GDF15 Mediates the Effect of Skeletal Muscle Contraction on Glucose-Stimulated Insulin Secretion

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Exercise is a first-line treatment for type 2 diabetes and preserves β-cell function by hitherto unknown mechanisms. We postulated that proteins from contracting skeletal muscle may act as cellular signals to regulate pancreatic β-cell function. We used electric pulse stimulation (EPS) to induce contraction in C2C12 myotubes and found that treatment of β-cells with EPS-conditioned medium enhanced glucose-stimulated insulin secretion (GSIS). Transcriptomics and subsequent targeted validation revealed growth differentiation factor 15 (GDF15) as a central component of the skeletal muscle secretome. Exposure to recombinant GDF15 enhanced GSIS in cells, islets, and mice. GDF15 enhanced GSIS by upregulating the insulin secretion pathway in β-cells, which was abrogated in the presence of a GDF15 neutralizing antibody. The effect of GDF15 on GSIS was also observed in islets from GFRAL-deficient mice. Circulating GDF15 was incrementally elevated in patients with pre- and type 2 diabetes and positively associated with C-peptide in humans with overweight or obesity. Six weeks of high-intensity exercise training increased circulating GDF15 concentrations, which positively correlated with improvements in β-cell function in patients with type 2 diabetes. Taken together, GDF15 can function as a contraction-induced protein that enhances GSIS through activating the canonical signaling pathway in a GFRAL-independent manner.

ARTICLE HIGHLIGHTS

- Exercise improves glucose-stimulated insulin secretion through direct interorgan communication.
- Contracting skeletal muscle releases growth differentiation factor 15 (GDF15), which is required to synergistically enhance glucose-stimulated insulin secretion.
- GDF15 enhances glucose-stimulated insulin secretion by activating the canonical insulin release pathway.
- Increased levels of circulating GDF15 after exercise training are related to improvements in β-cell function in patients with type 2 diabetes.

Type 2 diabetes is characterized by hyperglycemia and loss of pancreatic insulin secretion. The penultimate step in disease onset involves compensatory insulin secretion from pancreatic β-cells to overcome peripheral insulin resistance. The ensuing hyperinsulinemia leads to progressive β-cell fatigue, dysfunction, apoptosis, dedifferentiation, and senescence, resulting in reduced insulin secretory capacity and dysregulated glucose homeostasis (1). Exercise is a first-line therapy in the prevention and management of type 2 diabetes (2,3). Importantly, exercise enhances pancreatic β-cell function independent of changes in body weight, circulating lipids, or peripheral insulin sensitivity (4–8). However, the mechanisms underlying exercise-induced improvements in insulin secretory function remain unclear.

It has been proposed that biologically active factors secreted in response to muscle contraction may contribute to

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improved β-cell function (9,10). This is exemplified by recent observations in which exposure to conditioned medium (CM) derived from contracted skeletal muscle cells prevented β-cell apoptosis (11) and potentiated insulin secretion (12,13). However, the specific molecular transducers mediating cross talk between contracting skeletal muscle and pancreatic β-cells remain largely unknown. Growth differentiation factor 15 (GDF15) is a secreted ligand and member of the transforming growth factor-β (TGF-β) superfamily (14,15). Circulating levels of GDF15 are elevated in marathon runners and cyclists after strenuous physical exercise (16,17). Pharmacologic treatment and overexpression of GDF15 in mice reduce food intake and body weight while improving insulin sensitivity (18–27). Conversely, loss of GDF15 has been shown to increase body weight and impair glycemic control (25,28,29). It was recently demonstrated that clinical markers of insulin resistance and secretory function are associated with circulating GDF15 in patients with obesity and prediabetes or type 2 diabetes (30,31). More recently, GDF15 was also found to be expressed in mouse β-cells and human pancreatic islets and involved in glucose-stimulated insulin secretion (GSIS) (32,33). GDF15 expression was lower in islets of patients with type 1 diabetes, and treatment with exogenous GDF15 prevents cytokine-induced apoptosis (34). Furthermore, GDF15 was protective of β-cells, and administration of GDF15 prevented diabetes onset in mice (34). In addition, 12 weeks of GDF15 treatment improved exercise capacity in obese ZSF1 rats (35). Collectively, these reports indicate that skeletal muscle GDF15 may synergistically enhance β-cell function. Thus, in this study, we examined whether GDF15 secreted from skeletal muscle acts as a signaling peptide to regulate β-cell function.

RESEARCH DESIGN AND METHODS

In Vitro Studies

Muscle Contraction

Electrical pulse stimulation (EPS) was used to induce contraction in fully differentiated C2C12 myotubes as previously described (36).

RNA-seq

Following 16 h of stimulation, RNA was extracted from the cell pellet (RNAeasy; Qiagen) for untargeted RNA-seq. Libraries were prepared using the Illumina TruSeq Stranded Total RNA kit with 100-bp paired-end RNA-seq strategy. Illumina reads were mapped to mouse genome release mm10 using STAR Aligner, version 2.5.1. Differential expression was analyzed by Cuffdiff, version 2.2.1. Targets with a positive fold change were filtered through three secretome databases: SignalP (37), SPOCTUPUS (38), and Phobius (39).

CM Analysis

After 1, 2, 4, 8, 16, or 24 h of EPS, CM was collected for quantification of interleukin-6 by ELISA (Sigma). After 16 h of EPS in the culture medium from C2C12 or primary human skeletal muscle cells, GDF15 concentrations were determined by ELISA (R&D Systems).

GSIS

After treatment as described below, medium and cell lysates were harvested to determine insulin concentrations by ELISA (Mercodia). Total protein concentrations were determined by bicinchoninic acid assay (Thermo Fisher Scientific).

Glucose Titration. β-TC-6 cells were stimulated with increasing concentrations of glucose (0.5, 1, 3, 5, and 10 mmol/L) in KRH buffer for 3 h. Culture medium and cell lysates were harvested for analysis.

EPS-CM Treatment. β-TC-6 and MIN6 cells (AddexBio) were treated with CM from non-EPS or EPS-exposed C2C12 myotubes for 16 h. For GDF15-neutralizing experiment, 10 μg/mL anti-GDF15 monoclonal antibody (R&D Systems) was added to EPS-CM prior to a 16-h treatment in β-TC-6 cells. β-TC-6 cells were also treated with non-EPS CM supplemented with recombinant mouse GDF15 (rmGDF15) (R&D Systems) at 130 pg/mL or vehicle for 16 h. MIN6 cells were incubated with 0 or 10 mmol/L glucose in KRH buffer for 60 min. β-TC-6 cells were incubated with 0 or 1 mmol/L glucose in KRH buffer for 3 h.

GDF15 Treatment in β-Cells. β-TC-6 cells were treated with 300 pg/mL and 10 ng/mL rmGDF15 protein or vehicle. rmGDF15 was supplemented in 0.1% BSA in KRH buffer and used for β-cell treatment in the presence or absence of 1 mmol/L glucose for 3 h.

GDF15 Treatment in Islets. Human islets (Lonza) were obtained from a 33-year-old, Hispanic male donor, plated into cell culture inserts (MilliporeSigma), and equilibrated for 1 h in 2 mmol/L glucose–Krebs-Ringer bicarbonate buffer. Mouse islets were isolated from WT ($Gfral^{+/+}$) and Gfral-knockout ($Gfral^{-/-}$) mice as described previously (27,40). Islets were sequentially incubated for 1 h in Krebs-Ringer bicarbonate buffer plus 2 mmol/L glucose (basal) and Krebs-Ringer bicarbonate buffer plus 16 mmol/L glucose (stimulated), with or without 10 ng/mL recombinant human GDF15 (rhGDF15) (R&D Systems). Insulin was measured in supernatants and insulin content was determined in islet lysate.

Quantitative Real-Time PCR

Total RNA was extracted (RNAeasy; Qiagen) and converted to cDNA using an iScript cDNA synthesis kit (BIORAD). Quantitative real-time PCR was carried out with SYBR Green Master Mix (Thermo Fisher Scientific), as described previously (41). Gene expression was normalized to GAPDH and analyzed by the comparative cycle threshold method. Primer sequences are listed in Supplementary Table 1.

Glycolysis

Extracellular acidification rates were determined on a Seahorse XF24, as described previously (41). Maximal glycolytic

flux was determined in the presence of glucose (1 mmol/L) and oligomycin (2 μmol/L).

ADP to ATP Ratio

Cellular ATP to ADP ratios were determined using a commercially available bioluminescent luciferase assay (Biovision) according to manufacturer instructions.

Intracellular Calcium

Intracellular calcium flux was determined by incubation of β-TC-6 cells with Fura-2 A.M. (Molecular Devices) in 0.1% DMSO for 50 min prior to harvest. Fluorescent intensity was determined on a Flexstation3 at baseline and after addition of KCL (27 mmol/L). Data are reported as the ratio of 340:380 (maximum to minimum).

Viability, Proliferation, and Cytotoxicity

Cell viability (Dojindo), proliferation (Abcam), and lactate dehydrogenase cytotoxicity (Thermo Fisher Scientific) were determined via commercial colorimetric assays per manufacturer instructions.

Flow Cytometry

β-TC-6 cells were expanded in a 10-cm dish, harvested by scraping, transferred to flow cytometry tubes, and incubated at 37°C for 1 h to recover cell surface receptors. Cells were then washed, resuspended in FACS buffer (BD Biosciences), and stained with anti-ErbB2/Her2-PE (Bio-Techne R&D Systems), anti–CD44-FITC (eBioscience Invitrogen) and anti–CD48-PerCP/Cy5.5 (Biolegend). Data were acquired on an Aurora flow cytometer (Cytek). UltraComp eBeads (Thermo Scientific) were used for compensation, and FlowJo, version 10.8.1 software (Treestar Inc) was used for data analysis.

Mouse Studies

Animal Husbandry

Male C57BL/6J (catalog 000664, Jackson) mice were housed under a standard 12-h light/dark cycle at a temperature of 21–22°C and fed 5053 PicoLab standard Rodent Diet 20 (catalog 0007688, LabDiet) with ad libitum access to food and water. GFRAL-deficient mice ($Gfral^{-/-}$) and littermate controls ($Gfral^{+/+}$) were housed under a standard 12-h light/dark cycle at a temperature of 25°C and fed standard Rodent Diet (PicoLab) with ad libitum access to food and water. Mice were euthanized at 12 weeks of age for isolation of primary mouse islets.

In vivo Insulin Secretion

Twelve-week-old mice were fasted overnight for 12 h and, at 0800 the next day, were moved to the operating room for adaptation prior to treatment. After 1 h, the mice received either intraperitoneal injections of 0.25 mg/kg rhGDF15 (R&D Systems) or vehicle. After 2 h, the mice were given an intraperitoneal injection of 1 g/kg D-glucose. Blood was collected prior to injection, at 2 h after rhGDF15 injection via tail snip, and 30 min after glucose challenge via terminal cardiac puncture. Blood was collected in EDTA-treated tubes and plasma was isolated by centrifugation at 2000*g* for 20 min at 4°C. Insulin and C-peptide concentrations were determined by a multiplex assay (Millipore), and GDF15 by ELISA (R&D). All experiments and procedures involving animals were approved by the Pennington Biomedical Research Center and University of Michigan Institutional Animal Care and Use Committee.

Human Studies

All human data and specimens (vastus lateralis muscle samples, plasma samples) were obtained from previously approved and reported studies (42–47). In Human Study 1, skeletal muscle specimens were collected from older adults with obesity (mean ± SD: age 66.0 ± 4.4 years; BMI 33.7 ± 3.5) before and after 12 weeks of daily aerobic exercise training, as described previously (48). The supervised aerobic training program consisted of treadmill exercise set at 85% of heart rate maximum for 1 h/day, 5 days/week, over 12 weeks. Patient characteristics are provided in Table 1. Muscle cDNA samples were prepared as previously described (49). GAPDH was used as an internal standard. Data were calculated using the comparative cycle threshold method.

In Human Study 2, in the cross-sectional analysis, 62 sedentary, weight-stable (± 2.5 kg for prior 6 months) adults were assigned to the following groups: *1*) healthy controls: normal fasting glucose and glucose tolerance; *2*) prediabetes: impaired fasting glucose or glucose tolerance; and *3*) type 2 diabetes, based upon the American Diabetes Association guidelines for classification and diagnosis of diabetes (50). The participants' characteristics are described in Table 2. Fasting plasma samples were collected as previously reported (46) and assayed for GDF15 concentration by ELISA (R&D Systems).

In Human Study 3, plasma samples were collected from patients with type 2 diabetes before and after 6 weeks high-intensity exercise training intervention (46,47). The participants' characteristics are described in Table 1. Fasting plasma samples were analyzed for GDF15 by ELISA (R&D Systems) per manufacturer's instructions.

Quantification and Statistical Analysis

All data points are reported as biological replicates, not technical replicates. Technical replicates were averaged when appropriate. In figures, data are reported as mean ± SD, unless otherwise indicated in the figure legend. GraphPad Prism8 software was used for statistical analysis. Detailed descriptions of statistical procedures are provided in the respective figure legends. Normality was assessed by the Kolmogorov-Smirnov test. Pearson correlation was used to investigate the associations between the change in GDF15 and the disposition index. Significance was accepted at $P < 0.05$.

Data and Resource Availability

RNA-seq data sets were submitted to the National Center for Biotechnology Information's Gene Expression Omnibus database under the accession number GSE139872. All other

Table 1—Baseline participant characteristics for the clinical cohorts in Human Studies 1 and 3

Characteristic	Clinical cohort in Fig. 1*H* (Human Study 1)	Clinical cohort in Fig. 6*E* and *F* (Human Study 3)
Participants; male participants; female participants, *n*	19; 10; 9	12; 4; 8
Age, years	66 ± 4.4	54 ± 6.9
Weight, kg	98.5 ± 16.1	98.5 ± 12.5
Height, cm	170.6 ± 10.0	169.1 ± 10.4
BMI, kg/m^2	33.7 ± 3.5	34.5 ± 3.8
Fat, %	42.7 ± 6.5	44.0 ± 6.2
Fasting plasma glucose, mg/dL	98.6 ± 13.1	179.1 ± 57.5
Insulin, μU/mL	14 ± 4.8	23.1 ± 20.1
HbA_{1c}, %	5.6 ± 0.9	8.5 ± 2.1
HbA_{1c}, mmol/mol	38 ± 9.9	69 ± 23.1
Vo_{2max}, mL/kg/min	21.4 ± 3.9	23.5 ± 3.8
Type 2 diabetes, *n*	1	12

Data are reported as mean ± SD, unless otherwise indicated.

data sets generated and/or analyzed in this study are available from the corresponding author upon request.

RESULTS

The Skeletal Muscle Secretome Enhances Insulin Secretion in Pancreatic β-Cells

We first explored the relationship between skeletal muscle contraction and insulin secretion by using an in vitro model to induce muscle contraction (Supplementary Fig. 1). We and others have previously validated this EPS model and used it to demonstrate the effects of isolated cell contraction on glucose uptake and insulin signaling in C2C12 and primary human myotubes (36,51). In the present experiments, contraction was validated by accumulation of interleukin-6 in the CM from C2C12 myotubes (Supplementary Fig. 2*A*), a widely accepted, exercise-induced myokine (52). Cultured β-cells were then pretreated with EPS or non–EPS-CM for 16 h and evaluated for glucose-stimulated insulin secretion (GSIS) (Fig. 1*A*). EPS-CM increased GSIS by 30% in β-TC-6 cells and by 150% in MIN6 cells compared with non–EPS-CM–treated β-cells (Fig. 1*B*). To address anticipated concerns that EPS may produce non–cell-mediated effects (53), we assessed GSIS after EPS in cell-free conditions and confirmed that the phenotype observed was due to EPS-stimulated contraction of myotubes (Supplementary Fig. 2*B–D*). In addition, EPS-CM from C2C12 cells did not affect β-cell viability (Supplementary Fig. 2*E*). Together, these data demonstrate that EPS-CM from C2C12 myotubes potentiates GSIS.

GDF15 Is Increased in Response to Muscle Contraction and Exercise Training

To identify specific components of the secretome that may regulate β-cell function, we conducted untargeted RNA-seq on C2C12 myotubes after EPS (Fig. 1*A*). RNA-seq revealed 832 differentially regulated mRNA transcripts in response to

Table 2—Baseline characteristics of participants in the clinical cohort in Human Study 2 (Fig. 6*A–D*)

Characteristic	NFG/NGT	IFG/IGT	T2D
Participants; male participants; female participants, *n*	17; 12; 5	21; 11; 10	24; 19; 5
Age, years	45 ± 16.5	61 ± 13.7	55 ± 9.8
Weight, kg	82.2 ± 17.7	100.8 ± 15.1	93.9 ± 14.7
BMI, kg/m^2	29.1 ± 5.8	35.3 ± 3.7	34.3 ± 4.9
Fat, %	37.8 ± 13.6	45.9 ± 6.4	45.7 ± 5.4
Fasting plasma glucose, mg/dL	92.3 ± 7.0	101.4 ± 7.8	155 ± 49.0
Fasting plasma insulin, μU/mL	13.9 ± 6.6	25.0 ± 18.3	29.1 ± 23.5
HbA_{1c}, %	5.4 ± 0.4	5.6 ± 0.5	7.5 ± 2.0
HbA_{1c}, mmol/mol	36 ± 4.4	38 ± 5.5	58 ± 22
Vo_{2max}, mL/kg/min	28.6 ± 8.2	21.9 ± 4.6	21.8 ± 3.9

Data are reported as mean ± SD. IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NFG, normal fasting glucose; NGT, normal glucose tolerance; T2D, type 2 diabetes.

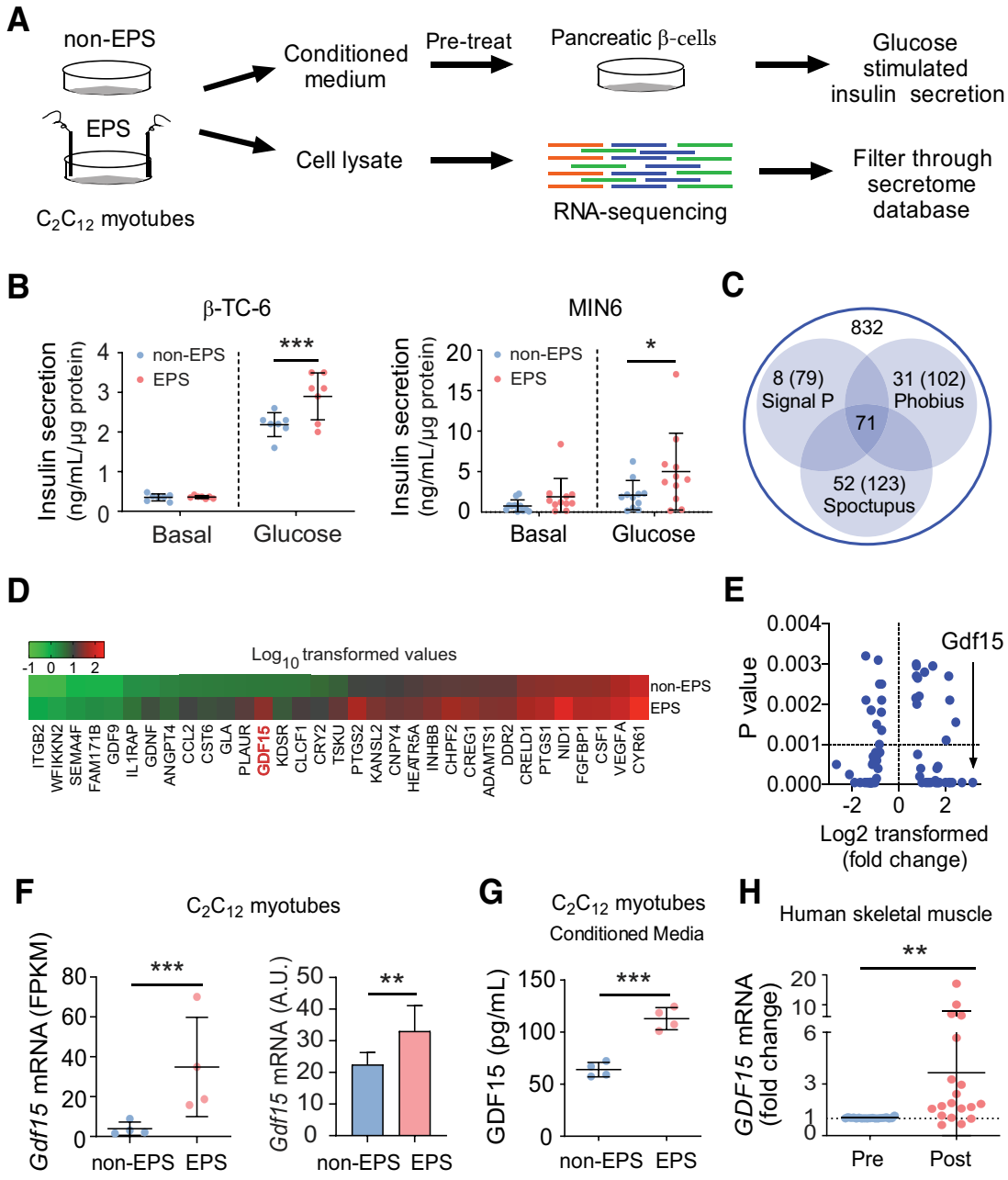

Figure 1—GDF15 secretion is increased in response to muscle contraction and exercise. *A*: Experimental flow to investigate cross talk between skeletal muscle and pancreatic β-cells. *B*: Insulin secretion in pancreatic β-cells after 16 h of treatment with CM collected from non-EPS or EPS-exposed C2C12 myotubes. Left: β-TC-6 cells treated with and without 1 mmol/L glucose for 3 h (n = 10). Right: MIN6 cells were treated with and without 10 mmol/L glucose for 1 h (n = 10). *C*: C2C12 myotubes were subjected to RNA-seq after 16 h of EPS or non-EPS, followed by filtration through a secretome database. A total of 71 secreted genes were significantly changed relative to the non-EPS condition (n = 4 repeated culture). *D*: Heat map of expression of 33 significantly upregulated genes. *E*: Scatterplot displaying the distribution of the significantly changed genes, which are presented as statistical significance (P value) vs. magnitude of change (fold change). *F*: *Gdf15* gene expression in EPS-stimulated C2C12 myotubes. Left: Results from transcriptome analysis expressed in fragments per kilobase of sequence per million (FPKM) mapped reads (n = 4 repeated culture). Right: Results from qPCR expressed in A.U. (arbitrary units) (n = 6). *G*: GDF15 protein secretion in the CM of EPS-exposed C2C12 myotubes (n = 4 repeated culture). *H*: *GDF15* gene expression in human skeletal muscle tissue. Gene expression was accessed in participants in Human Study 1 before and after 12-week aerobic exercise training (n = 19). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with non-EPS by two-way ANOVA with Bonferroni post hoc test for *B* and unpaired *t* test for *F* and *G*. **$P < 0.01$ compared with pretraining by paired *t* test for *H*.

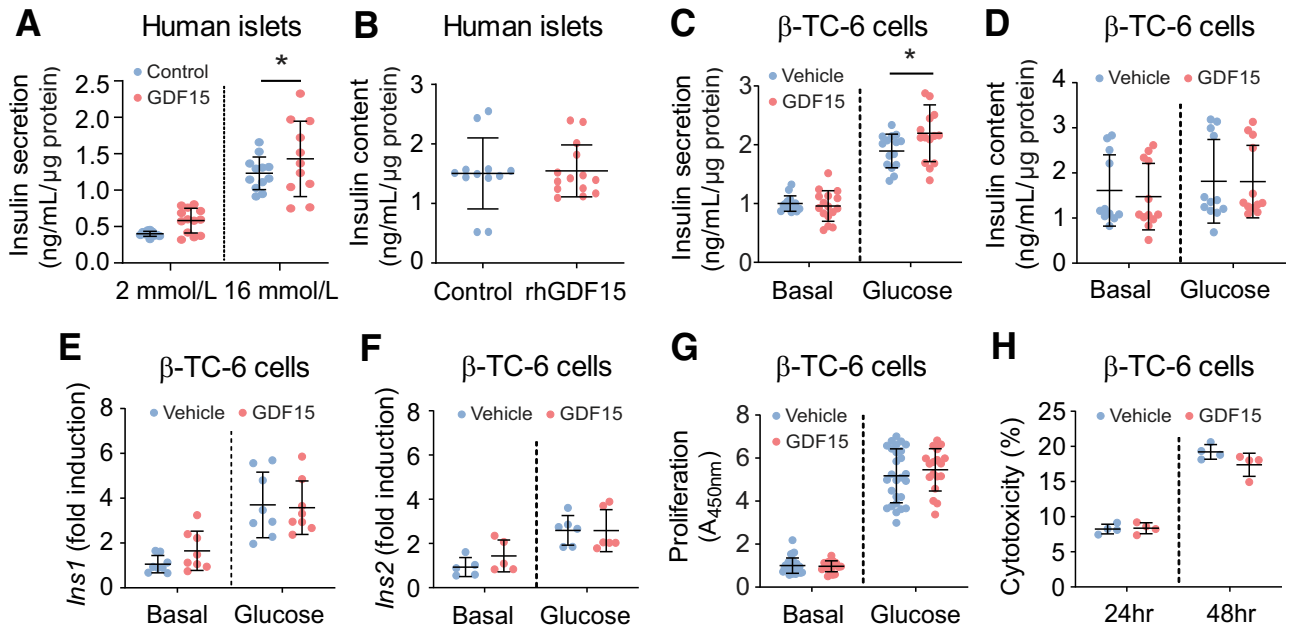

Figure 2—GDF15 increases GSIS in human islets and mouse pancreatic β-cells. *A* and *B*: Insulin secretion (*A*) and insulin content (*B*) in human islets treated with rhGDF15. Participants (n = 1); experiment replicates: n = 12/vehicle and n = 14/rhGDF15. *C* and *D*: Insulin secretion (*C*) and insulin content (*D*) in mouse pancreatic β-TC-6 cells after 3 h of rmGDF15 treatment in the presence or absence of 1 mmol/L glucose (n = 4 repeated culture, n = 4 replicates/culture). *E* and *F*: Gene expression of *Ins1* (*E*) and *Ins2* (*F*) in β-TC-6 cells treated with rmGDF15 in the presence or absence of 1 mmol/L glucose (n = 5–8). *G*: Effect of rmGDF15 on proliferation of β-TC-6 (n = 4 repeated culture, n = 4–10 replicates/culture). *H*: Toxicity of rmGDF15 in β-TC-6 cells (n = 4 repeated culture). Data are expressed as mean ± SD. *P < 0.05 compared with control by two-way ANOVA with Bonferroni post hoc test.

EPS. We then refined the pool of putative transcripts to those with known secretory functions by filtering through three protein databases: SignalP, SPOCTUPUS, and Phobius (Fig. 1*C*). This approach yielded 71 transcripts that were differentially expressed after EPS, 33 of which were upregulated (Fig. 1*D*). *Gdf15* was the transcript with the most significantly increased expression and was subsequently chosen for validation (Fig. 1*E*). Indeed, *Gdf15* expression was significantly increased in C2C12 myotubes after EPS, compared with non-EPS control myotubes (Fig. 1*F*). To validate protein expression after muscle contraction, CM from EPS-exposed myotubes was collected for assessment (Fig. 1*A*). GDF15 was increased by 76.3% in the CM of C2C12 myotubes (Fig. 1*G*) and by 57.6% in primary human muscle cells (Supplementary Fig. 3). To support these findings, we assessed changes in muscle *GDF15* expression after exercise training in humans. Characteristics of participants in Human Study 1 are listed in Table 1. In skeletal muscle tissue from this cohort of older adults with obesity, gene expression of *GDF15* was increased by 3.7-fold after 12 weeks of daily aerobic exercise training compared with the pretraining expression level (Fig. 1*H*). Taken together, these data suggest that *GDF15* gene expression is upregulated by muscle contraction and that exercise training promotes increased protein secretion.

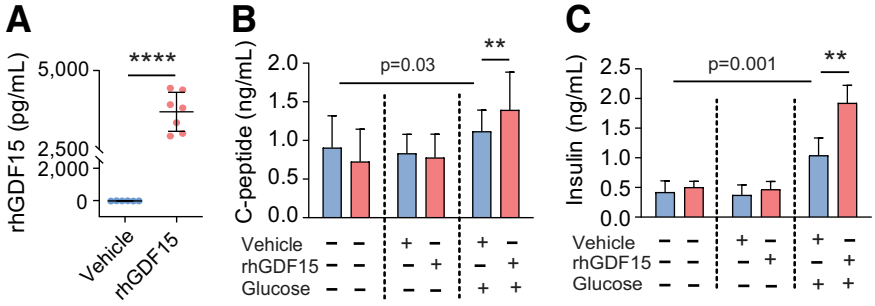

Figure 3—GDF15 increases GSIS in C57BL/6J mice. *A*: Fasting plasma rhGDF15 levels in C57BL/6J mice 2 h after intraperitoneal injection of vehicle or 0.25 mg/kg rhGDF15 (n = 6–7). *B*: Plasma C-peptide and (*C*) insulin levels in C57BL/6J mice pretreated with vehicle or rhGDF15 and after 1 g/kg D-glucose challenge (n = 6 in control group, n = 7 in the rhGDF15 treatment group). Data are expressed as mean ± SD. **P < 0.01 compared with control by unpaired t test for *A*, and two-way ANOVA with Bonferroni post hoc test for *B* and *C*.

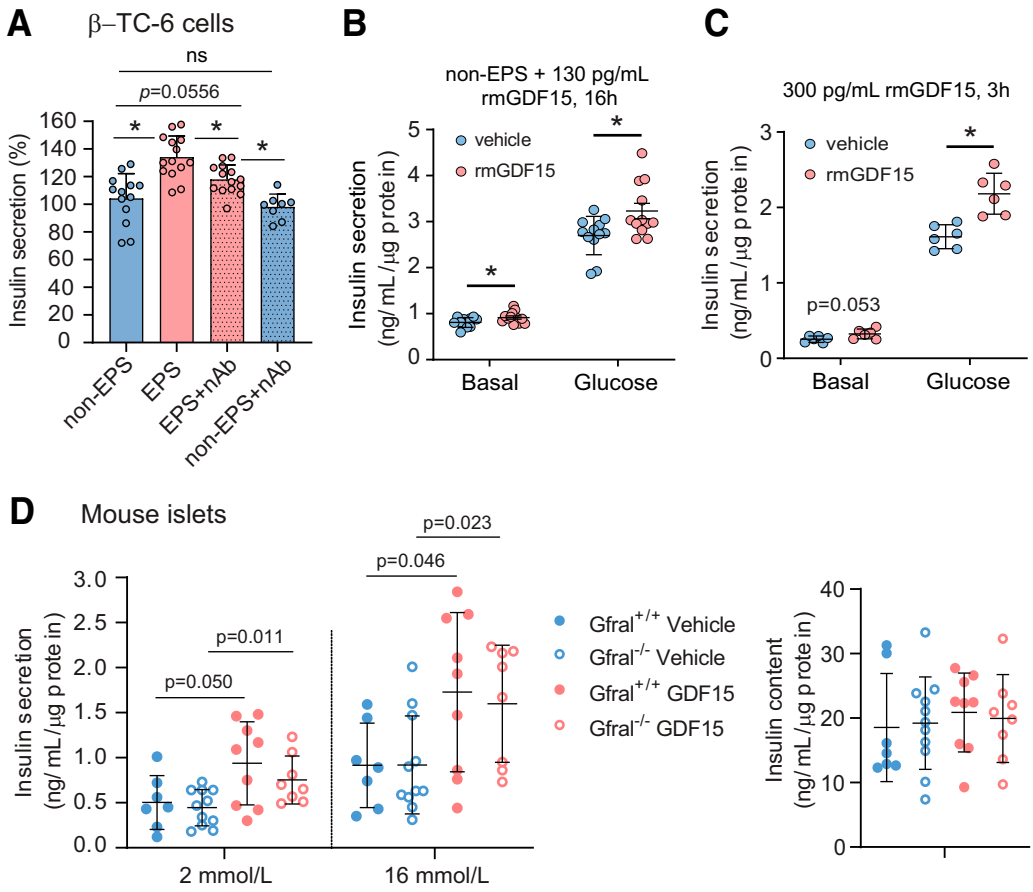

Figure 4—GDF15 at physiological doses increases GSIS in pancreatic β-cells. *A*: GSIS (1 mmol/L) in β-TC-6 cells after 16 h of treatment with CM: non-EPS, EPS, and supplemented with 10 μg/mL neutralizing antibody (nAb) anti-GDF15 at growth day 4. GSIS levels in non-EPC control cells were set as 100% (n = 2 repeated cultures, n = 6–7 replicates/culture). *B*: Insulin secretion in TC-6 cells in the presence or absence of 1 mmol/L glucose after 16 h of treatment with non–EPS-CM supplemented with vehicle or 130 pg/mL rmGDF15 at growth day 4 (n = 3 repeated cultures). *C*: Insulin secretion in β-TC-6 cells after 3 h of vehicle or 300 pg/mL rmGDF15 treatment in the presence or absence of 1 mmol/L glucose at growth day 3 (n = 3 repeated cultures, n = 4–6 replicates/culture). *D*: Insulin secretion in islets from *Gfral*$^{+/+}$ and *Gfral*$^{-/-}$ mice treated with rmGDF15 (n = 7–8 mice; n = 7–10 replicates/treatment). Data are expressed as mean ± SD. *$P < 0.05$ compared with vehicle-treated control by unpaired t test and two-way ANOVA with Bonferroni post hoc test.

GDF15 Potentiates GSIS

To determine whether GDF15 regulates β-cell function, isolated human islets and mouse β-TC-6 cells were exposed to rhGDF15. Under low-glucose (unstimulated) conditions, rhGDF15 treatment did not change insulin secretion in islets from a donor with prediabetes. Notably, in high-glucose (stimulated) conditions, insulin secretion was increased by 45% in rhGDF15-treated islets compared with control islets isolated from the same donor (Fig. 2*A*). This rhGDF15-induced increase in GSIS was independent of changes in total insulin content in human islets (Fig. 2*B*). To further explore the effect of GDF15 on β-cell function, pancreatic β-TC-6 cells were exposed to rmGDF15 for 3 h in the presence or absence of glucose. rmGDF15 alone did not promote insulin secretion but did potentiate GSIS (Fig. 2*C*). Like human islets, total insulin content was not changed in β-TC-6 cells with rmGDF15 treatment (Fig. 2*D*). In addition, *Ins1* and *Ins2* gene expression, as well as β-cell mass, were unaffected by rmGDF15 treatment under basal or glucose-stimulated conditions (Fig. 2*E–H*).

To determine the effect of GDF15 on GSIS in vivo, chow-fed male C57BL/6J mice were injected with saline or rhGDF15 and evaluated for insulin and C-peptide secretion (Fig. 3*A*). Mice were weight matched and glucose levels in circulation were not different in vehicle- or rhGDF15-injected mice (Supplementary Fig. 4*A* and *B*). We observed that GDF15 alone did not increase plasma C-peptide (Fig. 3*B*) or insulin concentrations (Fig. 3*C*). However, GDF15 increased GSIS and glucose-stimulated C-peptide release in mice (Fig. 3*B* and *C*). Collectively, these data support that GDF15 increases insulin secretion in isolated human islets, mouse β-cells, and chow diet–fed mice in a glucose-dependent manner.

GDF15 Is Required to Potentiate Contraction-Induced GSIS

To determine if GDF15 was required for EPS-CM to enhance GSIS, native protein was neutralized by incubation with an IgG anti-GDF15 antibody (54–56). Consistent with our previous experiments, GSIS increased by ∼30%

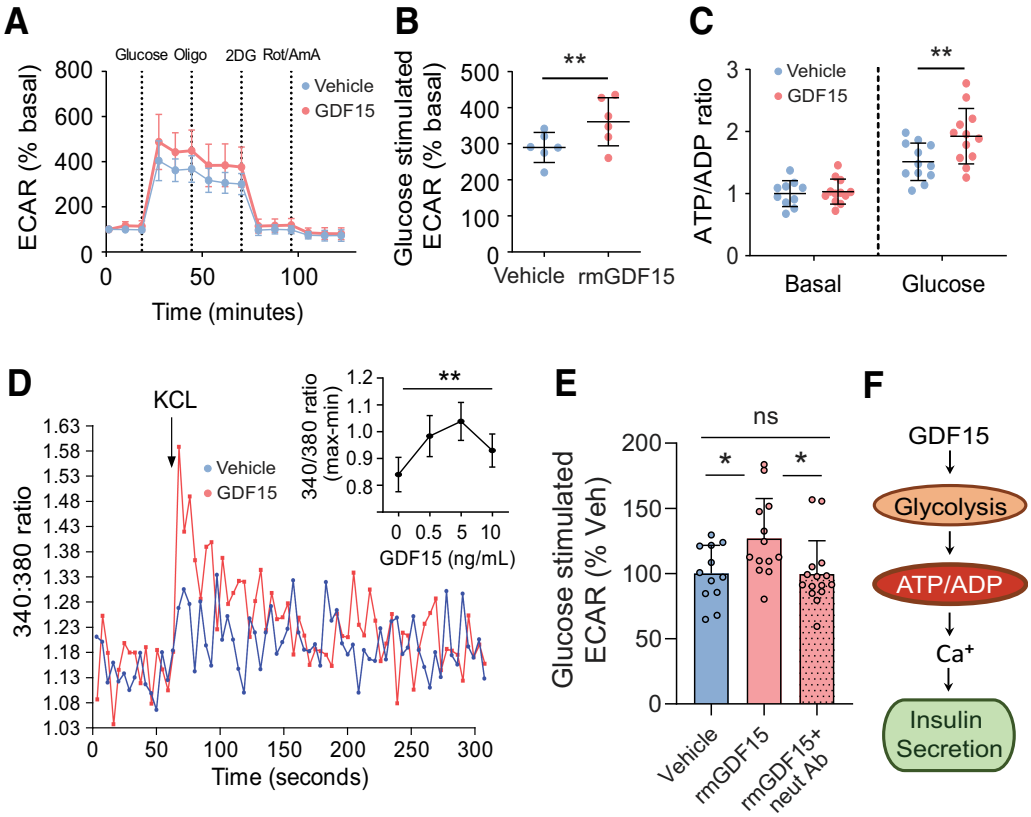

Figure 5— GDF15 upregulates the insulin secretion pathway. *A*: Extracellular acidification rate (ECAR) in β-TC-6 cells treated with rmGDF15 under 0.5 mmol/L glucose condition was determined using Seahorse XF24 extracellular flux analysis ($n = 6$). *B*: Glucose-stimulated (1 mmol/L) ECAR in β-TC-6 cells treated with rmGDF15 ($n = 6$). *C*: The ratio of ATP to ADP (ATP/APD) in β-TC-6 cells treated with rmGDF15 in the presence or absence of 1 mmol/L glucose ($n = 9$–12). *D*: Representative image of intracellular calcium influx in response to K^+ stimulation in β-TC-6 cells treated with vehicle or rmGDF15 ($n = 4$). Changes in intracellular calcium levels in β-TC-6 cells treated with rmGDF15 in response to K^+ stimulation are shown in the inserted graph ($n = 3$ repeated cultures, $n = 6$ replicates/culture). *E*: Glucose-stimulated ECAR in β-TC-6 cells at passage 5 treated with 5 ng/mL rmGDF15 under 1 mmol/L glucose conditions was determined by Seahorse XF24 extracellular flux analysis ($n = 4$ repeated cultures, $n = 7$–8 replicates/culture). *F*: Illustration of GDF15 effect on the insulin secretion pathway. Data are expressed as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$ compared with control by paired *t* test for (*A* and *B*), two-way ANOVA with Bonferroni post hoc test for *C*, and one-way ANOVA followed by unpaired *t* test for *D*. Ab, antibody; max, maximum; min, minimum; neut, neutralizing; Veh, vehicle. Rot, rotenone; AmA, antimycin A; Oligo, oligomycin.

after exposure to EPS-CM compared with β-cells exposed to non–EPS-CM. Blocking GDF15 with neutralizing antibody abrogated the effects of EPS-CM, and the insulin secretion rate did not differ significantly from non-EPS controls (Fig. 4*A*). Although, GDF15-neutralizing antibody in non–EPS-CM did not affect GSIS, the insulin secretion rate was still significantly lower compared with the effects of EPS-CM with the neutralizing antibody. Interestingly, replacement of non–EPS-CM with rmGDF15 to the concentration detected in EPS-CM (120 pg/mL) (Fig. 1*G* and Supplementary Fig. 6A) increased the basal insulin secretion rate by 13% and GSIS by 20% in β-TC-6 cells (Fig. 4*B*). Furthermore, short-term exposure of β-TC-6 cells with rmGDF15 at a physiological concentration increased GSIS by 30% (Fig. 4*C* and Supplementary Fig. 5*B*).

GDF15 has been shown to exert its peripheral metabolic actions via the GFRAL receptor (18–20). As such, we opted to evaluate whether GFRAL was required to potentiate the effects of GDF15 on GSIS. To this end, GDF15 increased GSIS in isolated islets obtained from GFRAL-deficient and control mice to a similar extent with no difference in insulin content in the islets (Fig. 4*D*). Interestingly, *Gfral* gene expression was not detected in β-TC-6 cells (Supplementary Fig. 6). However, we did detect expression of other putative GDF15 receptors, such as ErbB2, CD44, and CD48, in β-TC-6 cells (Supplementary Figs. 6 and 8). Taken together, these data demonstrate that GDF15 is required for muscle contraction–induced potentiation of GSIS and that replacement with recombinant protein at a physiological dose was sufficient to rapidly increase GSIS independent of GFRAL.

GDF15 Increases Glycolysis, ATP to ADP Ratio, and Calcium Release in β-cells

Having demonstrated that GDF15 increases GSIS in β-cells in vitro and in vivo, individual components of the canonical insulin-release pathway were investigated to identify how GDF15 was enhancing GSIS. First, glycolytic flux was assessed in real time by measuring extracellular acidification in response to glucose, oligomycin, 2-deoxy-D-glucose, and a

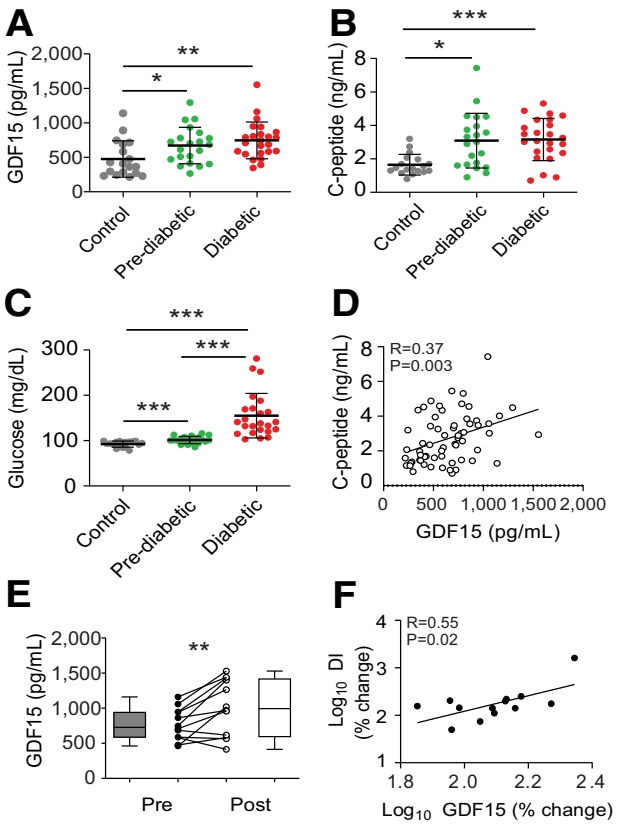

Figure 6—GDF15 is associated with improved insulin secretion in humans. *A*: Fasting plasma GDF15, (*B*) C-peptide, and (*C*) blood glucose levels in healthy control participants (n = 19), patients with prediabetes (n = 24), and those with type 2 diabetes (n = 27) from Human Study 2. *D*: Correlation between C-peptide and plasma GDF15 levels in participants in Human Study 2 (n = 62). *E*: Fasting plasma GDF15 levels in patients with type 2 diabetes in Human Study 3 before and after 6-weeks of high-intensity exercise training (n = 12). *F*: Association between exercise-induced circulating GDF15 levels and an improvement in the disposition index (DI) (β-cell function) in patients with type 2 diabetes (r = 0.69; P = 0.01; n = 12). Data are expressed as mean ± SD. *$P < 0.05$, **$P \leq 0.01$, ***$P < 0.001$ by one-way ANOVA followed by unpaired t test for *A–D* and by paired t test for *E*.

cocktail of rotenone and antimycin A in cultured β-cells (Fig. 5*A*). GDF15 increased glucose-stimulated extracellular acidification, a measure of net glycolytic activity (Fig. 5*B*). GDF15 treatment also increased the cytosolic ATP to ADP ratio (Fig. 5*C*) and the net intracellular calcium influx (Fig. 5*D*). Interestingly, the GDF15-stimulated increase in glycolysis was reversed with anti-GDF15 neutralizing antibody, and the glucose-stimulated extracellular acidification rate was not significantly different compared with the vehicle-treated rate (Fig. 5*E*). Additionally, GDF15 treatment did not significantly change expression of genes that maintain insulin secretion (e.g., *PDX-1*, *FOXO1*, *MafA*, *MafB*, *NeuronD1*, *NKX6.1*, *KLF11*, *GDF15*, *GLUT-2*, *GIPR*, *GLPIR*, *Kir6.1*, *SUR1*, and *Rab27a*) (Supplementary Fig. 7). Taken together, these data indicate that GDF15 enhances GSIS through activation of the canonical insulin secretion–signaling pathway (Fig. 5*F*).

GDF15 Is Associated With Enhanced β-Cell Function in Humans

To contextualize these findings, we then examined the relationship between GDF15 and β-cell function in humans. First, we conducted a cross-sectional analysis of circulating levels of GDF15 in sedentary adults across the spectrum of glucose tolerance (Human Study 2). We observed that fasting plasma GDF15 was increased in individuals with prediabetes and type 2 diabetes compared with healthy control participants (Fig. 6*A*). Of note, the relative increase in C-peptide (Fig. 6*B*) across the metabolic spectrum was accompanied by an incremental increase in GDF15. Fasting blood glucose was significantly greater in individuals with prediabetes compared with healthy control participants, although glucose levels in these individuals remained below 126 mg/dL (Fig. 6*C*). However, fasting glucose was further elevated in patients with type 2 diabetes compared with healthy control participants and individuals with prediabetes (Fig. 6*C*). Notably, C-peptide concentrations were positively correlated with circulating GDF15 in this cohort (Fig. 6*D*).

Next, we evaluated the effects of 6 weeks of high-intensity exercise training on circulating GDF15 and β-cell function in patients with type 2 diabetes (Human Study 3). Fasting plasma GDF15 was increased after training (from 768.5 ± 227.4 to 1,015.7 ± 397.6 pg/mL) (Fig. 6*E*), while fasting blood glucose (from 179.1 ± 57.5 to 157.0 ± 61.3, mg/dL; P = 0.2) and insulin (from 23.1 ± 20.2 to 25.8 ± 16.8 μU/mL; P = 0.5) remained relatively unchanged. Importantly, the change in circulating GDF15 levels between pre- and postexercise positively correlated with the insulin disposition index, a validated assessment of β-cell function (Fig. 6*F*). Together, these data provide clinical evidence to support a role for GDF15 in regulating glucose homeostasis and β-cell function.

DISCUSSION

GDF15 is a stress-sensitive cytokine (57) that regulates food intake, body weight, and glucose metabolism via direct action on the central nervous system (24,26,28,29). It has also been noted that GDF15 is highly enriched in hepatic tissue, suggesting that peripheral mechanisms may also contribute to its metabolic effects (58). Here, we provide comprehensive evidence that GDF15 is increased in response to skeletal muscle contraction and acts on pancreatic β-cells to improve GSIS. This observation is further supported by clinical studies demonstrating elevated GDF15 after exercise training and associations with β-cell function and glycemic control in patients with type 2 diabetes.

Using an in vitro contraction model, we found that GDF15 was secreted from C2C12 myotubes in response to EPS. These data are consistent with those in recent reports showing increasing GDF15 secretion after both acute and chronic exposure to EPS (56). Furthermore, others have now observed that circulating and skeletal muscle GDF15 is increased after exercise training in humans with obesity (45,56,59–61). In agreement with these observations, we

demonstrated that *GDF15* gene expression was increased in skeletal muscle tissue of adults with obesity and circulating GDF15 concentrations elevated in patients with type 2 diabetes after exercise training. In contrast, circulating GDF15 is unchanged in mice after acute treadmill running (62). Interestingly, acute cycling increased arterial and venous GDF15, whereas the arterial-venous difference was unaffected, indicating that exercise stimulates GDF15 secretion but that under these conditions, skeletal muscle may not substantially contribute to the circulating pool (59). It is likely that differences in exercise protocols, study populations, exercise duration, and intensity or timing of sample collections may influence GDF15 release from skeletal muscle and contribute to these disparate findings.

Exercise volume and mechanical loading are particularly significant (63); other studies have shown that C/EBP-homologous protein (CHOP), which is upstream in the unfolded protein response pathway, directly binds the GDF15 promoter to activate its transcription under endoplasmic reticulum stress conditions in the liver (64). Additionally, *GDF15*-deficient mice exposed to acute exercise exhibit hyperactivation of skeletal muscle *Atf3* and *Atf6*, and *Xbp1* (62), all of which induce unfolded protein response target gene expression (62). It is also known that muscle contraction upregulates synthesis, posttranslational modification, and signaling of membrane trafficking proteins (65). However, it was recently demonstrated that CHOP was not required for contraction-induced GDF15 release (66), whereas it was shown that AMPK is a central mediator when energy deficiency is present in the liver (67). Given that exercise activates AMPK signaling in skeletal muscle, there is the possibility that contraction-stimulated AMPK activation is a GDF15-driven mechanism in muscle. Whether GDF15 secretion from contracting muscle is mediated by other forms of stress, by changes in stress machinery or vesicle trafficking, or both, are areas ripe for further investigation.

We have previously shown that an acute bout of intense exercise increases GSIS in healthy young adults (68). After intense exercise, plasma insulin concentrations can increase two- to threefold to offset increased glucose production in response to prolonged glycogenolysis (69). In this situation, insulin secretion may be triggered by glucose-sensing mechanisms but may also involve direct communication between contracting muscle and pancreatic β-cells (13). There is also strong evidence that exercise training enhances β-cell function in humans with type 2 diabetes (4,6,46). Herein, we show that GDF15 is increased after exercise training in patients with type 2 diabetes and the increase correlates with improved β-cell function. Furthermore, our cell culture and animal data demonstrate that GDF15 stimulates insulin secretion in β-cells, human and mouse islets, and C57BL/6J mice. However, we recognize that GDF15's in vivo regulation of insulin secretion in humans was not directly confirmed in the present set of studies. This will require the development and validation of endogenous labeling techniques to trace GDF15 from source to site of action.

Presumably, GDF15 synergistically mediates insulin secretion by receptor-mediated signal transduction. However, GFRAL, the widely acknowledged receptor for GDF15, is not present in the human pancreas or β-TC-6 cells (18–21), though it appears to be present in pancreatic ductal adenocarcinoma (70). Nonetheless, our data rule out a direct GDF15/GFRAL signaling pathway in β-cells in normal conditions. In support of this, GDF15 is present in immortalized β-cell lineages, murine islets, and pancreatic sections from humans with type 1 diabetes and nondiabetic and type 2 diabetic human islets despite no detection of GFRAL (33,34).

Compellingly, recent work in rodents demonstrated that GDF15 activates the hypothalamic-pituitary-adrenal axis through hindbrain receptor, GFRAL (71). Endocrine responses including by the hypothalamic-pituitary-adrenal axis to exercise in humans vary depending upon the mode, frequency, and duration of exercise (72). Cortisol released in intensely exercising men suppressed the subsequent cortisol response to acute stressors (stress-buffering effect of exercise) (73,74). Thus, regardless of the absence of expression of GFRAL in the pancreas, involvement of GFRAL signaling in insulin secretion at the whole-body level as it relates to exercise-induced GDF15 and hypothalamic-pituitary-adrenal axis activation needs further investigation.

On the other hand, ErbB2 in cervical cancer cells and CD44 and CD48 in immune cells such as dendritic and regulatory T cells in a human cancer environment have been revealed recently as potential GDF15 receptors (75–77). CD44 also can function as a coreceptor to the ERBB family of receptor tyrosine kinases in cancer cells (78–80). Interestingly, we detected all three receptors in β-TC-6 cells expressed at the gene and surface protein levels. Thus, GDF15 may bind to an individual receptor or a complex of the coreceptors to synergistically enhance GSIS.

Conversely, GDF15 may regulate β-cell function through a glucose-dependent but nonreceptor-mediated mechanism, because GDF15 alone does not affect β-cell function. Previous studies have also reported that endogenous GDF15 is triggered in a glucose-dependent manner in HepG2 cells (81), which would be consistent with a glucose-coupled mechanism. Our data suggest that another potential mechanism may act via regulation of the insulin-release pathway. β-cells treated with GDF15 displayed enhanced glycolytic function, an ATP to ADP ratio, and intracellular calcium flux independent of altering insulin gene expression, insulin content, β-cell toxicity, or growth. These observations are consistent with studies showing that GDF15 amplifies intracellular calcium flux by increasing CaV1.3 expression (82) and promoting CaV3.1 and CaV3.3 α-subunit membrane trafficking in neurons (83).

Increased insulin secretion may also have consequences for how the β-cell itself functions, because increased glycolytic flux, decreased ADP concentration, and increased intracellular calcium concentrations can

all contribute to mitochondrial oxidative stress, in turn leading to a progressive decline in insulin secretion from β-cells (84). The clinical term for this phenomenon is secondary failure, and it manifests as short-term glycemic control but worse long-term type 2 diabetes disease progression (85). However, in our study, GDF15 treatment did not induce β-cell toxicity. This may be due to increased antioxidant activity or reduced oxidative stress resulting from β-cell detoxification (86). In fact, chronic GDF15 treatment of diet-induced obese mice for 1 year reduced islet hypertrophy and improved glucose homoeostasis (26), although it is not clear whether β-cell preservation was directly mediated by GDF15 or secondary to GDF15-induced weight loss. A similar phenotype was observed in *ob/ob* and diet-induced obese mice (18–26), making it more striking as a potential therapeutic target for type 2 diabetes.

We conclude that GDF15 secretion is increased in response to skeletal muscle contraction and exercise, and that the synergistic action of GDF15 and glucose enhances β-cell function. The mechanistic underpinnings are driven largely by acute potentiation of the insulin secretion pathway, independent of β-cell mass and GFRAL. Collectively, these findings advance our understanding of how GDF15 may contribute to regulation of glycemic control and provide important evidence for a role for GDF15 in the treatment of type 2 diabetes and related diseases.

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