Obesity-Induced Endothelial Dysfunction Is Prevented by Deficiency of P-Selectin Glycoprotein Ligand-1

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Obesity is epidemic in the United States and is associated with increased risk for cardiovascular complications (1). Excess visceral adipose tissue may be the primary driver of the vascular risk associated with obesity because visceral fat is strongly associated with a chronic, low-grade inflammatory state characterized by increased macrophage activity in adipose tissue (2,3). The mechanism(s) by which obesity promotes vascular disease is unclear, but endothelial dysfunction has been demonstrated even in children with obesity (4,5). Endothelial dysfunction due to impairment of nitric oxide (NO) activity represents an early stage of many cardiovascular diseases (6).

P-selectin glycoprotein ligand-1 (Psgl-1) is the primary leukocyte ligand for P-selectin (P-sel) and an important ligand for E-selectin (E-sel) (7). Psgl-1 deficiency has been shown to reduce leukocyte-endothelial interactions in obesity and to reduce macrophage accumulation in gonadal fat pads (8). The purpose of this study was to determine the effect of Psgl-1 deficiency on endothelial dysfunction associated with obesity.

RESEARCH DESIGN AND METHODS

Animals. Male C57BL/6j and Psgl-1+/− mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Psgl-1+/− were backcrossed to the C57BL/6j strain >16 generations before use in these experiments. Mice were fed a standard laboratory rodent diet (No. 5001, TestDiet, Richmond, IN) or a high-fat, high-sucrose diet (DIO diet; D12451, Research Diets Inc, New Brunswick, NJ) and tap water ad libitum in a temperature-controlled room with a 12:12-h light/dark cycle. HFD was given for 10 weeks, beginning at age 8 weeks. At age 18 weeks, blood pressure (BP) was measured in nonanesthetized mice by tail plethysmography using the BP-2000 Blood Pressure Analysis System (Visitech System, Apex, NC). All animal use protocols compiled with the Principle of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use of and Care of Animals.

Plasma measurements. Plasma samples were collected via ventricular puncture at time of euthanasia. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure plasma soluble P-selectin (sP-sel), soluble E-selectin (sE-sel), monocyte chemoattractant protein-1 (MCP-1), leptin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (Carlsbad, CA). Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with 100 ng RNA and 1 μL primer used per reaction. Results were analyzed using 7000 System SDS Software and the 2−ΔΔCt method (10) and were presented as fold-change of transcripts for target normalized to insulin resistance (HOMA-IR) index by HOMA-IR equation: [HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5] (9).

Real-time PCR. RNA from mouse mesenteric arteries was isolated using a QIAGEN RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). RNA from 100 mg mesenteric perivascular adipose tissue (mPVAT) surrounding mesenteric arteries was dissected in cold physiologic salt solution (KH2PO4, 1.18; NaHCO3, 25; glucose, 5.5; and EDTA, 0.026 (at pH 7.4). Surviving sections of mesenteric arteries were dissected in cold physiologic salt solution (PSS) containing (in mmol/L): NaCl, 120; KCl, 4.7; MgSO4, 1.18; CaCl2, 2.5; KH2PO4, 1.18; NaHCO3, 25; glucose, 5.5; and EDTA, 0.026 (at pH 7.4). Surrounding tissues and intravascular blood were removed. The vessels were frozen in optimal cutting temperature compound and transverse sections (10 μm) were produced using a cryostat. Sections were incubated in a 37°C incubator for 30 min with 2 μmol/L DHE (Invitrogen, Carlsbad, CA). Images were obtained with a Leica laser scanning confocal microscope with an

See accompanying commentary, p. 3070.
TABLE 1

|                  | Control  | Psgl-1+/+ | Psgl-1−/− | DIO  | Psgl-1+/+ | Psgl-1−/− |
|------------------|----------|-----------|-----------|------|-----------|-----------|
| Body weight (g)  | 28.6 ± 0.4 | 31.7 ± 3.0 | 44.6 ± 2.7* | 43.8 ± 3.2† |
| Blood glucose (mg/dL) | 110.9 ± 10.5 | 141.0 ± 21.9 | 270.2 ± 15.9* | 222.7 ± 14.7* |
| Plasma insulin (ng/mL) | 1.57 ± 0.3 | 1.13 ± 0.4 | 2.15 ± 0.3 | 2.0 ± 0.4 |
| HOMA-IR          | 0.47 ± 0.1 | 0.38 ± 0.1 | 5.85 ± 2.6* | 1.0 ± 0.2 |
| Plasma FFA (mEq/L) | 0.75 ± 0.1 | 0.71 ± 0.1 | 1.29 ± 0.1‡ | 0.86 ± 0.1 |
| Plasma adiponectin (µg/mL) | 34.75 ± 5.9 | 33.64 ± 6.6 | 36.16 ± 6.7 | 35.69 ± 3.9 |
| Gonadal fat (g)  | 0.39 ± 0.05 | 0.81 ± 0.23 | 2.30 ± 0.39* | 2.38 ± 0.17* |
| Perivascular fat (g) | 0.20 ± 0.02 | 0.25 ± 0.03 | 0.55 ± 0.06* | 0.56 ± 0.05* |
| Systolic BP (mmHg) | 121.0 ± 5.0 | 110.3 ± 7.5 | 112.0 ± 6.9 | 106.8 ± 2.5 |

*P < 0.01 compared with corresponding control. †P < 0.05 compared with corresponding control. ‡P < 0.01 compared with other groups.
FIG. 1. Circulating levels, expression of biomarkers, and macrophage content in control and DIO mice (*n* = 6–8 per group). A: Circulating levels of sP-sel, sE-sel, MCP-1, and leptin. B: Expression of IL-6, MCP-1, leptin, and Psgl-1 in mPVAT and Psgl-1 in mesenteric arteries (MA). C: Macrophage content in mPVAT. Arrows: Mac-3–positive macrophages. Scale bar: 100 μm. *P* < 0.01; **P* < 0.05. (A high-quality color representation of this figure is available in the online issue.)
cytokines, including IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), were tested but were below detection range. **Expression levels of IL-6, MCP-1, leptin, and Psgl-1.** To characterize the effect of DIO on mPVAT, the mRNA expression levels of IL-6, MCP-1, leptin, and Psgl-1 were assessed using real-time PCR. The expression levels of IL-6 and MCP-1 were significantly increased in DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice and were significantly reduced in DIO-Psgl-1+/+ mice compared with DIO-Psgl-1−/− mice (Fig. 1A). The leptin levels were significantly increased in DIO-Psgl-1+/+ and DIO-Psgl-1−/− mice compared with control groups (Fig. 1B). The levels of Psgl-1 in mPVAT and mesenteric arteries were significantly higher in DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice (Fig. 1B).

**Macrophage accumulation in perivascular adipose tissue.** To determine the effect of DIO and Psgl-1 deficiency on perivascular adipose tissue inflammation, macrophages were quantitated from fat surrounding mesenteric arteries using Mac-3 immunostaining (Fig. 1C). The macrophage content of mPVAT was increased in DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice, but the mPVAT macrophage content was significantly reduced in DIO-Psgl-1+/+ mice compared with DIO-Psgl-1−/− mice (Fig. 1F).

**Vascular superoxide and nitrotyrosine in mesenteric arteries.** As a measure of local oxidative stress, vascular superoxide was measured from cross-sections of mesenteric arteries using DHE staining (Fig. 2). Superoxide staining was significantly increased in mesenteric arteries of DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice; however, superoxide staining in DIO-Psgl-1−/− mice was reduced compared with DIO-Psgl-1+/+ mice and was not different between standard chow–fed Psgl-1+/+ or Psgl-1−/− mice.

Protein expression levels of nitrotyrosine were determined by immunoblotting. Nitrotyrosine protein expression in mesenteric arteries was increased in DIO-Psgl-1+/+ mice, but not in standard chow–fed Psgl-1+/+ mice and DIO-Psgl-1−/− mice (Fig. 2F). **Morphology and vascular function of mesenteric and carotid arteries.** The morphologic properties of mesenteric arteries were characterized in Ca2+-free conditions. No significant differences were detected in baseline lumen diameter, wall thickness, media-to-lumen ratio, and media cross-sectional area among the groups (Table 2).

NE-induced concentration-dependent contractile responses in mesenteric arteries were similar between Psgl-1+/+ mice and Psgl-1−/− mice fed both standard chow and HFD (Fig. 3A). Endothelium-independent vasorelaxation responses to SNP were also similar between the groups (Fig. 3B). Endothelium-dependent vasorelaxation was evaluated with Ach. Vasorelaxation responses to Ach were significantly reduced in DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice; however, DIO-Psgl-1−/− mice were protected against endothelial dysfunction with responses similar to standard chow–fed Psgl-1+/+ and Psgl-1−/− mice (Fig. 3C). Ach-induced vasorelaxation was inhibited in all groups after preincubation with L-NAME (Fig. 3D). The bioavailability of NO was determined by differences in Ach-induced maximal vasorelaxation in the presence and absence of L-NAME. NO-mediated relaxation was significantly reduced in DIO-Psgl-1−/− mice compared with standard chow–fed Psgl-1+/+ mice, whereas
NO-mediated relaxation in DIO Psgl-1−/− mice was not significantly different than in standard chow–fed Psgl-1−/− or Psgl-1+/+ mice (Fig. 3E).

To determine the role of superoxide in mediating endothelial dysfunction induced by DIO, vessels were incubated with the superoxide scavenger, TEMPO, for 30 min. Ach-induced vasorelaxation was significantly improved by TEMPO in DIO Psgl-1+/+ mice compared with vessels without TEMPO treatment (Fig. 3F). Endothelium-independent vasorelaxation responses to SNP were similar between the groups after TEMPO treatment (Fig. 3G). After treatment of TEMPO, superoxide staining by DHE in vessels of DIO Psgl-1+/+ mice was reduced compared with vessels without TEMPO treatment (Fig. 3H).

To assess the potential effect of systemic inflammation induced by DIO on carotid arteries, the pressure myograph was used to test vascular function on carotid arteries from standard chow–fed and DIO Psgl-1−/− mice. Phenylephrine-induced concentration-dependent contractile responses (Fig. 4A), and Ach-induced concentration-dependent relaxation responses (Fig. 4B) were similar between the groups.

**Direct local effect of mPVAT on vascular function.** To determine the local effect of mPVAT on vascular function, the mesenteric arteries from standard chow–fed Psgl-1−/− mice were incubated in mPVAT from DIO Psgl-1−/− mice or DIO Psgl-1−/− mice in vitro. After incubation in mPVAT from DIO Psgl-1−/− mice, the endothelial-dependent vasorelaxation responses to Ach were significantly impaired compared with responses after incubation in mPVAT from DIO Psgl-1−/− mice (Fig. 5F). Ach-induced vasorelaxation was inhibited in both groups after preincubation with l-NAME (Fig. 5C). Vasoconstriction responses to NE and endothelial-independent vasorelaxation responses to SNP were similar between the groups (Fig. 5A and D).

**Psgl-1 neutralization is protective against obesity-induced macrophage accumulation in perivascular adipose tissue and endothelial dysfunction.** To test the potential therapeutic strategy of Psgl-1 blockade, 5 weekly injections of a Psgl-1–blocking antibody or control isotype were given to DIO Psgl-1+/+ mice. After this regimen, the macrophage content of mPVAT was significantly reduced in DIO Psgl-1+/+ mice that received the antibody compared with control isotype-injected DIO Psgl-1+/+ mice (Fig. 6D). This reduced perivascular adipose tissue macrophage content did not affect NE-induced vasoconstriction responses (Fig. 6A) but did improve Ach-induced vasorelaxation (Fig. 6B). The endothelial-independent responses to SNP were similar between the groups (Fig. 6C).

**DISCUSSION**

Obesity is an inflammatory disease (11) and a risk factor for cardiovascular diseases (12,13). One of the earliest detectable vascular abnormalities associated with obesity is impaired vascular relaxation (4). Impaired vascular function has been shown to be predictive of later cardiovascular complications (14). This early endothelial dysfunction is associated with circulating markers of inflammation, suggesting interplay between leukocyte activity and endothelial properties (15). Currently, weight loss and physical activity are the most effective means to prevent or reduce these vascular abnormalities (16); however, sustained weight loss is difficult to achieve, so treatments designed to prevent the vascular effects of obesity are needed.

One of the mechanisms responsible for endothelial dysfunction in obesity may be accumulation of perivascular fat (17). Epicardial fat that overlies the heart and coronary arteries, as well as perivascular fat surrounding large arteries, are associated with vascular lesions (18–20) and may be causally related to local and systemic atherosclerosis. For example, transplantation of visceral fat to carotid arteries of atherosclerotic-prone mice triggered formation of a local atherosclerotic lesion, whereas transplantation of subcutaneous fat did not (21). An interaction between the visceral or perivascular adipocyte and infiltrating macrophages may be contributing to the adverse vascular effects of excess visceral adiposity.

Adipose tissue macrophage content increases in obesity, and this corresponds to increased expression of adipose tissue–related inflammatory cytokines (11). Inflammatory adipose tissue is preceded by increased leukocyte–endothelial interactions within the visceral fat depots (8,22). Adhesion molecules, including selectins, were up-regulated in obese adipose tissue and participated in leukocyte recruitment (22). Mice deficient in Psgl-1 had reduced visceral adipose tissue macrophage content in the setting of obesity (8). Leukocyte Psgl-1 deficiency may therefore protect against the adverse vascular consequences of excess visceral adiposity.

Because endothelial dysfunction represents an early manifestation of vascular disease, we tested whether Psgl-1 deficiency would be protective against endothelial dysfunction in the setting of obesity. In this research, we studied mesenteric arteries because they are particularly amenable to analyses with arterial pressure myography. These arteries also became surrounded by visceral adipose tissue in the setting of obesity and are prone to atherosclerosis (23). This model thus allows us to study inflammatory characteristics of endogenous visceral adipose tissue and endothelial function involving adjacent arteries.

We used a 10-week DIO protocol because mice gain considerable weight and develop clear evidence of adipose tissue inflammation at this time point (8). Compared with lean mice, DIO mice in this study developed marked impairment of endothelial function. To perform this measure,
FIG. 3. Vasoconstriction and vasorelaxation responses of mesenteric arteries (MA) and DHE fluorescence of MA after treatment with TEMPOL. Control, DIO Psgl-1<sup>+/+</sup>, and Psgl-1<sup>-/-</sup> mice (n = 5–7 per group). A: Concentration response to NE. B: Concentration response to SNP. C: Concentration response to Ach. D: Concentration response to Ach after preincubation in L-NAME. E: L-NAME–sensitive maximal vasorelaxation responses to Ach. F: Concentration response to Ach of MA from DIO Psgl-1<sup>+/+</sup> after treatment with TEMPOL. G: Concentration response to SNP of MA from DIO Psgl-1<sup>+/+</sup> after treatment with TEMPOL. H: DHE fluorescence of MA from DIO Psgl-1<sup>+/+</sup> without (left) and with (right) TEMPOL treatment. Scale bar: 50 μm. *P < 0.05 compared with DIO Psgl-1<sup>-/-</sup> mice, control Psgl-1<sup>-/-</sup> mice, and control Psgl-1<sup>+/+</sup> mice. #P < 0.05 compared with vessels without TEMPOL treatment. (A high-quality color representation of this figure is available in the online issue.)
it was necessary to clear the tissue surrounding the vessel, including the adipose tissue. Thus, the dysfunction observed does not require the immediate presence of the perivascular adipose tissue and likely reflects a chronic effect of the adipose tissue on the endothelium. Anatomic measurements of the mesenteric arteries harvested from the obese and lean mice were similar; however, more macrophages were present in the perivascular adipose tissue and adjacent arteries from the DIO mice. This was associated with increased vascular superoxide and nitrotyrosine protein expression supporting the hypothesis that local excessive oxidative stress in the vessel wall leads to reduced endothelial NO bioavailability. The current study showed that the expression levels of inflammatory biomarkers are significantly elevated in mesenteric perivascular fat from DIO Psgl-1+/+ mice. The carotid arteries,

![Graph A](image1)

**FIG. 4.** Vasoconstriction and vasorelaxation responses of carotid arteries from control Psgl-1+/+ and DIO Psgl-1+/+ mice (n = 4 per group). A: Concentration response to phenylephrine (PE). B: Concentration response to Ach.

![Graph B](image2)

**FIG. 5.** Vasoconstriction and vasorelaxation responses of mesenteric arteries (MA) from standard chow-fed Psgl-1+/+ mice after incubation in mPVAT from DIO Psgl-1+/+ mice or DIO Psgl-1+/− mice. A: Concentration response to NE. B: Concentration response to Ach. C: Concentration response to Ach after preincubation in l-NAME. D: Concentration response to SNP. *P < 0.01 compared with MA incubated in mPVAT from DIO Psgl-1+/+ mice.
which lack robust perivascular fat pads, did not show endothelial dysfunction, suggesting that local perivascular fat from DIO mice contributes more to vascular dysfunction than systemic effects in obesity.

Because we have previously shown that Psgl-1 deficiency leads to reduced adipose tissue inflammation in the setting of obesity (8) we tested the effect of Psgl-1 deficiency on endothelial dysfunction induced by obesity. As expected, Psgl-1 deficiency in DIO mice led to reduced accumulation of macrophages within the perivascular mesenteric adipose tissue. This was associated with reduced vessel wall superoxide content and nitrotyrosine. Previous research has demonstrated that excessive reactive oxygen species (ROS) are important in vascular dysfunction in different animal models of metabolic syndrome (24–26), and ROS are produced by multiple cell types, including endothelial cells, smooth muscle cells, and infiltrating inflammatory cells in the vasculature (25). NADPH oxidases and the mitochondria respiratory chain are major source of ROS in the vasculature (26,27).

The responsible cellular and enzymatic source of vascular superoxide in our study remains to be determined. The vessel wall superoxide appeared to be causally related to the endothelial dysfunction because the obesity-induced endothelial dysfunction was improved after treatment with the superoxide scavenger, TEMPOL. However, because TEMPOL only partially recovered endothelial dysfunction induced by the HFD, other factors may contribute to reduced NO bioavailability. Future in vivo experiments with TEMPOL or other antioxidants might be useful to reveal the relative importance of this mechanism.

Importantly, Psgl-1 deficiency prevented the endothelial dysfunction induced by DIO, presumably due to reduced perivascular adipose tissue inflammation and vessel wall oxidative stress. A supernatant prepared from obese homogenized fat was sufficient to induce endothelial dysfunction, and this effect was not present when the supernatant was prepared from obese Psgl-1-deficient fat. Although this fat supernatant experiment may not be reflective of the in vivo setting, because cells are homogenized, it supports the concept that an inflammatory factor derived from fat triggers local endothelial dysfunction and that this is reduced in the setting of Psgl-1 deficiency. Previous research reported that supernatants from inflammatory fat showed strong chemoattractant effects on leukocytes in vitro (28) and that several fat-derived inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are associated with increased NADPH oxidase expression and endothelial dysfunction (27,29–31). Identification of the specific downstream factor responsible for the Psgl-1 effect in our model will require further studies.

To determine whether Psgl-1 inhibition might be effective therapy for endothelial dysfunction associated with obesity, DIO Psgl-1+/− mice were treated with weekly injections of an antibody that blocks interactions between selectins and Psgl-1. Antibody injections were initiated at week 5, in the middle of the 10-week DIO protocol, to determine if Psgl-1 could prevent endothelial dysfunction while mice were in the process of gaining weight. Psgl-1 blockade in this setting was effective in reducing adipose tissue inflammation, endothelial dysfunction, and vascular superoxide production.
In the present study, we found that the HOMA-IR index was significantly increased in DIO Psgl-1+/− mice compared with lean control mice, as expected. In DIO Psgl-1−/− mice, HOMA-IR showed a trend toward protection, although it was not significant compared with DIO Psgl-1+/− mice. FFA levels were also significantly decreased in DIO Psgl-1−/− mice compared with DIO Psgl-1+/− mice, suggesting that Psgl-1 deficiency may correct other metabolic abnormalities in the setting of obesity. The underlying mechanisms responsible for this effect will also require additional study.

In conclusion, Psgl-1 deficiency is protective against obesity-induced endothelial dysfunction. Therapies designed to inhibit Psgl-1 binding to selectins may be useful to reduce preclinical vascular abnormalities associated with obesity.

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