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Original Article

Estrogen and G protein-coupled estrogen receptor agonist G-1 cause relaxation of human gallbladder

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ABSTRACT

Objective: Estrogen interacts with a membrane receptor, G protein-coupled estrogen receptor (GPER). It was reported that 17β-estradiol was able to inhibit contraction of the human colon and cause relaxation of the guinea pig gallbladder, however, the involvement of GPER was not clarified. The aim of the present study was to investigate the effect of estrogen on human gallbladder motility and the possible role of GPER.

Materials and Methods: Relaxation of human gallbladder strips were measured using isometric transducers. Expression of GPER was evaluated by reverse transcription polymerase chain reaction (PCR), real-time PCR, and immunohistochemistry.

Results: In human gallbladder strips, 17β-estradiol and G-1 elicited marked and rapid relaxation, whereas tamoxifen produced mild concentration-dependent relaxation. The relative efficacies to cause relaxation were as follows: 17β-estradiol = G-1 > tamoxifen. The relaxant response of 17β-estradiol was not attenuated by tetrodotoxin or conotoxin GVIA. This implies that nerve stimulation was not involved in the 17β-estradiol-induced gallbladder relaxation. Analysis by reverse transcription PCR and real-time PCR showed that GPER was expressed in the human gallbladder. Further analysis by immunohistochemistry revealed that GPER was expressed in the gallbladder muscle. This suggests that 17β-estradiol relaxes the human gallbladder via GPER.

Conclusion: These results demonstrate for the first time that 17β-estradiol and GPER agonist G-1 cause relaxation of the human gallbladder, probably through GPER. Estrogen might play an important role in the control of human gallbladder motility.

1. Introduction

Estrogen is an important sex hormone. The action of estrogen is mediated classically by two nuclear estrogen receptors, ERα and ERβ, which function as ligand-activated transcription factors and regulate gene expression. Recently, estrogen was found to be a ligand for a membrane receptor, G protein-coupled estrogen receptor (GPER), formerly known as GPR30 [1–4]. GPER mediates estrogen-dependent rapid signaling and transcriptional responses. Expression of GPER has been detected in cardiovascular, gastrointestinal, reproductive, and nervous tissues [1–4]. Interestingly, tamoxifen, which functions as a nuclear estrogen receptor antagonist and inhibits the activities of classical estrogen nuclear receptors, acts as a GPER agonist [1,5]. GPER may play an important role in the physiology of the reproductive, nervous, endocrine, immune, and cardiovascular systems as well as in the pathophysiology of cancer [1–4]. In the cardiovascular system, estrogen causes relaxation of vascular smooth muscle through GPER [6–9].

Conflicts of interest: none.

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GPER has been proposed as a novel therapeutic target in cardiovascular diseases [2,3,8,9]. In the gastrointestinal system, estrogen has been found to inhibit contraction of the human colon and guinea pig gallbladder as well as rat gastric emptying [10–12]. Females experience gallstones and constipation more frequently than males [13,14]. Impaired gallbladder motility may contribute to gallstone formation [15]. To date, GPER-mediated effects of estrogen in the gastrointestinal system are not clear [16]. Conversely, estrogen inhibits contraction of the human colon through unclear mechanisms. The mechanisms of estrogen action in the guinea pig gallbladder also remain unclear [17]. Little information is available on the effects of estrogen on the human gallbladder. The aim of the present study was to investigate the GPER-mediated effects of estrogen on human gallbladder motility.

2. Materials and methods

2.1. Materials

Compounds such as 17β-estradiol, tamoxifen, dimethyl sulfoxide (DMSO), and TRIzol reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). G-1 {1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone}, G-15 [(3aS*,4R*,9bR*)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta(c)quinolone], and tetrodotoxin were purchased from Tocris Cookson (Avonmouth Bristol, UK). ω-Conotoxin GVIA was obtained from Bachem (Bubendorf, Switzerland). Anti-human GPER rabbit polyclonal antibody (PAB22000, lot #A61748) was obtained from Abnova (Taipei City, Taiwan). The polymeric horseradish peroxidase–linker antibody conjugate system kit (Bond Polymer Refine Detection, DS9800) was obtained from Leica Biosystems (Nussloch, Germany). Reverse transcription polymerase chain reaction (RT-PCR) primers for human GPER and β-actin were purchased from Integrated DNA Technologies (Coralville, IA, USA). Klenow fragment was obtained from Level Biotechnology (New Taipei City, Taiwan). HincII was purchased from New England Biolabs (Hitchin, UK). The RNeasy Mini Kit (RNA purification kit) was purchased from Qiagen (Hilden, Germany). The RapidOut DNA Removal Kit, cDNA reverse transcriptase (RevertAid H Minus Reverse Transcriptase and High-Capacity cDNA Reverse Transcription Kit), and real-time PCR TaqMan primer/probe sets were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Methods

The protocol for this work was approved by the Research Ethics Committee of Buddhist Tzu Chi General Hospital, Hualien, Taiwan (IRB100-93, November 1, 2011). The study was performed in accordance with institutional ethical standards and the Helsinki Declaration. Human gallbladder tissues were obtained from 37 patients (22 men and 15 women, median age 59 years, range 28–80 years) undergoing surgery for gallstones (14 patients) or hepatocellular carcinoma (23 patients). Informed consent was obtained from the participants. Immediately after surgical removal of the gallbladder, a 3 cm × 5 cm section of tissue was excised from the middle portion of each gallbladder corpus and placed in oxygenated standard incubation solution for transportation to the laboratory, where the contraction and relaxation experiments were promptly initiated or tissues were frozen for other experiments.

2.2.1. Measurement of contraction and relaxation of isolated human gallbladder strips

Measurements of contraction and relaxation of muscle strips from the human gallbladder were performed according to procedures described previously [18,19]. In brief, human gallbladder muscle strips (1.0 cm × 0.3 cm) were suspended in organ baths and incubated at 37°C in standard incubation solution (118mM NaCl, 25mM $NaHCO_3$, 4.7mM KCl, 14mM glucose, 1.2mM NaH_2PO_4, 1.8mM $CaCl_2$, pH 7.4) gassed with 95% O_2–5% CO_2. The strips were connected to isometric transducers (FT.03; Grass Technologies, West Warwick, RI, USA), which were connected to amplifiers and a computer recording system (BIOPAC Systems, Goleta, CA, USA). Agents related to 17β-estradiol were added in a noncumulative fashion, i.e., with single dose administration. For measurements of relaxation in carbachol-precontracted strips, estrogen-related agents were added to muscle strips 15 minutes after the addition of carbachol. The relaxation responses were represented as a percentage (% papaverine) of the relaxation to 100μM papaverine. For studies using the receptor antagonist (G-15) and toxins (tetrodotoxin and ω-conotoxin GVIA), the muscle strips were exposed to the indicated concentration of these agents for 6 minutes and 15 minutes, respectively, and then to 17β-estradiol. Only one single-dose response, with or without a toxin or receptor antagonist, was studied with each preparation. 17β-estradiol, G-1, tamoxifen, and G-15 were dissolved in 100% DMSO and then serially diluted in DMSO–water mixtures. The final concentrations of DMSO were ≤1% in the gallbladder relaxation studies.

2.2.2. T-PCR for detection of mRNA of GPER in the human gallbladder

RT-PCR for the detection of the mRNA of GPER was performed as described previously with minor modifications [18,19]. Total RNA was isolated from the human gallbladder muscle using the TRIzol reagent, treated with RNase-free DNase I to remove genomic DNA contamination and reverse transcribed into cDNA using the RevertAid H Minus Reverse Transcriptase. The PCR amplification for GPER was performed in the GeneAmp PCR System 9700 (Applied Biosystems/Life Technologies, Grand Island, NY, USA) with Taq polymerase for one cycle at 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and, finally at 72°C for 5 minutes. After amplification, the PCR products were subjected to electrophoresis on a 1.5% agarose gel, which was stained with ethidium bromide and analyzed under UV light. The sequences of primers for human GPER were as follows: sense 5′-CTGCACGAGCGGTACTACGA-3′ and antisense 5′-CAGATGAGGCCACAGCTCAG-3′ (PCR product size 191 base pairs) [20]. β-Actin was used as an internal control. The sequences of primers for human β-actin were as follows: sense 5′-CACTCTTCCAGCCTTCCTTC-3′and antisense 5′-CTCGTCA-TACTCCTGCTTGC-3′ (PCR product size 314 base pairs) [21]. The PCR amplification for β-actin was performed with Taq polymerase for one cycle at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 62°C for 1 minute, 72°C for 1 minute, and finally 72°C for 5 minutes. For sequencing, the GPER PCR products were blunt ended by treating with Klenow fragment and ligated into the HincII cut pOk12 vectors. The ligation mixture was used to transform *Escherichia coli* strain Top10 to produce permanent clones for sequencing at Genomics BioSci & Tech (New Taipei City, Taiwan).

2.2.3. Real-time PCR for detection of mRNA of GPER in the human gallbladder

Total RNA was isolated from human gallbladder muscle using an RNA purification kit (RNeasy Mini Kit; Qiagen), treated with a recombinant DNase I (RapidOut DNA Removal Kit: Life Technologies), and reverse transcribed into cDNA with a recombinant Moloney murine leukemia virus reverse transcriptase (High-Capacity cDNA Reverse Transcriptase Kit; Life Technologies). The real-time PCR for detection of GPER mRNA was performed using the TaqMan Gene Expression Assay (Life Technologies) following the procedure described previously with minor modifications [22]. Real-time PCR

was performed using an ABI 7500 detection system (Applied Biosystems/Life Technologies) and the specific primer/probe set for GPER (Hs01922715_s1). Two housekeeping genes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Hs02758991_g1) and β-actin (Hs01060665_g1), were used as endogenous controls to standardize the amount of cDNA. The thermal cycling conditions were one cycle of 2 minutes at 50°C and one cycle of 10 minutes at 95°C, followed by 40 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C. Cycle threshold values were obtained, and quantification was carried out by the relative expression method using the geometric mean of two reference genes, GAPDH and β-actin. Control reactions with no reverse transcriptase added were performed in each experiment to determine that amplification was derived from the cDNA and not from genomic DNA contamination.

2.2.4. Immunohistochemistry for detection of GPER in the human gallbladder

To localize GPER in the human gallbladder, immunohistochemistry was performed as previously described with minor modifications [23]. Immunohistochemical staining was performed using an automated staining system (BOND-MAX; Leica Microsystems, Nussloch, Germany). Briefly, paraffinized human gallbladder tissue sections of 4 μm were deparaffinized, hydrated, and subjected to heat-induced antigen retrieval with citrate buffer, pH 6.0. The staining procedure involved peroxidase blocking with 3% hydrogen peroxide for 5 minutes, and sequential applications of the primary GPER antibody (1 μg/mL) for 30 minutes and an antirabbit horseradish peroxidase polymer for 8 minutes at room temperature. Subsequently, the tissue sections were treated with a chromogen, 3,3′-diaminobenzidine for 10 minutes and counterstained with hematoxylin for 5 minutes at room temperature. Normal rabbit immunoglobulin G (1 μg/mL) was used as a negative control.

2.2.5. Analysis of data

Results are expressed as mean values ± standard error of the mean. Statistical evaluation was performed using unpaired Student *t* test for two samples or one-way analysis of variance using Dunnett's procedure for more than two samples. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of estrogen-related agents on human gallbladder strips

To test the ability of estrogen-related agents to cause human gallbladder relaxation, muscle strips of human gallbladder were prepared and responses to 17β-estradiol, G-1, and tamoxifen were studied. Addition of 17β-estradiol and G-1 to the gallbladder strips precontracted with carbachol (1μM) caused marked, rapid, and concentration-dependent relaxation (Figs. 1 and 2). At 1μM, 17β-estradiol caused detectable relaxation of the carbachol-precontracted gallbladder strips. The highest concentration of 17β-estradiol tested, 100μM, produced 52 ± 8% ($n = 7$) relaxation of papaverine (100μM)-induced relaxation. In contrast, the vehicle DMSO caused only 23 ± 5% ($n = 4$) of papaverine-induced relaxation (Fig. 2). Similarly, G-1 caused detectable relaxation of the carbachol-precontracted human gallbladder strips at 1μM. The highest concentration of G-1 tested, 100μM, produced 48 ± 6% ($n = 4$) relaxation of papaverine-induced relaxation. Tamoxifen was less effective than 17β-estradiol and G-1, and caused detectable relaxation of the carbachol-precontracted human gallbladder strips at 10μM. At 30μM, tamoxifen produced 23 ± 4% ($n = 4$) relaxation of the human gallbladder (Fig. 2). The relative efficacies for estrogen-related agents to cause relaxation of carbachol-precontracted gallbladder were as follows: 17β-estradiol = G-1 > tamoxifen. The 17β-estradiol-induced relaxation was not altered by tetrodotoxin or ω-conotoxin GVIA. In the presence of 1μM tetrodotoxin and ω-conotoxin GVIA, 17β-estradiol (30μM) produced 33 ± 6% ($n = 5$) and 31 ± 2% ($n = 4$) papaverine-induced relaxation of carbachol-precontracted human gallbladder, respectively ($p = 0.38$ and $p = 0.26$, respectively, compared with 17β-estradiol alone, 36 ± 2%, $n = 11$). At 3μM, G-15, a GPER receptor antagonist [1], could inhibit human gallbladder relaxation caused by 17β-estradiol (30μM), but it was not of statistical significance (data not shown).

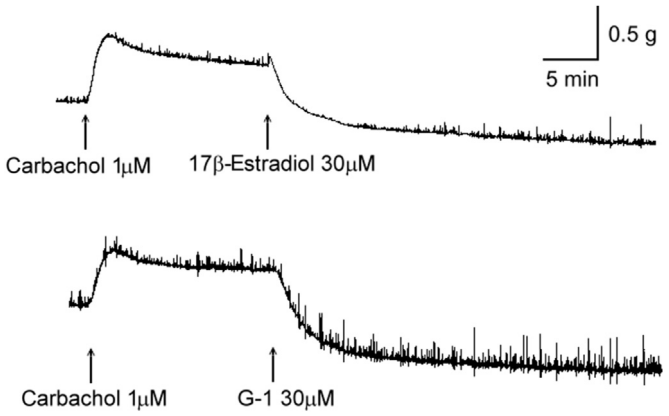

Fig. 1. Typical tracings showing the relaxation of carbachol-precontracted human gallbladder strips caused by 17β-estradiol (upper panel) and G-1 (lower panel).

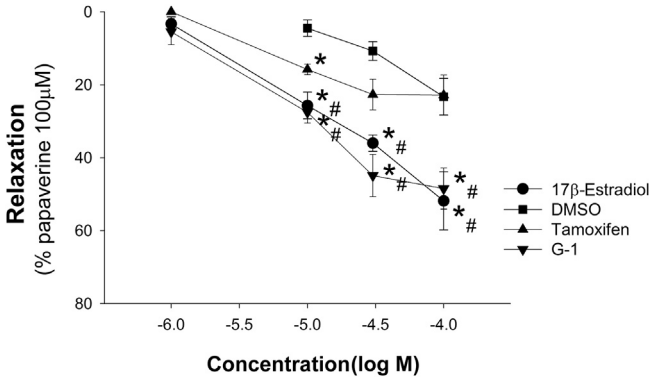

Fig. 2. The ability of 17β-estradiol, G-1, and tamoxifen to cause relaxation of human gallbladder strips precontracted with carbachol (1μM). Values are expressed as percent of papaverine (100μM)-induced relaxation. The results given are from at least four experiments. Vertical bars represent ± standard error of the mean. * Significant difference from DMSO vehicle (ANOVA; $p < 0.05$). # Significant difference from tamoxifen (ANOVA; $p < 0.05$). ANOVA = analysis of variance; DMSO = dimethyl sulfoxide.

3.2. RT-PCR analysis of GPER expression in the human gallbladder

RT-PCR was used to examine mRNA expression of GPER in the human gallbladder. As shown in Fig. 3, amplification of human gallbladder cDNA yielded the predicted 191 base-pair product for GPER ($n = 3$) [20]. In addition, sequencing of the GPER PCR products and blasting against the National Center for Biotechnology Information nucleotide database revealed that the following PCR products represented a partial sequence of human GPER.

Nucleotide sequence of the GPER PCR products:

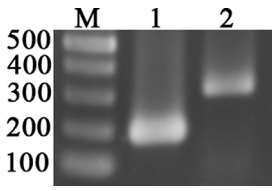

Fig. 3. Reverse transcription polymerase chain reaction analysis of expression of GPER mRNA in the human gallbladder. Total RNA was reverse transcribed and amplified using GPER and β-actin specific primers. The amplified products were electrophoresed on agarose gel, stained with ethidium bromide, and analyzed under UV light. The results presented are representative of three experiments. Lane M represents molecular weight markers; Lane 1, GPER; and Lane 2 β-actin. GPER = G protein-coupled estrogen receptor.

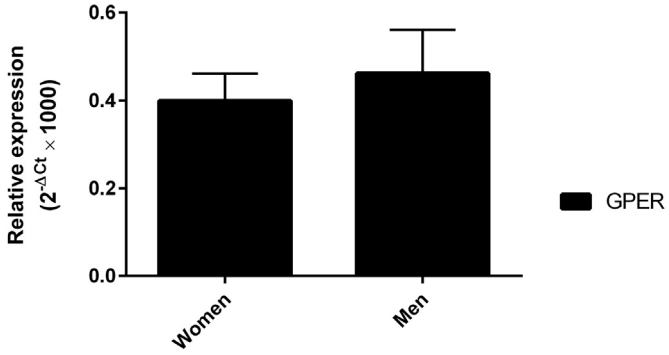

Fig. 4. Real-time PCR analysis of GPER mRNA expression in the human gallbladder. The relative GPER expression levels in human gallbladders from women and men were calculated using the comparative Ct method and normalized against two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase and β-actin. Vertical bars represent ± standard error of the mean (n = 3/group). Ct = cycle threshold; GPER = G protein-coupled estrogen receptor; PCR = polymerase chain reaction.

5′…**GATGAGGCCACAGCTCAG**CCGGGCGTGGTGCTTGGTGCGGA-ACAGGCTGCAGCGCATGGCCCTGGCCAGGGCGATGTAGCGGTCGAA-GCTCATCCAGGTGAGGAAGAAGACGCTGCTGTACATGTTGACCTGC-AGGAAGAGCGACATGAAGGTGCACAGGACGGCGATG **TCGTAGTAC-CGCTCGTGCA**…3′ (Sections in bold indicate sequences of primers, partially excised.)

3.3. Real-time PCR analysis of GPER expression in the human gallbladder

The relative mRNA expressions of GPER in the human gallbladder are shown in Fig. 4. Real-time PCR analysis showed that the relative expression levels of GPER were 0.40 ± 0.08 × 10^{-3} and 0.46 ± 0.12 × 10^{-3} for human gallbladders from women (n = 3) and men (n = 3), respectively.

3.4. Immunohistochemical analysis of GPER expression in the human gallbladder

In the immunohistochemical study, GPER was detected in the smooth muscle of the human gallbladder (Fig. 5A). In contrast, incubation of the human gallbladder with nonimmune rabbit immunoglobulin G followed by the secondary antibody complex resulted in a lack of staining (Fig. 5B).

4. Discussion

Previous studies showed that 17β-estradiol causes relaxation of the human colon and guinea pig gallbladder [10,12]. However, the role of GPER in the relaxation of the gallbladder and colon was not clear. The present study provides the first evidence that GPER-related agents, such as 17β-estradiol and G-1, can cause relaxation of the human gallbladder. In addition, we demonstrated the expression of GPER in the human gallbladder.

G-1 is a GPER-selective agonist and does not bind to estrogen nuclear receptors [1,5,24]. Tamoxifen is an estrogen nuclear receptor antagonist but a weak GPER agonist [1,5]. In the human gallbladder, 17β-estradiol and G-1 elicited a marked and fast relaxation, while tamoxifen produced a mild relaxation. This indicates that GPER mediates the relaxant response. The existence of GPER in the human gallbladder was confirmed by immunohistochemistry, RT-PCR, and real-time PCR. Thus, the present study demonstrates that 17β-estradiol may cause relaxation of the human gallbladder via GPER. However, G-15, a GPER receptor antagonist [1], could inhibit the human gallbladder relaxation caused by 17β-estradiol, but not with statistical significance. More potent GPER antagonists are needed to further clarify the involvement of GPER in human gallbladder motility.

In human gallbladder strips, the ability of 17β-estradiol to cause relaxation was not altered by tetrodotoxin and ω-conotoxin GVIA. This suggests that 17β-estradiol interacts directly with receptors on the gallbladder smooth muscle to cause the relaxation.

Natriuretic peptides, bile salts, pituitary adenylate cyclase-activating peptide, and vasoactive intestinal peptide have been reported to cause relaxation of the human and guinea pig gallbladder [17,19]. The present study demonstrates that 17β-estradiol causes relaxation of the human gallbladder. Estrogen might play an important role in regulating the muscle tone of the human gallbladder. GPER antagonists, which may increase gallbladder muscle

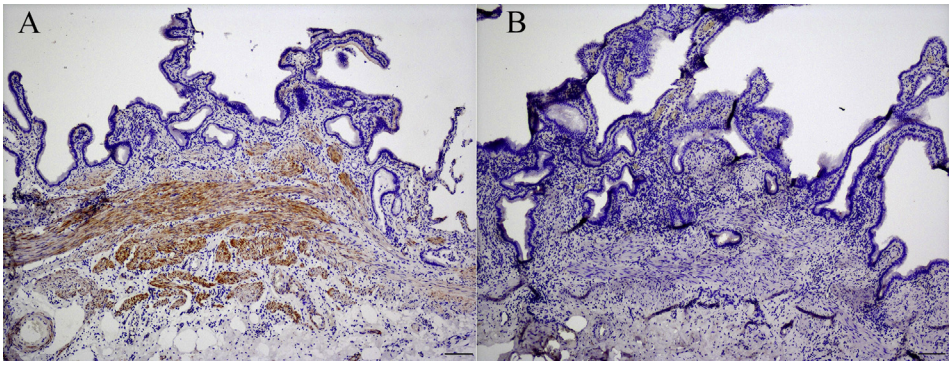

Fig. 5. Microscopic images (100×) showing (A) immunohistochemical localization of GPER in the human gallbladder muscle. (B) No immunostaining was observed in the negative control with normal rabbit immunoglobulin G. The results presented are representative of three experiments. Scale bar = 100 μm. GPER = G protein-coupled estrogen receptor.

tone, might be of potential therapeutic value in gallstone disease. In human colon strips, 17β-estradiol was reported to interact with a membrane receptor, which might be GPER [10]. Therefore, it is likely that estrogen may modulate human gastrointestinal and hepatobiliary tract motility via GPER.

Gallbladder motility is involved in the pathogenesis of gallstone formation [15,25]. From our *ex vivo* study, GPER might mediate the relaxation of human gallbladder, and GPER probably expresses in the human gallbladder of both women and men. It is not clear whether there are potential differences in gallbladder expression of GPER and estrogen-induced gallbladder relaxant responses between the two sexes. Further studies, including estrogen-induced human gallbladder relaxation *in vivo*, are needed to clarify the possible involvement of GPER in gallstone formation.

In conclusion, our results demonstrate for the first time that 17β-estradiol and GPER agonist G-1 cause relaxation of the human gallbladder, probably through GPER. Estrogen might play an important role in the control of human gallbladder motility.

Acknowledgments

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