



LUND UNIVERSITY

Insulinotropic and Antidiabetic Effects of 17{beta}-Estradiol and the GPR30 Agonist G-1 on Human Pancreatic Islets.

Kumar, Rajesh; Balhuizen, Alexander; Amisten, Stefan; Lundquist, Ingmar; Salehi, S Albert

Published in:
Endocrinology

DOI:
[10.1210/en.2010-1361](https://doi.org/10.1210/en.2010-1361)

2011

[Link to publication](#)

Citation for published version (APA):

Kumar, R., Balhuizen, A., Amisten, S., Lundquist, I., & Salehi, S. A. (2011). Insulinotropic and Antidiabetic Effects of 17{beta}-Estradiol and the GPR30 Agonist G-1 on Human Pancreatic Islets. *Endocrinology*, 152(7), 2568-2579. <https://doi.org/10.1210/en.2010-1361>

Total number of authors:
5

General rights

Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Insulinotropic and Antidiabetic Effects of 17 β -Estradiol and the GPR30 Agonist G-1 on Human Pancreatic Islets

Rajesh Kumar, Alexander Balhuizen, Stefan Amisten, Ingmar Lundquist, and Albert Salehi

Department of Clinical Science, Division of Islet Cell Physiology (R.K., A.B., S.A., I.L., A.S.) and Department of Experimental Medical Science (I.L.), University of Lund, S-205 02 Malmö, Sweden

We have recently shown that 17 β -estradiol (E2) and the synthetic G protein-coupled receptor 30 (GPR30) ligand G-1 have antiapoptotic actions in mouse pancreatic islets, raising the prospect that they might exert beneficial effects also in human islets. The objective of the present study was to identify the expression of GPR30 in human islets and clarify the role of GPR30 in islet hormone secretion and β -cell survival. GPR30 expression was analyzed by confocal microscopy, Western blot, and quantitative PCR in islets from female and male donors. Hormone secretion, phosphatidylinositol hydrolysis, cAMP content, and caspase-3 activity in female islets were determined with conventional methods and apoptosis with the annexin-V method. Confocal microscopy revealed GPR30 expression in islet insulin, glucagon, and somatostatin cells. GPR30 mRNA and protein expression was markedly higher in female vs. male islets. An amplifying effect of G-1 or E2 on cAMP content and insulin secretion from isolated female islets was not influenced by the E2 genomic receptor (ER α and ER β) antagonists ICI 182,780 and EM-652. Cytokine-induced (IL-1 β plus TNF α plus interferon- γ) apoptosis in islets cultured for 24 h at 5 mmol/liter glucose was almost abolished by G-1 or E2 treatment and was not affected by the nuclear estrogen receptor antagonists. Concentration-response studies on female islets from healthy controls and type 2 diabetic subjects showed that both E2 and G-1 displayed important antidiabetic actions by improving glucose-stimulated insulin release while suppressing glucagon and somatostatin secretion. In view of these findings, we propose that small molecules activating GPR30 could be promising in the therapy of diabetes mellitus. (*Endocrinology* 152: 0000–0000, 2011)

An important pathogenic factor in diabetes is hyperglycemia, which often arises from the loss of insulin production/secretion from pancreatic islet β -cells. In type 1 and the late stages of type 2 diabetes, proinflammatory cytokines and/or chronic elevation of blood glucose might generate oxidative stress in pancreatic islets, which ultimately provokes β -cell death by apoptosis (1, 2).

Estrogen is a steroid hormone in females being secreted mainly from the ovaries and exerting important functions on the regulation of female secondary sexual characteristics. In males, relatively small amounts of

estrogen is produced by Leydig and germ cells of the testis, as well as from liver, adrenal glands, and adipocytes (3). A well-described site of action for estrogen is the binding to and activation of nuclear estrogen receptors (ER)- α and - β in turn resulting in the stimulation of transcriptional events in various tissues. Previous data from human and animal studies suggest that 17 β -estradiol (E2) might promote insulin production in diabetic individuals and prolong β -cell survival (4, 5). Importantly, in addition to ER α and ER β , estrogen has recently been shown to be a natural ligand for the newly discovered orphan G protein-coupled receptor 30

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Copyright © 2011 by The Endocrine Society

doi: 10.1210/en.2010-1361 Received November 30, 2010. Accepted April 5, 2011.

Abbreviations: AKT, Serine/threonine protein kinase; BMI, body mass index; CREB, cAMP response element-binding; db-cAMP, dibutyl cAMP; E2, 17 β -estradiol; ER, estrogen receptor; GPR30, G protein-coupled receptor 30; IFN γ , interferon- γ ; IP $_3$, inositol triphosphate; JNK, c-Jun N-terminal kinase; p, phosphorylated; PI, phosphatidylinositol; PKA, protein kinase A; PLC, phospholipase C; PVDF, polyvinylidene difluoride; qPCR, quantitative PCR; SAPK, stress activated protein kinase.

(GPR30) (6, 7). We have shown that GPR30-deficient mice display a reduced estrogen-stimulated insulin release *in vitro* as well as a decreased response to a glucose challenge performed *in vivo* (8), suggesting the involvement of GPR30 in islet biology. Moreover, we recently found in female mouse islets that activation of GPR30 is associated with potentiation of glucose-stimulated insulin release in parallel with an inhibition of glucagon and somatostatin secretion. In addition, activation of GPR30 also counteracted cytokine-induced apoptosis in islets from female mice (9).

However, the expression pattern and involvement of GPR30 in mediating modulatory actions of estrogen and G-1 (a synthetic GPR30 agonist, a tetrahydroquinoline) (10) on human pancreatic islet cell function and hormone secretion has not been documented. Because GPR30 expression was found to be higher in female mouse islets (9), we decided to perform our study mainly in female islets. The objective of this study was thus to explore with different approaches the influence, if any, of GPR30 on islet hormone release and whether GPR30 activation by G-1 and E2 plays a protective role in female human islets. Moreover, hormone secretion studies were performed in islets isolated from both diabetic and nondiabetic female donors. To exclude the effect of estrogen on its main nuclear receptors (ER α and ER β), we used two different nuclear receptor antagonists, *i.e.* ICI 182,780 (fulvestrant) and/or EM-652 (acolbifene), which have been documented to selectively inhibit these receptors (11, 12). Moreover, the effects of E2 on pancreatic islet cell function were compared with GPR30 agonist, *i.e.* G-1 (10). With our experimental design and findings, we now demonstrate the importance of E2 and G-1 in human pancreatic islet cell function and survival.

Materials and Methods

Drugs and chemicals

Fatty acid-free BSA and fatty acid-free fetal BSA were from Roche Molecular Biochemicals, Mannheim, Germany. G-1 {1-[4-(6-benzo (1,3) dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-yl]-ethanone} and ICI 182,780 (fulvestrant) were purchased from Cayman Chemical, Ann Arbor, MI. EM-652 (12), acolbifene {2(S)-3-(4-hydroxyphenyl)-4-methyl-2-[4-(2-piperidin-1-ylethoxy)phenyl]-2H-chromen-7-ol} was kindly provided by Professor F. Labrie, University of Quebec, Quebec, Canada. Glibenclamide, clonidine, the protein kinase A (PKA) inhibitor H-89 dihydrochloride hydrate, the phospholipase C (PLC) inhibitor RHC80267, dibutyryl cAMP (N6,2'-O-dibutyryladenine 3',5'-cyclic monophosphate) were from Sigma-Aldrich (St. Louis, MO). All other chemicals were from Merck AG (Darmstadt, Germany) or Sigma-Aldrich.

Isolation of pancreatic islets and measurement of islet hormone secretion

Isolated human pancreatic islets from nondiabetic males and females [glycosylated hemoglobin, 4.3–6.2; body mass index (BMI), 20.1–30.2 kg/m²; age, 26–71 yr; n = 19] and diabetic females (glycosylated hemoglobin, 6.8–7.0; BMI, 29.4–33.1 kg/m²; age, 43–55 yr; n = 7) were provided by the Nordic network for clinical islet transplantation (O. Korsgren, Uppsala University, Uppsala, Sweden). The human islets had been cultured at 37 C (5% CO₂) for 1–5 d before the experiments in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mmol/liter HEPES, 2 mmol/liter L-glutamine, 50 μ g/ml gentamicin, 0.25 μ g/ml Fungizone (Life Technologies, Inc., BRL, Gaithersburg, MD), 20 μ g/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/liter nicotinamide. The islets had 70–90% purity when they arrived; the islets were then handpicked under stereomicroscope before use. All procedures were approved by the ethical committees at Uppsala and Lund Universities.

On the experiment day, the islets were incubated as previously described (9). The secreted hormones were measured by using RIA kits following the manufacturer's recommendations for insulin (Millipore), glucagon and somatostatin (Millipore, Malmö, Sweden) (13–15). The inter- and intraassay coefficients of variation were for insulin 3.0 and 2.7%, for glucagon 6.3 and 3.6%, and for somatostatin 8.1 and 3.2%. We investigated the concentration-dependent (0–5000 nmol/liter) effects of G-1 and E2 on insulin secretion from diabetic and nondiabetic subjects. In addition, we also tested the effect of the ATP-sensitive potassium channel inhibitor glibenclamide (3 μ mol/liter) and the α 2-adrenergic agonist clonidine (1 μ mol/liter) with and without G-1 (100 nmol/liter) and E2 (100 nmol/liter). For the GPR30 insulin secretion pathway analysis, we used the PKA inhibitor H-89 (10 μ mol/liter) and the PLC inhibitor RHC80267 (10 μ mol/liter) as well G-1 (100 nmol/liter) and E2 (100 nmol/liter).

Detection of mRNA for GPR30 by quantitative real-time PCR

RNA from islets was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to a modified RNA extraction protocol and transcribed into cDNA as described elsewhere (16). To confirm the results obtained by agarose gel electrophoresis, additional experiments were performed on the original samples containing the total RNA of the isolated islets. Concentration and purity of total RNA was measured with a NanoDrop ND-1000 spectrophotometer (A260/A280 > 1.9 and A260/A230 > 1.4) (NanoDrop Technologies, Wilmington, DE) and RNA Quality Indicator (RQI) higher than 8.0 (Experion Automated Electrophoresis, Bio-Rad, Hercules, CA), which could be considered as a high-quality total RNA preparation and thus giving a consistent extraction procedure.

Quantitative PCR (qPCR) was performed on a HT7900 system (Applied Biosystems, Foster City, CA) using QuantiTect primer assays (ESR1/ER α , QT00044492; ESR2/ER β , QT00060641; GPR30, QT00201040; GAPDH, QT01192646) and QuantiFast SYBR Green PCR (all from QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. The specificity of all primer assays was validated by melting curve analysis and gel electrophoresis. Gene expression relative to GAPDH was calculated using the $\Delta\Delta$ Ct method (17). Primer efficiency (E) (expressed as percent) was calculated from the slope of the standard curve using the formula $E = (10^{-1/\text{slope}} - 1) \times 100$.

100 (18), which resulted in the following primer efficiencies: ER α , 97.5 \pm 2.5%; ER β , 95.7 \pm 2.1%; and GPR30, 95.3 \pm 2.2%.

Expression of GPR30 mRNA in female pancreatic islets analyzed by microarray technique

Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany), and RNA quality and concentration were measured as described elsewhere (19). The microarrays were performed following the Affymetrix standard protocol and as described elsewhere (19). The array data were summarized and normalized with the robust multiarray (RMA) analysis method using the software Expression Console (Affymetrix, Santa Clara, CA).

Western blot

Approximately 400 islets per vial from male or female donors ($n = 5$ in each group) were suspended in 100 μ l PBS containing a cocktail of protease and phosphate inhibitors, frozen, and sonicated on ice on the day of analysis. In a parallel experiment, additional vials with the same amount of islets were incubated for 1 h at 37 C, 5% CO₂ with 5 mmol/liter glucose in Krebs-Ringer bicarbonate buffer containing a cocktail of cytokines IL-1 β (100 ng/ml), TNF α (125 ng/ml), and interferon- γ (IFN γ) (125 ng/ml) in the absence or presence of E2 (100 nmol/liter) or G-1 (100 nmol/liter) alone or in combination with ICI 182,780 (100 nmol/liter) or EM-652 (100 nmol/liter). The protein content of the homogenates was determined according to the bicinchoninic acid method (BCA) (Pierce, Rockford, IL). Homogenate samples representing 15 μ g total protein were run on 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). After electrophoresis, proteins were transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked in blocking buffer (LI-COR, Lincoln, NE). Subsequently, the membranes were incubated overnight at 4 C with one or two of the following primary antibodies: goat-raised polyclonal anti-hGPR30 (1:150) (R&D Systems, Minneapolis, MN), rabbit-raised polyclonal ER α (1:200), rabbit-raised polyclonal ER β (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse-raised monoclonal phosphorylated (p)-ERK1/2 (1:2000), rabbit-raised monoclonal phosphorylated serine/threonine protein kinase (pAKT) (1:1000), mouse-raised monoclonal p38 (1:2000), rabbit-raised monoclonal phosphorylated cAMP response element-binding (pCREB) (1:1000), and mouse-raised monoclonal phosphorylated cAMP response element-binding (pCREB) (1:2000) (Cell Signaling, Boston, MA). Loading control antibodies against α -tubulin (1:500) (Invitrogen, Carlsbad, CA) and β -actin (1:5000) (Sigma-Aldrich, St. Louis, MO) were also used. After the incubation, the membranes were washed three times with Tris-buffered saline with Tween 20, and the following secondary antibodies were added: polyclonal antirabbit conjugated with IRDye 800CW (1:4000), donkey antigoat conjugated with IRDye 680 (1:4000), and donkey antimouse conjugated with IRDye 680 (1:4000) (LI-COR). For stripping of the PVDF membrane, Newblot PVDF stripping buffer was used (LI-COR) according to the manufacturer's instruction with a subsequent blocking of the membrane in blocking buffer during 1 h. Quantitative analysis of bands was performed by Odyssey version 2.1 software (LI-COR) with automatic background correction, and the band's density was correlated with a loading control, either α -tubulin or β -actin. The specificity of the anti-hGPR30 anti-

body was verified using transfected HeLa cells with the hGPR30⁺ vs. control nontransfected HeLa cells (data not shown).

Confocal microscopy

Isolated islets from female donors were washed with PBS, fixed with 4% formaldehyde, and permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). GPR30 was detected with the same antibody as used for Western blot (1:50). For staining of insulin, glucagon, and somatostatin, we used the following antibodies: guinea pig anti-insulin (Eurodiagnostica, Malmö, Sweden) (1:500), guinea pig antiglucagon (1:500) and rat anti-somatostatin (1:250) (both from Jackson ImmunoResearch Laboratories), followed by treatment with fluorescent-conjugated secondary antibodies (1:150) from Jackson ImmunoResearch. The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2), 543/570 (Cy3), and 633/>650 nm (Cy5). The colocalization of the different hormones and GPR30 was quantified pixel by pixel with Manders' overlap coefficient; noise and cross talk were corrected, using Zen 2008 (Carl Zeiss, Oberkochen, Germany) software.

cAMP measurement

For the measurement of cAMP, human islets from female donors were incubated as previously described (9) with 12 mmol/liter glucose in the absence or presence of the following test agents, E2 (100 nmol/liter), G-1 (100 nmol/liter), ICI 182,780 (100 nmol/liter), and EM-652 (100 nmol/liter). On the day of analysis, samples were sonicated on ice and cAMP was measured using a cAMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The protein level was analyzed by the Bradford method (20).

Phosphatidylinositol (PI) hydrolysis

Receptor activation by G-1 and E2 on PLC signaling was assayed in islets from female donors by measuring hydrolysis of PI (21). Briefly, islets were dispersed into single cells in Ca²⁺-free medium (approximately 100,000 cells per well) and were then preloaded with myo-[³H]inositol (Perkin-Elmer, Boston, MA) for 16–20 h and then thoroughly washed and incubated in KRB buffer at 5.0 mmol/liter glucose in the presence of E2 (100 nmol/liter), G-1 (100 nmol/liter), ICI 182,780 (100 nmol/liter), or EM-652 (100 nmol/liter). After incubation, the cells were lysed with formic acid on ice and the inositol phosphates were isolated using anion exchange chromatography. The PI hydrolysis was expressed as PI hydrolysis per well. The coefficient of variation (interassay differences) was 7%.

Measurement of apoptosis and caspase-3 activity

Isolated islets from female donors were dispersed into single cells using Ca²⁺-free medium. The cells were incubated with and without a cocktail of cytokines: IL-1 β (100 ng/ml), TNF α (125 ng/ml), and IFN γ (125 ng/ml). The effects of E2 (100 nmol/liter) or G-1 (100 nmol/liter) on cytokine-induced apoptosis were studied in the absence or presence of ICI 182,780 (100 nmol/liter) or EM-652 (100 nmol/liter) after 24 h culture of the cells in RPMI 1640 medium with 5 mmol/liter glucose supplemented with 10% fetal bovine serum. Apoptotic cells were detected by using a

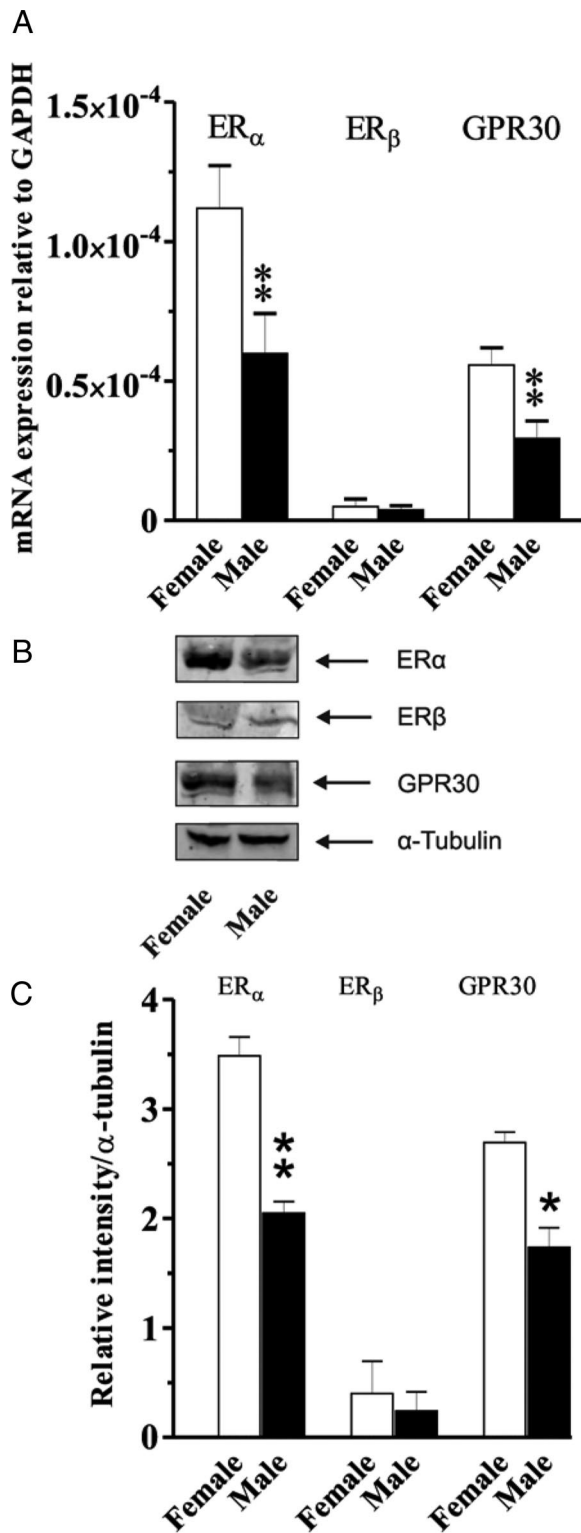


FIG. 1. A, Relative mRNA expression of ERα (n = 6 in each group), ERβ (n = 6 in each group), and GPR30 (n = 10 in each group) analyzed by qPCR in islets from female and male donors. The expression levels of ERα and GPR30 are higher in the isolated islets from female donors. B, A representative Western blot of the ERα, ERβ, and GPR30 protein expression in pancreatic islets from female and male donors. C, The mean intensity (means ± SEM) of the Western blots (n = 5 in each group) is presented as relative value of the band intensity toward the endogenous control α-tubulin. *, *P* < 0.05; **, *P* < 0.01.

fluorescence-activated cell sorting system with annexin V staining (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions, and the flow cytometry data were analyzed using the FlowJo and CellQuest software packages.

The activity of caspase-3 was determined by monitoring the cleavage of a specific fluorogenic caspase-3 substrate, Ac-N-Acetyl-ASP-Glu-Val-Asp-Asp-Glu-Val-Asp-7-Amino-4-Methylcoumarin (Ac-DEVD-AMC) (Upstate Cell Signaling Solutions, Lake Placid, NY). Dispersed islet cells were cultured for 24 h in a medium containing 5 mmol/liter glucose with a combination of cytokines as described above. For comparison, the effect of H-89 (10 μmol/liter) and dibutyryl-cAMP (db-cAMP), a cAMP analog (10 μmol/liter), on caspase-3 activity was also studied. After culture, the cells were washed with PBS and then lysed with lysis buffer. Thereafter, the homogenates were allowed to react with the fluorogenic caspase-3 substrate in a 96-well plate (3 μl to

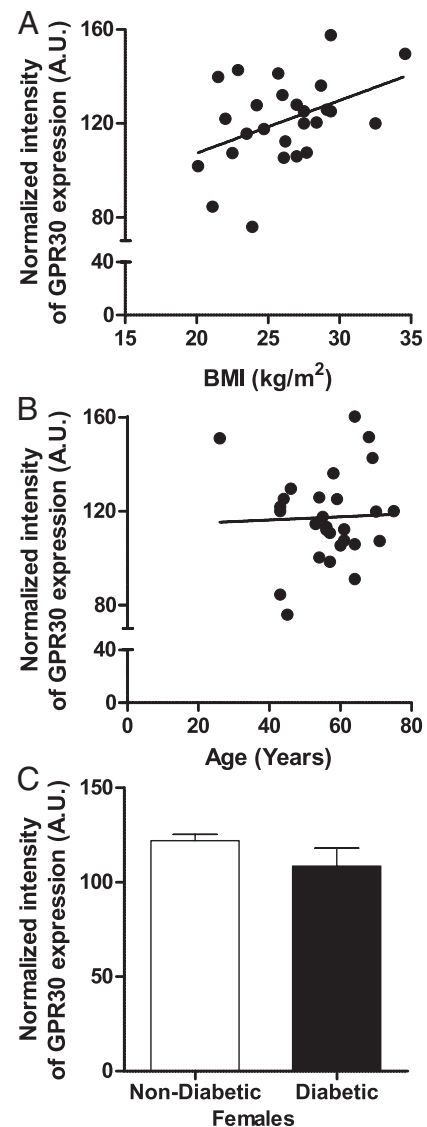


FIG. 2. A, Microarray data showing expression level of GPR30 mRNA in relation to BMI from female pancreatic islets (n = 26); B, expression level of GPR30 mRNA in relation to age from female pancreatic islets (n = 26); C, quantitative microarray data (means ± SEM) showing relative expression levels of GPR30 in nondiabetic (n = 19) and diabetic (n = 7) female pancreatic islets.

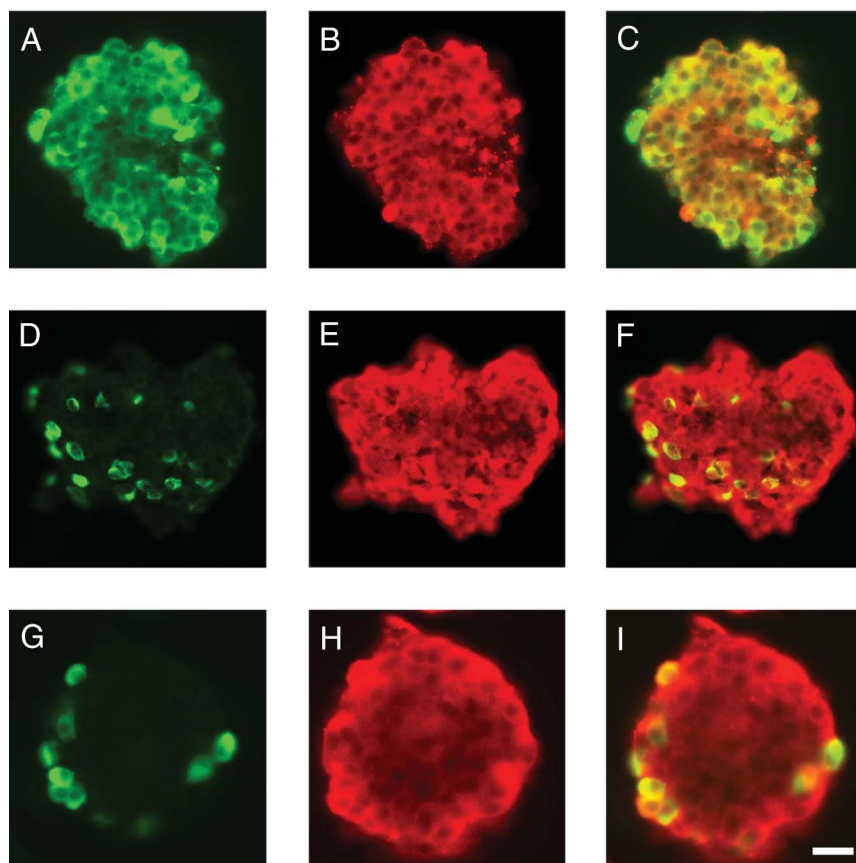


FIG. 3. Confocal microscopy of whole mounted islets from female donors and double immunolabeled for insulin, glucagon, and somatostatin (green fluorescence) (A, D, and G) and for GPR30 (red fluorescence) (B, E, and H). Colocalization of GPR30 and the different hormones is seen as orange-yellowish fluorescence (C, F, and I). Bar, 20 μm .

each well) in a reaction buffer containing 20 mmol/liter HEPES, 10% glycerol, and 2 mmol/liter dithiothreitol. The mixtures were maintained at 37 C for 60 min (in the dark) and subsequently analyzed on a Fluostar system at the wavelength of 390/460 nm. The results were correlated to the protein concentration of each well measured according to the Bradford method (20).

Statistics

All results are expressed as means \pm SEM for the indicated number of observations or illustrated by a representative observation for a number of different experiments (Western blots and confocal microscopy). Statistical significance was assessed by using ANOVA with Tukey-Kramer's multiple-comparison posttest or Student's *t* test where appropriate. The EC₅₀ values were calculated from a nonlinear sigmoid curve fit using GraphPad Prism version 4. A *P* value < 0.05 was considered significant.

Results

mRNA and protein expression of GPR30 in female and male human islets

Control experiments showed no differences between female and male human islets with regard to mRNA expression for ER β , whereas ER α was lower in males (Fig.

1A). Notably, ER α expression was much higher than ER β in islets from both sexes (Fig. 1A). The expression of GPR30 mRNA relative to the reference gene *GAPDH* was found to be higher in islets from females [(5.67 \pm 0.6) \times 10⁻⁵] compared with males [(2.95 \pm 0.6) \times 10⁻⁵] (*P* < 0.01; *n* = 10 in each group) (Fig. 1A).

Densitometric analysis of Western blots revealed that also on the protein level, GPR30 displayed a strong expression in islet homogenates from females (2.7 \pm 0.16 relative intensity of GPR30/ α -tubulin) compared with males (1.7 \pm 0.20 relative intensity GPR30/ α -tubulin), (*P* < 0.05) (*n* = 4 in each group) (Fig. 1, B and C). Protein expression of ER α was also stronger in females *vs.* males (3.45 \pm 0.30 relative intensity of ER α / α -tubulin in females *vs.* 2.1 \pm 0.18 in males), whereas ER β expression was not different although six to eight times lower than ER α expression.

Expression of GPR30 in pancreatic islets analyzed by microarray technique

To study the relationship between the expression of GPR30 and BMI, we used a microarray data analysis. As shown in Fig. 2A, the expression of GPR30 was positively correlated to the BMI in female subjects (*r* = 0.42, with a slope of 2.246 \pm 0.98; *P* < 0.05; *n* = 26). Notably, there was no correlation between age and the expression of GPR30 (Fig. 2B), which was also confirmed by qPCR showing no differences concerning GPR30 expression in the islet from premenopausal (20–50 yr) and postmenopausal (55–75 yr) female subjects. Thus, the expression of GPR30 mRNA relative to the *GAPDH* was [(5.35 \pm 0.388) \times 10⁻⁵] (*n* = 10) and [(4.90 \pm 0.321) \times 10⁻⁵] (*n* = 15), respectively. There was also not any significant difference in GPR30 expression in nondiabetic compared with diabetic females (Fig. 2C), which was also confirmed by qPCR analysis of the expression of GPR30 mRNA relative to the *GAPDH* mRNA from nondiabetic [(5.58 \pm 0.319) \times 10⁻⁵] (*n* = 12) and diabetic [(4.83 \pm 0.679) \times 10⁻⁵] (*n* = 6) islets from female subjects.

GPR30 protein expression in different islet cell types

To investigate the cell-specific expression of GPR30 protein in human pancreatic islet cells, islets isolated from

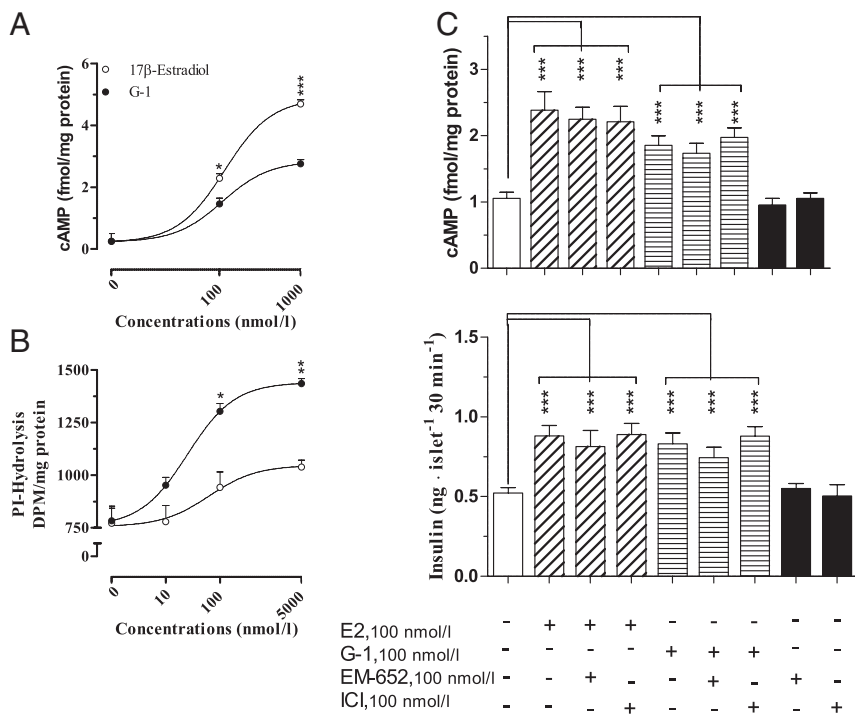


FIG. 4. Concentration-dependent induction of secondary messengers via GPR30 by E2 and G-1 in human islet cells and E2 and G-1 effects on cAMP content in isolated female pancreatic islets at 12 mmol/liter glucose. A, Stimulation of cAMP production by E2 and G-1; B, induction of PI hydrolysis by E2 and G-1; C, effects of G-1 and E2 on cAMP content in isolated islets and insulin secretion at 12 mmol/liter glucose. Islets were incubated with G-1 (100 nmol/liter) or E2 (100 nmol/liter) in the absence or presence of ER α and ER β inhibitors ICI 182,780 or EM-652 (100 nmol/liter). Results are shown as means \pm SEM; $n = 4$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

females were simultaneously immunolabeled for insulin, glucagon, or somatostatin together with GPR30 and analyzed by confocal microscopy. As seen in Fig. 3, GPR30 could be visualized in the different islet cell types and showed a strong colocalization with insulin (Fig. 3, A–C) ($85 \pm 2.2\%$ of the insulin positive cells, $n = 8$), glucagon (Fig. 3, D–F) ($75 \pm 1.5\%$ of the glucagon-positive cells, $n = 8$), and somatostatin (Fig. 3, G–I) ($86 \pm 1.2\%$ of the somatostatin-positive cells, $n = 8$).

Influence of GPR30 activation on β -cell second messengers and insulin release

E2 and G-1 were then tested for their effects on the second messenger systems producing cAMP and PLC-stimulated PI hydrolysis, respectively, in female islets. Figure 4 shows that E2 as well as G-1 concentration-dependently stimulated both the formation of cAMP (Fig. 4A) and the PI hydrolysis (Fig. 4B) in the presence of 12 mmol/liter glucose. Interestingly, G-1 was found to be less potent than E2 with regard to cAMP formation, whereas it was more potent with regard to PI hydrolysis.

Because we suspected that the cAMP system rather than the PLC pathway was linked to the rapid potentiating effects of GPR30 activation on glucose-stimulated insulin

release (9), we performed an experiment at 12 mmol/liter glucose with parallel measurements of the islets cAMP content and insulin release in the presence and absence of E2, G-1, and the nuclear receptor antagonist EM-652 or ICI 182,780. Figure 4C shows that E2 and G-1 stimulated cAMP generation in parallel with insulin release both in the absence and presence of EM-652 and/or ICI 182,780. EM-652 or ICI 182,780 did not amplify cAMP or insulin release (Fig. 4C).

We have previously observed in mice islets (9) and now in human islets (Fig. 4A) that an important signal from GPR30 activation by E2 and G-1 is transduced through the cAMP system. We now tested the influence of the PKA inhibitor H-89 and, with regard to our present results on PI hydrolysis, also the influence of the PLC inhibitor RHC80267 on insulin release at 12 mmol/liter glucose in the presence and absence of E2 and G-1 (Fig. 5A). As expected, both H-89 and RHC80267 markedly reduced glucose-stimulated insulin release (Fig. 5A). Figure 5A also shows that the potentiating

effects of E2 and G-1 on glucose-stimulated insulin release were greatly impaired by the PKA inhibitor. Moreover, GPR30 activation by E2 and G-1 surprisingly reversed the suppressive effect exerted by the PLC inhibitor on insulin release (Fig. 5A). The nuclear ER antagonist EM-652 did not influence the effects of E2 or G-1 (Fig. 5A).

To further study the influence of E2 and G-1 on insulin secretory pathways induced by glucose, we tested whether they would modulate the potentiating effect of glibenclamide and the inhibitory effect of clonidine. Figure 5B shows that E2 and G-1 significantly amplified glibenclamide-stimulated insulin release at 12 mmol/liter glucose. Interestingly, the potent inhibitory effect of clonidine on glucose-stimulated insulin release was almost abolished by both E2 and G-1 (Fig. 5B). The presence of EM-652 did not influence these effects (Fig. 5B).

Influence of GPR30 activation on islet cell survival

We studied the downstream activation of MAPK by GPR30 (Fig. 6). Islets from female donors were incubated in a cocktail of cytokines at 5 mmol/liter glucose with E2 or G-1 in the absence or presence of EM-652 or ICI 182,780. Compared with control samples, E2 or

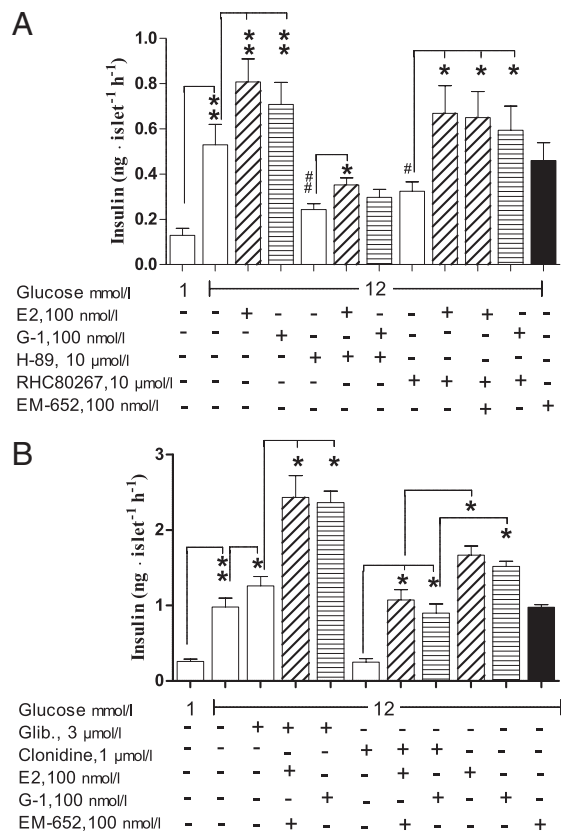


FIG. 5. A, Insulin release from female islets at 12 mmol/liter glucose showing effects of H-89 (a PKA inhibitor) and RHC80267 (a PLC inhibitor) after GPR30 stimulation with G-1 (100 nmol/liter) or E2 (100 nmol/liter) in the absence and presence of EM-652 (100 nmol/liter). For comparison, insulin release at 1 mmol/liter glucose is also included. B, Insulin secretion from islets incubated at 12 mmol/liter glucose with either glibenclamide (Glib.) (3 μmol/liter) or clonidine (1 μmol/liter) together with E2 (100 nmol/liter) or G-1 (100 nmol/liter) in the presence or absence of EM-652 (100 nmol/liter). The basal insulin release at 1 mmol/liter glucose is also included. The values are displayed as means ± SEM; n = 4. #, $P < 0.05$; ##, $P < 0.01$, comparing effects of H-89 or RHC80267 with the 12 mmol/liter glucose in control group (white bar); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

G-1 suppressed the cytokine-induced phosphorylation of pSAPK/JNK and p38 also in the presence of the nuclear ER blockers (Fig. 6, A and B), whereas both E2 and G-1 induced an increased phosphorylation of pCREB, pAKT, and pERK1/2 (Fig. 6, C–E). Figure 6F shows an example of the quantified blots.

Effects of E2 or G-1 on cytokine-induced apoptosis in islet cells in the absence or presence of ICI 182,780 or EM-652

Dispersed female islet cells were cultured for 24 h in 5 mmol/liter glucose with or without a mixture of cytokines (IL-1β, TNFα, and IFNγ). As illustrated in Fig. 7A, addition of E2 or G-1 to the culture medium markedly prevented the apoptotic effects of cytokines ($P < 0.001$) (Fig. 7A). The antiapoptotic effect of E2 or G-1 was also clearly

evident in the presence of either ICI 182,780 or EM-652 (Fig. 7A). In this context, it is important to point out that the high basal values of apoptosis in human islet cells shown in Fig. 7A as compared with rodent islet cells (9) are most likely the result of the more complex and protracted isolation procedures for human islets. The cytokine-induced increase in apoptosis is thus approximately the same for human and rodent islet cells.

To study the effect of GPR30 on apoptosis via the caspase cascade, we measured the activity of caspase-3 in pancreatic islets from female donors (Fig. 7B). Islets incubated with E2 (100 nmol/liter), G-1 (100 nmol/liter), or db-cAMP (10 μmol/liter) in the presence of cytokines showed a highly significant decrease in caspase-3 activity compared with islets incubated with cytokines alone ($P < 0.001$). Islets incubated with the PKA inhibitor H-89 plus cytokines showed no significant decrease in caspase-3 activity in the presence of E2 (100 nmol/liter) or G-1 (100 nmol/liter) (Fig. 7B). Addition of H-89 alone did not significantly change the basal caspase-3 activity (Fig. 7B).

Effect of GPR30 activation on islet hormone release in islets from type 2 diabetic female subjects

Finally, a series of concentration-response experiments was designed to compare the hormone secretory response of nondiabetic and diabetic female islets to E2 and G-1 at 12 mmol/liter glucose concentration (Fig. 8, A–C). Both agents concentration-dependently potentiated insulin secretion in the presence of 12 mmol/liter glucose regardless of whether islets were from nondiabetic or diabetic subjects. The potentiating effect of E2 on insulin secretion was markedly higher compared with the effects of G-1, and the EC_{50} values were 3.78×10^{-9} (E2) and 1.03×10^{-8} (G-1). Neither E2 nor G-1 had any significant effect on insulin release at low glucose (1 mmol/liter) (data not shown). As seen in Fig. 8D, both E2 and G-1 suppressed glucagon release at 1 mmol/liter glucose in both nondiabetic and diabetic islets. The IC_{50} values for the inhibitory action of E2 (9.78×10^{-11}) and G-1 (4.6×10^{-10}) on glucagon release in diabetic islets were almost of the same magnitude. Importantly, the glucagon release from diabetic islets was markedly higher compared with nondiabetic islets (Fig. 8D). The suppressive effect of high glucose (12 mmol/liter) on glucagon release from nondiabetic islets was only slightly modulated by E2 or G-1 (Fig. 8B), whereas the exaggerated glucagon release seen in the diabetic islets was efficiently suppressed by both agents. A concentration-dependent suppressive effect of E2 and G-1 on somatostatin secretion at 12 mmol/liter glucose was also observed (Fig. 8C). The IC_{50} values for the suppressive effects on somatostatin secretion were for E2 ($4.45 \times$

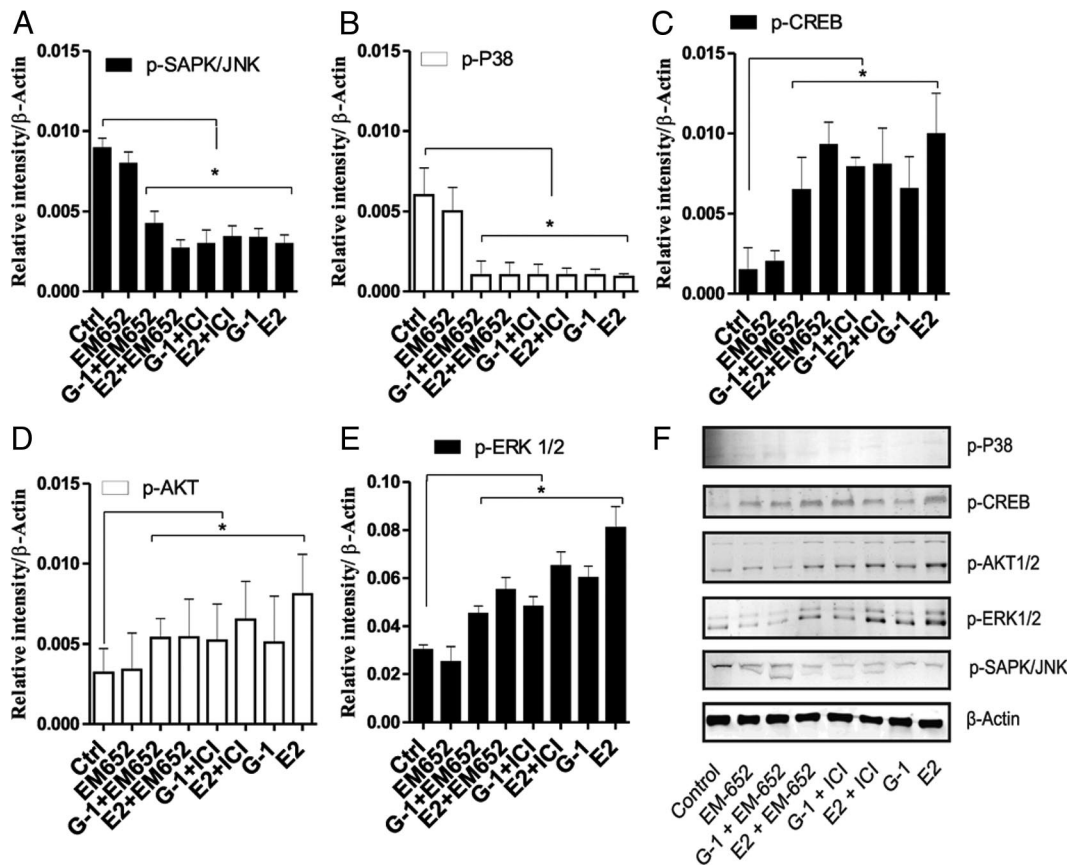


FIG. 6. Effects of E2 (100 nmol/liter) or G-1 (100 nmol/liter) on the MAPK signaling pathways induced by the cytokine cocktail [IL-1 β (100 ng/ml) plus TNF α (125 ng/ml) plus IFN γ (125 ng/ml)] in the presence or absence of nuclear ER antagonists EM-652 (100 nmol/liter) or ICI 182,780 (ICI) (100 nmol/liter). A–E, Displays the mean intensity of the Western blots ($n = 5$ in each group) presented as the relative values of the band intensity toward the endogenous control (Ctrl) β -actin from the following proteins: A, pSAPK/JNK; B, pP38; C, pCREB; D, pAKT; E, pERK1/2. F, Example of the quantified blots. The values are displayed as means \pm SEM; $n = 4$; $P < 0.05$.

10^{-8}) and for G-1 (1.3×10^{-7}). Somatostatin release at low glucose (1 mmol/liter) was not affected by E2 or G-1 (data not shown).

Discussion

The G protein-coupled ER1 (GPER1), also known as GPR30, has recently been recognized as a membrane receptor for estrogens that mediate nongenomic signals (6–9, 22). The present study clearly shows that GPR30 is expressed in human pancreatic islets isolated from both female and male subjects. Interestingly, the expression of GPR30 was higher in islets from females compared with males. Moreover, the potent antiapoptotic effect of estrogen in female islets was found to be mediated, at least in part, through the activation of GPR30. Finally, we found that E2 and G-1 displayed a marked antidiabetic action on the secretion of insulin, glucagon, and somatostatin from female diabetic islets by improving glucose-stimulated insulin release while suppressing glucagon and somatostatin secretion. Hence, our present findings on human pancreatic islets are in good agreement with our recent reports indicat-

ing that both pharmacological manipulations and genetically deletion of GPR30 (GPR30 $^{-/-}$) in mice are associated with an altered estrogen-stimulated insulin release (8, 9).

The mechanisms by which E2 alters cellular function have been the subject of intense investigation. The data presented here on human islets show that E2 and G-1 influence the generation of both PLC/inositol triphosphate (IP $_3$) and the cAMP/PKA signaling pathways. Although E2 acts more efficiently via the cAMP/PKA pathway, the G-1 response seems to favor the PLC/IP $_3$ pathway, which could be due to interaction of different conformational changes of GPR30. These results indicate that GPR30 activation is important for the observed signaling events in the islet cells, as has also been shown previously in other tissues (23). In the β -cells, cAMP seems to be the predominant GPR30 messenger for potentiation of glucose-stimulated insulin release. This, however, does not exclude the possibility that GPR30 signaling pathways might differ in different pancreatic islet cell types.

We have recently reported in mouse islets that G-1 enhances insulin secretion while inhibiting glucagon

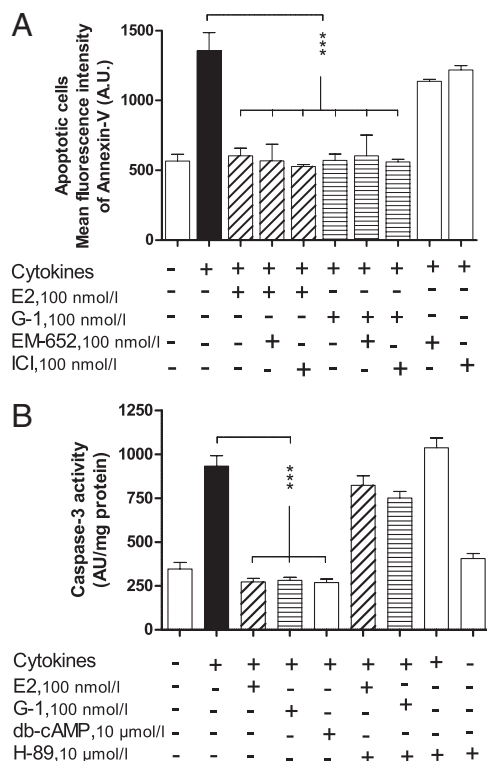


FIG. 7. A, Cytokine-induced apoptosis in islet cells from female donors performed on five different occasions ($n = 8$). The levels of apoptotic cells induced by cytokines [IL-1 β (100 ng/ml) plus TNF α (125 ng/ml) plus IFN γ (125 ng/ml)] are strongly reduced when E2 (100 nmol/liter) or G-1 (100 nmol/liter) is added in the presence or absence of either ICI 182,780 (ICI) (100 nmol/liter) or EM-652 (100 nmol/liter). ***, $P < 0.001$. B, Effects of E2 (100 nmol/liter), G-1 (100 nmol/liter), or db-cAMP (10 μ mol/liter) on cytokine-induced activation of caspase-3 in female islets in the absence or presence of H-89. Means \pm SEM are shown for $n = 8$ in each group. ***, $P < 0.001$. AU, Arbitrary units.

and somatostatin secretion (9). As we have shown in the current study in isolated islets from female humans, E2 and G-1 stimulate cAMP in parallel with increased insulin release even in the presence of the nuclear receptor ER α and ER β antagonists ICI 182,780 or EM-652. Both E2 and G-1 potentiate insulin secretion and inhibit glucagon and somatostatin release in a concentration-dependent manner from both diabetic and normal female human islets. These findings clearly indicate that GPR30 plays a functional role in insulin secretion via a cAMP-dependent pathway, suggesting that pharmacotherapy with small G-1-like molecules that induce GPR30-mediated cAMP-PKA signaling might constitute novel potential remedies against diabetes. There are some reports challenging the role of GPR30 as an extranuclear ER that mediates the rapid action of estrogen, because in a recent study, it has been shown that G-1 also binds to a new membrane receptor for estrogen, *i.e.* ER- α 36 in cancer cells (24). In accordance with our recently published data (8, 9), our current findings suggest that GPR30 is the main player at least in human islets, but it does not exclude the notion that G-1

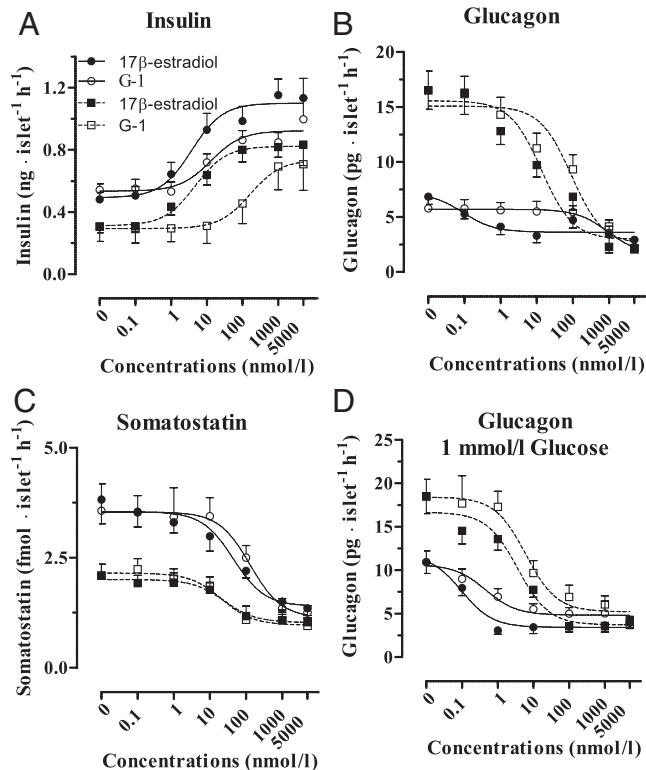


FIG. 8. Concentration-response effects of E2 (●/■) and G-1 (○/□) on insulin (A), glucagon (B), and somatostatin (C) secretion from isolated pancreatic islets of nondiabetic (*solid line*) and diabetic (*dotted line*) female donors at 12 mmol/liter glucose. Identical concentration ranges for both compounds were used (see x-axis). G-1 mimics the effects of E2. Both compounds amplified glucose-stimulated insulin release, whereas glucagon and somatostatin secretions were suppressed. D, Glucagon secretion at 1 mmol/liter glucose. Values are presented as means \pm SEM for eight observations in each group from three different donors performed at three separate occasions.

might bind to ER- α 36 and thus that ER- α 36, if present in normal islet tissue, might somehow be implicated in the insulinotropic effects of G-1 in certain situations (25).

To further illustrate the importance of the PLC- and cAMP-dependent pathways in mediating the GPR30-induced insulin secretion, we used a specific PLC inhibitor to block the formation of IP $_3$ and diacylglycerol and the PKA inhibitor H-89 to block the PKA effects. The inhibition of the PLC pathway suppressed glucose-stimulated insulin secretion but did not influence the beneficial effect of E2 on insulin release; in contrast, H-89 suppressed the stimulatory action of E2 on insulin secretion. These data showed that the cAMP pathways play a major role in estrogen potentiation of glucose-stimulated insulin secretion and even may compensate for inhibitory effects on the PLC pathways. Moreover, we also noticed that insulin secretion amplified by the K $_{ATP}$ channel inhibitor glibenclamide at 12 mmol/liter glucose was further potentiated by both E2 and G-1 and that the inhibitory effect of the α_2 -adrenergic agonist clonidine on glucose-stimulated in-

sulin release was markedly reversed even in the presence of the estrogen nuclear receptor blockers. These observations suggest that the GPR30-mediated effects on the cAMP pathways are most likely exerted at more distal events in the secretory process. The fact that E2 and G-1 abolished the inhibitory effects of clonidine on insulin secretion is in the good accordance with previous reports showing that clonidine exerts its inhibitory effects on glucose-stimulated insulin release through inhibition of adenylate cyclase (26, 27). Notably, in this situation, E2 and G-1 might also increase the activation of the PLC pathways.

Several mechanisms may contribute to β -cell apoptosis and the development of type 2 diabetes. It is generally accepted that β -cell dysfunction, at least in part, is a consequence of an increased production of proinflammatory cytokines such as IL-1 β , TNF α , and IFN γ . These cytokines in synergy then lead to induction of apoptotic signaling cascades in the pancreatic β -cells (28).

Under normal physiological conditions, different MAPK signaling cascades are thought to be balanced through pathways in which ERK signaling mediates growth and survival and p38 and JNK kinases that are associated with apoptosis (29). Previous studies have suggested that p38 and ERK1/2 phosphorylation are involved in either islet cell damage or islet cell survival, highlighting the intricate balance between p38 *vs.* ERK1/2 activity (30, 31). Our present result indicates that E2 and G-1 by down-regulation of JNK and p38 activity could be an efficient novel approach to increase β -cell survival.

Our present results also show that activation of GPR30 mediates, at least in part, antiapoptotic effects of estrogen via phosphorylation and activation of CREB, which appears to be crucial for pancreatic β -cell survival (32). This is in good agreement with a number of recent studies indicating that CREB activation precedes the up-regulation of Bcl2, which is known to be mediating the antiapoptotic action of various agents (33). In addition, we have observed that estrogen markedly up-regulates the antiapoptotic signaling properties of AKT, which suppress B-cell lymphoma related protein, a proapoptotic member of the Bcl family (29). Our present findings also provide evidence for a beneficial effect of estrogen on female human islets being mediated via activation of ERK1/2 and AKT, in parallel with the inhibition of the p38 MAPK pathway. To further clarify the mechanisms mediating the antiapoptotic action of estrogen, we measured caspase-3 activity in the presence of the PKA inhibitor H-89. The lack of suppressive effect of E2 and G-1 on caspase-3 activity in the presence of H-89 clearly indicates that the cAMP/PKA-dependent mechanism is an important pathway through which activation of GPR30 signaling provides protection

against proapoptotic effects of cytokines on female human islets.

Ovarian endocrine insufficiency after menopause has been reported as a diabetogenic factor (34), and in animal studies, it has been shown that estrogen provides a protection against streptozotocin-induced diabetes (35). Furthermore, GPR30 deletion abolished the estrogen-stimulated insulin release both *in vivo* in ovariectomized female adult mice and *in vitro* in isolated islets (8). These findings suggest that GPR30 is necessary for normal insulin production and glucose homeostasis (8, 9). Notably, our results suggested that GPR30 expression was not different in pre- and postmenopausal pancreatic islets of female donors.

Finally, it should be emphasized that although our present data speak much in favor of GPR30 being involved in estrogenic signaling and regulation of islet function in female human subjects, there are still many controversies to be resolved. Notably, the importance of GPR30 as a true functional receptor in different tissues has been questioned (36, 37), and although the expression level of ER β is very low both in human and rodent islets (9), it does not necessarily exclude ER β having an essential role in islet biology. Hence, ER β has been suggested to be implicated in the rapid nongenomic regulation of β -cell K_{ATP} channel activity and atrial natriuretic peptide receptor activation (38), and estrogen- β -cell interactions have been discussed in a recent review by Nadal *et al.* (39) who point out that the influence of cytosolic parts of the classical ER α and ER β receptors should not be overlooked. Hence, we cannot exclude that certain interaction between GPR30, ER α , and ER β receptors are still to be elucidated with regard to mechanisms of action and effects in short-term *vs.* long-term aspects.

In conclusion, the present study identifies GPR30 as a new target to prevent pancreatic islet dysfunction. The feminizing effects of estrogens limit their clinical application to promote islet survival in male subjects. However, GPR30 agonistic compounds such as G-1 which use extranuclear and membrane pathways that are sex nonspecific may help to identify and develop new ligands that stimulate and protect β -cells while lacking the mitogenic actions predisposing to hormone-dependent cancers. The present study adds a novel dimension to estrogen biology in β -cells and identifies GPR30 as a new target to increase β -cell function and survival.

Acknowledgments

We thank Britt-Marie Nilsson for skillful technical assistance.

Address all correspondence and requests for reprints to: Dr. Albert Salehi, Department of Clinical Science, Skåne Universitetssjukhus, Division of Islet Cell Physiology, Clinical Research Center, Building 91, Plan 11, Entrance 72, S-205 02 Malmö, Sweden. E-mail: S_Albert.Salehi@med.lu.se.

This work was supported by the Swedish Research Council; Medical Faculty, Lund University; Novo Nordic Foundation; and Øresund Diabetes Academy.

Disclosure Summary: The authors have nothing to disclose.

References

- Mathis D, Vence L, Benoist C 2001 β -Cell death during progression to diabetes. *Nature* 414:792–798
- Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H 2003 Glucose toxicity in β -cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes* 52:581–587
- Hess RA 2003 Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* 1:52
- Wild S, Roglic G, Green A, Sicree R, King H 2004 Global prevalence of diabetes. *Diabetes Care* 27:1047–1053
- Louet JF, LeMay C, Mauvais-Jarvis F 2004 Antidiabetic actions of estrogen: insight from human and genetic mouse models. *Curr Athroscler Rep* 6:180–185
- Fissore F, Fortunati N, Comba A, Fazzari A, Gaidano G, Berta L, Frairia R 1994 The receptor-mediated action of sex steroid binding protein (SBP, SHBG): accumulation of cAMP in MCF-7 cells under SBP and estradiol treatment. *Steroids* 59:661–667
- Filardo EJ, Quinn JA, Frackelton Jr AR, Bland KI 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 16:70–84
- Mårtensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grände PO, Owman C, Rosen CJ, Adamo ML, Lundquist I, Rorsman P, Nilsson BO, Ohlsson C, Olde B, Leeb-Lundberg LM 2009 Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* 150:687–698
- Balhuizen A, Kumar R, Amisten S, Lundquist I, Salehi A 2010 Activation of G protein-coupled receptor 30 modulates hormone secretion and counteracts cytokine-induced apoptosis in pancreatic islets of female mice. *Mol Cell Endocrinol* 320:16–24
- Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI, Prossnitz ER 2006 Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2:207–212
- Wade GN, Blaustein JD, Gray JM, Meredith JM 1993 ICI 182,780: a pure antiestrogen that affects behaviors and energy balance in rats without acting in the brain. *Am J Physiol Regul Integr Comp Physiol* 265:R1392–R1398
- Tremblay A, Tremblay GB, Labrie C, Labrie F, Giguère V 1998 EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors α and β . *Endocrinology* 139:111–118
- Heding L 1966 A simplified insulin radioimmunoassay method. In: Donato L, Milhaud G, Sirchis J eds. *Labelled proteins in tracer studies*. Brussels: Euratom; 345–350
- Ahrén B, Lundquist I 1982 Glucagon immunoreactivity in plasma from normal and dystrophic mice. *Diabetologia* 4:258–263
- Panagiotidis G, Salehi AA, Westermark P, Lundquist I 1992 Homologous islet amyloid polypeptide: effects on plasma levels of glucagon, insulin and glucose in the mouse. *Diabetes Res Clin Pract* 18:167–171
- Amisten S, Braun OO, Bengtsson A, Erlinge D 2008 Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thromb Res* 122:47–57
- Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45
- Ramakers C, Ruijter JM, Deprez RH, Moorman AF 2003 Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62–66
- Jonsson A, Isomaa B, Tuomi T, Taneera J, Salehi A, Nilsson P, Groop L, Lyssenko V 2009 A variant in the KCNQ1 gene predicts future type 2 diabetes and mediates impaired insulin secretion. *Diabetes* 58:2409–2413
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Meidute Abaraviciene S, Lundquist I, Galvanovskis J, Flodgren E, Olde B, Salehi A 2008 Palmitate-induced β -cell dysfunction is associated with excessive NO production and is reversed by thiazolidinedione-mediated inhibition of GPR40 transduction mechanisms. *PLoS ONE* 3:e2182
- Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P 2007 Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology* 148:3236–3245
- Kanda N, Watanabe S 2003 17 β -Estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression. *J Invest Dermatol* 121:1500–1509
- Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, Wang ZY 2010 Involvement of estrogen receptor variant ER- α 36, not GPR30, in nongenomic estrogen signaling. *Mol Endocrinol* 24:709–721
- Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF 2006 A variant of estrogen receptor- α , hER- α 36: Transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc Natl Acad Sci USA* 103:9063–9068
- Yamazaki S, Katada T, Ui M 1982 α 2-Adrenergic inhibition of insulin secretion via interference with cyclic AMP generation in rat pancreatic islets. *Mol Pharmacol* 21:648–653
- Leclercq-Meyer V, Herchuelz A, Valverde I, Couturier E, Marchand J, Malaisse WJ 1980 Mode of action of clonidine upon islet function: dissociated effects upon the time course and magnitude of insulin release. *Diabetes* 29:193–200
- Mandrup-Poulsen T 2001 β -Cell apoptosis: stimuli and signaling. *Diabetes* 50(Suppl 1):S58–S63
- Boutros T, Chevet E, Metrakos P 2008 Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol Rev* 60:261–310
- Khoo S, Gibson TB, Arnette D, Lawrence M, January B, McGlynn K, Vanderbilt CA, Griffen SC, German MS, Cobb MH 2004 MAP kinases and their roles in pancreatic β -cells. *Cell Biochemistry and Biophysics* 40:191–200
- Abaraviciene SM, Lundquist I, Salehi A 2008 Rosiglitazone counteracts palmitate-induced β -cell dysfunction by suppression of MAP kinase, inducible nitric oxide synthase and caspase 3 activities. *Cell Mol Life Sci* 65:2256–2265
- Kim SJ, Nian C, Widenmaier S, McIntosh CH 2008 Glucose-dependent insulinotropic polypeptide-mediated up-regulation of β -cell antiapoptotic Bcl-2 gene expression is coordinated by cyclic AMP (cAMP) response element binding protein (CREB) and cAMP-responsive CREB coactivator 2. *Mol Cell Biol* 28:1644–1656
- Szego EM, Barabás K, Balog J, Szilágyi N, Korach KS, Juhász G, Abrahám IM 2006 Estrogen induces estrogen receptor α -dependent cAMP response element-binding protein phosphorylation via mito-

- gen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. *J Neurosci* 26:4104–4110
34. **Lenzen S, Bailey CJ** 1984 Thyroid hormones, gonadal and adrenocortical steroids and the function of the islets of Langerhans. *Endocr Rev* 5:411–434
 35. **Rossini AA, Williams RM, Appel MC, Like AA** 1978 Sex differences in the multiple-dose streptozotocin model of diabetes. *Endocrinology* 103:1518–1520
 36. **Levin ER** 2009 G protein-coupled receptor 30: estrogen receptor or collaborator? *Endocrinology* 150:1563–1565
 37. **Langer G, Bader B, Meoli L, Isensee J, Delbeck M, Noppinger PR, Otto C** 2010 A critical review of fundamental controversies in the field of GPR30 research. *Steroids* 75:603–610
 38. **Soriano S, Ropero AB, Alonso-Magdalena P, Ripoll C, Quesada I, Gassner B, Kuhn M, Gustafsson JA, Nadal A** 2009 Rapid regulation of K(ATP) channel activity by 17 β -estradiol in pancreatic β -cells involves the estrogen receptor β and the atrial natriuretic peptide receptor. *Mol Endocrinol* 23:1973–1982
 39. **Nadal A, Alonso-Magdalena P, Soriano S, Ripoll C, Fuentes E, Quesada I, Ropero AB** 2011 Role of estrogen receptors α , β and GPER1/GPR30 in pancreatic β -cells. *Front Biosci* 16:251–260