Hynes, 2002; Rose et al., 2007; Roman et al., 2004; Graves and Roman, 1996), suggesting that Angptl2 may activate monocytes via such integrins. It remains to be clarified whether only Angptl2 among the Angptl family shows a stimulatory effect on adipose tissue inflammation, because some other members of this family bind to integrins (Camenisch et al., 2002), and Angptl4 is also abundantly expressed in adipose tissue. The skin tissue of K14-Angptl4 mice showed no inflammatory changes (Ito et al., 2003), unlike that of K14-Angptl2 mice. Moreover, there was no correlation between the serum Angptl4 concentration and Angptl2-related metabolic factors. These findings suggest that the effects of Angptl4 on endothelial cells and/or immune cells are different from those of Angptl2.

In this study, we demonstrated that Angptl2 deletion not only ameliorated inflammation in adipose tissue but also improved systemic insulin resistance in mice with dietary obesity, although it did not completely normalize their insulin sensitivity to the level seen in mice fed a normal chow diet (Figure S11I). The restoration of insulin sensitivity related to Angptl2 deletion may be attributable to the difference of body fat accumulation between the two genotypes. Since adipose tissue volume was not correlated with macrophage infiltration in Angptl2−/− mice, some mechanism other than the difference of adiposity may also have contributed to reducing adipose tissue inflammation in Angptl2−/− mice. Actually, constitutive Angptl2 overexpression in adipose tissue induced both local inflammation and systemic insulin resistance in nonobese mice. Since adipose tissue inflammation can be a cause of systemic insulin resistance via the secretion of several inflammatory factors (Apovian et al., 2008; Neels and Olefsky, 2006; Schenk et al., 2008), it is suggested that Angptl2 probably influenced systemic insulin sensitivity by exacerbating adipose tissue inflammation (Figure S13).

Although a reduction of adipose tissue inflammation could well be the main reason for improvement of insulin sensitivity in Angptl2−/− mice, some other possible mechanisms remain. Adiponectin can potentially increase insulin sensitivity, and the adiponectin level is usually decreased in obesity (Kadowaki and Yamauchi, 2005). However, there was no difference of circulating adiponectin levels between Angptl2−/− and control mice (Figure S11H). On the other hand, Angptl2−/− mice had a reduced triglyceride content in both skeletal muscle and liver, which could improve insulin sensitivity in these two organs (Schenk et al., 2008).

Angptl2−/− mice showed reduced body fat and tissue triglyceride accumulation when fed a high-fat diet, although there was no obvious difference of daily food intake and energy expenditure estimated from the O2 consumption rate. Interestingly, the respiratory quotient of Angptl2−/− mice was significantly lower.
than that of wild-type mice, suggesting that Angptl2−/− mice were more likely to use lipids than carbohydrates for oxidation to create energy. There was also a trend of increased expression of lipid oxidation genes in the skeletal muscle of Angptl2−/− mice and increased UCP1 expression in brown adipose tissue (Figures S11D and S11F), which may account for the lower respiratory quotient, decreased triglyceride content of skeletal muscle, and decrease of whole-body fat in Angptl2−/− mice. On the other hand, the hepatic expression of lipogenic genes (SREBP-1c, FAS, and SCD1) was significantly decreased in Angptl2−/− mice (Figure S11G), which explains the decreased triglyceride content in the liver of these mice, although further studies will be needed to clarify the molecular mechanisms involved.

In summary, this study provided evidence that Angptl2 plays a key role in inflammation of adipose tissue via inflammatory vascular remodeling and recruitment of macrophages into adipose tissue. These findings suggest that Angptl2 may be an important part of the mechanism underlying adipose tissue inflammation that is involved in the pathogenesis of systemic insulin resistance related to obesity. The present findings should also lead to new treatment strategies for obesity and related insulin resistance.

**EXPERIMENTAL PROCEDURES**

Materials and additional methods are available in the Supplemental Experimental Procedures.

**Animal Study**

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University. Only male mice were used for the experiments. For the metabolic analyses, mice at 8 weeks of age were fed either a normal diet (CE-2; CLEA, Japan) or a high-fat diet (HFD-32; CLEA) for a period of 8 weeks. During the analyses, mice continued to feed on the same diet.

**Human Studies**

White adipose tissue samples were obtained from the intact adipose tissue surrounding the tumor resected from a patient with pancreatic carcinoma. Samples were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Sections 5 μm thick were cut and stained with an anti-Angptl2 polyclonal antibody (#383). Nuclei were counterstained with hematoxylin. A total of 98 volunteers working at Kumamoto University were enrolled in the study as the healthy group (persons with obesity [body mass index > 30] or diabetes were excluded). Blood samples were collected, and the plasma glucose, insulin, and CRP levels were measured. A total of 89 patients with type 2 diabetes were enrolled as the DM group. Their abdominal fat content was evaluated by magnetic resonance imaging. The HOMA-R index was calculated as the product of fasting plasma insulin (μU/ml) and fasting plasma glucose (mg/dl) divided by 405 (Matthews et al., 1985). The euglycemic-hyperinsulineemic clamp test was carried out according to a protocol described elsewhere (DeFronzo et al., 1979). A total of 109 patients with coronary artery disease (diagnosed by coronary angiography) were enrolled as the CAD group, and blood samples were collected. Twenty-seven obese diabetic men who had not previously received any antidiabetic agents, antihypertensive agents, or lipid-lowering drugs were treated with pioglitazone at a dose of 30 mg/day for 3 months. Before and after treatment, the abdominal fat content was evaluated by CT scanning, and fasting blood samples were collected to measure the levels of glucose, insulin, and CRP. Blood samples were also collected from 935 consecutive volunteers aged 27–84 years, who underwent medical checkups at the Japanese Red Cross Kumamoto Health Care Center. Serum or plasma levels of Angptl2 and Angptl4 were measured by ELISA. This study was approved by the Ethics Committees of Kumamoto University (healthy and CAD groups), Kobe University (DM group), Ryukyu University (pioglitazone study), and the Japanese Red Cross Kumamoto Health Care Center. Written informed consent was obtained from each subject.

**SUPPLEMENTAL DATA**

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, 13 figures, one table, and four movies and can be found with this article online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00232-0.

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