Human islet expression levels of Prostaglandin E_2 synthetic enzymes, but not prostaglandin EP3 receptor, are positively correlated with markers of β-cell function and mass in non-diabetic obesity.

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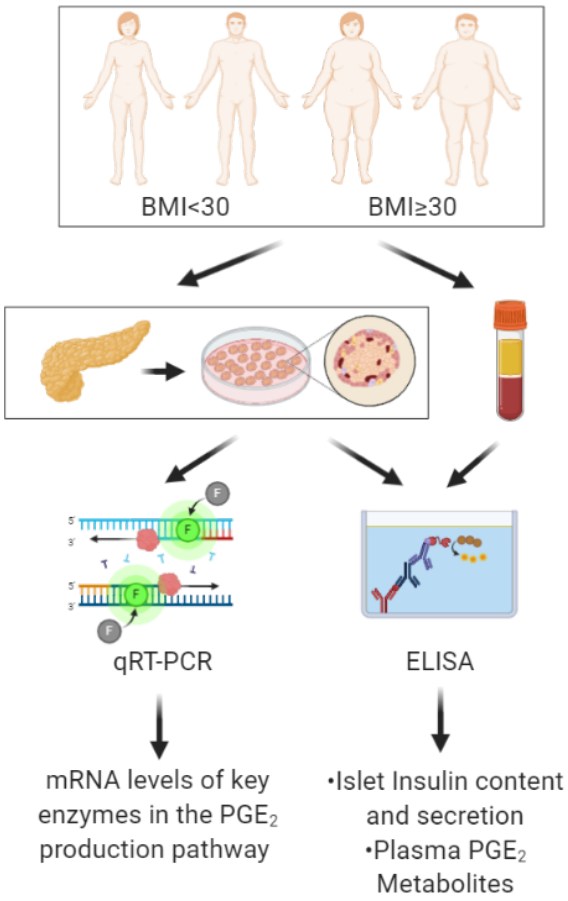
Keywords: IL-6; EP3 receptor; obesity; insulin resistance; β-cell compensation; prostaglandin E_2; insulin secretion; human islets

Abstract: We and others previously reported increased signaling through the Prostaglandin E3 Receptor (EP3), a G protein-coupled receptor (GPCR) for the arachidonic acid metabolite, prostaglandin E_2 (PGE_2), is associated with β-cell dysfunction of type 2 diabetes (T2D). Yet, the relationship between PGE_2 production and signaling and β-cell function during the progression to T2D remains unclear. In this work, we assessed gene expression from a panel of cadaveric human islets from 40 non-diabetic donors with BMI values spanning the spectrum from lean to high-risk obesity. Interleukin-6 (gene symbol: *IL6*) and cyclooxygenase-2 (COX-2) (gene symbol: *PTGS2*) mRNA levels were positively correlated with donor body mass index (BMI), while EP3 (gene symbol: *PTGER3*) was not. *IL6* was itself strongly correlated with *PTGS2* and all but one of the other PGE_2 synthetic pathway genes tested. About half of the islet preparations were used in glucose-stimulated- and incretin-potentiated insulin secretion assays using an EP3-specific antagonist, confirming functionally-relevant up-regulation of PGE_2 production. Islets from obese donors showed no inherent β-cell dysfunction and were at least equally as glucose- and incretin-responsive as islets from non-obese donors. Furthermore, insulin content, a marker of islet size known to be associated with donor BMI, was also significantly and positively correlated with islet *PTGS2* expression. We conclude up-regulated islet PGE_2 production and signaling may be a necessary part of the β-cell adaption response, compensating for obesity and insulin resistance. Analysis of plasma PGE_2 metabolite levels from a clinical cohort reveal these findings are not in conflict with the concept of further elevations in PGE_2 production contributing to T2D-related β-cell dysfunction where islet EP3 expression has also been up-regulated.

Graphical Abstract:



Introduction

Fundamentally, type 2 diabetes mellitus (T2D) results from a failure of the insulin-secreting pancreatic β-cells to compensate for peripheral insulin resistance. Obesity is the most common co-morbidity found in individuals with insulin resistance. While there exists debate about whether one condition precedes the other, the physiological changes that often occur in the obese, insulin-resistant state (e.g., systemic inflammation, dyslipidemia, hyperinsulinemia, mild hyperglycemia) simultaneously induce β-cell stress and increase β-cell workload, forcing the β-cell to initiate a compensatory response in order to function, replicate, and survive[1-3]. Whether the β-cell can initiate and continue this adaptive program is the key determinant of the progression to T2D[2].

Cyclic adenosine monophosphate (cAMP) is a well-characterized potentiator of glucose-stimulated insulin secretion (GSIS) and promotes a number of proliferative and survival pathways in the β-cell[4-8]. Numerous changes in cAMP homeostasis occur in a highly compensating β-cell, all with the central theme of increasing cAMP production, decreasing cAMP degradation, or promoting signaling through downstream effectors[7, 9-12]. Our and others' previous studies suggest that signaling through the Prostaglandin EP3 receptor (EP3), a G protein-coupled receptor for the arachidonic acid metabolite, prostaglandin E_2 (PGE_2), is up-regulated by the pathophysiological conditions of T2D, negatively influencing β-cell function and mass through its inhibition of adenylyl cyclase[13-21]. Islets isolated from T2D mice and human organ donors express more EP3 and produce more PGE_2 than islets isolated from non-diabetic controls[13, 18], and treating these islets with an EP3 receptor antagonist, L798,106, potentiates GSIS and restores their secretory response to glucose and glucagon-like peptide 1 receptor (GLP1R) agonists[13]. As GPCRs form the largest class of druggable targets in the human genome, we and others have previously proposed EP3 as

a putative therapeutic target for the β-cell dysfunction of T2D[9, 13, 14, 18, 22-25]. Yet, other investigators have found no correlation of EP3 with human organ donor obesity and/or T2D status, and PGE_2 has other downstream effects shown to be important in β-cell replication and/or survival[9, 14]. Finally, while the effects of EP3 signaling in metabolically healthy islets and T2D islets has been well-characterized, little is known about the role of PGE_2 production and signaling in the β-cell highly compensating for obesity and insulin resistance. As many of the same metabolic derangements of T2D are present in severe insulin resistance (e.g., hyperglycemia, dyslipidemia, systemic inflammation, etc.), this is a critical issue to address.

To our knowledge, a comprehensive analysis of the PGE_2 production and EP3 signaling pathways and their correlations with β-cell function and mass have never been completed in pancreatic islets of non-diabetic human donors spanning the obesity spectrum. In this work, we profiled the islet mRNA expression of genes encoding EP3 and PGE_2 production pathway enzymes using a comprehensive set of islets obtained from 40 non-diabetic human organ donors distributed across a BMI range of ~22-45 kg/m^2. Just over half of these islet preparations were also used in glucose-stimulated- and incretin-potentiated insulin secretion assays to determine the impact of endogenous EP3 signaling on β-cell function with insulin content serving as a surrogate of β-cell mass. When combined with a strong association of plasma PGE_2 metabolite levels with T2D in a clinical cohort even in non-obese subjects, our results suggest differential effects of PGE_2 signaling depending on the extent to which PGE_2 production is enhanced and islet EP3 expression is up-regulated.

Results

Relative mRNA levels of key enzymes in the PGE_2 production pathway are positively correlated with non-diabetic donor BMI, obesity status, and islet IL6 *expression.*

Relative quantitative PCR (qPCR) for PGE_2 synthetic pathway genes, along with interleukin-6 (*IL-6*), known to be associated with adipose inflammation and insulin resistance[26-28], was performed on cDNA samples from a panel of islet preparations from non-diabetic human organ donors spanning a BMI range of 22.8-44.7 (**Table S1**). The set included 27 male and 13 female donors, with a mean age of 42.6 years and mean BMI of 31.5 kg/m^2 (**Table 1**). The difference between the mean ages of non-obese (40.7 years) and obese donors (44.0 years) was not statistically different. Seventeen islet preparations were from donors with BMI < 30. Five of these were from lean donors (BMI < 25), with no donors being underweight (BMI < 18.5), and 12 were from overweight donors (BMI 25-29.9). Of the 23 obese islet donors, 13 were in the low-risk obesity category (BMI < 35), 8 were in the moderate-risk category (BMI 35 – 39.9), and were 2 classified as high-risk obesity (BMI ≥ 40). This BMI distribution suggests our panel is a good representation of the normal physiological changes islets undergo during the progression from lean to overweight to obesity in both sexes. (see **Table 2** for primer sequences).

Table 1: Donor demographics of islet preparations used in gene expression and insulin secretion assays (i.e., Islet Set 1). NR, not reported

Donor Demographics	All	BMI < 30	BMI ≥ 30	P-value
Subjects (N)	40	17	23	-
Male, N (%)	27 (68%)	12 (70%)	15 (65%)	-
Female, N (%)	13 (32%)	5 (30%)	8 (35%)	-
Age (y), mean ± SD (range)	42.6 ± 11.6 (29 – 73)	40.7 ± 12.3 (19 – 61)	44.0 ± 7.6 (21 – 63)	0.3829
BMI (kg/m^2), mean ± SD (range)	31.5 ± 5.5 (22.8 – 44.7)	26.4 ± 2.2 (22.8 – 29.6)	35.2 ± 3.6 (30.6 – 44.7)	<0.0001
HgA1c (%), mean ± SD (range)	-	NR	NR	-

Table 2: Primer sequences used for quantitative gene expression analyses.

Protein	Gene Symbol	Primer Sequences	Species Selectivity
β-actin	*ACTB*	F: ACCACACCTTCTACAATGAGC R: GATAGCACAGCCTGGATAGC	human
Interleukin-6 (IL-6)	*IL6*	F: GTGCTCTTGGTGAGGAAGTT R: TTCTGGGACTCCTGGGAATA	human

Cyclooxygenase 1 (COX-1) (aka PTGS1)	*PTGS1*	F: GTTCAACACCTCCATGTTGG R: CCACAGCCACATGCAGGATG	human
Cyclooxygenase 2 (COX-2) (aka PTGS2)	*PTGS2*	F: CGAGGTGTATGTATGAGTGTGG R: CAAAAATTCCGGTGTTGAGCAG	human
Prostaglandin E synthase (PTGES)	*PTGES*	F: TGGTCATCAAGATGTACGTGGTGGC R: TAGATGGTCTCCATGTCGTTCCGGT	human
Prostaglandin E synthase 2 (PTGES2)	*PTGES2*	F: ACCTCTATGAGGCTGCTGACAAGT R: CATACACCGCCAAATCAGCGAGAT	human
Prostaglandin E synthase 3 (Ptges3)	*PTGES3*	F: AAAGGAGAATCTGGCCAGTCATGG R: TCCTCATCACCACCCATGTTGTTC	human
Prostaglandin EP3 receptor (EP3)	*PTGER3*	F: TCACCTTTTCCTGCAACCTG R: ACGCACATGATCCCCATAAG	human

The relationship between gene expression and BMI was determined using two methods: (1) linear regression and (2) binning samples by donor obesity status (BMI < 30 and BMI > 30; N=17 non-obese and N=23 obese donors in each set) and performing a two-tail T-test. The complete results of this analysis can be found in **Table S2**. Besides IL-6 (*IL6*) and COX-2 (*PTGS2*) (**Figure 1A,B**), none of the other genes were significantly correlated with BMI, including EP3 (*PTGER3*) (**Figure 1C**). Because the latter finding is partially in conflict with previously-published work[13, 14], *PTGER3* expression was measured from an independent set of non-diabetic human donor islets

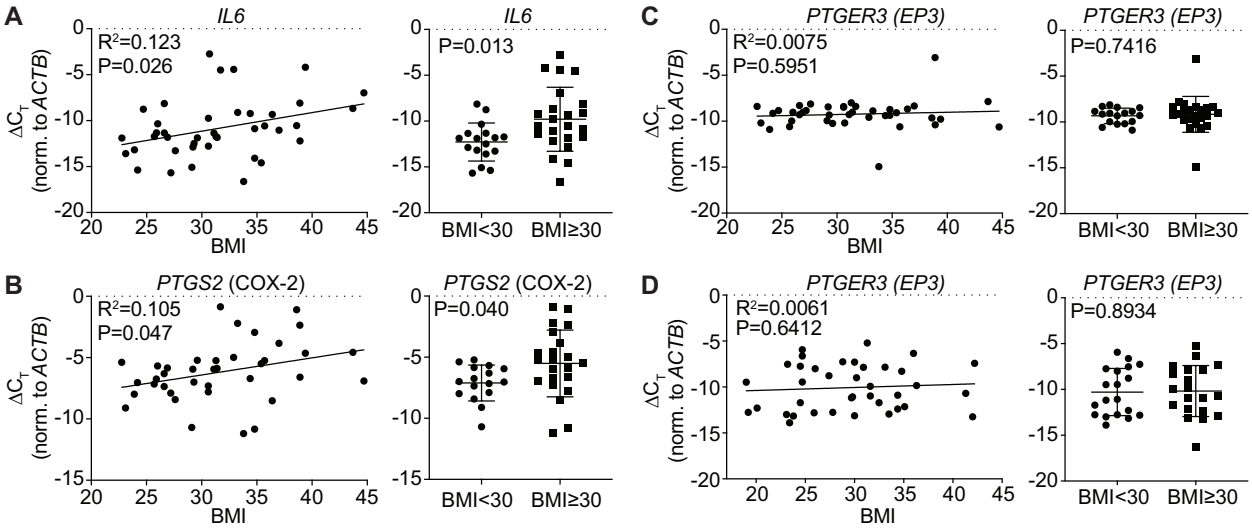


Figure 1. Islet *IL6* and *PTGS2* expression, but not that of *PTGER3*, positively correlates with donor BMI. A-C: Quantitative PCR results from Islet Set 1 for (A) *IL6*, (B) *PTGS2*, and (C) *PTGER3*. D: Quantitative PCR results from Islet Set 2 for *PTGER3*. Data are represented as cycle time normalized to that of β-actin (*BACT*) (ΔC_T). Data were analyzed by linear regression vs. donor BMI (left panels) or two-tailed t-test by donor obesity status (right panels). The Goodness-of-fit (R^2) and P-value after linear regression analyses and P-value after two-tailed t-test are indicated.

with almost identical sex distribution, age range, and BMI range (**Table 3 and Table S3**), with nearly identical results (**Figure 1D**).

Table 3: Donor demographics of islet preparations used for confirmation of PTGER3 expression (i.e., Islet Set 2). NR, not reported

Donor Demographics	All	BMI < 30	BMI ≥ 30	P-value
Subjects (N)	40	17	23	-
Male, N (%)	24 (60%)	12 (71%)	12 (52%)	-
Female, N (%)	16 (40%)	5 (29%)	11 (47%)	-
Age (y), mean ± SD (range)	41.4 ± 13.2 (16 – 64)	43.4 ± 12.1 (20 – 65)	39.7 ± 12.8 (16 – 64)	0.3603
BMI (kg/m^2), mean ± SD (range)	30.4 ± 6.0 (19.0 – 42.2)	25.0 ± 3.2 (19.0 – 29.8)	34.4 ± 3.6 (30.0 – 42.2)	<0.0001
HgA1c (%), mean ± SD (range)	-	NR	NR	-

PTGS2 expression was highly correlated with *IL6*, as were all of the other PGE_2 production genes, save *PTGES3* (**Figure 2**). No relationship was observed between *IL6* and *PTGER3* expression (**Table S2**).

An EP3 antagonist promotes glucose-stimulated and incretin-potentiated insulin secretion in islets from obese donors only.

Activated EP3 acts as a non-competitive antagonist of GLP1R, blocking its maximal potentiating effect on insulin secretion[13]. In order to test the impact of endogenous EP3 signaling on human β-cell function in non-diabetic obesity, we performed glucose-stimulated insulin secretion (GSIS) assays with and without the addition of the GLP1R agonist, exendin-4 (Ex4) and the EP3 antagonist, L798,106.

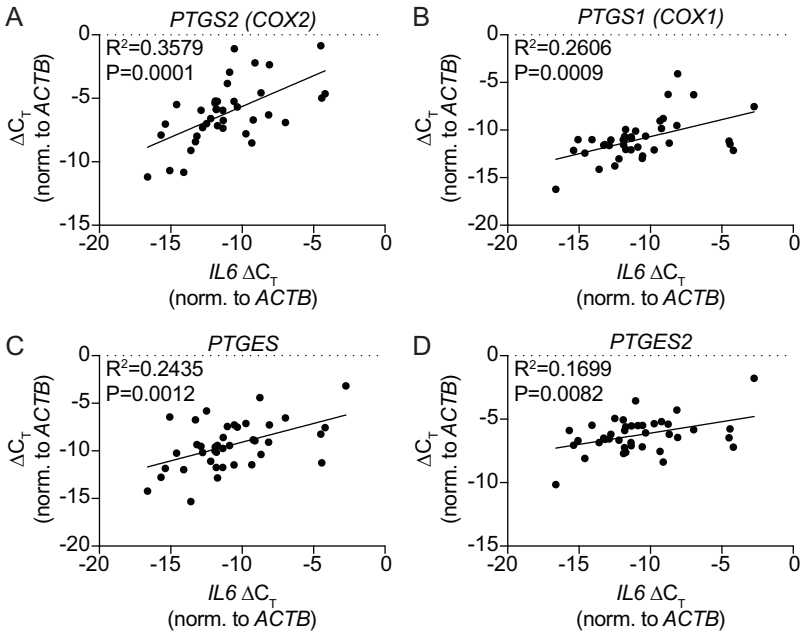


Figure 2. PGE_2 production genes are positively correlated with *IL6* expression. A-D: Quantitative PCR results from Islet Set 1 for (A) *PTGS2*, (B) *PTGS1*, (C) *PTGES*, and (D) *PTGES2*. Data are represented as cycle time normalized to that of β-actin (*BACT*) (ΔC_T) and were analyzed by linear regression. The Goodness-of-fit (R^2) and P-value are

Islets from non-obese and obese donors were both glucose responsive, with significantly more insulin secreted as a percent of content in stimulatory glucose (16.7 mM) vs. basal glucose (1.7 mM) (**Figure 3**). Comparing the results from non-obese to obese donor islets, GSIS was higher in every condition tested, with GSIS in 1.7 mM glucose and 16.7 mM glucose plus L798,106 being significantly so. Finally, only in islets from obese donors did L798,106 and Ex4 treatment synergize to enhance GSIS above 16.7 mM glucose alone. These results confirm the elevated expression of PGE_2 synthetic genes in non-diabetic obesity is of functional consequence, although islets from the obese donors in the cohort used in our studies were not dysfunctional.

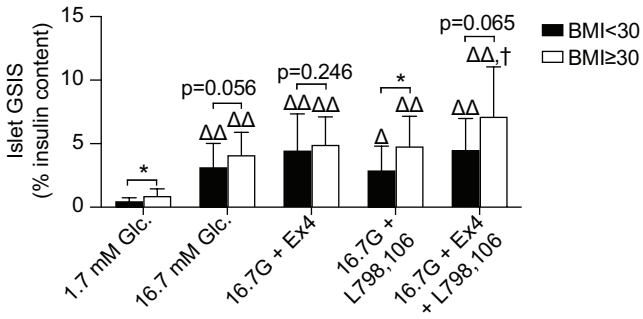


Figure 3. An EP3 antagonist promotes glucose-stimulated and incretin-potentiated insulin secretion in islets from obese donors only. Acute islet insulin secretion by donor obesity status (N=11, BMI < 30 and N=10, BMI ≥ 30) in the presence of 1.7 mM glucose, 16.7 mM glucose, or 16.7 mM glucose with 10 nM Ex4, 20 μM L798,106, or both. Data are represented as % insulin secreted as normalized to content and plotted as mean ± SD. Within treatments, BMI groups were compared by two-way t-test. Within BMI groups, data were compared by one-way paired ANOVA with Holm-Sidak test post-hoc to correct for multiple comparisons. *, $p<0.05$ for BMI < 30 vs. BMI ≥ 30. Δ, $P<0.05$ and ΔΔ, $P<0.01$ as compared to 1.7 mM glucose. †, $p<0.05$ as compared to 16.7 mM glucose. Unless indicated, a comparison was not statistically significant.

Islet insulin content is positively correlated with donor BMI and PTGS2 *expression.*

The GSIS assays in Figure 3 were performed on single islets adhered to the vertex of a 96-well V-bottom tissue-culture treated microtiter plate, and each islet's insulin secretion was normalized to its own insulin content. This method has been previously optimized by our laboratory to produce high-quality, reproducible results from mouse and human islets[29]. With the standard curve, this provides up to 76 biological replicates per islet preparation to calculate mean islet insulin content. Insulin content itself is significantly correlated with BMI, both linearly ($p=0.0018$; $R^2=0.408$) and

by obesity status (p<0.01) (**Figure 4A,B**). Notably, insulin content was also significantly correlated with *PTGS2* expression (P=0.0005; R^2=0.4766). None of the other genes had a significant correlation with islet insulin content, although *IL6* was the second-most highly correlated after *PTGS2* (p=0.172; R^2=0.096) (**Table S2**).

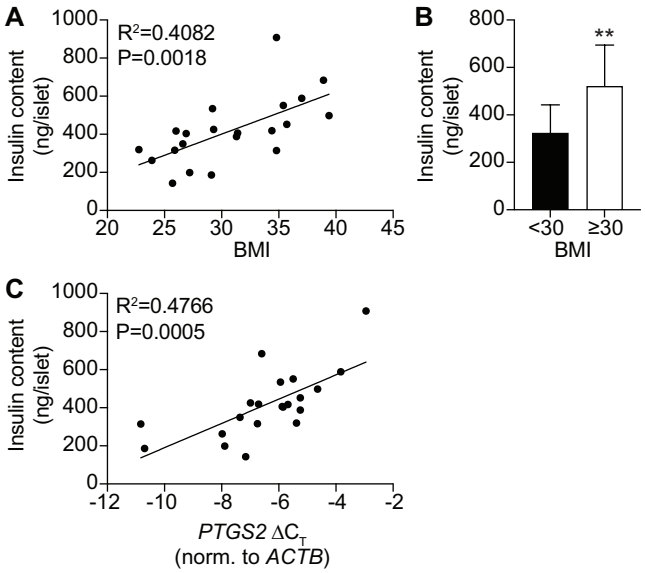


Figure 4. Islet insulin content is positively correlated with BMI and *PTGS2* expression. A & B, Islet insulin content (ng/islet) was subject to linear curve-fit analysis vs. donor BMI (A) or two-tailed t-test by donor obesity status (B). C, Islet insulin content was subject to linear curve-fit analysis vs. *PTGS2* expression. In A and C, the goodness-of-fit (R^2) and p-value for deviation from zero of each of the analyses are indicated. In (B), N=11, BMI < 30 and N=10, BMI ≥ 30. Data represent mean ± SD and were compared by two-way t-test. N=10-11 per group. **, p < 0.01

Plasma PGE_2 metabolite (PGE-M) levels correlate strongly with T2D disease status in a clinical cohort.

132 subjects with diagnosed T2D and 35 non-diabetic (ND) controls were enrolled at University of Wisconsin Hospitals and Clinics to provide biometric parameters, routine clinical labs, and a plasma sample for research purposes. The total cohort was equally representative of male (51%) and female (49%) subjects, with a higher percentage of female subjects in the ND group (63%) and a higher percentage of male subjects in the T2D group (55%) (**Table 4**). The mean age of ND subjects was lower than that of T2D subjects (ND,

Table 4: Demographics of Non-diabetic (ND) and T2D study subjects recruited to provide a plasma sample for PGE metabolite analysis.

Cohort Demographics	All	Non-Diabetic (ND)	T2D	P-value
Subjects (N)	167	35	132	-
Male, N (%)	85 (51%)	13 (37%)	72 (55%)	-
Female, N (%)	82 (49%)	22 (63%)	60 (45%)	-
Age (y), mean ± SD (range)	54.1 ± 10.5 (29-73)	47.8 ± 11.5 (29-70)	55.8 ± 9.6 (29-73)	<0.0001
BMI (kg/m^2), mean ± SD (range)	35.2 ± 8.3 (19.5-61.9)	30.0 ± 6.4 (19.5-50.5)	36.8 ± 7.7 (21.8-61.9)	<0.0001
HgA1c (%), mean ± SD (range)	-	5.4 ± 0.2 (4.8-5.9)	8.2 ± 1.2 (6-11.5)	<0.0001

47.8 ± 11.5 y vs. T2D, 55.8 ± 9.6 y; $p<0.0001$), but both means represented mid-late middle-age and the age ranges of the two groups were nearly identical (ND, 20-70 y vs. T2D, 29-73 y). The mean BMI was also lower in non-diabetic subjects vs. T2D subjects (ND, 30.0 ± 6.4 kg/m^2 vs. T2D, 36.8 ± 7.7 kg/m$_2$; $p<0.0001$), but both means represented low-to-moderate risk obesity (BMI 30-39.9) and both groups had BMI ranges that spanned the continuum from lean (18.5-24.9) to high-risk obesity (>40). Therefore, we viewed these statistically-significant differences in group age or BMI likely to have limited biological relevance to our analysis. Besides daily aspirin therapy for cardiovascular health, which was higher in T2D subjects vs. ND (50.0% vs. 7.7%, respectively), chronic COX inhibitor use within the past 90 days was an exclusion criterion of our study.

PGE_2 in clinical biosamples undergoes rapid metabolism to form 13,14-dihydro-15-keto prostaglandin E_2, which undergoes a variable degree of degradation to form 13,14-dihyrdro-15-keto prostaglandin A_2 depending on the type of biofluid, processing, and storage conditions. The Prostaglandin E Metabolite (PGEM) ELISA (Cayman Chemical Company) converts both of these metabolites to a single, stable derivative correlating with initial PGE_2 levels. Mean plasma PGEM was significantly elevated in the T2D group vs. ND group (an approximate 200% increase; $p<0.0001$) (**Figure 5A**). Within the T2D group, degree of glucose control did not affect PGEM levels, with no statistically significant differences in plasma PGEM in subjects with excellent, good, or poor glucose control (HbA1c < 6.9%, HbA1c 7.0 – 8.9%, or HbA1c ≥ 9.0%, respectively) (**Figure 5B**). When both groups were binned by BMI, the mean plasma PGEM levels were higher in obese subjects, but in neither case was this statistically-significant (**Figure 5C**). T2D was a

much stronger predictor of PGEM levels, with non-obese T2D subjects having significantly elevated PGEM levels as compared to non-obese T2D subjects (**Figure 5C**).

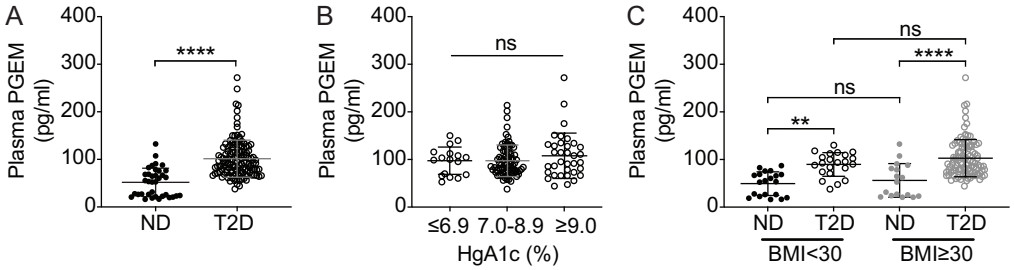


Figure 5. Plasma PGE_2 metabolite (PGEM) levels are significantly associated with T2D status but not glucose control or obesity in a human clinical cohort. A, Plasma PGEM levels in a cohort of 132 T2D subjects and 35 non-diabetic (ND) controls. B, Plasma PGEM levels in T2D subjects binned by excellent (HgA1c >7%), good (HgA1c 7.0-8.9), or poor (HgA1c ≥ 9.0), glucose control. C, Plasma PGEM levels in ND and T2D subjects by obesity status. In (A), data were compared by two-way t-test. In (B) and (C), data were compared by one-way ANOVA with Holm-Sidak test post-hoc to correct for multiple comparisons. **, $p < 0.01$; ****, $p < 0.0001$. ns, not significant.

Discussion

Insulin resistance, often found with obesity, necessitates the hyperproduction and processing of proinsulin into mature insulin in order to augment insulin secretory capacity, causing significant mitochondrial oxidative and ER stress[3, 30, 31]. Together with hormonal dysregulation, dyslipidemia, and systemic inflammation, which also impact the β-cell, downstream adaptive processes need to unfold in order to prevent progression to T2D[2]. Many of the factors linked with β-cell adaptation in the obese and/or insulin resistant state(s) have also been proposed as contributing to the development and/or pathophysiology of T2D itself, including our and others' previously published work on PGE_2 production and EP3 signaling[13, 14, 16-22, 24, 32].

PGE_2 is the most abundant natural ligand for the EP3 receptor (encoded by the *PTGER3* gene). COX-1 and COX-2 (officially known as prostaglandin-endoperoxidase 1 and 2: *PTGS1* and

PTGS2), catalyze the rate-limiting step in the production of PGE_2 derived from arachidonic acid (AA) incorporated in plasma membrane phospholipids. High glucose, free fatty acids, and/or pro-inflammatory cytokines have all been shown to upregulate the expression and/or activity of enzymes involved in the PGE_2 synthetic pathway, including phospholipase A2 (PLA2: which cleaves AA from membrane phospholipids), COX-1 and COX-2 (which sequentially convert arachidonic acid to the intermediates PGG_2 and PGH_2), and *PTGES*, *PTGES2*, and *PTGES3*, which convert PGH_2 to PGE_2[13, 14, 17-21, 24, 33-37]. Using BMI as a marker of obesity, we found a positive correlation with *PTGS2* expression, consistent with previously-published results[13]. More surprising was the lack of correlation of EP3 gene expression with BMI. We previously published or contributed to two studies characterizing *PTGER3* expression in human islet cDNA samples from non-diabetic human organ donors of varying BMI with seemingly disparate results[13, 14]. In Kimple and colleagues, when islets were binned by obesity status, *PTGER3* expression was significantly correlated with donor BMI[13]. In the current study, *PTGER3* expression was not correlated with the BMI of the donor, whether linearly or by binning islets by obesity status. This finding was replicated in a separate cohort of 40 non-diabetic human donor islets, and is also consistent with the findings of Carboneau and colleagues[14]. As the mean difference in *PTGER3* expression by obesity status in Kimple and colleagues was relatively small ($\Delta\Delta C_T \approx 1.5$; fold-change ≈ 2.8), it is likely this change is of limited biological relevance even if statistically-significant.

Pro-inflammatory cytokines have long been understood as contributing to β-cell death and decreased viability[19-21, 23, 24, 34, 38]. IL-1β is up-regulated in islets from T2D mice and humans as compared to non-diabetic controls, and treating islets with IL-1β ex vivo significantly up-regulates PGE_2 production, EP3 expression, or both[18-21, 33]. On the other hand, recent reports suggest IL-6 as

an obesity-associated "anti-inflammatory" cytokine that stimulates β-cell adaptation. IL-6 promotes β-cell survival, reduce oxidative stress, and promote glucagon-like peptide 1 (GLP-1)-potentiated insulin secretion[39-42]. IL-6 is required for the adaptive proliferative response to high fat diet-induced glucolipotoxicity by paracrine effects on the alpha-cell[43]. IL-6 also promotes β-cell autophagy—a survival mechanism working in concert with the adaptive UPR—through stimulation of alpha-cell GLP-1 production[41]. In the current work, we chose islet IL-6 expression as a physiologically-relevant indication of adiposity and the need to induce beta-cell compensation. Our results, although correlative, suggest the possibility of a mechanistic link between islet IL-6 expression and PGE_2 production. In support of this concept, IL-6 expression has been specifically linked with PGE_2 produced by COX-2 and not COX-1[44]. COX-2 is of particular relevance to the β-cell, as in contrast to other cell types, β-cell COX-2 expression is constitutive[37] and is the major isoform responsible for both endogenous and stimulated β-cell PGE_2 production[35, 37, 45, 46].

Previous studies, including our own, have shown little-to-no effect of EP3 agonists or antagonists on human islet β-cell function, proliferation, or survival, unless the islets were cultured in glucolipotoxic conditions[13, 14, 32]. The comprehensive gene expression and functional assays in the current work confirm up-regulation of PGE_2 production enzymes in non-diabetic obesity is of functional consequence. Because L798,106 is an EP3-specific competitive antagonist, it has no biological activity of its own: it simply displaces the native agonist. Yet, islets from obese donors were not dysfunctional. In fact, they secreted at least as much insulin as a percent of content as islets from non-obese donors, particularly in basal glucose, in addition to having significantly elevated islet insulin content. Increased fractional basal insulin secretion in larger islets or islets from higher BMI organ donors is consistent with a β-cell compensation response to obesity and insulin resistance, which has been observed previously[47, 48]. In contrast, the strong relationship of

islet *PTGS2* expression with enhanced insulin secretion and islet insulin content in non-diabetic donors by BMI was unexpected, particularly in light of the body of literature supporting elevated PGE_2 production and β-cell dysfunction in T2D. Yet, as PGE_2 is rapidly metabolized in circulation, a much higher level of systemic and cellular PGE_2 production must be associated with T2D vs. non-diabetic obesity in order to obtain the results we found. Our findings from the PGEM ELISA are consistent with quantitative assessment of the levels of PGE_2 and its metabolites in plasma of T2D subjects vs ND subjects via mass spectrometry approaches[49, 50]. In conclusion, we suggest a dose-dependent effect of PGE_2 production and signaling in obesity and T2D that has important implications in studies of EP3 as a therapeutic target for the prevention and treatment of T2D.

Materials and Methods

Materials and Reagents

Sodium chloride (S9888), potassium chloride (P3911), magnesium sulfate heptahydrate (M9397), potassium phosphate monobasic (P0662), sodium bicarbonate (S6014), HEPES (H3375), calcium chloride dehydrate (C3881), exendin-4 (E7144) and radioimmunoassay (RIA)-grade bovine serum albumin (A7888) were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-insulin antibodies (Insulin + Proinsulin Antibody, 10R-I136a; Insulin + Proinsulin Antibody, biotinylated, 61E-I136bBT) were from Fitzgerald Industries (Acton, MA, USA). The 10 ng/ml insulin standard (8013-K) and assay buffer (AB-PHK) were from MilliporeSigma (Burlington, MA, USA). RPMI 1640 medium (11879–020: no glucose), penicillin/streptomycin (15070–063), and fetal bovine serum (12306C: qualified, heat inactivated, USDA-approved regions) were from Life Technologies (Carlsbad, CA, USA). Dextrose (D14–500) was from Fisher Scientific (Waltham, MA, USA). The RNeasy Mini Kit and RNase-free DNase set were from Qiagen (Valencia, CA,

USA). The High-Capacity cDNA Reverse Transcription Kit was from Applied Biosystems (Foster City, CA, USA). FastStart Universal SYBR Green Master mix was from Roche (Indianapolis, IN, USA).

Human islet preparations

Cultured human islets were obtained from the Integrated Islet Distribution Program (IIDP) under an approved exemption (UW 2012–0865) for non-human subjects research by the UW Health Sciences IRB. One set of 40 islet preparations was used for gene expression and insulin secretion assays, while a second set of 40 was used for confirmation of gene expression only (Set 2 included three flash-frozen islet samples purchased from Beta-Pro LLC, Charlottesville, VA, USA). The unique identifiers for each islet preparation, along with age, sex, BMI, HbA1c, and islet isolation center are listed in Supplementary Tables 1 and 2. All but two islet preparations were associated with a donor HbA1c value. Islet samples for quantitative PCR analysis were hand-picked and pelleted on the day of arrival and flash-frozen prior to RNA isolation. Prior to *ex vivo* insulin secretion assays, human islets were cultured for at 24 h in human islet medium (RPMI 1640 medium containing 8.4 mM glucose, supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin).

Quantitative PCR assays

150-200 islets from each human islet preparation were washed with PBS and used to generate RNA samples via Qiagen RNeasy Mini Kit according to the manufacturer's protocol. Copy DNA (cDNA) was generated and relative qPCR performed via SYBR Green assay using primers validated to provide linear results upon increasing concentrations of cDNA template, as previously described [35]. The primer sequences used in the qPCR experiments are listed in Table 2.

Ex vivo islet insulin secretion assays

The day before assay, islets were hand-picked into 96-well V-bottom tissue culture plates and incubated overnight in RPMI growth medium to adhere islets to the bottom of the plate and single-islet GSIS assays performed with and without the addition of the indicated compounds to the stimulation medium, as previously described[29]. The secretion medium was collected for insulin secretion assessment and islets were lysed in RLT buffer to measure the insulin content using in-house-generated insulin sandwich ELISA as described previously[29]. In general, secretion medium was diluted 1:20 and content medium diluted 1:200 in order for readings in the linear range of the assay.

Clinical Study Participant Recruitment and Sample Collection

All human subjects research was conducted in accordance with the standards set out by the World Medical Association (WMA) Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" as approved by the UW Health Sciences IRB (UW 2013-1082). Study subjects were recruited from the University of Wisconsin Hospitals and Clinics (UWHC) in Madison, Wisconsin between June 2014 and August 2015. Potentially eligible subjects were UWHC Endocrinology or Internal Medicine Clinic patients with an upcoming morning outpatient appointment who met baseline inclusion and exclusion criteria by consent-waived electronic medical record (EMR) search. Inclusion criteria were 18-74 years old, not pregnant or lactating, no anemia or grossly abnormal kidney or liver function tests, no known autoimmune diseases or inflammatory disorders, and no diagnosis of diabetes besides T2D. Exclusion criteria were history of transplant, chronic steroid use, or use of COX inhibitors other than prophylactic aspirin more than twice per week during the past 90 days. Patients meeting all inclusion and exclusion criteria

were contacted via telephone for verification of EMR data, including dose and frequency of aspirin therapy. The background and aims of the study were described, including the need for an overnight fast and a laboratory blood draw for research purposes. Patients interested in participating provided initial verbal consent by phone and were instructed to fast for 12 h prior to their upcoming outpatient appointment. If an iatrogenic hypoglycemic event occurred, patients were instructed to treat per standard medical protocol until resolution. The patient's care provider was contacted to coordinate laboratory test orders, as our study protocol included complete blood count (CBC), complete metabolic panel (CMP), fasting lipid panel, HbA1c, C reactive protein (CRP), and erythrocyte sedimentation rate (ESR).

Upon presentation at the UWHC lab, a study coordinator described the aims of the study and that participation was voluntary and confirmed eligibility criteria. The study coordinator confirmed the patient had fasted for 12 h. If no greater than 15 grams of carbohydrates were consumed as treatment, the patient remained eligible to provide a blood sample. If greater than 15 grams of carbohydrates were consumed and the patient remained interested in participating, the clinical laboratory appointment was re-scheduled. Consent forms were reviewed and signed by both participants and study coordinators prior to sample procurement and filed in a locked area thereafter within each respective clinic. Height, weight, blood pressure, and pulse were measured, and daily omega-3 supplement use was noted. Current T2D medications were confirmed and recorded. A UWHC staff phlebotomist drew blood for the clinical lab test panel, followed by a blood draw for research purposes into a 8.5 ml BD™ P800 blood collection tube coated with potassium EDTA and a proprietary mix of protease and esterase inhibitors (BD Biosciences, cat. no. 366421). The research blood tube was gently mixed and immediately refrigerated until transported to the UWHC Central Lab via cold pack, where plasma was separated, aliquoted, and

stored at -80°C until used in research lab assays. All subjects providing consent received remuneration in the form of a $25 gift card.

Based on means and SDs from our pre-clinical models, our target recruitment was 137 T2D subjects and 35 non-diabetic (ND) subjects for a power of 80% to see significant differences at alpha = 0.05. One patient carrying a T2D diagnosis prior to their intake appointment being treated with diet/lifestyle modifications only had a HgA1c value less than 6% and was re-assigned to the ND cohort. One patient initially enrolled in the ND group who was being monitored for pre-diabetes had a HbA1c value over 6.5%, and, in coordination with their care provider, informed of their T2D diagnosis and re-assigned to the T2D cohort. Finally, four subjects were excluded from final analysis, all in the T2D group. One subject withdrew consent. One subject did not have clinical labs run. One subject had an elevated white-cell count and, upon follow-up, had been acutely ill on the day of presentation. Lastly, one subject had a low potassium level that triggered the endocrinologist on call to adjudicate. This subject was found to have a history of bariatric surgery and referred to follow-up with their surgeon. Therefore, the final cohort includes 132 T2D subjects and 35 ND subjects.

Prostaglandin E Metabolite (PGEM) Assay

Plasma PGE_2 levels were quantified using a Prostaglandin E Metabolite (PGEM) ELISA kit (Cayman Chemical Company, cat. no. 514531) according to the manufacturer's protocol. Briefly, samples were purified by acetone precipitation followed by drying under a gentle nitrogen stream before being re-suspended in an equivalent volume of ELISA buffer. Samples and standards were derivatized overnight and loaded onto 96-well assay plates at a 1:5 dilution in duplicate. No

deviations from the ELISA protocol were made, and the optional acidification and ethyl acetate extraction step after derivatization was not necessary.

Statistical analyses

Statistical analyses were performed with GraphPad Prism v. 9 (GraphPad Software, San Diego, CA) according to the methods described in the figure legends. In all cases, $p < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

We wish to thank the many present and former members of the Kimple Laboratory who contributed technical assistance or scientific discussion during the course of these experiments, most notably, Jackson Moeller, Renee Buchanan, and Stephanie Blaha.

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Conceptualization, DBD and MEK; data curation, NAT, RJF, AMW, CP, RN, MD, SP, MP, DBD, and MEK; formal analysis, NAT, RJF, HKS, AMW, CP, DBD, and MEK; funding acquisition, RJF,

AMW, ALB, SP, DBD, and MEK; investigation, NAT, RJF, HKS, AMW, CP, AR, JMH, ALB, DCP, RN, MD, SP, MP, and MEK; methodology, NAT, RJF, AMW, DBD, and MEK; project administration, AMW, DBD, and MEK; supervision, NAT, RJF, AMW, DBD, and MEK; visualization, RJF, CP, and MEK; writing—original draft, RJF, CP, and MEK; writing—review and editing, RJF, CP, MD, DBD, and MEK. All authors have read and agreed to the published version of the manuscript.

Funding Sources

This work was supported in part by Merit Review Awards I01 BX003700 from the United States (U.S.) Department of Veterans Affairs Biomedical Laboratory Research and Development Service (BLR&D) (to MEK), I01 BX001880 (to DBD), and I01 BX004715 (to DBD). This work was also supported in part by NIH Grants R01 DK102598 (to MEK), R01 DK110324 (to DBD), and F31 DK109698 (to RJF); JDRF Grant 17-2011-608 (to MEK), American Diabetes Association (ADA) Grant 1-16-IBS-212 (to MEK), UW Institute for Clinical and Translational Research Type 1 and Translational Pilot Grant ICTR-UWHC-20120919 (to MEK), a Research Starter Grant in Translational Medicine and Therapeutics from the PhRMA Foundation (to MEK), and a UW2020 Discovery Initiative Award from the Wisconsin Alumni Research Foundation (WARF) (to MEK and DBD). Allison Brill was supported in part by an American Society for Pharmacology and Experimental Biology (ASPET) Summer Undergraduate Research Fellowship. Alicia Weeks was supported by a VA Advanced Fellowship in Women's Health. Samantha Pabich was supported by a Pearl Stetler Research Fund for Women Physicians Fellowship. The funding bodies had no role in any aspect of the work described in this manuscript. This manuscript is the result of work supported with resources and the use of facilities at the William S. Middleton Memorial Veterans

Hospital in Madison, WI. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Conflict of Interest — RJF, HKS, CP, AR, JMH, DCP, RN, MD, SP, MP, DBD, and MEK declare that they have no conflicts of interest with the contents of this article. NAT is currently a Nanomedicine Innovation Center LLC employee (46701 Commerce Center Drive, Plymouth, MI 48170). AMW is currently an Allena Pharmaceuticals employee (One Newton Executive Park, Suite 202, Newton, MA 02462). ALB is currently a Pfizer employee (235 East 42nd Street, New York, NY 10017). This work was completed in full during their research training with Dr. Kimple and are not related to their current positions.

Supporting Information. The following files are available free of charge.

Table S1: Sources and Donor Demographics of islet preparations from Set 1, used for gene expression analyses and insulin secretion assays.

Table S2: Complete Correlation analysis of BMI/gene/Insulin content analyses.

Table S3: Sources and Donor Demographics of islet preparations from Set 2, used to confirm the relationship of *PTGER3* expression with obesity.

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