Increasing Circulating IGFBP1 Levels Improves Insulin Sensitivity, Promotes Nitric Oxide Production, Lowers Blood Pressure, and Protects Against Atherosclerosis

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Low concentrations of insulin-like growth factor (IGF) binding protein-1 (IGFBP1) are associated with insulin resistance, diabetes, and cardiovascular disease. We investigated whether increasing IGFBP1 levels can prevent the development of these disorders. Metabolic and vascular phenotype were examined in response to human IGFBP1 overexpression in mice with diet-induced obesity, mice heterozygous for deletion of insulin receptors (IR+/−), and ApoE−/− mice. Direct effects of human (h) IGFBP1 on nitric oxide (NO) generation and cellular signaling were studied in isolated vessels and in human endothelial cells. IGFBP1 circulating levels were markedly suppressed in dietary-obese mice. Overexpression of hIGFBP1 in obese mice reduced blood pressure, improved insulin sensitivity, and increased insulin-stimulated NO generation. In nonobese IR+/− mice, overexpression of hIGFBP1 reduced blood pressure and improved insulin-stimulated NO generation. hIGFBP1 induced vasodilation independently of IGF and increased endothelial NO synthase (eNOS) activity in arterial segments ex vivo, while in endothelial cells, hIGFBP1 increased eNOS Ser1177 phosphorylation via phosphatidylinositol 3-kinase signaling. Finally, in ApoE−/− mice, overexpression of hIGFBP1 reduced atherosclerosis. These favorable effects of hIGFBP1 on insulin sensitivity, blood pressure, NO production, and atherosclerosis suggest that increasing IGFBP1 concentration may be a novel approach to prevent cardiovascular disease in the setting of insulin resistance and diabetes.

Insulin resistance, obesity, and type 2 diabetes are associated with reduced bioavailability of endothelial nitric oxide (NO) and predisposition to atherosclerosis. Cardiovascular disease develops ∼15 years earlier in the presence of diabetes and accounts for the majority of deaths in these individuals (1). The current epidemic of diabetes (2) and the predicted increase in diabetes prevalence by the year 2030 (3) represent major challenges to global healthcare resources. Disappointing results from recent clinical trials investigating a strategy of intensive blood glucose control to reduce cardiovascular events argue for novel approaches to reduce cardiovascular risk in individuals with diabetes (4).

Insulin-like growth factor (IGF) binding proteins (IGFBPs) comprise a family of proteins that modulate IGF bioactivity through high-affinity binding (5). IGFBP1 is a 30 kDa circulating protein expressed predominantly in the liver that has been implicated in reproductive physiology and metabolic homeostasis (5–7). Both inhibitory and stimulatory effects of IGFBP1 on IGF bioactivity are recognized. In addition, in certain cell types, IGFBP1 regulates cellular actions independently of IGFs (8). Specifically, IGFBP1 modulates cell migration independently of IGF via an interaction of the Arg-Gly-Asp sequence in its COOH-terminal domain with cell surface integrin receptors (9–12). Under physiological conditions, insulin-mediated suppression of hepatic IGFBP1 production in response to nutritional cues confers dynamic regulation of IGF bioavailability (13). IGFBP1 has therefore been proposed as a player in glucose counterregulation and metabolic homeostasis (14,15). In cross-sectional studies, circulating IGFBP1 concentrations correlate with insulin sensitivity (16–22), while in longitudinal studies, low circulating IGFBP1 concentrations predict the development of glucose intolerance and diabetes (23,24). In addition to these data supporting a role of IGFBP1 in metabolism, accumulating evidence suggests that IGFBP1 may contribute to cardiovascular pathophysiology. First, IGFBP1 concentrations are inversely associated with cardiovascular risk (16), carotid artery intima thickening (25), and overt macrovascular disease (26) in cross-sectional studies in humans. Second, we previously reported that transgenic (tg) overexpression of human IGFBP1 in mice was associated with increased vascular NO generation and lower blood pressure (27). Therefore, we hypothesized that low IGFBP1 levels may be permissive for the development of both metabolic diseases and related cardiovascular complications and that IGFBP1 could be a target for therapeutic manipulation.

Here, we investigated whether increasing IGFBP1 in mice subjected to obesity, insulin resistance, or atherosclerosis could ameliorate the development of overt metabolic and cardiovascular disease.

RESEARCH DESIGN AND METHODS

Mice and diet. The tg mice overexpressing human (h)IGFBP1 (GenBank Accession Number NM_000596.2; transgene copy number ∼20) and generated as previously described (28) were backcrossed for a minimum of six generations onto a C57BL/6 background. Heterozygous hIGFBP1tg mice were

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Diabetes. Diabetes Journals.org Diabetes Publish Ahead of Print, published online February 22, 2012

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used for experiments. Sequence similarity between human and murine IGFBP1 is 66%. However, because the NH2- and COOH-terminal domains of IGFBP1 (which participate in interactions with IGFs) remain highly conserved between species, hIGFBP1 is fully compatible with the murine-IGF axis (28). IR+/− mice were bred from a colony originally obtained from the Medical Research Council (Harwell, U.K.). IGF1R−/− mice were from Columbia University (New York, NY) and were crossed with Tie2-Cre tg mice (The Jackson Laboratory) for two to six generations to generate mice homozygous for the Tie-2-Cre transgene. ApoE−/− mice were from a colony at our institution originally purchased from The Jackson Laboratory. Mice were housed in a conventional animal facility (12-h light/dark cycle) at constant temperature (22°C). Genotype was determined by PCR of ear samples. All mice were established on a C57BL/6 background for at least six generations. To avoid confounding by alterations in sex hormones, experiments were performed exclusively in male mice with littermates used as controls.

To induce obesity, mice received a high-fat/high-energy diet (F3282; Bio-Serv, Frenchtown, NJ) with the following composition: protein 20.5%, fat 36%, ash 3.5%, carbohydrate 35.7%, moisture <10%, and energy 5.49 kcal/g (60% of energy from fat). Control mice received standard rodent chow (BK001; Special Diets Services, Witham, Essex, U.K.) composed of protein 19.64%, fat 7.52%, ash 6.21%, moisture 10%, fiber 3.49%, nitrogen-free extract 54.90%, and energy 3.29 kcal/g. In accordance with our previous observation that diet F3282 rapidly induces an adverse cardiometabolic phenotype in mice (29), the diet was administered ad libitum from weaning for 8 weeks. Food intake was estimated by weighing food on a daily basis during the first 2 weeks of the feeding period. To induce atherosclerotic plaques, ApoE−/− mice received Western diet containing 21% pork fat and supplemented with 0.15% cholesterol (Special Diets Services). Animal experiments were approved by the ethical review committee at the University of Leeds and were performed in accordance with the Animals (Scientific Procedures) Act 1986.

Metabolic studies and plasma assays. In glucose tolerance tests, mice were fasted overnight before injection with 1 mg/kg glucose i.p. For insulin tolerance tests, mice were fasted for 4 h before injection with 0.75 units/kg recombinant human insulin i.p. (Actrapid; Novo Nordisk). Blood glucose was measured with a portable glucometer. Plasma insulin was quantified by an ultrasensitive mouse ELISA kit (Crystal Chem, Downers Grove, IL). IGFBP1 was measured by an ELISA kit (Diagnostic Systems Laboratories, Webster, TX) that is unaffected by phosphorylation status and does not cross-react with other binding proteins, and IGF-I was quantified by a mouse/rat Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Lipoprotein analyses were carried out in terminal blood samples using an automated analyzer in the Department of Clinical Chemistry, Leeds Teaching Hospitals National Health Service Trust, U.K.

Blood pressure measurement. Blood pressure was measured by tail volume-pressure recording (CODA2; Kent Scientific, Torrington, CT) in conscious animals in a temperature-controlled restrainer as previously described (27,30,31). Animals were acclimatized to the restrainer during six training sessions before blood pressure measurement.

Obesity analysis. Body mass was measured after 4 and 8 weeks of feeding high-fat diet. Epididymal fat pads were weighed as a conventional measure of obesity in rodents. Samples of epidymal adipose tissue were fixed in 10% formalin and embedded in paraffin. Adipocyte area was calculated by hematoxylin-eosin stained sections as previously described (32).

Vasomotor studies. Segments of thoracic aorta were suspended in physiological salt solution from strain gauge in an eight-chamber organ bath at 37°C as described (27,30,31). Dose-response curves were constructed (Chart V5.6, ADInstruments) for cumulative addition of phenylephrine after preincubation with insulin (100 mU/mL Actrapid; Novo Nordisk), hIGFBP1 (500 ng/mL with 1 mg/mL BSA carrier protein or BSA alone; Novozymes Biopharma AU Limited, Thebarton, Australia), IGF-I (100 nmol/L), Nω-monomethyl-L-arginine (L-NMMA; 0.1 mmol/L), or LY294002 (10 μmol/L).

FIG. 1. Diet-induced obesity and IGFBP1 expression and circulating plasma concentrations in C57BL/6 mice fed high-fat diet or chow (n = 8 per group). Hepatic murine IGFBP1 expression was quantified by real-time RT-PCR (A), and plasma concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (B). *P < 0.02 vs. chow. C and D: Plasma IGFBP1 and IGF-I concentrations, measured using ELISA, in WT and hIGFBP1tg (TG) diet-induced obese mice (n = 8 per group). **P = 0.01 vs. WT. E: Body mass of WT and TG mice during induction of dietary obesity. F–I: Representative sections of epididymal fat pads (magnification ×10) and distribution of adipocyte cross-sectional area in obese WT (F and H) and TG (G and I) mice (n = 8 per group). (A high-quality digital representation of this figure is available in the online issue.)
Cell culture. Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) (PromoCell) were cultured in M199 medium supplemented with 20% FCS, 15 μg/mL endothelial cell growth factor, 1% L-glutamine, 20 mmol/L HEPES, 1 μmol/L sodium pyruvate, 5 units/mL heparin, 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin and maintained in a humidified incubator with 5% CO2 at 37°C.

Immunoblotting and immunofluorescence microscopy. Protein expression and phosphorylation status were analyzed in cell lysates or homogenized aorta by immunoblotting using the following antibodies: endothelial NO synthase (eNOS), Ser1177 p-eNOS (BD Biosciences), Akt, Ser473 p-Akt, GSK-3α, Ser9 p-GSK-3α, and β-actin (Santa Cruz Biotechnology). All primary antibodies were used at 1:1,000 dilution with the exception of β-actin at 1:3,000. Horseradish peroxidase-conjugated secondary antibodies were visualized using an enhanced chemiluminescence kit (Pierce). For immunofluorescence, intact cells were fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, blocked with fish-skin gelatin, and probed with primary anti-Ser1177 phospho-eNOS antibodies, using anti-mouse IgG (Invitrogen) for immunofluorescence detection. Slides were imaged under ×100 magnification (Olympus, Hamburg, Germany). Fluorescence intensity was estimated using CellB software (Version 3.0, Olympus Soft Imaging Solutions, Hamburg, Germany) by placing regions of interest within the cytoplasm of ~100 cells per high-power field. Exposure times and number of cells counted were standardized across experiments.

IGFBP1 mRNA expression. A total of 0.5 μCi/mL 3H-arginine was added for 5 min to quiescent washed cells bathed in 0.25% HEPES-BSA, followed by hIGFBP (500 ng/mL) for 30 min. Chilled 5 mmol/l-arginine per 4 mmol/L EDTA was used to stop the reaction, and cells were trypsinized, alcohol denatured, and centrifuged. Cell pellets were dissolved in 20 mmol/L HEPES-Na (pH 5.5) and soluble component separated by centrifugation. Equilibrated DOWEX resin was used to separate Arg from citrulline, with the latter then quantified by liquid-scintillation counting and normalized to total cell protein content.

RESULTS

Increasing hIGFBP1 concentrations improves insulin sensitivity in obesity. To investigate whether increasing hIGFBP1 levels can reverse the metabolic and vascular sequelae of obesity, we fed an obesogenic diet to mice overexpressing hIGFBP1. In control mice, obesity was associated with suppression of hepatic IGFBP1 mRNA expression (Fig. 1A) and a marked reduction in total circulating IGFBP1 (Fig. 1B). Glucose and insulin tolerance test: obese hIGFBP1tg mice had lower fasting glucose concentrations than obese WT mice (6.3 ± 0.3 vs. 7.6 ± 0.6 mmol/L, P = 0.02) and were more glucose tolerant. Area under the curve: 1287 ± 28 mmol/L per 120 min in obese WT mice (n = 8 per group). *P = 0.02. B: Insulin tolerance test: obese TG animals were more insulin sensitive than obese WT animals. Area under the curve: 753 ± 28 mmol/L per 120 min in obese TG vs. 926 ± 31 mmol/L per 120 min in obese WT mice (n = 8 per group). *P = 0.01. C: Plasma insulin concentrations in fasting animals and repeated 30 min after injection of glucose (1 mg/kg i.p.) (n = 8 per group). D–F: Isometric tension studies in thoracic aorta from obese WT and TG mice (n = 8 per group). Phenyloenphrine (PE)–induced constriction was unaltered by insulin preincubation in WT mice (D) but was attenuated by insulin preincubation in TG mice (E), *P = 0.01 vs. WT two-way ANOVA. F: Relaxation of preconstricted aorta to acetylcholine (Ach) was unaltered. G: Ser phosphorylation of eNOS was quantified by immunoblotting of aorta harvested from diet-induced obese mice 15 min after injection of insulin (0.75 IU/kg i.p.) or vehicle. Ser1177 phospho-eNOS expression was normalized to expression of total eNOS and β-actin. *P < 0.05 for insulin-induced eNOS phosphorylation in TG mice vs. WT.
IGFBP1 concentration (Fig. 1B). As previously published, tg overexpression of hIGFBP1 in chow-fed animals results in a fivefold increase in circulating IGFBP1 concentration (28). In obesity, we found that tg overexpression of hIGFBP1 similarly increased plasma IGFBP1 concentration (Fig. 1C) without altering total plasma IGF-I (Fig. 1D). hIGFBP1tg mice developed a similar increase in body mass compared with wild-type (WT) littermates (Fig. 1E). Because constitutive overexpression of IGFBP1 has been reported to inhibit adipocyte expansion (33), we measured adipocyte diameter in epididymal fat depots after induction of obesity. In our mice, in which hIGFBP1 is expressed under the control of its native promoter and regulatory sequences, we found no effect on epididymal fat pad mass (28 ± 9 in WT vs. 34 ± 2 mg in tg mice; P = 0.47) or adipocyte cross-sectional area (Fig. 1F–I). Food intake was similar in hIGFBP1 mice and WT controls receiving the obesogenic diet (2.54 ± 0.07 vs. 2.57 ± 0.12 g/day; P = 0.85). hIGFBP1tg mice were at least partially protected from the metabolic consequences of obesity. Diet-induced obesity increased fasting blood glucose and induced glucose intolerance in WT animals; these effects were ameliorated in hIGFBP1tg mice (Fig. 2A). Insulin sensitivity, as indicated by an insulin tolerance test, was significantly enhanced in hIGFBP1tg animals (Fig. 2C). Plasma insulin concentrations were not significantly altered by IGFBP1 overexpression (Fig. 2C).

Increasing hIGFBP1 concentrations enhances vascular NO production and lowers blood pressure in obesity. We next tested whether favorable effects on vascular function accompanied the protective effect of hIGFBP1 on glucose regulation in obesity. Impaired insulin-mediated NO production (34) is implicated in insulin resistance–associated vascular dysfunction. In nonobese mice, insulin induces a hypocontractile response to phenylephrine in aorta by enhancing NO generation (30,31). This effect was absent

|                  | Systolic blood pressure (mmHg) | hIGFBP overexpression | P value |
|------------------|--------------------------------|------------------------|---------|
| Chow diet        | 103 ± 3                         | 102 ± 3                | 0.58    |
| Nutritional obesity | 122 ± 2                       | 108 ± 5               | 0.03    |
| IR<sup>+/−</sup>  | 120 ± 3                         | 105 ± 4               | 0.01    |

Data are mean ± SEM unless noted otherwise. Systolic blood pressure was measured by tail-cuff plethysmography in control and hIGFBP1tg mice (n = 6–7 per group).

FIG. 3. hIGFBP1 overexpression lowers blood pressure and enhances vascular insulin sensitivity in hemizygous insulin receptor null mice. A and B: Glucose and insulin tolerance tests. Blood glucose was measured at intervals after injection of glucose (1 mg/g i.p.) or insulin (0.75 IU/kg i.p.) in mice hemizygous for deletion of insulin receptors (IR<sup>+/−</sup>) and littermate IR<sup>+</sup> mice with tg overexpression of hIGFBP1 (IR<sup>+/−</sup> hIGFBP1tg) (n = 6–8 per group). There was no difference in the response to glucose tolerance test (area under the curve: 993 ± 42 vs. 840 ± 39 mmol/min/L) or insulin tolerance test (area under the curve: 513 ± 21 vs. 503 ± 18 mmol/min/L) between IR<sup>+/−</sup> and IR<sup>+/−</sup> hIGFBP1tg mice. C and D: Ex vivo isometric tension studies in thoracic aorta from IR<sup>+/−</sup> and IR<sup>+/−</sup> hIGFBP1tg mice (n = 6–8 per group). Phenylephrine (PE)-induced constriction was unaltered by insulin preincubation in IR<sup>+</sup> mice (C) but was attenuated by insulin preincubation in IR<sup>+/−</sup> hIGFBP1tg mice (D). *P = 0.01 vs. IR<sup>+</sup> by two-way ANOVA.
aorta harvested from obese mice (Fig. 2D), consistent with vascular insulin resistance. Overexpression of hIGFBP1 restored hypocontractility to insulin in vessels from obese mice (Fig. 2E), indicating increased insulin-induced NO production in tg animals. Vasodilation of aorta to acetylcholine (Fig. 2F) or sodium nitroprusside (not shown) was unchanged. Insulin stimulates NO production via phosphorylation of eNOS at Ser residues (35). Aortic Ser1177 eNOS phosphorylation after insulin injection was significantly upregulated in hIGFBP1tg animals (Fig. 2G). hIGFBP1 overexpression ameliorated the increase in systolic blood pressure observed in WT obese animals (Table 1). Increasing hIGFBP1 concentrations enhances vascular NO production and lowers blood pressure in insulin resistance.

We next examined whether increasing hIGFBP1 improves vascular function in a nonobese model of insulin resistance. We crossed hIGFBP1tg mice with mice heterozygous for deletion of the insulin receptor (IR+/2). IR+/2 mice have increased blood pressure and endothelial dysfunction despite maintaining normal glucose regulation through increased insulin secretion (31). Glucose tolerance and insulin sensitivity were unchanged when hIGFBP1 was overexpressed in IR+/2 mice (Fig. 3A and B), but systolic blood pressure was increased (120 ± 3 vs. 102 ± 4 mmHg; P = 0.01). Overexpression of hIGFBP1 protected IR+/2 mice from increased blood pressure (Table 1). Insulin did not alter the contractile response of aorta from IR+/2 mice (Fig. 3C), in keeping with vascular insulin resistance. Overexpression of hIGFBP1 restored insulin-mediated hypocontractility (Fig. 3D), indicating that IGFBP1 reverses vascular insulin resistance. Vasodilatation to acetylcholine or sodium nitroprusside (data not shown) was unchanged by hIGFBP1 overexpression.

hIGFBP1 increases endothelial NO production independently of IGF-I by stimulating eNOS phosphorylation via the phosphatidylinositol 3-kinase pathway. To determine whether these phenotypes resulted from a direct effect of hIGFBP1, we carried out experiments in isolated segments of WT mouse aorta. Incubation of aorta with hIGFBP1 reduced contractility to phenylephrine (Fig. 4A). This was abolished by endothelial denudation (Fig. 4B) and coincubation with L-NMMA (Fig. 4C), indicating that hIGFBP1 stimulates endothelial NO generation. Coincubation with LY294022, an inhibitor of phosphatidylinositol 3-kinase (PI3K), abolished this response (Fig. 4D), indicating that PI3K activation is required for hIGFBP1-induced NO production. These effects were observed in a serum-free preparation, suggesting that hIGFBP1 stimulates vascular NO production independently of IGFs. To exclude a contribution from IGF contamination or local IGF secretion, we repeated our experiments in mice with endothelial-specific deletion of type 1 IGF receptors generated by crossing IGF1Rflxed mice (36,37) with Tie2-Cre tg mice (36). As expected, aortas from these animals did not vasodilate in response to IGF-I (Fig. 4E). However, hIGFBP1 did reduce contractility to phenylephrine (Fig. 4F), reaffirming that hIGFBP1-induced NO generation is not mediated by type 1 IGF receptors.

**FIG. 4.** hIGFBP1 induces vasodilatation by increasing NO bioavailability in isolated vessels. A–D: Ex vivo isometric tension studies in thoracic aorta from WT mice (n = 6–8 per group). In A, phenylephrine (PE)-induced constriction was attenuated by preincubation with hIGFBP1 (500 ng/mL 2 h). *P = 0.02 vs. vehicle control containing 1 mg/mL BSA. This vasodilatory effect was blocked by mechanical removal of the endothelium (B) and by coincubation with the NO synthase inhibitor L-NMMA (0.1 mmol/L) (C) or the PI3K inhibitor LY294002 (10 μmol/L) (D). E and F: Isometric tension studies were repeated in aorta from mice with deletion of type 1 IGF receptors in endothelial cells (generated by crossing IGF1Rflxed mice with Tie2-Cre tg mice) (n = 6 per group). PE-induced constriction was unaltered by incubation with IGF-I (100 nmol/L) (E) but was attenuated by incubation with hIGFBP1 (500 ng/mL) (F). *P = 0.01.
To confirm a direct effect of hIGFBP1 on endothelial NO production, we undertook experiments in primary human endothelial cells. hIGFBP1 induced concentration- and time-dependent eNOS Ser1177 phosphorylation in HCAECs (Fig. 5A–D). hIGFBP1 similarly stimulated dose-dependent Ser1177 phosphorylation of Akt and Ser473 phosphorylation of its downstream substrate GSK-3β (Fig. 5E and F) but had no effect on total levels of expression of eNOS, Akt, or GSK-3β. hIGFBP1 induced Ser1177 eNOS phosphorylation in HUVECs (Fig. 5G–I), an effect blocked by coincubation with LY294022 (Fig. 5J). To confirm that the changes in eNOS phosphorylation resulted in changes in NO production, we demonstrated that hIGFBP1 stimulated LY294022-inhibitable eNOS activity in HUVECs (Fig. 5K).

**IGFBP1 overexpression reduces atherosclerosis.** Finally, we hypothesized that increasing IGFBP1 levels would result in protection from atherosclerosis. To address this, we crossed hIGFBP1tg mice with ApoE2/2 mice. After feeding a Western-type diet for 12 weeks, plaque burden was significantly reduced in ApoE2/2/IGFBP1tg mice, both in en face preparations of aorta (Fig. 6A and B) and in aortic sinus sections (Fig. 6C and D). Overexpression of hIGFBP1 did not affect plaque complexity as measured by analysis of necrotic core area (33.8 ± 3.7% in ApoE2/2 vs. 29.1 ± 3.2% in ApoE2/2/IGFBP1tg; P = 0.32) or fibrous cap thickness (36 ± 4 μm in ApoE2/2 vs. 28 ± 4 μm in ApoE2/2/IGFBP1tg; P = 0.17). There were no significant differences in plasma lipoprotein concentrations between ApoE2/2/IGFBP1tg mice and ApoE2/2 mice (Fig. 6E).

To ascertain whether IGFBP1 is expressed in the vascular wall, we carried out real-time PCR to assess expression of IGFBP1 in atherosclerotic aorta. Murine IGFBP1 expression was detectable at low levels in the aorta of ApoE2/2 and ApoE2/2/IGFBP1tg mice (Fig. 6F). hIGFBP1 was detectable only in aorta from hIGFBP1tg mice (Fig. 6F), as expected.

**DISCUSSION**

During the past 2 decades, a series of epidemiological studies has linked low circulating IGFBP1 concentrations with insulin resistance, type 2 diabetes, and cardiovascular disease. We previously demonstrated that hIGFBP1 overexpression increased vascular NO generation in mice (27), suggesting that low IGFBP1 concentrations may play a mechanistic role in vascular pathophysiology. However, whether the effects of IGFBP1 on the vascular endothelium were direct or indirect, and whether increasing IGFBP1 concentrations could be of therapeutic potential in preventing disease,

A

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C

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FIG. 5. hIGFBP1 increases eNOS activity in human endothelial cells by stimulating eNOS phosphorylation via a PI3K/Akt-mediated signaling pathway. A–C: eNOS phosphorylation in HCAECs was assessed by immunoblotting (β-actin is shown as a loading control). hIGFBP1 induced dose-dependent (A) and time-dependent (B) Ser1177 eNOS phosphorylation, which was partially blocked by the PI3K inhibitor wortmannin (C). D–F: Mean data for hIGFBP1-induced dose-dependent phosphorylation of eNOS, Akt, and GSK-3β, expressed as a ratio of the phosphorylated to nonphosphorylated form, each normalized for β-actin. *P < 0.03; **P < 0.05 vs. control. G–J: hIGFBP1-induced eNOS phosphorylation was confirmed in HUVECs with fluorescence microscopy using anti–phospho-Ser1177-eNOS antibodies. Representative untreated cells are shown in G, with hIGFBP1-stimulated cells shown in H. Mean fluorescence was increased by incubation with hIGFBP1 (500 ng/mL) (20.3 ± 1.9 fluorescence units vs. 16.4 ± 1.1 units in untreated [Con] cells, P = 0.03) (I). This was blocked by coincubation with LY294002 (10 μmol/L) (J). K: hIGFBP1 (500 ng/mL) increased eNOS activity in HUVECs by 46%, *P = 0.001 vs. control cells. This effect was blocked by coincubation with LY294002 (10 μmol/L). (A high-quality digital representation of this figure is available in the online issue.)
was unknown. In this study, we demonstrate for the first time, using a series of mouse models, that elevating IGFBP1 levels improves whole-body insulin sensitivity and glucose tolerance, lowers blood pressure, enhances vascular NO production, and reduces susceptibility to atherosclerosis.

A critical novel finding of the current study is that hIGFBP1 has a direct effect on vascular endothelium, resulting in increased NO generation. Mechanistically, although the proximal signaling steps are yet to be elucidated, we have shown that hIGFBP1 stimulates dose-dependent NO production in isolated murine vessels and in primary human endothelial cells. hIGFBP1-stimulated NO generation is mediated by activation of the PI3K/Akt signaling pathway, resulting in Ser phosphorylation of eNOS (Fig. 7). This mirrors the pathway by which insulin and growth factors stimulate NO generation in endothelial cells (38). hIGFBP1 mRNA was readily detectable in aorta from ApoE−/− hIGFBP1tg mice but was undetectable in aorta from ApoE+/− mice, as expected. Murine (m)IGFBP1 mRNA was expressed at low levels in aorta from both ApoE−/− and ApoE+/− hIGFBP1tg mice. (A high-quality digital representation of this figure is available in the online issue.)

**FIG. 6.** hIGFBP1 overexpression reduces atherosclerosis in ApoE-null mice. A: Representative images of Oil Red O–stained aorta demonstrating atherosclerotic lesions in ApoE−/− mice and ApoE−/− hIGFBP1tg mice (magnification ×200) (n = 7–15 per group). B: Mean aortic plaque area (expressed as percent of total vessel area) was significantly reduced in ApoE−/− hIGFBP1tg mice compared with ApoE+/− mice. *P < 0.001. C: Representative images of aortic sinus sections stained with Miller stain (magnification ×400) (n = 9–13 per group). D: Mean aortic sinus plaque area (expressed as percent of total sinus area) was significantly reduced in ApoE−/− hIGFBP1tg mice compared with ApoE+/− mice. **P < 0.05. E: Plasma total cholesterol (TC), LDL, HDL, and triglyceride concentrations were similar in ApoE−/− and ApoE+/− hIGFBP1tg mice. F: hIGFBP1 mRNA was readily detectable in aorta from ApoE−/− hIGFBP1tg mice but was undetectable in aorta from ApoE+/− mice, as expected. Murine (m)IGFBP1 mRNA was expressed at low levels in aorta from both ApoE−/− and ApoE+/− hIGFBP1tg mice. (A high-quality digital representation of this figure is available in the online issue.)
The contribution of IGFBP1 to glucose regulation has previously been attributed to modulation of IGF-I bioavailability (7). Our observations, however, indicate that hIGFBP1 functions as an insulin-sensitizing peptide per se (8). Our observations, however, indicate that hIGFBP1 previously been attributed to modulation of IGF-I bioavailability in the absence of change in body weight or fat cell size. hIGFBP1 reduces glucose intolerance in the setting of obesity in the absence of change in body weight or fat cell size.

In the current study, we have demonstrated that IGFBP1, which is expressed in the vascular wall as well as present in the circulation, stimulates NO production independently of IGF and the type 1 IGF receptor. Although the receptor for IGFBP1 (6) and the PI3K/Akt signaling pathway, culminating in phosphorylation of eNOS (7) and an increase in NOS activity. In addition to direct effects on endothelial cells, IGFBP1 also enhances insulin sensitivity in endothelial cells potentially by upregulating signaling via the insulin receptor (8). It is likely that this is a chronic effect, considering that we observed enhanced insulin signaling in isolated vessels ex vivo in the absence of hIGFBP1. Enhanced NO bioavailability may contribute to the lower blood pressure, reduced atherosclerosis, and improved insulin sensitivity we observed in hIGFBP1-overexpressing mice. It is also possible that IGFBP1 improves whole-body insulin sensitivity by stimulating the PI3K/Akt pathway in canonical insulin-sensitive tissues. IRS, insulin receptor substrate.

The robust effect of IGFBP1 overexpression we observed on atherosclerotic plaque development in ApoE−/− mice was striking. It is tempting to speculate that the inhibition of atherosclerosis we observed in ApoE−/− mice overexpressing hIGFBP1 is attributable to enhanced NO bioavailability as a result of upregulation of Akt-mediated eNOS phosphorylation. Certainly, the evidence that Akt signaling and eNOS-derived NO play fundamental roles in atherosclerosis is compelling (48,49). However, we cannot exclude the possibility that other factors may have contributed to the reduced atherosclerosis we observed in hIGFBP1tg mice.

Although our observations of increased NO generation, reduced blood pressure, and reduced atherosclerotic plaque in IGFBP1tg mice are in keeping with the inverse associations between IGFBP1 and cardiovascular disease demonstrated in cross-sectional studies, we acknowledge that not all human data support a favorable association between IGFBP1 concentrations and cardiovascular risk. Specifically, high circulating concentrations of IGFBP1 have been linked with the development of heart failure in the elderly (50) and with an adverse prognosis after myocardial infarction (51,52). Associated changes in COOH-terminal propeptide (copeptin) may explain the apparent prognostic importance of IGFBP1 concentrations highlighted in these trials (53); however, further studies of the molecular effects of IGFBP1 in these clinical scenarios are clearly warranted.

In conclusion, our findings demonstrate that hIGFBP1 has insulin-sensitizing, blood pressure-lowering, and antiatherosclerotic properties. We have identified a novel function of hIGFBP1 as a vasomodulatory molecule in its own right, here acting as a regulator of endothelial NO production independent of the type 1 IGF receptor by activating the PI3K/Akt/phospho-eNOS pathway. Our observations have
significant clinical implications, raising the possibility that enhancing IGFBP1 levels may be a therapeutic option to protect individuals from insulin resistance, hypertension, and atherosclerosis.

ACKNOWLEDGMENTS

This work was funded by the British Heart Foundation and supported in part through a Yorkshire Enterprise Fellowship for S.B.W.; S.B.W. is a British Heart Foundation Intermediate Clinical Research Fellow. A.R., V.E., E.R.D., R.M.C., M.B.K., A.Ab., and A.Az. held British Heart Foundation Clinical Research Training Fellowships. A.M.S. is a British Heart Foundation Professor of Cardiology.

No potential conflicts of interest relevant to this article were reported.

A.R. and M.B.K. prepared the manuscript and performed the in vitro and in vivo experiments. V.E., J.S., N.Y.Y., E.R.D., M.G., H.I., A.Ab., H.V., A.Az., and P.S. performed in vitro and in vivo experiments. A.V.-F., P.J.G., and K.E.P. supervised the project. R.M.C. and J.K.S. critically reviewed the manuscript. S.B.W. conceived the study, secured funding, designed the experiments, and supervised the project. S.B.W. conceived the study, secured funding, designed the experiments, supervised the project, and prepared the manuscript. S.B.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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