Diet-induced obesity, adipose inflammation, and metabolic dysfunction correlating with PAR2 expression are attenuated by PAR2 antagonism

Junxian Lim,*† Abishek Iyer,*† Ligong Liu,* Jacky Y. Suen,* Rink-Jan Lohman,* Vernon Seow,* Mei-Kwan Yau,* Lindsay Brown,†,‡ and David P. Fairlie,*†

*Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland, Australia; and †Department of Biological and Physical Sciences, University of Southern Queensland, Toowoomba, Queensland, Australia

ABSTRACT Excessive uptake of fatty acids and glucose by adipose tissue triggers adipocyte dysfunction and infiltration of immune cells. Altered metabolic homeostasis in adipose tissue promotes insulin resistance, type 2 diabetes, hypertension, and cardiovascular disease. Inflammatory and metabolic processes are mediated by certain proteolytic enzymes that share a common cellular target, protease-activated receptor 2 (PAR2). This study showed that human and rat obesity correlated in vivo with increased expression of PAR2 in adipose tissue, primarily in stromal vascular cells (SVCs) including macrophages. PAR2 was expressed more than other PARs on human macrophages and was increased by dietary fatty acids (palmitic, stearic, and myristic). A novel PAR2 antagonist, GB88 (5-isoxazoyl-Cha-Ile-spiroindene-1,4-piperidine), given orally at 10 mg/kg/d (wk 8–16) reduced body weight by ~10% in obese rats fed a high-carbohydrate high-fat (HCHF) diet for 16 wk, and strongly attenuated adiposity, insulin resistance, and cardiac fibrosis and remodeling; while reversing liver and pancreatic dysfunction and normalization of secretion of PAR2-directed glucose-stimulated insulin secretion in MIN6 β cells. In summary, PAR2 is a new biomarker for obesity, and its expression is stimulated by dietary fatty acids; PAR2 is a substantial contributor to inflammatory and metabolic dysfunction; and a PAR2 antagonist inhibits diet-induced obesity and inflammatory, metabolic, and cardiovascular dysfunction.—Lim, J. Iyer A., Liu L., Suen J. Y., Lohman R. J., Seow V., Yau M. K., Brown, L., Fairlie, D. P. Diet-induced obesity, adipose inflammation, and metabolic dysfunction correlating with PAR2 expression are attenuated by PAR2 antagonism. FASEB J. 27, 4757–4767 (2013). www.fasebj.org

Key Words: protease-activated receptor · adipocyte · macrophage · biomarker · inhibitor

Obesity, type 2 diabetes, and cardiovascular diseases have been attributed to nutritional overload from modern diets high in saturated fats and carbohydrates; reduced energy expenditure due to sedentary lifestyles; and saturation of nutrient storage, processing, and excretion systems responsible for managing intake of lipids and carbohydrates (1). Obesity is now considered to be a chronic low-grade inflammatory stress, mediated by infiltration of adipose tissue by immune cells, coupled with metabolic stress in adipocytes oversupplied with lipids and glucose (2). The causal relationship between excessive nutrient uptake (fatty acids, lipids, and sugars) and metabolic disturbances is mediated through many cell types, including adipocytes and resident or infiltrating immune cells, such as macrophages, monocytes, T cells, and mast cells, that indirectly alter adipocyte function and dysfunction (3–6). During obesity, elevated concentrations of prothrombotic factors, such as plasminogen activator inhibitor 1, tissue factor, and other proteases, including mast cell tryptase, cathepsins, caspases, and kallikreins, are implicated in metabolic and cardiovascular complications (7–10). In addition, multiple G-protein-coupled receptors (GPCRs) regulate obesity, immune cell–dependent inflammation in adipose tissue, and metabolic functions in organs and tissues (11).

Abbreviations: Adipoq, adiponectin (gene); Atgl, adipose triglyceride lipase (gene); BMI, body mass index; CS, corn starch; BSA, bovine serum albumin; DEXA, dual-emission X-ray absorptiometry; FBS, fetal bovine serum; GB88, 5-isoxazoyl-Cha-Ile-spiroindene-1,4-piperidine; GPCR, G-protein-coupled receptor; GSIS, glucose stimulated insulin secretion; HCHF, high-carbohydrate high-fat; HMDM, human macrophage; IMDM, Iscove’s modified Dulbecco’s medium; PAR2, protease-activated receptor 2; Pparγ, peroxisome proliferator-activator receptor-γ (gene); SGBS, Simpson-Golabi-Behmel syndrome; SVC, stromal vascular cell
Extracellular serine proteases, such as trypsin, trypsinogen, tissue factor VIIa, and kallikreins, activate protease-activated receptor 2 (PAR2), which belongs to a unique class of GPCRs that are N-terminally truncated to generate a functional receptor by proteolytic activation of the tethered ligand (12). Although serine proteases were shown to activate PAR2, few members of the serine protease family, including neutrophil elastase, cathepsin G, and proteinase 3, have been shown to inactivate PAR2 by cleaving downstream of the tethered ligand (13–15). PAR2 activation is generally proinflammatory both in vitro and in vivo (12). The PAR2-activating tissue factor VIIa can also activate adipose tissue macrophages, and cause insulin resistance and metabolic dysfunction in mice (16), but little is known about other extracellular signals or dietary ligands that may influence expression or activation of human PAR2 in diet-induced obesity. No PAR2-directed therapies have yet been devised for treating metabolic dysfunction (12, 17, 18).

We recently discovered the first potent, selective, and orally bioavailable small-molecule antagonists of PAR2 (17, 18) and characterized their functional responses in inflammatory cellular and animal models (19–21). We report here that expression of PAR2 is increased in vivo in adipose tissue of obese humans and rats; is stimulated in vitro in human macrophages by dietary fatty acids; and is inhibited in vivo and in vitro by a PAR2 antagonist (21), GB88 (5-isoxazolyl-Cha-Ile-3-spiroindene-1,4-piperidine). This antagonist was used as a tool to identify the role of PAR2 in mediating metabolic dysfunction in human monocyte-derived macrophages (HMDMs), human and rodent adipocytes, and rats with diet-induced obesity. Oral treatment of obese rats with GB88 (10 mg/kg/d) attenuated PAR2 signaling in adipose tissue and inhibited adipose inflammation, insulin resistance, diet-induced obesity, and cardiovascular abnormalities. This is the first report that a PAR2 antagonist improves obesity, glucose homeostasis, and obesity-associated chronic inflammation in vivo. These findings indicate that increased expression of PAR2 may be a valuable new biomarker for metabolic dysfunction and, further, that PAR2 antagonism can be an effective intervention for treating metabolic dysfunction and obesity.

MATERIALS AND METHODS

PAR2 ligands

The PAR2 agonists SLIGRL-NH₂ and 2-furoyl-LIGRLO-NH₂ and the antagonist GB88 were synthesized and characterized by analytical HPLC and mass and NMR spectroscopy (17, 21).

Human adipose tissue RNA

Paired omental and subcutaneous adipose RNAs (n=11) were obtained from Associate Professor Jon Whitehead ( Mater Medical Research Institute, Brisbane, QLD, Australia). The samples were categorized according to body mass index (BMI) as defined by the World Health Organization: lean subjects (n=2; BMI, 21.4±1.2 kg/m²), overweight subjects (n=5; BMI, 27.3±1.8 kg/m²), and obese subjects (n=4; BMI, 32.9±1.5 kg/m²). Neither the subjects’ identities nor their personal information was known to us. The protocol was approved by the Research Ethics Committees of The University of Queensland and Princess Alexandra Hospital (Brisbane, QLD, Australia).

Gene analysis

Samples were homogenized using Qiashredder and Qiazol reagent (Qiagen Pty. Ltd., Chadstone Centre, VIC, Australia). RNA was extracted from the homogenate with the RNeasy mini kit (Qiagen). Real-time PCR was run on a Prism 7900HT (Applied Biosystems, Scoresby, VIC, Australia) with cycle conditions as described elsewhere (22), and the target gene was expressed relative to housekeeping 18S rRNA or cyclophilin. The cycle conditions used were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and completed with 25°C for 2 min and 72°C for 10 min. Data were analyzed with SDS2.3 (Applied Biosystems). The primer sequences are shown in Supplemental Table S1.

Immunoblot analysis

The samples were homogenized in 50 mM Tris (pH 7.4), 1.7 mM SDS, and protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Equal amounts of proteins were loaded and separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Protein levels were detected with antibodies against PAR2 (SAM11; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Sigma-Aldrich, Castle Hill, NSW, Australia). Relative densitometry analysis on protein bands was performed with Imagej 1.42q software (U.S. National Institutes of Health, Bethesda, MD, USA) in ≥3 independent experiments.

Animals and diets

Male Wistar rats were bred at The University of Queensland Biological Resources Facility (n=6–9/group; age, 8–9 wk; weight, 300–320 g). Before specific diets were started, all the rats were fed standard laboratory chow. The experimental protocols were approved by the Animal Experimentation Ethics Committee of The University of Queensland, under the guidelines of the National Health and Medical Research Council of Australia. The rats were given ad libitum access to food and water and housed in 12-h light-dark cycles. The corn starch (CS) and high-carbohydrate high-fat (HCHF) diets and their effects over a 16-wk period have been described previously (23). GB88 (10 mg/kg/d suspended in olive oil) was administered daily by oral gavage to HCHF-fed rats between wk 8 and 16. Control HCHF-fed rats received equal amounts of olive oil by oral gavage.

Body composition measurements

The body weight and food and water intakes of the different groups were measured daily. Dual-emission X-ray absorptiometry (DEXA; XR36 DXA instrument; Norland Corp., Fort Atkinson, WI, USA) was used to measure the rats after 16 wk of feeding. 2 d before the rats were euthanized for pathophysiological assessments (23). DEXA scans were analyzed with the manufacturer’s recommended software for use in laboratory animals (Small Subject Analysis Software, version 2.5.3/1.3.1; Norland Corp.). After euthanasia, the total visceral adipose tissue was removed and weighed.
HMDMs isolation and activation

HMDMs were harvested from anonymous human donors by the buffy coat protocol (Australian Red Cross Blood Service, Brisbane, QLD, Australia). Monocytes were isolated and purified by Ficoll-Paque Plus (GE Healthcare, Waukesha, WI, USA) density centrifugation and repeated washings with ice-cold water to remove contaminating erythrocytes. CD14+ magnetic beads (MACS; Miltenyi Biotec Australia Pty. Ltd., Macquarie Park, NSW, Australia) were used to select CD14+ monocytes. After successive magnetic sorting and washing, the CD14+ monocytes were plated at a density of 1.5 × 10^6 cells/ml and supplemented with 10 ng/ml macrophage colony-stimulating factor for 7 d. A palmitic acid–bovine serum albumin (BSA) complex was prepared (24) with fatty-acid–free BSA (Sigma-Aldrich). HMDMs were seeded at 1 × 10^6/ml and pretreated with GB88 (10 μM) for 1 h before stimulation with 2f-LIGRLO-NH2 (10 μM) and palmitic acid (500 μM). The compounds and palmitic acid were dissolved in medium, and medium alone was used as the vehicle control. After overnight incubation, culture supernatants were collected, and cytokine levels were assessed by using specific ELISA kits (BD-PharMingen, San Diego, CA, USA).

Cell culture

HMC-1 cells were cultured in Iscove’s modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, 10 U/ml streptomycin, 2 mM l-glutamine, (Invitrogen, Mulgrave, VIC, Australia), and 20 μM 2-mercaptoethanol. Jurkat and THP-1 cells were cultured in Rosewell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 10 U/ml penicillin, 10 U/ml streptomycin, 2 mM l-glutamine, 2 mM nonessential amino acids (NEAAs), and 1 mM HEPES. HMDMs were cultured in IMDM supplemented with 10% FBS, 10 U/ml penicillin, 10 U/ml streptomycin, and 2 mM l-glutamine, supplemented with 10 ng/ml recombinant human macrophage colony-stimulating factor, for differentiation. Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were the kind gift of Jon Whitehead (Mater Medical Research Institute) and were cultured and differentiated (25). MIN6 β cells were cultured in DMEM (25 mM glucose) supplemented with 15% FBS, 10 U/ml penicillin, 10 U/ml streptomycin, 2 mM l-glutamine, and 60 μM 2-mercaptoethanol.

Confocal microscopy of the HMDMs

The HMDMs were seeded on sterile glass coverslips and allowed to adhere overnight. The cells were treated with palmitic acid (500 μM) overnight and fixed with 4% formaldehyde for 15 min on ice. They were then blocked with 3% BSA for 30 min and incubated with goat anti-PAR2 N-19 (Santa Cruz Biotechnology) for 2 h followed by anti-goat Alexa-Fluor 488 (Invitrogen). The slides were counterstained in DAPI, mounted with Mowiol 4–88 reagent, and examined by an LSM-510 META inverted microscope (Zeiss, North Ryde, NSW, Australia).

Histologic analysis of adipose tissue

Visceral adipose tissue was harvested, snap frozen in optimal cutting temperature (OCT) embedding compound (TissueTek; Sakura Finetek, Tokyo, Japan), and stored at −80°C. Blocks were cut on a cryostat (CM3050S; Leica Microsystems Pty. Ltd., Gladesville, NSW, Australia) at 10 μm and air dried. Before staining, all tissues were fixed in 4% paraformaldehyde (15 min) and then in xylene (20 s) to clear the lipids.

Calcium mobilization

Intracellular calcium mobilization assays were performed as described elsewhere (21). MIN6 β cells were seeded at 1 × 10^6/ml and allowed to adhere overnight. The next day, the medium was removed, and the cells were incubated in dye loading buffer (HBSS with 4 mM Fluo-3, 25 mM pluronic acid, 1% FBS, and 2.5 mM probenecid) for 1 h at 37°C. The cells were washed before addition of compounds, and a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA, USA) was used to monitor intracellular Ca^{2+} release via fluorescence measurement for ≥300 s (excitation 495 nm, emission 520 nm).

Glucose-stimulated insulin secretion

The MIN6 β cells were a gift from Dr. Brad Marsh (University of Queensland). They were passaged and harvested by using nonenzymatic cell dissociation solution (Sigma-Aldrich), and
glucose-stimulated insulin secretion (GSIS) was measured (26). Briefly, the MIN6 β cells were seeded at 0.8 × 10⁶/ml in a 96-well plate and cultured for 48 h. The cells were washed twice with glucose-free Krebs buffer (119 mM NaCl; 4.74 mM KCl; 2.54 mM CaCl₂; 1.19 mM MgCl₂; 1.19 mM KH₂PO₄; 25 mM NaHCO₃; 10 mM HEPES, pH 7.4; and 0.05% BSA) and pretreated with GB88 for 30 min with Krebs buffer supplemented with 2.5 mM glucose. After incubation, they were washed twice with the Krebs buffer. Glucose (2.5 and 25 mM) and the compounds were added at the specified concentrations and incubated for 1 h. The compounds were dissolved in Krebs buffer, which was also used as the vehicle control. The supernatants were collected, and insulin concentrations were determined by ELISA (Abcam, Cambridge, UK).

Statistical analysis

Data were analyzed with Prism 5.0d for Mac OSX (GraphPad Software, San Diego, CA, USA). Statistical differences between treatments were assessed with Student's t test between 2 groups or 1-way ANOVA for multiple groups. Values of P < 0.05 were considered significant. All independent parameters are the mean ± SEM of results in ≥3 independent experiments.

RESULTS

Human and rat obesity increased PAR2 expression

PAR2 mRNA in human omental adipose tissue was found to increase with body weight in a small cohort (n=11) of lean, overweight, or obese people (Fig. 1A). Increases in their BMI correlated with increased expression of PAR2 in their omental (Fig. 1A) and subcutaneous (Supplemental Fig. S1) adipose tissue, suggesting that increased expression of PAR2 is a promising newly recognized biomarker of human obesity. This relationship between obesity and PAR2 mRNA expression was corroborated in the current study in Wistar rats fed an HCHF diet (23) for 16 wk. The results showed a 15-fold increase in PAR2 mRNA (Fig. 1B) and a 2-fold increase in PAR2 protein (Fig. 1C) in adipose tissue relative to rats fed a low fat CS diet. Three-quarters of this increase in PAR2 was associated with the non-adipocyte stromal vascular cell (SVC) fraction of rat adipose tissue (Fig. 1B), which contains extensive infiltrated macrophages and other immune cells implicated in the pathogenesis of obesity-associated chronic inflammation (6). To further validate the roles of PAR2 signaling on adipocytes, and establish the links between activation of PAR2 and inflammation, we stimulated human SGBS adipocytes with the PAR2 agonist (2f-LIGRLO-NH₂). 2f-LIGRLO-NH₂ induced mRNA expression of TNF, IL6, and IL8 in SGBS adipocytes, while treatment with GB88 attenuated the increases in these important inflammatory mediators associated with development of metabolic disorders (Supplemental Fig. S2).

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Dietary fatty acids amplified PAR2 expression in human macrophages

There was substantially higher expression of PAR2 than PAR1, -3, or -4 in primary HMDMs, as well as in other immune cells (THP-1, U937, Jurkat, and HMC-1; Supplemental Fig. S3), suggesting a dominant regulatory role for PAR2 compared with other PARs in immune cell–mediated inflammation. Moreover, myristic,
stearic, and palmitic acids, the common dietary fatty acids in Western-style human diets, dose dependently (10–500 μM) increased PAR2 mRNA by up to 20-fold in HMDMs (Fig. 2A–C). Previous studies have shown that palmitic acid is the most abundant fatty acid in plasma and contributes to insulin resistance (27–29), so we briefly investigated its effects on HMDMs. Palmitic acid represents 50% (47.8 g/100 g) of total plasma fatty acids in HCHF-fed rats (23). Analysis of the palmitic acid–stimulated HMDMs showed that PAR2 mRNA remained highly expressed when compared with PAR1, -3, and -4 (Fig. 2D). Stimulation with palmitic acid translated into increased expression of PAR2 protein, as measured by confocal microscopy using the anti-PAR2 antibody N-19 (Fig. 2E and Supplemental Fig. S4), and into increased susceptibility to activation of surface PAR2 by the extracellular agonist 2f-LIGRLO-NH2 (Fig. 2F).

**PAR2 antagonist attenuated diet-induced obesity in rats**

To discover whether PAR2 was a therapeutic target for intervention in obesity or just a downstream marker of obesity, we studied the effects of a PAR2 antagonist GB88 in rats fed an HCHF diet for 16 wk. Our previous studies had demonstrated that GB88 alone does not cause weight loss in Wistar rats (19–21). Daily oral administration of GB88 (10 mg/kg/d) from wk 8 to 16 attenuated or reversed many of the indicators of obesity and of metabolic and inflammatory stress (Figs. 3–5). GB88 prevented a 10% increase in body weight without appetite suppression (Fig. 3A and Supplemental Fig. S5) by inhibiting the accumulation of fat mass (Fig. 3B) and diet-induced visceral abdominal fat deposition (Fig. 3C), as determined by DEXA and organ weight analyses, respectively. PAR2 mRNA was overexpressed 15-fold in adipose tissue (mostly in the non-adipocyte SVC fraction) after HCHF feeding, but this was reduced by 70% after treatment with GB88 (Fig. 3D). The antagonist treatment also decreased PAR2 protein expression in adipose tissue of the HCHF-fed rats, as observed by immunofluorescence detection (Fig. 3E). The density and distribution of PAR2+ cells observed with immunofluorescence staining in the HCHF-fed rats were higher, and in clusters, compared with those in the GB88-treated, HCHF-fed rats. This observation of PAR2+ cells correlates with macrophage accumulation and crown structures in adipose tissue of obese subjects (6).

**GB88 modulated inflammation in rat adipose tissue and HMDMs**

Knowing that recruitment of immune cells to adipose tissue is a feature of chronic inflammatory and metabolic dysfunction (4, 6), we analyzed the distribution of immune cells in adipose tissue by using immunohistochemistry for ED1 (CD68), a marker for monocytes and macrophages. Consistent with the immunofluorescence data, the immunohistochemical localization of ED1 in adipose tissue showed similar crown structures in the HCHF-fed rats. In contrast, the GB88-treated, HCHF-fed rats displayed markedly reduced immune cell infiltration and crown structures (Fig. 4A, B). Since PAR2 is

![Figure 2. Dietary fatty acids amplified expression and activation of PAR2 in vitro in HMDMs. A–C] Myristic acid (A), stearic acid (B), and palmitic acid (C) (10–500 μM) induced dose-dependent increases of PAR2 mRNA in primary HMDMs. mRNA was normalized against cyclophilin, and fold change was calculated relative to untreated samples. D) PAR2 mRNA (PAR1–4) in HMDMs after stimulation with palmitic acid (500 μM). E) Representative images of immunofluorescence detection of PAR2 protein, by confocal microscopy with the PAR2 antibody N-19, on HMDMs before and after addition of palmitic acid (500 μM) (see Supplemental Data). F) Dose-dependent activation of PAR2 on HMDMs (reflecting relative surface-expressed PAR2) by the extracellular agonist 2f-LIGRLO-NH2 before (open squares) and after (solid squares) treatment with palmitic acid (500 μM). Bars represent means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.
activated by mast cell tryptase, and mast cells are associated with diet-induced obesity (4), we also examined mast cells in adipose tissue by differential histological staining for their different activation states (Supplemental Fig. S6). The total number of mast cells and those degranulated (stained blue) in adipose tissue of the HCHF-fed rats were strikingly reduced by the GB88 treatment in vivo (Supplemental Fig. S6). This reduction was one indication of the benefits of a PAR2 antagonist for treating metabolic and inflammatory dysfunction.

Adipose tissue macrophages are classified in two distinct polarization states, M1 and M2, depending on the extent of obesity (30, 31). An analysis of adipose tissue from the HCHF-fed rats showed increased expression of M1-specific proinflammatory genes (Il6, Tnfa, Ccl2, and Ccl9), known to exacerbate the state of metabolic dysfunction and to alter metabolism (32–34). These genes were substantially decreased in adipose tissue of the GB88-treated, HCHF-fed rats (Fig. 4C). By contrast, the expression of M2-specific genes Mcr2 (C-type mannose receptor 2), MgIl (macrophage galactose-type C-type lectin 1), and Argl (arginase 1) was suppressed in HCHF-fed rats, but normalized by GB88 treatment (Fig. 4C). The modulation of these genes is consistent with PAR2 activation contributing to adipose inflammation (16). However, it is not certain that these changes were entirely due to adipose tissue macrophages. Other genes involved in lipid metabolism, such as Pparg (peroxisome proliferator-activator receptor-γ), Adipog (adiponectin), Atgl (adipose triglyceride lipase), and Hsl (hormone-sensitive lipase), were down-regulated in HCHF-fed rats but normalized by the PAR2 antagonist in vivo (Fig. 4D). Adiponectin is known to activate AMPK, whereas Pparg, Atgl, and Hsl are active in lipid metabolism (35). Consistent with previous studies, the normalization of these genes suggests that attenuation of PAR2 activation promotes AMPK activation, leading to increased fatty acid oxidation (16, 36).

Together, these data strongly indicate that treatment with the PAR2 antagonist prevented or reduced markers of tissue inflammation and metabolic dysfunction in adipose tissue from the HCHF-fed rats. Supporting in vitro experiments showed that the secretion of the proinflammatory cytokines IL-1β and IL-6 from HMDMs was induced by the PAR2 agonist 2f-LIGRLO-NH₂ (Fig. 4E), albeit only after stimulation with palmitic acid (500 μM). GB88 (10 μM) specifically abolished the effects of 2f-LIGRLO-NH₂, but not those of palmitic acid (Fig. 4E), which triggered expression of PAR2 in the HMDMs (Fig. 2C). In the absence of palmitic acid, activation of PAR2 in HMDMs by 2f-LIGRLO-NH₂ did not induce IL-1β or IL-6 secretion (Fig. 4E).
PAR2 regulated cardiovascular structure and function in rats with diet-induced obesity

HCHF feeding for 16 wk changed cardiovascular structure and function (Supplemental Fig. S8). Left ventricular mass, systolic blood pressure, and diastolic and systolic volumes were increased in the HCHF-fed rats, together with decreased fractional shortening and cardiac output without changes in heart rate and E/A ratio, and increased deposition of interstitial collagen in the left ventricle of the heart (Supplemental Fig. S8A–D). Consistent with some studies suggesting a possible important role for PAR2 in heart diseases (37, 38), treatment with GB88 in the current study improved most of the diet-induced structural and functional changes and notably prevented the increased deposition of collagen in the heart (Supplemental Fig. S8). This antifibrotic effect of GB88 could be a beneficial therapeutic property in cardiovascular and inflammatory diseases in which collagen deposition causes tissue or organ dysfunction.

PAR2 regulated glucose homeostasis

Chronic nutritional surplus and elevated fatty acids are linked to the pathogenesis of pancreatic dysfunction, so in vivo and in vitro effects of GB88 were evaluated on rat pancreas and mouse MIN6 β cells. Rats fed the HCHF diet developed impaired glucose tolerance with insulin resistance, but GB88 treatment improved glucose tolerance in an oral glucose tolerance test and improved responsiveness to insulin in an insulin tolerance test (Fig. 5A). Following treatment with GB88, the HCHF-fed rats had insulin sensitivity comparable to that of the CS-fed rats. Real-time PCR analysis revealed that the pancreatic genes Glut2 (glucose transporter 2) and Mll (basic helix-loop-helix transcription factor), which are involved in metabolism and transcription, were suppressed in the HCHF-fed rats relative to the CS-fed rats but were normalized by GB88 in vivo (Fig. 5B). The genes involved in insulin production and mediation of stress responses, Iapp (islet amyloid polypeptide), Atf3 (activating transcription factor 3), Fas (CD95), and Id-1 (inhibitor of DNA binding 1), were all up-regulated in the HCHF-fed rats, but attenuated by treatment in vivo with GB88. The PAR2 agonists and GB88 were further evaluated in vitro for GSIS (Fig. 5C) and intracellular calcium mobilization (Supplemental Fig. S9) in mouse MIN6 β cells. 2f-LIGRLO-NH₂ inhibited GSIS in MIN6 cells, while pretreatment with GB88 normalized insulin secretion (Fig. 5C). Three different PAR2 agonists...
(2f-LIGRLO-NH₂, trypsin, and SLIGRL-NH₂) each activated intracellular calcium release in MIN6 cells in a concentration-dependent manner (Supplemental Fig. S9). These data support a close association between PAR2 activation, insulin modulation, and glucose homeostasis.

DISCUSSION

Obesity is a multifaceted disorder involving complex crosstalk between metabolic and immune systems. These systems involve proteases that contribute to nutrient sensing, protein degradation and metabolism, recruitment of immune cells to sites of infection or inflammatory and metabolic stress, and elimination of infectious organisms and infected or damaged cells (39, 40). Extracellular proteases control formation and degradation of many proteins that regulate metabolism and inflammation, including cell surface–expressed PARs (12) that activate intracellular signaling cascades. Mast cell tryptase is one important proinflammatory serine protease that is elevated in obese human subjects and activates PAR2, leading to chronic inflammatory conditions such as arthritis and asthma (19, 41). Tissue factor VIIa is another protease reported to be important in the development of obesity and insulin resistance in mice (16). Using knockouts of tissue factor and PAR2, or antibody blockade of tissue factor, PAR2 activation in adipocytes suppressed Akt and AMPK signaling, as well as downstream metabolic genes, leading to insulin resistance and obesity. PAR2 activation in adipose macrophages induced M1 polarization and increased proinflammatory mediators (16).

However, many proteases signal through PAR2, and the roles for PAR2 activation by each protease in inflammation, metabolism, and obesity remain poorly understood. Antibodies and siRNA have been used unsuccessfully to define the pathogenesis of PAR2-driven inflammation, while the use of knockout animals does not take into account the many redundant pathways in inflammation or provide information relevant to drug action in a clinical setting (42). Previously reported PAR2 antagonists were not selective, potent, bioavailable, or effective against protease-activated PAR2 and thus have not been useful for inhibiting PAR2-mediated disease (43–45). The current study used the novel, orally active, selective PAR2 antagonist GB88, which blocks activation of PAR2 by all known PAR2 agonists, whether they are proteases, peptides, or small molecules (18, 21). Small-molecule PAR2 antagonists provide new opportunities to investigate roles for PAR2 in vivo in metabolism, immunity, and disease, while also validating this strategy as a prospective treatment for PAR2-mediated disease.

Our findings show that expression of PAR2, a target shared by many serine proteases, was up-regulated in human adipose tissue, positively correlating with BMI; in human macrophages, stimulated by fatty acids commonly present in human diets; and in rat adipose tissue, correlating with diet-induced obesity in rats where most of the increased expression of PAR2 was associated with the non-adipocyte immune cell fraction of adipose tissue. The finding that palmitic and other fatty acids can up-regulate the expression of PAR2 mRNA, and augment PAR2-activated secretion of the proinflammatory cytokines IL-1β and IL-6 in HMDMs, connects diet to expression of PAR2, supporting PAR2 as an important regulator in metabolic homeostasis and inflammatory pathways. A previous study reported intra-
cellular calcium mobilization in unstimulated macrophages (46), but we did not detect intracellular calcium release in HMMDs in response to PAR2 agonists. This difference may be due to the different methods used to isolate HMMDs. Unlike Colognato et al. (46), who used only the monocytes that adhered to plastic tissue culture dishes, thus possibly skewing subsequent differentiation rather than representing the whole population of monocytes, we used the whole monocyte population for differentiation. Further, our study showed that unstimulated HMMDs do not express cell surface PAR2, unless stimulated with palmitic acid (Fig. 2) or trichostatin A (19). Palmitoylation is a post-translational protein modification known to influence activation of PAR2, intracellular signaling, endocytosis, and degradation (47, 48). However, inhibiting palmitoylation or mutating palmitoylation sites in GPCRs does not seem to affect GPCR expression or G-protein signaling (49–52). Thus, palmitic acid may instead enhance expression of PAR2 via another receptor instead of palmitoylating PAR2 directly.

In this study, oral administration of GB88 to rats with diet-induced obesity ameliorated classic symptoms of metabolic dysfunction, with marked reduction in obesity, central adiposity, adipose tissue expression of PAR2, insulin resistance, glucose intolerance, inflammation, metabolic and pancreatic dysfunction, and cardiovascular abnormalities, including collagen deposition and fibrosis in the heart. The GB88-treated, HCHF-fed rats showed normalization of genes related to lipid metabolism (Pparg), showed reduction in PAR2 mRNA expression and activation of PAR2 in adipose tissue, indicating that PAR2 plays a role in modulating inflammation and metabolism. The finding of higher expression in vivo of PAR2 over other PARs in human immune cells (HMDM, THP-1, U937, Jurkat, and HMC-1) supports the dominant role of PAR2 in modulating inflammation. Serine proteases are vital regulators in glucose homeostasis. Most mammalian trypsin is synthesized and secreted from the pancreas, while PAR2, but not other PARs, is highly expressed in mouse pancreatic islet cells (55). Our finding that a PAR2 agonist inhibited GSIS in MIN6 β-cells is consistent with a beneficial antagonist effect of GB88 in normalizing GSIS to levels comparable to those in control animals. In the GSIS experiments, a higher concentration of GB88 (30 μM) was used because 2f-LIGRLO-NH2 has lower efficacy in these cells in Ca2+ studies, but GB88 is a competitive and surmountable antagonist (21).

It is now evident that metabolism and inflammation are highly integrated and that a dynamic balance between metabolic homeostasis and immunity is needed for optimal physiological functions (56). However, it is currently unclear whether nutrients such as glucose or lipids initiate inflammation in obesity, or whether inflammation is a secondary reactive event that occurs after the onset of obesity. It is known that excess nutrients and obesity trigger cellular stress, leading to activation of downstream inflammatory responses in adipocytes and infiltration of macrophages. This creates a mutual feedback loop in metabolic dysfunction. The in vivo reduction in PAR2 expression and macrophage activity in adipose tissue of GB88-treated, HCHF-fed rats is consistent with a phenotypic switch from an M1- to M2-polarized state of macrophages. However, the results point to PAR2 antagonism ablating adipose inflammation by preventing this feedback loop and the inflammation promoted by both adipocytes and macrophages. Our results strongly suggest that PAR2 is an important convergent point in the integration of metabolism and inflammation, and that the therapeutic benefits of GB88 involve synergistic responses from both metabolic tissues and immune cells.

In summary, this study has shown that PAR2 in human and rat adipose tissue is a novel biomarker of obesity, that PAR2 activation is important in regulating inflammation and metabolic pathways in diet-induced obesity, and that an orally active antagonist of human and rodent PAR2 has beneficial effects in metabolic syndrome. The PAR2 antagonist ablated inflammation in adipose tissue by reducing infiltrating macrophages, attenuated central adiposity and obesity, reversed insulin resistance and glucose intolerance, modulated liver and pancreatic metabolic parameters, and inhibited cardiac fibrosis and cardiovascular abnormalities. These new
findings validate PAR2 antagonism as a promising new therapeutic intervention strategy for treating diet-induced obesity and metabolic dysfunction.

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Junxian Lim, Abishek Iyer, Ligong Liu, et al.

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