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Research paper

Activation of G protein-coupled receptor 30 modulates hormone secretion and counteracts cytokine-induced apoptosis in pancreatic islets of female mice

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The role of the newly discovered estrogen receptor GPR30 in islet physiology and pathophysiology is unclear. We examined GPR30 expression in relation to hormone secretion and possible anti-apoptotic effects in isolated mouse islets using the synthetic GPR30 ligand G-1. The mRNA and protein expression of GPR30 was analyzed by qPCR, Western blot and confocal microscopy. Hormone secretion and cAMP content were determined with RIA and apoptosis in islet cells with the Annexin-V method.

GPR30 mRNA and protein expression was markedly higher in islets from females compared to male. This gender difference was not found for the genomic estrogen receptors ERα and ERβ, the ERα expression being 10-fold higher than ERβ in both genders. Confocal microscopy revealed abounden GPR30 expression in insulin, glucagon and somatostatin cells. Dose-response studies of G-1 *vs* 17β-estradiol in isolated islets at 1 or 12 mM glucose showed an almost identical pattern in that both compounds increased insulin and inhibited glucagon and somatostatin secretion. ICI-182,780 and EM-652, potent antagonists of the 17β-estradiol receptors (ERα and ERβ) did not influence the amplifying effect of G-1 or 17β-estradiol on cAMP content or insulin secretion from isolated islets. Cytokine-induced (IL-1β + TNFα + INFγ) apoptosis in islets, cultured for 24 h at 5 mM glucose, was almost abolished by G-1 or 17β-estradiol treatment. Addition of ICI-182,780 or EM-652 did not affect this beneficial effect of G-1 or 17β-estradiol.

Taken together, our findings show that GPR30 is expressed in most islet endocrine cells. The synthetic GPR30 ligand G-1 mimics the nongenomic effects of 17β-estradiol on islet hormone secretion, cAMP content in islets and its anti-apoptotic effects. G-1 or analogs thereof might be new potential candidates in the therapeutic strategy for type 2 diabetes in women.

1. Introduction

Previous studies have shown that women display not only increased insulin sensitivity, and a better glucose tolerance but also a better insulin secretory response to nutrients compared to men, (Boyns et al.,1969; Yki-Järvinen,1984) and are more resistant to nutrition-induced insulin resistance (Frias et al.,2001; Hevener et al.,2002; Soeters et al.,2007). Likewise, female mice are less prone to diet induced insulin resistance (Corsetti et al.,2000; Zierath et al.,1997) and many genetically induced forms of insulin resistance have milder phenotypes in female compared with male mice (Corsetti et al.,2000; Li et al.,2000).

Genomic effects of estrogens through the nuclear estrogen receptors (ER) ERα and ERβ have been recognized for a long time in several tissues including the islets of Langerhans (Prossnitz et al.,2007).

Recently, however, several reports have described rapid effects by 17β-estradiol elicited through a non-classical membrane receptor, which has been shown to share the characteristics of a G protein-coupled receptor (GPCR). This receptor has been labeled GPR30 (Filardo et al.,2007; Martensson et al.,2009; Prossnitz et al.,2007).Very recently it was shown that deletion of GPR30 in female mice resulted in impaired glucose tolerance, reduced bone growth and increased vascular resistance (Martensson et al.,2009). Moreover, in that study we could show that glucose-stimulated insulin release was greatly impaired in GPR30$^{(-/-)}$ mice both *in vitro* and *in vivo* (Martensson et al.,2009). In addition, the estrogen-induced suppression of glucagon secretion was almost abolished in GPR30$^{(-/-)}$ mice (Martensson et al.,2009). Recent results from different studies suggest that 17β-estradiol protects insulin secretory processes in various diabetic states and that the prevalence of diabetes is known to be lower in females than in males (Wild et al.,2004). Hence, we found it of interest to study whether GPR30 was expressed in the different pancreatic endocrine cells and if GPR30 activation could influence insulin, glucagon and somatostatin secretion as well as islet cell survival. To this end we used a non-steroidal, high affinity, selective agonist of GPR30 referred to as G-1 (GPR30-specific compound 1; a substituted dihydroquinoline) (Prossnitz et al.,2008). For comparative purposes, we also studied the influence of 17β-estradiol.

2. Experimental procedures

2.1. Materials

Female and male mice of the NMRI strain (B&K, Sollentuna, Sweden) weighing 25-30g were used for the experiments. They were given a standard pellet diet (B&K) and tap water *ad libitum*. All animals were housed in metabolic cages with constant temperature (22ºC) and 12-h light/dark cycles. The local animal welfare committee, (Lund, Sweden) approved the experimental protocols and all procedures using animals.

Collagenase (CLS 4) was from Sigma St. Louis, MO, USA. Fatty acid free bovine serum albumin (BSA) was from Boehringer Mannheim, Germany. G-1 (1-[4-6-benzo [1, 3] dioxol-5-yl)-3a, 4, 5, 9b-tetrahydro-34-cyclopenta[c]quinoline-8-yl]-ethanone) and ICI 182,780 (Fulvestrant) were purchased from Cayman, CA, USA. EM-652 ((2S)-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, Canada. All other chemicals were from Merck AG, (Darmstadt, Germany) or Sigma (USA).

2.2. Isolation of pancreatic islets and islet hormone secretion measurements

Preparation of mouse pancreatic islets was performed by clamping the distal end of the pancreatic duct followed by a retrograde injection of ice-cold collagenase solution *via* the bile pancreatic duct. (Gotoh et al.,1985) Thereafter pancreas was dissected out and placed in tubes and incubated at 37°C for 17 min. The pancreatic islets were then separated from acinar tissue by vigorous shaking in ice-cold Hanks´ solution for 3 minutes. Next the isolated islets were handpicked under a stereomicroscope at room temperature. The isolated islets were then subjected to different experimental procedures (see below).

The freshly isolated islets were pre-incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mM HEPES, 0.1 % bovine serum albumin and 1.0 mM glucose. After pre-incubation the buffer was changed and the islets were incubated with different concentrations of test agents for 60 min at 37°C unless otherwise stated. Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. All incubations were performed in an incubation box at 30 cycles/min. Immediately after incubation an aliquot of the medium was removed and frozen for subsequent assay of the hormones by using commercial available radioimmunoassy kits following the manufacturer's recommendations for insulin (Millipore, USA), glucagon and somatostatin (Diagnostika, Falkenberg, Sweden) (Ahrén,1982; Heding,1966; Panagiotidis et al.,1992). The inter- and intra-assay coefficients of variation were; for insulin 4.2 and 3.2 %; glucagon 8.3 and 6.8 % and somatostatin 3.2 and 6.1 %. MIN64c cells (kindly donated by Professor J. Miyazaki, Osaka University Medical School) were grown in Dulbecco´s modified Eagle´s medium (DMEM) with Glutamax-1 supplemented with 15 % heat-inactivated FBS (Invitrogen, Paisley, UK), 60 μM β-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin. After acquiring ~95% confluent growth, the cells (~2 x 10^6) were harvested and used for qPCR and Western blot analysis.

2.3. cAMP measurement

For the measurement of cAMP, the female islets were first preincubated for 30 min at 1 mM glucose (see above) and then incubated for 30 min at 37°C with 12 mM glucose in the absence or presence of

different test agents, 17β-estradiol (100 nM or 5 µM), G-1 (100nM), ICI 182,780 (100nM) and EM-652 (100nM). Each incubation vial contained 40 islets in 1.5 ml of KRB-buffer. After the incubation the islets were treated with 100 mM HCl for 5 min and then frozen. At the day of analysis samples were sonciated on ice and cAMP was measured using a cAMP EIA kit (Cayman Chemical, USA) according to manufacturer's instructions. The protein level was analyzed by the Bradford method (Bradford,1976).

2.4. Detection of mRNA for GPR30 by quantitative real-time PCR

RNA from islets and MIN6c4 cells (~2 x 10^6) was isolated using TRIzol (Invitrogen) according to a modified RNA extraction protocol and transcribed into cDNA as described elsewhere (Amisten et al.,2008). qPCR was performed on a HT7900 system (Applied Biosystems) using QuantiTect primer assays and QuantiFast SYBR Green PCR (all from Qiagen) 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 DDCt method (Pfaffl,2001).

2.5. Western blot

Approximately 400 islets or MIN6c4 (~2 x 10^6) per vial (n=5 in each group) were suspended in 100 µl of SDS-buffer (50mM Tris-HCl, 1mM EDTA) containing a cocktail of protease inhibitors, frozen and sonicated on ice on the day of analysis (Jimenez-Feltstrom et al.,2004).The protein content of the homogenates was determined according to the Bradford method (Bradford,1976). Homogenate samples representing 20 µg of total protein were run on 7,5% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked in LS-buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk powder for 40 min at 37°C. Subsequently the membranes were incubated over night with a rabbit-raised polyclonal anti-GPR30 antibody (Acromed Invest, Sweden) against the C-terminal peptide: NH2-AVIPDSTEQSEVRFSSAV-COOH (1:150) at room temperature. The specificity of the antibody was evaluated in islets from GPR30 $^{(-/-)}$ mice (data not shown). After washing (three times) in LS-buffer the membrane was finally incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:500). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). For detection of β-actin as endogenous control a commercially available antibody was used (BioGenesis, UK)

2.6. Confocal microscopy

The freshly isolated female mouse islets were washed with PBS and fixed with 4% formaldehyde, 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 anti-glucagon (1:500) and rat anti-somatostatin (1:250) both from Jackson Immunoresearch Laboratories, followed by treatment with fluorescent-conjugated secondary antibodies (1:50-75) from Jackson Immunoresearch Laboratories. 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).

2.7. Apoptosis measurement

Islets isolated from female mice were dispersed into single cells using Ca^{2+} free-medium. The cells were incubated with and without a cocktail of cytokines *i.e.* IL-1β (100 ng/ml), TNFα (125 ng/ml), and INFγ (125 ng/ml). The effects of 17β-estradiol (100 nM or 5 μM) or G-1(100 nM) on the cytokine-induced apoptosis were studied in the absence or presence of ICI 182,780 (100 nM) or EM-652 (100 nM) after 24 h culture of the cells in RPMI1640 medium with 5 mM glucose and 10% FSB. The detection of apoptosis was performed on a FACS-system with the Annexin V-staining method (BDPharmigen, USA) according to manufacturer's instructions, and the flow cytometry data was analysed using the FlowJo and CellQuest software packages.

2.8. Statistics

All results are expressed as means ± SEM for the indicated number of observations or illustrated by an observation representative for a number of different experiments (Western blots, confocal microscopy). Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramers' multiple comparisons test. The EC50 values (Fig. 3) were calculated from a non-linear sigmoid curve fit using GraphPad Prism 4. A P- value of <0.05 was considered significant (*P < 0.05; ** < 0.01; ***P < 0.001).

3. Results

3.1. *mRNA and protein expression of GPR30 in female and male mice islets*

Control experiments showed no differences between female and male mice islets with regard to mRNA expression for ERα and ERβ (Figure 1A). Notably ERα expression was almost 10-fold higher than ERβ ($p<0.01$) in both female and male mice islets (Figure 1A). However, the expression of GPR30 mRNA relative to house keeping gene GAPDH was found to be higher in female [$(9.5 \pm 1.0) \times 10^{-3}$] compared to male mice islets [$(5.5 \pm 0.7 \times 10^{-3})$]; ($p<0.01$; n=10 in each group) (Figure 1A). The mRNA expression of GPR30 in the β-cell line MIN6c4 was comparatively much lower (n=10) ($p<0.001$) (Figure 1A).

Densitometric analysis of Western blots revealed that also on the protein level, GPR30 was more abundant in islet homogenates from female (0.20 ± 0.027 relative intensity of GPR30/β-actin) compared to male (0.13 ± 0.018 relative intensity GPR30/β-actin) mice ($p<0.05$). The quantified protein level of GPR30 in MIN6c4 cells (0.012 ± 0.016 relative intensity GPR30/β-actin) was markedly lower compared to islets from both female and male mice ($p<0.001$) (n=5 in each group) (Figure 1B - C).

3.2. GPR30 expression in different islet cell types

In order to investigate the cell-specific expression of GPR30, isolated islets from female mice were simultaneously immunolabelled for either insulin, glucagon or somatostatin together with GPR30 and analyzed by confocal microscopy. As seen in Figure 2A-I, GPR30 could be visualized in the different islet cell types and showed a strong co-localization with insulin (2A-C), (89 ± 3.2 % of the insulin positive cells, n=8), glucagon (2D-F), (82 ± 1.1 % of the glucagon positive cells, n=8) and somatostatin (2G-I) (87 ± 0.7 % of the somatostatin positive cells, n=8) could be observed in all investigated islets.

3.3. Effect of GPR30 activation on islet hormone release

Next experiment we compared the effects of G-1, a specific GPR30 agonist, (Bologa et al.,2006) and 17β-estradiol on islet hormone secretion at both low (1 mM) (Figure 3A, C and E) and high (12 mM) (Figure 3B, D and F) glucose concentrations (Figure 3A-F). Neither 17β-estradiol nor G-1 had any significant effect on insulin release from isolated female islets at basal glucose (1 mM) under static incubation conditions (1 h) (Figure 3A) whereas both agents dose-dependently potentiated insulin secretion in the presence of 12 mM glucose (Figure 3B). The potentiating effect of 17β-estradiol was almost one order of magnitude higher compared to the effects of G-1 on insulin secretion at 12 mM glucose. The calculated EC50 values for the modulatory action of 17β-estradiol and G-1 on insulin release were 1.88×10^{-8} (17β-estradiol) and 1.22×10^{-7} (G-1). In contrast, glucagon secretion at 1 mM glucose was dose-dependently inhibited by both 17β-estradiol and G-1 in a similar way (Figure 3C). The calculated EC50 values for the inhibitory action of 17β-estradiol (8.79×10^{-11}) and G-1 (9.58×10^{-11}) on glucagon release were of the same magnitude. The suppressive effect of high glucose (12 mM) on glucagon release, however, was not further modulated by G-1 (Figure 3D). Somatostatin release at basal glucose (1 mM) was not notably affected by 17β-estradiol or G-1 whereas somatostatin secretion at 12 mM glucose was dose-dependently inhibited by both 17β-estradiol and G-1 (Figure 3D and E). The calculated EC50 values for the suppressive effects of 17β-estradiol (1.93×10^{-9}) and G-1 (5.79×10^{-9}) on somatostatin secretion were of similar magnitude.

3.4. Effect of GPR30 activation on cAMP generation in relation to insulin release

To further study the rapid effect of GPR30 activation by 17β-estradiol or G-1 we incubated isolated islets for 30 min and measured islet content of cAMP as well as insulin release in the presence and absence of the nuclear estrogen receptor antagonists ICI 182,780 or EM-652. Figure 4 shows that, in the presence of 12 mM glucose, 17β-estradiol as well as G-1 strongly stimulated cAMP generation in parallel with insulin release both in the absence ($p<0.001$) and presence ($p<0.001$) of ICI 182,780 and EM-652. Regarding 17β-estradiol, a more pronounced effect was observed at 5 μM compared to a lower concentration of 17β-estradiol (100 nM) (Figure 4).

3.5. Effect of 17β-estradiol or G-1 on cytokine-induced apoptosis in islet cells in the absence and presence of ICI 182,780 or EM-652

Dispersed female islet cells were cultured for 24 h in 5 mM glucose with and without a mixture of cytokines (IL-1β, TNFα and INFγ) (Figure 5A). Addition of 17β-estradiol or G-1 to the culture medium almost abolished the cytokine-induced apoptosis (Figure 5B-C). The estrogen nuclear receptor antagonists ICI 182, 780 and EM-652 did not influence the cytokine-induced apoptosis (Figure 6A). The potent antagonist EM-652 did not influence the anti-apoptotic effects of either 17β-estradiol (100 nM or 5 μM) or G-1 (100 nM) (Figure 6B-C). Figure 7 illustrates quantitatively the sum of the anti-apoptotic effect of both 17β-estradiol and G-1, as shown in figure 5 and 6, from 8 different observations performed at 5 different occasions ($p<0.001$). As shown, the protective effect against apoptosis was clearly evident also in the presence of either ICI 182,780 or EM-652 (Figure 7).

4. Discussion

It has been described recently that the putative membrane-associated estrogen receptors either are intimately linked to or distinct from the classical nuclear receptors (Filardo et al.,2007; Filardo et al.,2002; Filippo Acconcia,2003; Govind and Thampan,2003). Although still controversial, current studies point at the involvement of a G protein-coupled receptor for non-genomic, rapid effects of estrogen in different tissues including excitable cells such as neurons and endocrine cells (Filardo et al.,2007; Martensson et al.,2009; Prossnitz et al.,2007; Prossnitz et al.,2008; Qiu et al.,2008)

The present study was focused on the expression and function of GPR30 in endocrine pancreas. While islet mRNA expression for ERα and ERβ did not differ between genders, ERα being markedly higher than ERβ in both female and male mice islets, we demonstrated here that GPR30 mRNA and protein expression was much higher in isolated islets from female than male mice and very low in the β-cell line MIN6c4. Importantly, MIN6c4 cells should be considered less suitable for GPR30 screening and functional studies. Further, we found that GPR30 protein expression, as visualized by confocal microscopy, was abundant in insulin, glucagon and somatostatin cells comprising 82-89 % of the different cell types. Moreover, functional studies on isolated islets in short-time incubations showed that the GPR30 specific ligand G-1 stimulated insulin release and inhibited glucagon and

somatostatin release in a manner almost identical to 17β-estradiol. Incubation of isolated islets at 12 mM glucose in the presence of either G-1 or 17β-estradiol induced an increase of cAMP content in parallel with an amplified insulin release. These effects were neither influenced by ICI 182,780 a blocker of the classical estrogen receptors ERα and ERβ nor by EM-652 which has a most potent inhibitory activity on both ERα and ERβ compared to other anti-estrogens tested. (Labrie et al.,1999).

In order to asses a potential independent role of GPR30; we have used the new antagonist EM-652 which has no agonistic effect on GPR30. This is in contrast to ICI 182,780 which in previous studies has been shown to be a nuclear receptor blocker as well as having partial agonistic properties on GPR30 in other tissues (Rae and Johnson,2005; Smith et al.,2009). Apparently ICI 182,780 might be tissue specific with regard to its agonistic activity, since we could not find any agonistic effect on GPR30 in female mice islets.

Altogether, the present data speak in favour of GPR30 being an important receptor for rapid estrogen effects. In the present work we also demonstrate for the first time that the long-term anti-apoptotic effects of 17β-estradiol can be mediated via activation of GPR30 in the presence of the ERα and ERβ antagonists ICI 182,780 and EM-652 after culturing islets for 24 hours with a mixture of cytokines.

It has previously been shown that 17β-estradiol exerts a rapid insulinotropic effect through activating of a plasma membrane receptor (Nadal et al.,1998). The authors showed that 17β-estradiol, in synergy with glucose, depolarized the β-cell membrane and elicited electrical activity and $[Ca^{2+}]_i$ signals to promote insulin release. By specific competitive binding studies they concluded that a novel 17β-estradiol receptor was localized at the plasma membrane and was responsible for K_{ATP} channel regulation and Ca^{2+} signals. Later on the same group reported that also the glucagon producing α-cells were provided with non-classical estrogen receptors at the plasma membrane and that activation of these receptors prevented the low-glucose-induced $[Ca^{2+}]_i$ oscillations in these cells (Ropero et al.,2002). Furthermore, competition studies with 17β-estradiol-linked horseradish peroxidase suggested that this estrogen receptor at the plasma membrane was neither ERα nor ERβ (Ropero et al.,2002). The results of the present investigation are in accordance with these earlier data and strongly suggest that this estrogen receptor is identical to the G protein-coupled receptor 30 (GPR30), recently described in other tissues (Prossnitz et al.,2007) and we very recently found that deletion of GPR30 ($GPR30^{-/-}$) in female mice resulted in a marked impairment of glucose stimulated insulin release both *in vivo* and from *in vitro* isolated islets (Martensson et al.,2009). Moreover, estrogen-stimulated insulin release from islets of $GPR30^{(-/-)}$ mice was abolished although serum estrogen levels were apparently normal in these mice (Martensson et al.,2009). We could now show that GPR30 is expressed not only in the insulin-producing β-cells and the glucagon-producing α-cells but also in the somatostatin-producing δ-cells. The functional studies presented here using the newly developed GPR30-specific agonist G-1 lend further support to GPR30 being an important plasma membrane-

associated estrogen receptor in that short-time incubation of isolated islets in the presence of a wide range of G-1 concentrations showed a dose-response pattern of hormone release similar to 17β-estradiol i.e. a gradual increase of insulin secretion and a gradual suppression of glucagon and somatostatin secretion. This modulatory effect of GPR30 on insulin and glucagon secretion is apparently dependent on ambient glucose levels. As seen in Figure 3 there is no effect on insulin secretion at low glucose, while glucagon secretion is suppressed. At 12 mM glucose, however, GPR30 stimulation amplifies insulin release and suppresses somatostatin release thus manifesting an anti-diabetic action. The potentiating effect of 17β-estradiol was almost one order of magnitude higher compared to G-1 on glucose-stimulated insulin secretion while no difference was found with regard to the effect on glucagon and somatostatin secretion. Although not directly tested in the present study it seems not inconceivable that 17β-estradiol, has a greater efficacy at GPR30 than the synthetic compound, G-1 and/or 17β-estradiol might exert another short-time action independent of GPR30 (Kelly and Ronnekleiv,2009). Moreover, although ligand receptor-binding studies have suggested that G-1 compared to 17β-estradiol has stronger affinity for GPR30 (Prossnitz et al.,2008; Prossnitz and Maggiolini,2009; Prossnitz and Maggiolini,2009; Prossnitz et al.,2008) our data show that 17β-estradiol is more potent than G-1 to stimulate insulin release. This is in good agreement with previous dose-response studies showing that affinity and efficacy are separate quantities *i.e.* pharmacological response to a drug with a lower receptor-affinity could comparatively be of the same magnitude or even higher (Colquhoun,1998; Williams and Sewing,2005). Furthermore, a certain effect with regard to glucagon secretion cannot be excluded, since 17β-estradiol action on the glucagon cells is obviously exerted at lower concentrations compared to the 17β-estradiol effect on insulin release. This might be due to a higher receptor density on the glucagon cells.

The lack of amplifying effects by 17β-estradiol or G-1 on insulin release at low glucose concentration could be explained by the fact that low glucose levels are not capable of elevating/generating intracellular signals such as Ca^{2+} and cAMP that are important for insulin and somatostatin secretion. Regarding the absence of suppressive effects of 17β-estradiol or G-1 on glucagon release at high glucose level, it should be mentioned that an intermediate high glucose concentration (12 mM), as previously shown, is a potent suppressor of glucagon release (Vieira et al.,2007). The suppressive effects of 17β-estradiol or G-1 on glucagon secretion could, however, be masked by the presence of a high glucose concentration in the incubation media (Figure 3D). Hence, these dose-dependent effects of G-1, a GPR30 specific ligand, which mimics the 17β-estradiol effect on pancreatic hormone release further support GPR30 being an important plasma membrane-associated estrogen receptor. These cumulative data highlight a positive anti-diabetic role of GPR30 agonists such as G-1 on islet hormone secretion.

There has been extensive work done in other tissues which showed that 17β-estradiol increases cAMP levels as a result of its ability to stimulate adenylate cyclase (Aronica et al.,1994; Nakhla et al.,1990). These findings together with the observation that 17β-estradiol stimulates Ca^{2+} (Morley et

al.,1992), inositol trisphosphate (Le Mellay et al.,1997) and heterotrimeric G proteins (Le Mellay et al.,1999) led these investigators to speculate that GPCRs might be implicated in rapid estrogen signalling. Moreover, estrogen-stimulated insulin release from islets of GPR30$^{(-/-)}$ mice was abolished although serum estrogen levels were apparently normal in GPR30$^{(-/-)}$ mice (Martensson et al.,2009). However, the intracellular signalling following G-1 activation of GPR30 in the different islet cells is far from elucidated. As previously mentioned, 17β-estradiol triggers opposite $[Ca^{2+}]_i$ responses in β- and α-cells (Ropero et al.,2008) which would lead to increased insulin and decreased glucagon secretion. In the present study we found that, in the presence of glucose, G-1 as well as 17β-estradiol stimulated the generation of cAMP content in parallel with increased insulin release. These data further support that cAMP plays an important role in Ca^{2+}-induced exocytosis of insulin (Eliasson et al.,2003) and that the adenylate cyclase-cAMP system is stimulated through activation of GPR30 in other tissues (Prossnitz et al.,2007). Neither cAMP generation nor insulin release was affected by the presence of ICI 182,780 and EM-652, showing that the classical estrogen receptors ERα and ERβ were not involved.

Finally we found for the first time that also long-term effects by G-1 and 17β-estradiol could be exerted through GPR30 activation in that cytokine-induced apoptosis in islets cultured for 24 h was almost abolished by these estrogen compounds, with and without the presence of ICI 182,780 or EM-652. A functional failure of insulin producing pancreatic β-cells is a common characteristic of type 1 (insulin-dependent) and type 2 (non insulin-dependent) diabetes mellitus. Accumulating evidence suggests that programmed cell death (apoptosis) is a main form of β-cell death in these disorders (Mandrup-Poulsen,2003). It has been reported in previous studies that 17β-estradiol prevents β-cell apoptosis in the absence of ERα (Le May et al.,2006). One possibility to explain this finding is that 17β-estradiol may activate a non-genomic pathway *via* membrane GPR30 receptors unrelated to ERs and thus prevent β-cell apoptosis. Therefore we tested whether 17β-estradiol and G-1, a selective agonist of GPR30, could protect pancreatic islets from cytokine-induced apoptosis when the nuclear ERα and ERβ were not functional. The results were quite striking in that G-1 or 17β-estradiol markedly reduced the level of apoptosis induced by a mixture of IL-1β, TNFα and INFγ in the presence of the nuclear ER receptor antagonists ICI 182,780 and EM-652. Our data thus clearly indicate that GPR30 plays a main role in mediating the beneficial effects of estrogen in preventing the cytokine induced pancreatic β-cell apoptosis.

In conclusion, our present findings suggest that drugs targeting GPR30 specifically might be a new therapeutic option in the management of type 2 diabetes, not only by potentiating insulin secretion and inhibiting glucagon and somatostatin secretion but also by suppressing inflammatory injuries induced by cytokines on pancreatic islets. Indeed successful treatment of type 2 diabetes has been achieved by using IL-1β antagonists (Maedler and Donath,2004) suggesting an inflammatory component in the pathogenesis of type 2 diabetes.

1

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Foot notes

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Abbreviation list

ER, Nuclear estrogen receptor; G-1, GPR30 specific ligand**,** (1-[4-6-benzo [1, 3] dioxol-5-yl)-3a, 4, 5, 9b-tetrahydro-34-cyclopenta[c]quinoline-8-yl]-ethanone); ICI 182,780 Fulvestrant, antagonist of ERα and ERβ, 7-alpha-[9-(4,4,5,5,5-pentafluoropentylsulphinyl)nonyl]estra-1,3,5-(10)-triene-3,17-beta-diol. EM-652, antagonist ERα and ERβ, ((2S)-3-(4-hydroxyphenyl)-4-methyl-2-[4-(2-piperidin-1-ylethoxy)phenyl]-2H-chromen-7-ol)

Figure legends

Figure 1. A) Relative expression of mRNA for 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 mice as well as in MIN6c4-cells. The expression level of GPR30 is higher in the isolated islets from female mice. In MIN6c4 cells the GPR30 expression is almost negligible. B) A representative Western blot of the GPR30 expression in female and male mouse islets as well as in MIN6c4 cells with an antibody against GPR30. The female islets display a much stronger band than the male islets. C) The mean intensity (means ± SEM) of the Western blots (n=5 in each group) is presented as relative value of the band intensity towards the endogenous control β-Actin. *$p<0.05$, ***$p<0.001$.

Figure 2. Confocal microscopy of female mouse islets double immunolabelled for insulin, glucagon and somatostatin (green fluorescence) (A,D,G) and for GPR30, (red fluorescence) (B,E,H). Co-localization of GPR30 and the different hormones is seen as orange-yellowish fluorescence(C,F,I). Bar indicates length (20 µm).

Figure 3. Dose-response studies of hormone secretion from female mouse islets modulated by G-1 and 17β-estradiol at 1 mM glucose (A,C,E) and at 12 mM glucose (B,D,F) with the same range of concentrations for both compounds as shown on the x-axis. G-1 mimics the effects of 17β-estradiol.

Both compounds amplified glucose-stimulated insulin release while glucagon and somatostatin secretion was reduced. The values are means ± SEM for 8 observations in each group performed at 3 different occasions.

Figure 4. Effects of G-1 and 17β-estradiol (E2) on islet cAMP content and insulin secretion at 12 mM glucose. The female islets were incubated with G-1 (100 nM) or 17β-estradiol (100 nM or 5μM) in the absence and presence of ICI 182,780 (100 nM) or EM-652 (100 nM). Means ± SEM for 8 observations in each group are shown. ***p <0.001

Figure 5. A-C, Representative examples of the suppressive effect of 17β-estradiol (E2) and G-1 on cytokine-induced apoptosis (IL-1β; 100 ng/ml + TNFα; 125 ng/ml + INFγ; 125 ng/ml) from dispersed female islet cells cultured for 24h, in the presence of test agents and then stained with Annexin V. A) Shows an example of control tubes with and without cytokines. B-C, Shows representative examples of 17β-estradiol (5μM) and G-1 (100nM) effects on cytokine-induced apoptosis.

Figure 6. A-C, representative examples of the suppressive effect of 17β-estradiol and G-1 on cytokine-induced apoptosis (IL-1β (100 ng/ml), TNFα (125 ng/ml), and INFγ (125 ng/ml)) from dispersed female pancreatic islets cells cultured for 24h, in the presence of test agents and then stained with Annexin V. A) Shows an example of cytokine-cultured tubes in the presence or absence of the ERα and ERβ inhibitors ICI 182,780 (100 nM) or EM-652 (100 nM). B-C) Shows representative examples of the effects of17β-estradiol (E2) (100 nM or 5μM) or G-1 (100nM) together with EM-652 (100 nM) on cytokine-induced apoptosis..

Figure 7. This graph shows the collected results from n=8 observations on cytokine-induced apoptosis in female islet cells performed at 5 different occasions. The data reveal that the levels of apoptotic cells in cytokine-induced apoptosis (IL-1β; 100 ng/ml + TNFα; 125 ng/ml + INFγ; 125 ng/ml) are strongly reduced when 17β-estradiol (E2) (100 nM or 5μM) or G-1 (100nM) is added in presence and absence of either ICI 182,780 (100 nM) or EM-652 (100 nM) ***p <0.001.

Acknowledgements

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Figure 1

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Fig. 1

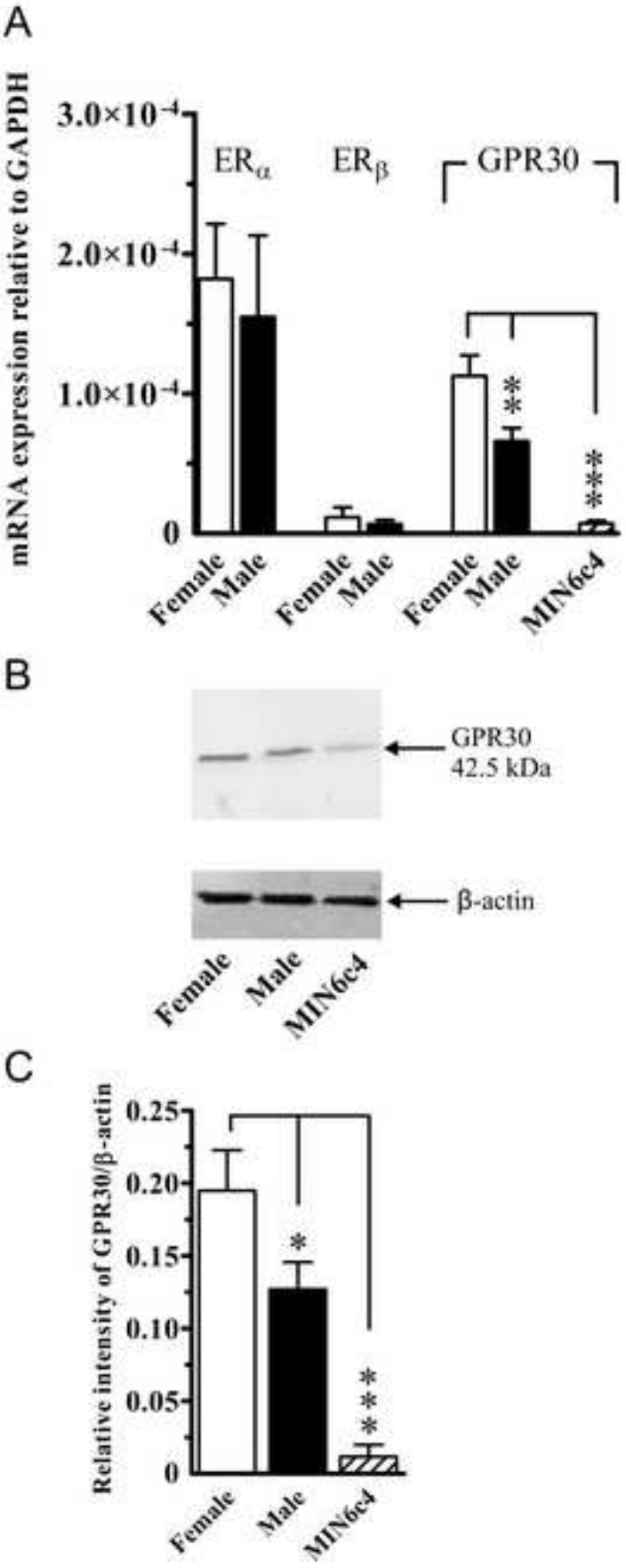

Figure 2

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Fig. 2

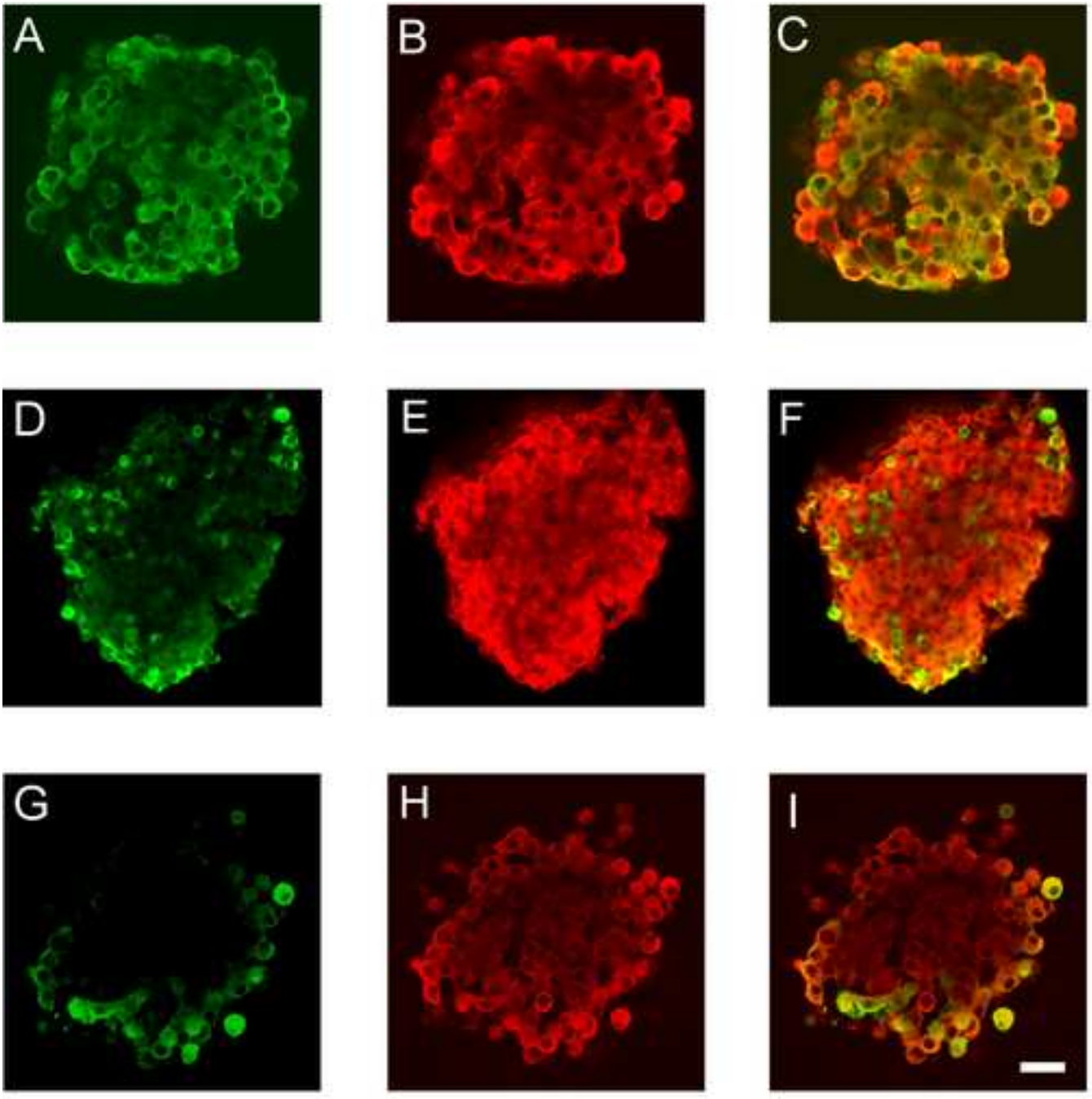

Figure 3

Fig. 3

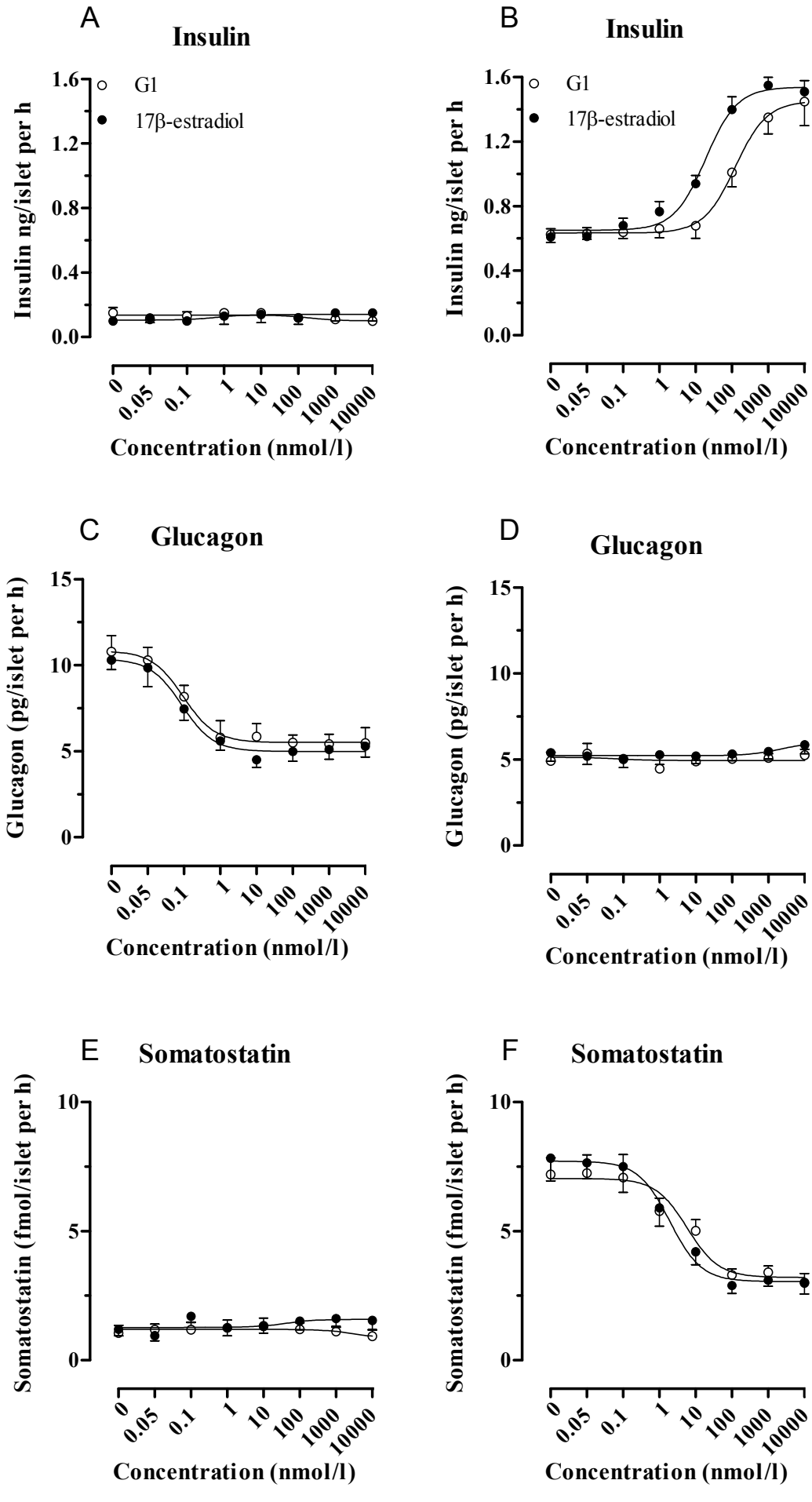

Figure 4

Fig. 4

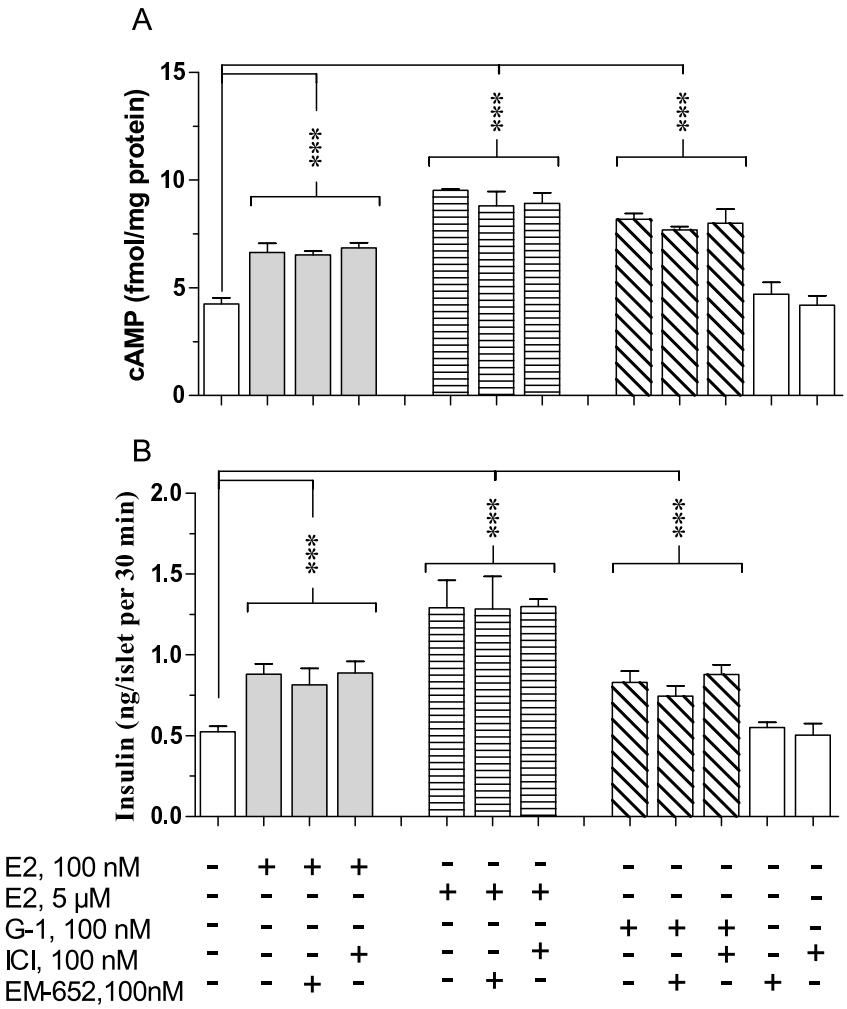

Figure 5

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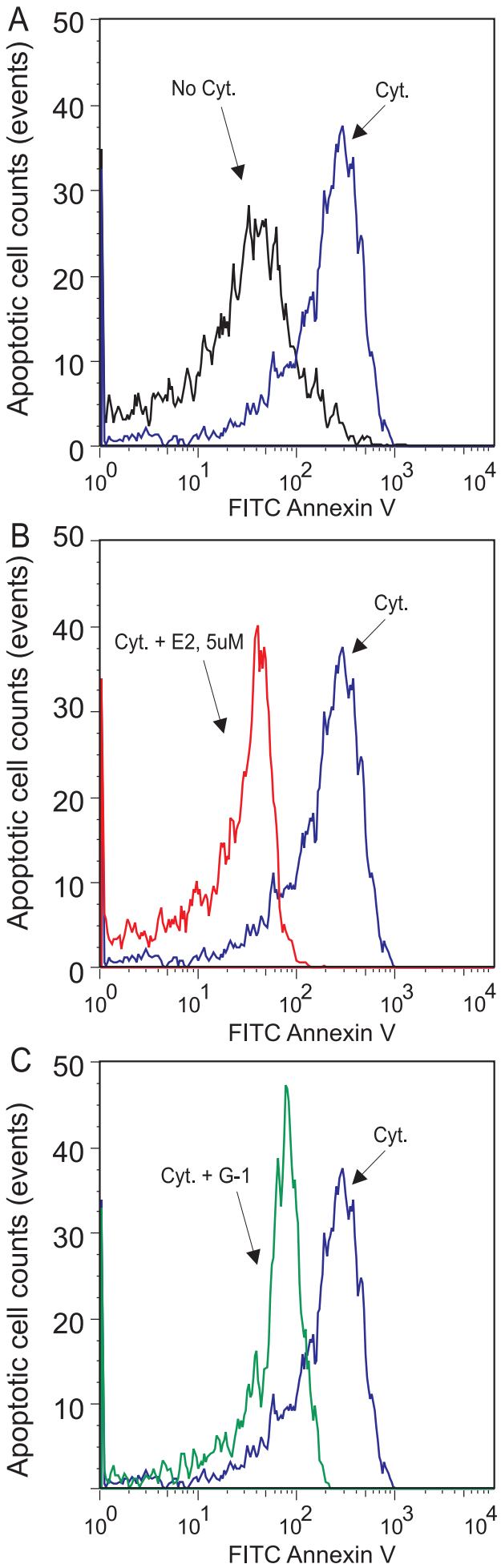
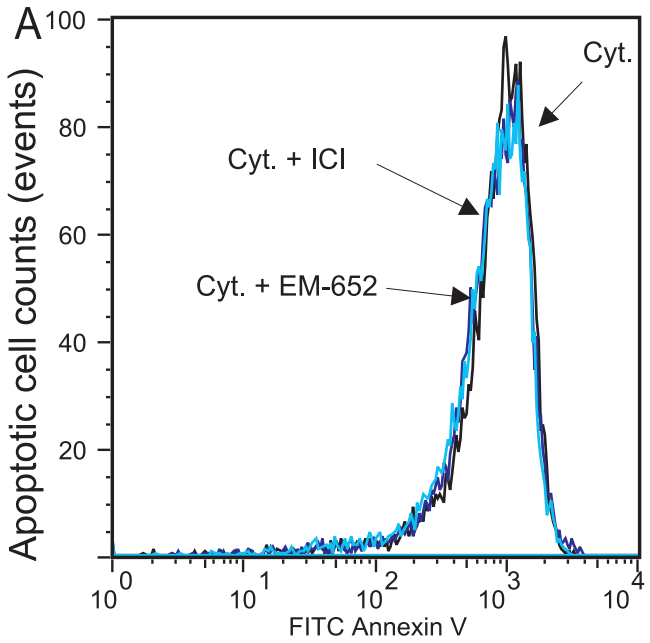
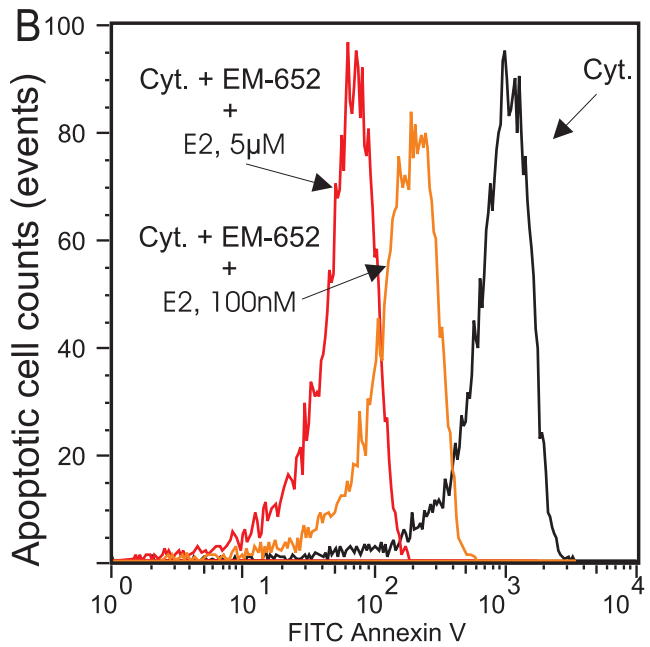


Figure 6

Fig. 6





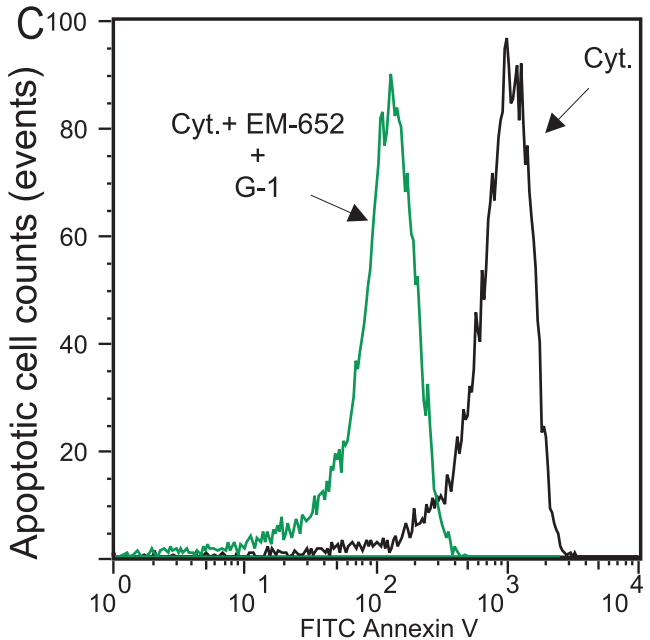


Figure 7

Fig. 7