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Communication

Estradiol 17-β Induces Pancreatic Beta-Cell Proliferation through Distinct Estrogen Receptors in a Glucose Dependent Manner

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Abstract

***Background*:** Estradiol 17-beta (E2) enhances the function and survival of pancreatic beta-cells, but its clinical use has been questioned due to concerns regarding oncogenic potential and feminizing effects in males. The G-protein coupled estrogen receptor (GPER), expressed in pancreatic islets, exhibits estrogenic beta-cell protective effects without the feminizing effects of the nuclear ERs. Here, we examine the outcome of selective activation of the three estrogen receptors ERα, ERβ, and GPER on replication in human pancreatic islets and the INS1-E rodent β-cell line under hyperglycemic conditions, such as occur in diabetes mellitus.

***Methods*:** Pancreatic islets from nine human donors and INS1-E cells were grown at glucose concentrations of 11mM and 25mM and examined for DNA synthesis using ^{3}[H]-thymidine incorporation after 24 hour treatment with E2, specific agonists for ERα, ERβ, and GPER (10nM PPT, 10nM DPN, and 100nM G1, respectively), and antagonists for ERα and ERβ (100nM MMP and 150nM PTHPP, respectively). Expression of the three ERs was examined by qRT-PCR.

***Results*:** In human islets at 11mM glucose, agonists to ERα and GPER induced a significant approximate twofold increase in ^{3}[H]-thymidine incorporation ($p < 0.01$), while the ERβ agonist DPN enhanced proliferation by approximately 50%, which was not significant. However, only the GPER agonist G1 retained its proliferative effect ($p < 0.001$) while under still higher glucose concentrations (25mM). Concurrently, expression of ERα and ERβ but not of GPER was reduced by approximately 15% at 25mM glucose ($p < 0.05$). In INS1-E cells, all ER agonists enhanced ^{3}[H]-thymidine incorporation by two- to threefold under 11mM glucose, but at 25mM glucose only the ERα-specific agonist elicited a similar response ($p < 0.001$). Concordantly, expression of ERβ and GPER but not of ERα was reduced by approximately 50% at 25mM glucose ($p < 0.001$). E2 enhanced static insulin secretion by 1.2-fold in human islets and by 1.8-fold in INS1-E cells ($p < 0.05$).

***Conclusions*:** These findings suggest that GPER may comprise an attractive target in the therapy of human diabetes and point to the phenomenon of species specificity regarding effects of glucose on estrogen receptor subtype expression and proliferative activity.

Keywords

Diabetes mellitus; hyperglycemia; β-cells; human islets; estradiol-17beta; estrogen; estrogen receptor

1. Introduction

Diabetes mellitus types 1 and 2 are characterized by loss of functional beta-cell mass. Estradiol 17-beta (E2), the most potent female sex hormone, has been shown to exert diverse beta-cell protective effects. E2 enhances insulin secretion, protects beta-cells from apoptosis, increases beta-cell mass, and improves transplantation of human islets into diabetic nude mice [1-3]. E2 has also been shown to delay autoimmune diabetes through promotion of immune tolerance [4]. Thus far, these attributes have not translated into clinical practice due to concerns related to the feminizing and oncogenic effects of E2 on reproductive tissues.

E2 exerts its effects through three receptors: two classic, ligand-dependent transcription factors ERα and ERβ, and the novel membrane-bound G-protein coupled estrogen receptor GPER [5]. Effects of E2 on the reproductive system are mediated primarily by ERα and ERβ. Therefore, selective stimulation of GPER has been suggested as a means of achieving E2 mediated beta-cell protection while avoiding undesired systemic effects. Recent studies in mice using GPER knockout and the specific GPER agonist G1 have shown that GPER, similar to E2, enhances glucose stimulated insulin secretion (GSIS) and islet survival and prevents the development of glucose intolerance and diabetes in streptozotocin treated mice [6, 7].

While substantial evidence exists with regard to GPER protective effects in rodent beta-cells/islets, relatively little is known about GPER in human islets. Kumar et al. [8] demonstrated that GPER is expressed in all three islet cell types (i.e., insulin, glucagon, and somatostatin-secreting cells), with higher expression in female than male islets, which parallels similar findings in mice [9]. GPER expression did not differ between diabetic and non-diabetic or pre- and post-menopausal women. Stimulation of GPER with the G1 agonist conferred anti-diabetic effects

through prevention of cytokine-induced apoptosis, enhanced glucose-stimulated insulin secretion, and reduced glucagon and somatostatin secretion [6, 8].

We have previously shown that ERα promotes replication of human islets and rat insulinoma INS1-E cells in a FOXO1-dependent manner [10]. Here, we examined the involvement of all three ERs in the replication of human islets and INS1-E cells under hyperglycemic conditions, such as that which occur in diabetes.

2. Materials and Methods

2.1 Cell Line Culture and Drug Stimulation

2.1.1 Human Pancreatic Islets

Human islets from nine non-diabetic donors (seven males, aged 19 – 58 years, and two females, aged 58 and 68 years) were kindly provided by Prof. Shapiro (University of Alberta, Canada). Islets were cultured in CMRL 1066 (Biological industries, Israel) according to standard protocol and transferred to phenol free medium with 1% charcoal stripped FBS (Biological industries, Israel) for 12-24h before transferring to a high glucose media and treating with one of the following: 17β-estradiol ($3X10^{-8}$M, $3X10^{-7}$M, and $3X10^{-6}$M, Sigma-Aldrich Rehovot, Israel), the ERα agonist 4,4',4'-[4-propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (10^{-8}M PPT, Tocris, Bristol-UK), the ERβ agonist 2,3-bis (4-hydroxyphenyl)-propionitrile (10^{-8}M DPN, Tocris, Bristol-UK), or the GPER agonist G1 (10^{-7}M, Sigma-Aldrich, Rehovot, Israel) [10].

2.1.2 Rat Insulinoma Cell Line

The rat pancreatic insulinoma cell line INS-1E was kindly provided by Michael Walker (Department of Biological Chemistry, Weizmann Institute, Israel) with permission from Dr. Wolheim (University of Geneva). Cells were cultured in RPMI according to standard protocol, treated as described above, and additionally treated with the ERα antagonist MPP at 10^{-7}M and the ERβ antagonist PTHPP at $15x10^{-8}$ M (Tocris, Bristol) [11].

2.2 Assessment of Cell Proliferation

Proliferation was evaluated by ^{3}[H]-thymidine incorporation as previously described [10].

2.3 Quantitative RT-PCR

RNA was extracted from INS-1E cells and human islets using the PerfectPure RNA Cultured Cell Kit (5-Prime, Inc., Gaithersburg, MD, USA). cDNA was synthesized from 500ng total RNA using the qScript kit (Quanta Biosciences, Gaithersburg, MD, USA). PCR amplification was carried out with PerfeCTa SYBR Green FastMix (Quanta Biosciences) on a StepOnePlus system (Applied Biosystems) with gene-specific primers purchased from IDT syntezza (Coralville, Iowa, USA). Samples were analyzed in triplicates. Primers used for *Rattus norvegicus* are as follows: *ERα* forward 5'-GCTTCAACATTCTCCCTCCTC-3', *ERα* reverse 5'-CAATGCACCATCGATAAGAACC-3', *ERβ* forward 5'-CAGATGTTCCATGCCCTTGT-3', *ERβ* reverse 5'-GGTGATTGCGAAGAGTGGTAT-3', GPER forward 5'-TCTACCTAGGTCCCGTGTGG-3', and GPER reverse 5'-AGAGAGGTCCCCAGTGAGGT-3'. Primers used

for human islets are as follows: *ERα* forward 5'-TGGGAATGATGAAAGGTGGGAT-3', *ERα* reverse 5'-AGGTTGGCAGCTCTCATGTC-3', *ERβ* forward 5'-CGCTCGCTTTCCTCAACAGG-3', *ERβ* reverse 5'-GGGCAAGTATAATGGCTTGCAG-3', GPER forward 5'-TCCAACATCTGGACGGCAGC-3', and GPER reverse 5'-CTGGGTACCTGGGTTGCAG-3'. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA binding protein (TBP) mRNA in INS1-E and human islets, respectively.

2.4 Insulin Secretion

GSIS in INS1-E cells was performed according to previously published protocols [12]. In short, following 24h incubation with E2, 30nM cells were washed twice with glucose-free Krebs-Ringer Bicarbonate buffer (KRB), preincubated for 1 hour at 37°C in 500 µL glucose-free KRB, and then incubated for 2h in 500 µL KRB under low (2.8 mM) and high (16.7 mM) glucose. After 2 hours, media was collected and samples were assessed for insulin concentration. In human islets, insulin was measured at the baseline and after 1h, 4h, and 24h treatment with 30nM E2 at 11mM glucose. Insulin was detected by an electro chemiluminescence immunoassay using the Cobas e411 (Roche Diagnostics, Mannheim, Germany). Data were normalized to cell number in INS1-E and protein content in human islets, and presented as fold changes of the control.

2.5 Statistical Analysis

Results are presented as mean ± SEM of at least three independent repeats in triplicates unless otherwise stated. Data were analyzed by SPSS25 using the Student's t test or ANOVA for multivariate analysis. Thymidine incorporation in human islets was analyzed with the Kruskal–Wallis test (pairwise comparison), and the significance level was adjusted for multiple comparisons by the Bonferroni method. A $p < 0.05$ was considered statistically significant.

2.6 Ethics Statement

This research was approved by the Institutional Review Board of the Tel-Aviv Sourasky Medical Center, Helsinky approval number 459-14-TLV.

3. Results

3.1 Human Pancreatic Islets

3.1.1 Severe Hyperglycemia Affects E2 Induced Proliferation

Human pancreatic islets were treated with E2 at concentrations of 30nM, 300nM, and 3000nM under conditions of 11mM and 25mM glucose (also termed severe hyperglycemia) for 24 hours to assess dose response (Figure 1A). At 11mM glucose, E2 enhanced proliferation (approximately twofold, $p < 0.001$) at a concentration of 300nM, a 10-fold higher E2 concentration than that previously shown by us to induce proliferation of human islets under 5.5mM glucose [10]. Under 25mM glucose, E2 induced proliferation (approximately 1.5-fold, $p < 0.01$) at a concentration of 3000nM, once again a 10-fold higher concentration than that needed to induce proliferation at 11mM glucose (Figure 1A).

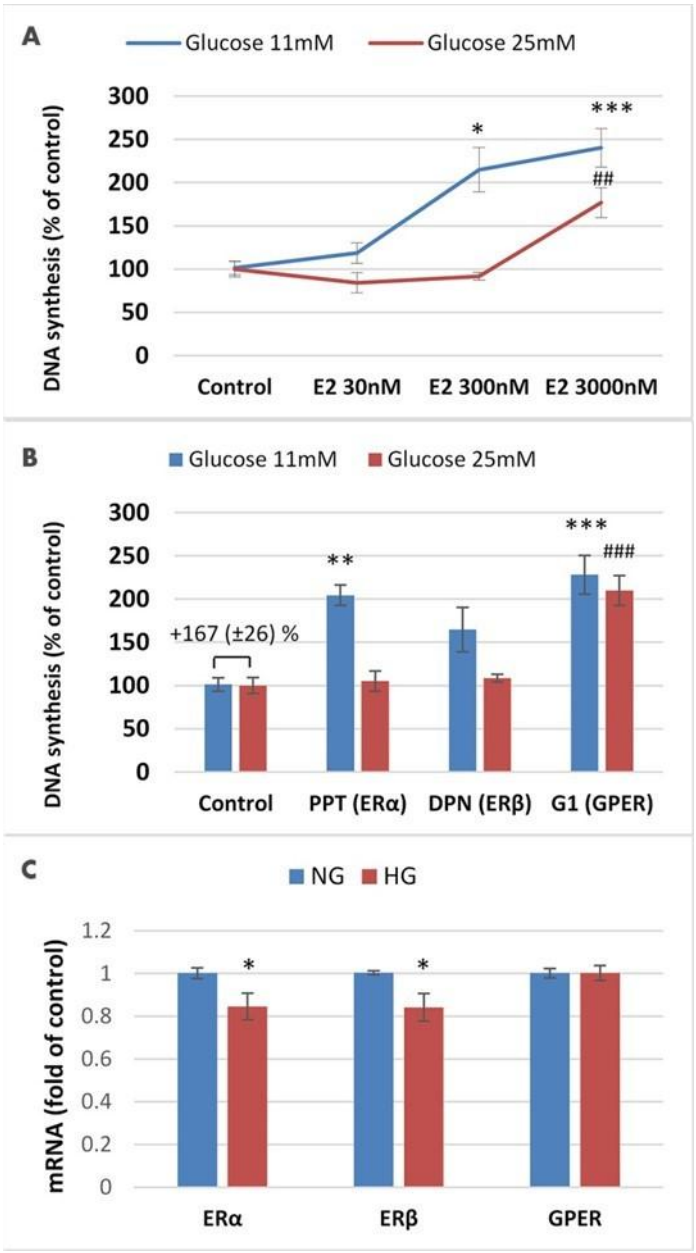

Figure 1 Effect of high glucose on E2 and ER mediated DNA synthesis and ER subtype expression in human pancreatic islets. **A.** Dose response of E2 effect on DNA synthesis. Islets were treated for 24h with E2 30nM-3000nM in glucose 11mM (blue bars) or glucose 25mM (red bars) and measured for proliferation by ^{3}[H] -thymidine incorporation. **B.** Estrogen receptor agonists mediated DNA synthesis. Islets were treated for 24h with PPT 10nM, DPN 10nM or G1 100nM (agonists to ERα, ERβ and GPER respectively) in glucose 11mM (blue bars) or glucose 25mM (red bars) and measured for proliferation. Glucose 25mM per se increased ^{3}[H]-thymidine incorporation to 167±26% of the 11mM control, as depicted in the chart above controls. **C.** Expression of ERα, ERβ and GPER under high glucose as assessed by qRT-PCR. Results represent mean ± SEM (n⩾3, in duplicates or triplicates), hormonal treatment vs. control. *p<0.05, **p<0.01, ***p <0.001 for the comparison with control values at glucose 11mM; $^{\#\#}$p <0.01, $^{\#\#\#}$p <0.001 for the comparison with control values at glucose 25mM.

3.1.2 Severe Hyperglycemia Affects ER Agonists-Induced Proliferation

Human pancreatic islets were treated with agonists for the three ERs: ERα (10nM PPT), ERβ (10nM DPN), and GPER (100nM G1) under conditions of 11mM and 25mM glucose (Figure 1B). At 11mM glucose, the ERα agonist PPT and the GPER agonist G1 increased proliferation by approximately twofold ($p < 0.01$ for PPT and $p < 0.001$ for G1). The ERβ agonist DPN increased proliferation approximately 1.5-fold, which was not significant. At a glucose concentration of 25mM, basal proliferation was increased approximately 1.6-fold, however, only the GPER agonist G1 increased proliferation (approximately twofold, $p < 0.001$), while stimulation of ERα and ERβ did not enhance proliferation further (Figure 1B).

3.1.3 Severe Hyperglycemia Affects the Expression of Estrogen Receptors

We next examined the expression profile of the three estrogen receptors under growth conditions of 11mM and 25mM glucose for 24 hours. As depicted in Figure 1C, expression of ERα and ERβ was reduced by approximately 15% ($p < 0.05$), while expression of GPER was unchanged.

The functionality of human islets treated with E2 was assessed by the measurement of insulin secretion under static incubation. Human islets were incubated with 30nM E2 at 11mM glucose, and insulin concentration was determined after 1h, 4h, and 24h. A peak of approximately a 1.2-fold increase ($p < 0.05$) in insulin concentration was seen at 4h, with a return to the baseline at 24h (Figure S1).

3.2 INS1-E (Insulinoma Cell Line)

3.2.1 Severe Hyperglycemia Affects E2 and ER-Induced Proliferation

INS1-E cells were treated with E2 (30nM) and with agonists for the three ERs, including ERα (10nM PPT), ERβ (10nM DPN), and GPER (100nM G1), at glucose concentrations of 11mM and 25mM (Figure 2A). At 11mM glucose, E2 and all three ER agonists induced an approximately threefold increase in ^{3}[H]-thymidine incorporation (***$p < 0.001$). At a glucose concentration of 25mM, basal proliferation increased approximately 2.8-fold, however, only the ERα agonist PPT elicited an additional response, increasing DNA synthesis by approximately 2.5-fold ($p < 0.001$). Stimulation of ERβ and GPER did not induce proliferation. Stimulation with E2 enhanced proliferation by approximately 1.3-fold at 25mM glucose ($p < 0.05$), but this effect was abrogated upon addition of the ERα antagonist MMP (100nM; Figure S2). Addition of the ERβ antagonist PTHPP (150nM) did not affect E2-mediated DNA synthesis, supporting that ERα plays a dominant role in high glucose environments (Figure S2).

3.2.2 Severe Hyperglycemia Affects the Expression of Estrogen Receptors

Expression of the three estrogen receptors under high glucose demonstrated a different profile than that seen in human islets. Severe hyperglycemia (25mM glucose) led to a significant reduction in mRNA of ERβ and GPER by approximately 50% (***$p < 0.001$), while expression of ERα was not reduced. This was in line with the retained effect observed of the ERα agonist PPT on proliferation under severe hyperglycemia.

Functionality of INS1-E cells under E2 treatment was assessed by the measurement of glucose stimulated insulin secretion. E2 (30nM) enhanced insulin secretion approximately 1.8-fold at basal glucose concentrations (2.8mM), but did affect GSIS (Figure S3).

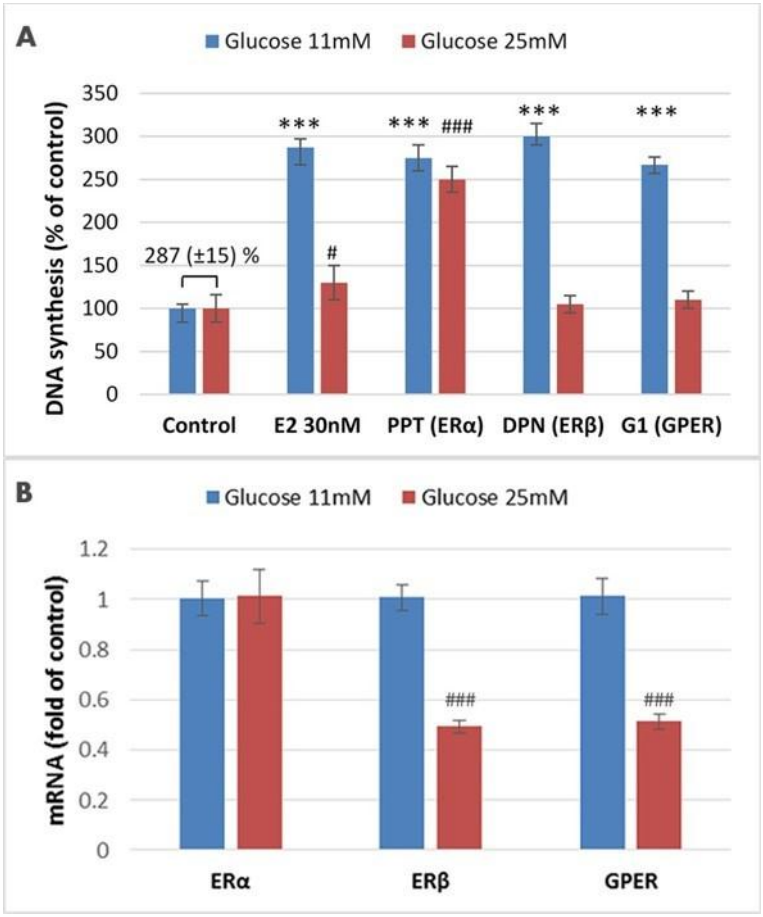

Figure 2 Effect of high glucose on E2 and ER mediated DNA synthesis and ER subtype expression in INS1-E cells. **A.** E2 and ER induced DNA synthesis. INS1-E cells were treated for 24h with E2 30nM, DPN 10nM, PPT 10nM or G1 100nM, in glucose 11mM (blue bars) or glucose 25mM (red bars) and measured for proliferation as percent of control by ^{3}[H] -thymidine incorporation assay. Glucose 25mM per se increased ^{3}[H]-thymidine incorporation to 287±15%, as depicted in the chart above controls. **B.** Expression of ERs under high glucose as assessed by qRT-PCR. Results represent mean ± SEM (n = 4 in triplicates). ***p <0.001 for the comparison with control values at glucose 11mM; #p <0.05, ###p <0.001 for the comparison with control values at glucose 25mM.

4. Discussion

E2, through its three cognate receptors, has been shown to enhance beta-cell function and viability, but its ability to promote beta-cell replication, especially in humans, is still controversial. GPER, the membrane bound estrogen receptor, has recently come into focus as a potential pharmacologic target, conveying favorable E2 metabolic effects while evading ERα- and ERβ-mediated reproductive effects [13].

In the present study, we show that E2 and ER agonists can enhance proliferation of human islets and INS1-E rat insulinoma cells, but under severe hyperglycemia (25mM glucose) this effect is fully retained only by GPER in human islets and by ERα in INS1-E cells. Concordantly, in the presence of severe hyperglycemia, expression of ERα and ERβ was reduced in human islets while in INS1-E cells expression of ERβ and GPER was reduced. The more prominent effects seen in INS1-E cells may arise from the uniformity in cell type and higher proliferative potential of this rodent cell line as compared to human islets. E2 treatment under hyperglycemia slightly enhanced (approximately 1.2-fold) insulin secretion in human islets but had no effect on GSIS in INS1-E cells.

GPER has been implicated in the expansion of beta-cell mass during pregnancy in rodents through the downregulation of miR-338-3p. This effect, however, was not repeated *in vitro* in human beta-cells [14]. GPER, independent of ERα and ERβ, has been shown to bestow protective metabolic effects in multiple tissues involved in diabetic complications: CVS, renal, adipose, immune, and neuronal cells (reviewed in [15]). However, there is evidence that GPER may promote breast cancer progression in certain settings [13]. Still, tamoxifen, which is currently recognized as a GPER agonist [16], is broadly used as first-line treatment for human breast cancer. While concerns could be raised that the proliferative estrogenic effects in the pancreas might affect the exocrine pancreas as well, the limited clinical and epidemiological information accrued thus far does not support this possibility [17-19].

Ongoing attempts aimed at translating the beneficial effects of E2 into therapeutically safe targets for diabetes are currently in progress [20]. Here, we report for the first time to our knowledge that hyperglycemia affects the proliferative activity and expression of estrogen receptor subtypes in human islets, and that this phenomenon is species specific, lending support to the concept of GPER as a therapeutic target in humans. The experiments in human islets are limited by the small number of specimens, which does not allow for reliably extracting potential sex-specific effects. Our findings should be extended to a larger number of donors and refined by the use of additional estrogenic and estrogen-mimetic agonists and antagonists. Additionally, antagonizing GPER and combining agonists and antagonists of the different receptors should be explored to test potential interactions between the individual receptors, which may also play a role in the net effect of E2 on beta-cell proliferation. Further studies are needed to decipher the exact mechanisms by which hyperglycemia affects ER activity and expression, as well as whether it is influenced by sex, in order to promote E2-based therapies.

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Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Figure S1: E2 enhances static insulin secretion in human islets.
2. Figure S2: E2 mediated proliferation in INS1-E upon high glucose is abrogated by ERα antagonism.

3. Figure S3: E2 enhances basal insulin secretion but does not affect GSIS in the INS1-E cell line.

Author Contributions

SS designed, performed the experiments and wrote the manuscript, GCM designed and performed the experiments, SO performed the experiments, GS provided technical support and conceptual advice, SD performed the experiments, analysed and interpreted data and NS provided conceptual advice, interpreted data and supervised the manuscript. All authors contributed to the drafting of the manuscript and approved the final version of the manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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