Published in final edited form as:

Pharmacol Res. 2015 October ; 100: 309–320. doi:10.1016/j.phrs.2015.08.014.

The effects of (–)-epicatechin on endothelial cells involve the G protein-coupled estrogen receptor (GPER)

Aldo Moreno-Ulloa,

University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA, USA..
Laboratorio de Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

David Mendez-Luna,

Laboratorio de modelado Molecular y Diseño de Fármacos, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

Ernesto Beltran-Partida,

Universidad Autónoma de Baja California, Facultad de Odontología, Mexicali, BC, México

Carmen Castillo,

Laboratorio de Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

Gustavo Guevara,

Laboratorio de Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

Israel Ramirez-Sanchez,

University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA, USA..
Laboratorio de Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

José Correa-Basurto,

Laboratorio de modelado Molecular y Diseño de Fármacos, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México. Laboratorio de
Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado, Escuela Superior de
Medicina, Instituto Politécnico Nacional, México

Guillermo Ceballos*, and

Address correspondence to: F.V. MD, PhD, Professor of Medicine, UCSD School of Medicine, 9500 Gilman Dr. BSB4028, La Jolla, CA 92093-0613J, Tel: (858)-534-3630, Lab: (858)-534-1317, Fax: (858)-534-0522, fvillarr@ucsd.edu.

*Guillermo Ceballos, and Francisco Villarreal co-senior this work

Conflict of interest None

Authorship contributions: Participated in research design: A.M.U., D.M.L., J.C.B., I.R.S., G.C., F.V. Conducted experiments: A.M.U., D.M.L., G.G., C.C. Contributed new reagents or analytic tools: D.M.L., J.C.B. Performed data analysis: A.M.U. Wrote or contributed to the writing of the manuscript: All authors

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Laboratorio de Investigación Integral Cardiometabólica, Sección de Estudios de Posgrado,
Escuela Superior de Medicina, Instituto Politécnico Nacional, México

Francisco Villarreal*

University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA, USA

Abstract

We have provided evidence that the stimulatory effects of (–)-epicatechin ((–)-EPI) on endothelial cell nitric oxide (NO) production may involve the participation of a cell-surface receptor. Thus far, such entity(ies) has not been fully elucidated. The G protein-coupled estrogen receptor (GPER) is a cell-surface receptor that has been linked to protective effects on the cardiovascular system and activation of intracellular signaling pathways (including NO production) similar to those reported with (–)-EPI. In bovine coronary artery endothelial cells (BCAEC) by the use of confocal imaging, we evidence the presence of GPER at the cell-surface and on F-actin filaments. Using *in silico* studies we document the favorable binding mode between (–)-EPI and GPER. Such binding is comparable to that of the GPER agonist, G1. By the use of selective blockers, we demonstrate that the activation of ERK 1/2 and CaMKII by (–)-EPI is dependent on the GPER/c-SRC/EGFR axis mimicking those effects noted with G1. We also evidence by the use of siRNA the role that GPER has on mediating ERK1/2 activation by (–)-EPI. GPER appears to be coupled to a non $G\alpha_{i/o}$ or $G\alpha_s$, protein subtype. To extrapolate our findings to an *ex vivo* model, we employed phenylephrine pre-contracted aortic rings evidencing that (–)-EPI can mediate vasodilation through GPER activation. In conclusion, we provide evidence that suggests the GPER as a potential mediator of (–)-EPI effects and highlights the important role that GPER may have on cardiovascular system protection.

Graphical abstract

Keywords

(–)-epicatechin; Cacao; GPER; ERK 1/2; EGFR; Docking

1. Introduction

The consumption of moderate amounts of cacao-based products (i.e. cocoa and dark chocolate) has been associated with a reduced risk for cardiovascular diseases (CVD) (1–5).

Evidence suggests that the beneficial effects of cacao-based products may be mediated by improving endothelial function primarily, through an increase in the production of nitric oxide (NO) (3). Cacao composition is complex and includes flavonoids present as monomers and polymers mainly of (–)-epicatechin ((–)-EPI) and (+)-catechin. The oral ingestion of pure (–)-EPI stimulates the production of NO by the vasculature (6, 7), mimics the effects of cocoa consumption (7) and suggests (–)-EPI (a flavan-3-ol) as the molecule responsible of the aforementioned effects on the cardiovascular system. Using endothelial cells in culture, we have reported on the ability of (–)-EPI to stimulate NO production mediated by endothelial nitric oxide synthase (eNOS) stimulation through the activation of the PI3K/AKT and calcium (Ca^{2+}) (e.g. activation of Calmodulin and Ca^{2+} /Calmodulin-dependent kinase II [CaMKII]) pathways (8). These pathways are known to be linked with the activation of plasma membrane receptors that either possess intrinsic tyrosine kinase activity or couple to heterotrimeric G-proteins (9, 10). By using a cell-surface impermeable (–)-EPI-dextran conjugate, we provided further evidence suggesting that (–)-EPI activates eNOS through a cell-surface receptor (11). However, the exact nature of such entity(ies) has not been elucidated.

Flavonoids that are structurally similar to estrogen (e.g. the isoflavone genistein) are known to bind to the classical estrogen receptors (ERs) α and β leading to stimulation of the transcriptional activity of both receptors in the low nanomolar range (12, 13). While (–)-EPI is structurally similar to estrogen, it does not appear to trigger transcriptional activity indicating that it does not interact with α or β ERs (14).

The G protein-coupled estrogen receptor (GPER, once known as an orphan receptor) is a membrane-bound receptor that can couple to either $\text{G}\alpha_s$ or $\text{G}\alpha_{i/o}$ proteins, and binds 17 β -estradiol (15) as well as the high-affinity highly selective synthetic agonist G1 (16). Activation of GPER leads to c-SRC-dependent transactivation of the epidermal growth factor receptor (EGFR) via $\text{G}\beta\gamma$ and consequently, stimulation of ERK 1/2 (17) and PI3K/AKT/eNOS signaling pathways (18, 19). Studies ascribe in part, the beneficial cardiovascular effects of estrogen to the stimulation of GPER (20). Interestingly, recent evidence indicates that flavonoids such as s-equol can activate GPER linked signaling pathways (19).

Given the similarities between GPER intracellular signaling linked pathways activation and those activated by (–)-EPI, we wished to test the hypothesis that GPER may act as a cell-surface receptor that can partly mediate the effects of (–)-EPI.

2. Materials and Methods

2.1 Chemicals and Reagents

Fetal bovine serum (FBS), charcoal stripped FBS, antibiotic-antimitotic solution, ActinGreen Alexa Fluor-488, Lipofectamine RNAiMAX, Opti-MEM 1 reduced serum media, and Dulbecco's Modified Eagle's Media (DMEM) with and without phenol red were from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) was from Tissue Culture Biologicals (Long Beach, CA, USA). Hank's Balanced Salt Solution (HBSS) phenol red free was from Mediatech Cellgro, Inc. (Herndon, VA, USA). MISSION® siRNA

universal negative control #1, GPER specific siRNA, (–)-EPI, PP2, Tyrphostin AG 1498, pertussis toxin (PT), protease, and phosphatase inhibitor cocktails, Isobutylmethylxanthine (IBMX), and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies, anti-phospho (p)-CaMKII (Thr²⁸⁶), anti-pERK 1/2 (Thr²⁰²/Tyr²⁰⁴), anti-CaMKII, anti-ERK 1/2, anti-GAPDH, and anti-rabbit and -mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling (Danvers, MA, USA). Anti-GPER was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary donkey anti-rabbit IgG conjugated to Alexa Fluor 594 was from Abcam (Cambridge, MA, USA). Lab-Tek II chamber slides and methanol-free formaldehyde (16 % in solution) were from Thermo Scientific (Waltham, MA, USA). VECTASHIELD mounting medium with DAPI was from Vector Laboratories Inc. (Burlingame, CA, USA). Immobilon-P Membrane, PVDF transfer membrane was from EMD Millipore (Bedford, MA, USA). Enhanced chemiluminescence (ECL) Plus Western Blot detection kit was from Amersham Biosciences (Piscataway, NJ, USA). G15 and G1 were from Cayman Chemical (Ann Harbor, MI, USA). cAMP ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Molecular modeling and molecular dynamic (MD) simulations

We used as a target the tridimensional (3-D) model of GPER previously developed, refined by molecular dynamics (MD) simulations and validated by docking studies (21). This 3-D model of GPER was built using the GPCR I-Tasser web site (22) (a specialized server for building 3-D models of GPCRs). The 3-D model was evaluated by both the ERRAT (<http://nihserver.mbi.ucla.edu/ERRAT/>) and PROCHECK (<https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/>) servers. The NAMD 2.9 program was used to perform the GPER refinement by MD simulations. The 14 and 70 ns snapshots derived from MD were used for docking studies, as these snapshots appeared to evidenced key molecular recognitions of G1 (agonist) and G15 (antagonist) in the binding site of GPER (21).

2.3. Molecular Docking studies

Docking studies were performed as previously described (21), with slight modifications. Docking parameters employed here consisted on a grid box of 60 X 60 X 60 Å³ centered at the Ca of F208, as this residue has been suggested by us and others (23) to be the protein active site centre, other parameters were kept the same. All analyzed ligands were drawn with ACD/CHEMSKETCH (<http://www.acdlabs.com/resources/freeware/chemsketch/>) and converted into 3-D structures (Z-matrix) with the Gauss view 5.0 program. Ligands were energetically and geometrically optimized at the AM1 semi-empirical level using the Gaussian 03 package. The prediction and description of GPER binding site with all ligands were carried out on AutoDock 4.2 software using the graphic interface AutoDock Tools 1.5.2.

2.4. Cell culture

Bovine Coronary Artery Endothelial Cells (BCAECs) were purchased from Cell Applications, Inc. (San Diego, CA, USA). BCAECs obtained from healthy bovine coronary arteries from 4 hearts were used in this study. A mouse skeletal muscle cell line (C2C12 myoblasts) were purchased from American Type Culture Collection (ATCC, Manassas, VA,

USA). Both cell types were maintained at 37°C in an incubator with humidified atmosphere of 5 % CO₂ and cultured in DMEM supplemented with 10 % FBS and 1 % antibiotic and antimetabolic solution.

2.5. Immunofluorescence

Cells were seeded onto glass slides pre-coated with 0.1 % gelatin, and incubated for 24 hr in growth medium. Thereafter, cells were washed with PBS and then fixed with a freshly-prepared cold 4 % formaldehyde solution in PBS for 10 min. Cells were permeabilized/blocked with 3 % BSA, 0.3 M glycine and 0.1 % saponin in PBS for 1 h. Next, slides were incubated with anti-GPER (1:100, 3 % BSA in PBS) for 1 h at room temperature and thereafter washed 3x with PBS. To stain F-actin filaments, Alexa Fluor-488 phalloidin was used for 30 min and slides washed 3x with PBS. Next, Alexa Fluor 594-labeled anti-rabbit (1:400, PBS) was then used as secondary antibody for 1 hr at room temperature and washed 3x with PBS. Finally, slides were vertically dried for 10 min and mounted on VECTASHIELD mounting medium with DAPI. 0.3 µm z-stacks were collected using an Olympus Confocal Microscope model FV1000-IX81 at 1024 x 1024 pixel resolution with a 60X objective lens.

2.6 siRNA Treatment

BCAEC were used at 40-50 % confluence in 6-well plates. Templates for siRNA were designed on the basis of the GPER bovine sequence and ordered from Sigma-Aldrich. Three designed siRNAs were pooled and their sequences are illustrated in Supplemental Table 2. Negative control and specific GPER siRNAs (40 nM) were initially incubated with Lipofectamine RNAiMAX in Opti-MEM 1 media for 5 min to allow for the formation of siRNA-Lipofectamine complexes. As an additional control, Lipofectamine RNAiMAX in Opti-MEM 1 medium without any siRNA was used. Accordingly, the complexes and controls were added to cells that contained 2 ml of regular growth media per well. After 12 h of incubation, the transfection media was replaced by regular growth media and cells allowed to grow for 6 days. GPER knockdown efficacy was evaluated by Western Blotting.

2.7. Cell treatment

When cells reached 80 % confluence, growth media was replaced with phenol red-free DMEM supplemented with 1 % FBS (charcoal treated) and 1 % antibiotic and antimetabolic solution and incubated for 12 h. Next, cells were maintained in phenol red-free HBSS supplemented with 0.4 mM L-arginine for 1 h. Inhibitors were used as follows (concentration, preincubation time): G15 (0.01-1 µM, 30 min), PP2 (1-5 µM, 30 min), Tyrphostin AG1498 (5-10 µM, 30 min) and PT (100 ng/mL, 12 hr). Inhibitors were present during ligand stimulation. (-)-EPI was used at 0.1 µM for 10 min based on previous studies (24). As positive controls for select experiments forskolin and the GPER agonist, G1, were used at 1 µM and 0.1 µM for 10 min, respectively. (-)-EPI, G1, G15, AG1478 and forskolin were dissolved in DMSO (at a final concentration of 0.1 % v/v in the cells). PT was dissolved in water. DMSO at equal concentration was used as vehicle for control cells.

2.8. Total protein extraction

After cells were treated as above they were washed three times with cold HBSS (5 ml/well) and lysed in 100 μ l of ice cold buffer (1% Triton X-100, 20 mmol/l Tris, 140 mmol/l NaCl, 2 mmol/l EDTA, and 0.1% SDS) with protease and phosphatase inhibitor cocktails supplemented with 1 mmol/l PMSF, 2 mmol/l Na₃VO₄, and 1 mmol/l NaF. Homogenates were sonicated for 10 min at 4°C, and centrifuged (12,000 g) for 10 min to remove cell debris. The total protein concentration was measured in the supernatant using the Bradford method.

2.9. Western Blot

Cells were treated and proteins extracted as described above. A total of 40 μ g of protein was loaded onto a 4–15% SDS-PAGE, electrotransferred, incubated for 1 h or overnight in blocking solution (5% nonfat dry milk [NFM] or 5 % BSA in TBS [tris-buffered saline solution] plus 0.1% Tween 20 [TBS-T]), and followed by either 3 h incubation at room temperature or overnight incubation at 4°C with primary antibodies. Primary antibodies were typically diluted 1:1,000 or 1:2,000 in TBS-T plus 5% bovine serum albumin or 5 % NFM. Membranes were washed (3X for 5 min) in TBS-T and incubated 1 h at room temperature in the presence of HRP-conjugated secondary antibodies diluted 1:5,000 in blocking solution. Membranes were again washed 3X in TBS-T, and the immunoblots developed using an ECL detection kit. The band intensities were digitally quantified and phospho-proteins normalized against the total protein in study (i.e. relative phosphorylation was calculated).

2.10. cAMP assay

BCAEC were seeded on 24-well plates a density of 5×10^4 cells/well until they reached 80% confluence and prepared as noted above for agonist and antagonist stimulation. HBSS medium was supplemented with 100 μ M of the phosphodiesterase inhibitor, IBMX for 15 min. After cells were stimulated, the media was aspirated by vacuum and cAMP was extracted twice with 0.5 mL ice-cold ethanol for 5 min at room temperature. The collected solvent was evaporated using a SpeedVac concentrator and the dry residue was resuspended in 0.2 mL of assay buffer. The amount of cAMP in the supernatants (using 0.1 mL and done in duplicate) was assayed using an ELISA kit according to the manufacturer's instructions. Cell density was assessed using the methylene blue method (25), and results expressed as pmol/ 10^6 cells.

2.11 Animals

The aortas of male Wistar rats (250 to 300 g) were employed for ex vivo evaluations of the vascular effects of (–)-EPI. Rats were maintained at room temperature (18–25 °C) and on a 12 h light/dark cycle, with food and water provided *ad libitum*. Animals were handled in accordance with Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) and the good practices of the Comité Interno para el Cuidado y Uso de los Animales de Laboratorio (CICUAL) in regard to research with experimental animals.

2.12 Vascular reactivity

Under anesthesia (pentobarbital 60 mg/kg) animals were decapitated and the thoracic aorta from the diaphragm to the aortic arch was isolated. As previously done by us (26), aortas were immediately submerged in cold Krebs solution to remove all adjacent connective tissue, then cut into ring segments (4–5 cm long), which were then mounted on two stainless steel hooks within an isolated organ chamber. One of the hooks was fixed to the bottom of the chamber and the other to a transducer linked to a Biopac System apparatus for registering changes in tension (force). The isolated organ chamber contained 10 ml of Krebs bicarbonate solution with the following composition in (mM): NaCl 11; KCl 4.7; KH_2PO_4 1.2; MgSO_4 7; H_2O 1.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5; NaHCO_3 25; dextrose 11.7 and calcium disodium EDTA 0.026. The chamber was maintained at a constant temperature of 37°C, pH of 7.4 and a continuous bubbling with a mixture of 95% O_2 and 5% CO_2 .

Compounds were dissolved in DMSO and sequentially diluted in buffer to a final DMSO concentration of less than 0.1%. Standard concentration-response curves were constructed to analyze vascular reactivity to (–)-EPI and G1 [1×10^{-11} to 1×10^{-5} M] on pre-contracted rings (phenylephrine [1×10^{-6} M]) and compared to the effects induced by the GPER agonist G1. A second series of experiments were performed in the presence of the GPER antagonist G15 [1 μM]. Arteries were incubated during 30 minutes with the antagonist before concentration-response curves to (–)-EPI effects were performed. To avoid the oxidation of (–)-EPI bubbling with 95% O_2 was suspended just before its addition to the bath chamber, this maneuver did not affect the contraction of phenylephrine nor the dilation to acetylcholine (data not shown). No effect was observed with DMSO at the final concentration used with the compounds.

2.13. Statistical analysis

Data are presented as mean $\pm$ SEM derived from at least three independent experiments performed each in triplicate. Statistical analysis of data was performed using one-way ANOVA followed by the Tukey post hoc test or Student's t-test for unpaired data, as appropriate. All data was graphed and analyzed using Prism 3.0 (GraphPad Software, San Diego, CA). Data was considered significant when p values obtained were <0.05 .

3. Results

3.1. Molecular modeling, MD simulations and molecular docking

In order to evaluate the plausible binding mode between (–)-EPI (Fig. 1A) and GPER, we employed *in silico* studies. As previously reported by us, we have combined MD simulations and docking studies to explore the potential ligand binding sites of GPER (21). We have focused on 14 and 70 ns conformers retrieved from MD simulations for the subsequent docking analysis as these conformers were capable to accept G1 and G15 in the same binding pose under a blind docking procedure (21). The docking results on 14 ns GPER conformer (Fig. 2A–D) show that G1 and (–)-EPI possess a similar binding pose and both reached the amino acid residues L137, Q138, M141, Y142, F208, Q215, E218, V219 (Supplemental Table 1). Conversely, in this study (using a focused docking) G15 reaches an adjacent site (Fig. 2C and 2D), contrary to our previous blind docking study (21).

Results suggest that the chemical nature of the interactions between (–)-EPI and 14 ns GPER conformer are mainly π - π interactions with the aromatic residues F208 and Y142 and an additional hydrogen bond with the side chain of E218 (Fig. 2A and supplemental Fig. S1, which importantly contributes to the binding free energy value ($\Delta G = -7.9$ kcal/mol) (Supplemental Table 1). Using the 70 ns GPER conformer, we show that G1 (Fig. 2B) and G15 (Fig. 2C) possess the same binding pose and reach the same aminoacid residues (Fig. 2D) as previously described under blind docking procedure (21). (–)-EPI reaches the same binding site of G1 and G15 (Fig. 2D and 2H) although; it makes interactions with more aminoacid residues (Supplemental Table 1). Specifically, (–)-EPI makes π - π interactions with W272 and F208, as well as hydrophobic interactions with the side chains of E218, C207 and Q215, and an additional hydrogen bond with E275 (Fig. 2E). G1 makes π - π interactions with F208 and hydrophobic interactions with the side chains of M141, Q138, M133 (Fig. 2F). G15 has the same structural binding pose and the same non-bond interaction than G1. Remarkably, although G15 lacks an acetyl group compared to G1, its binding energy is more favorable than G1 (Fig. 2G). The specific distances between the interacting ligand and residues atoms are shown in Supplemental Table 3.

3.2. GPER expression

Receptor expression at the protein level was evaluated by immunoblotting using a polyclonal antibody against the N-terminal domain of GPER. The data shows the presence of a band at ~38 KDa in BCAEC (Fig. 3A, **lane 1**) that corresponds to the predicted molecular weight of GPER. Moreover, as positive and negative controls we used lysates from C2C12 and HEK293 cells, respectively. For C2C12 (Fig. 3A, **lane 3**) a band at ~38 KDa was also detected. In HEK293, there was a relative very low immunodetection compared to the other cell types (Fig. 3A, **lane 2**) as reported by others (27).

3.3. GPER localization

In order to determine the subcellular localization of GPER we performed a z-stack analysis using confocal immunofluorescence microscopy. GPER immunostaining of fixed and permeabilized BCAEC denoted a web-like appearance suggesting receptor localization on a cytoskeletal structure (Fig. 4A). To further address this issue, we used phalloidin conjugated to Alexa Fluor-488 to stain F-actin (Fig. 4B). As shown in Fig. 4C there appears to be extensive co-localization between GPER and F-actin filaments. Images also show localization of GPER at the cell-surface (Fig. 4D).

3.4. (–)-EPI-induced GPER-mediated activation of ERK 1/2 and CaMKII

We evaluated the activation of ERK 1/2 and CaMKII kinases that are associated to GPER stimulation (17, 28, 29). (–)-EPI effects on ERK 1/2 and CaMKII activation were assessed in the presence or absence of the selective GPER antagonist, G15 (30). Cells were stimulated with (–)-EPI 0.1 μ M for 10 min, which evokes peak effect on endothelial cell signaling as previously reported by us (24). (–)-EPI treatment led to the activation of ERK 1/2 and CaMKII as denoted by their phosphorylation (Fig. 5A). G15 at 1 μ M completely blocked the effects of (–)-EPI on ERK 1/2 and CaMKII activation as shown in Fig. 5A and Figs. 6A and 6B.

3.5. EGFR expression

To further elucidate the mechanism associated to GPER activation by (–)-EPI, we examined the presence of EGFR, which is known to be trans-activated by GPER, and can lead to the activation of ERK 1/2 (17). As illustrated in Fig 5.B, all immunoblots demonstrated the presence of a band at ~175 KDa in BCAEC (**lane 1**), HEK293 (**lane 2**) and C2C12 (**lane 3**) that corresponds to the molecular weight of EGFR.

3.6. (–)-EPI-induced activation of ERK 1/2 and CaMKII is EGFR- and c-SRC-dependent

Reports indicate that GPER can trans-activate EGFR through the participation of c-SRC (17). To examine this issue in our model, we first blocked EGFR activity using its selective antagonist AG1478 (5-10 μ M) (31). AG1478 at 5 μ M completely abrogated the effects of (–)-EPI on ERK 1/2 and CaMKII, as noted in Fig. 5C and Figs. 6C and 6D. We also inhibited c-SRC by using a selective antagonist PP2 (1-5 μ M) (32). PP2 at 3 μ M completely blocked the effects of (–)-EPI on the kinases as shown in Fig. 5D and Figs. 6A and Fig. 6B.

3.7. G1-induced activation of ERK 1/2 is GPER/c-SRC/EGFR dependent

We employed the GPER agonist, G1, as a positive control to compare its effects with those of (–)-EPI. We used the lowest concentration of the antagonists that completely blocked the effects of (–)-EPI on ERK 1/2. At equimolar concentrations (0.1 μ M) G1 activated ERK 1/2 with no significant differences in the magnitude of the effect compared with (–)-EPI. As expected, G15 blocked G1 activation of ERK 1/2 (Fig.7A). AG1478 (Fig.7B) and PP2 (Fig. 7C) also blocked G1 activation of ERK 1/2.

3.8. ERK 1/2 activation by (–)-EPI is not PT-sensitive

GPCRs can couple to PT-sensitive $G_{i/o}$ -proteins leading to the activation of ERK 1/2 (33). Apparently, GPER-mediated activation of ERK 1/2 can be dependent on $G_{i/o}$ -proteins sensitive to PT (17, 34). By the use of PT we therefore examined the role that $G_{i/o}$ may have on EPI- and G1-mediated ERK 1/2 activation. As shown in Fig. 8A and Fig. 8B PT did not block ERK 1/2 activation by either (–)-EPI or G1, respectively.

3.9. Effects of (–)-EPI and G1 on cAMP levels

GPER can also couple to G_{α_s} , which in turn can lead to increased cAMP levels (35–37). We thus, addressed the capacity of (–)-EPI and G1 to increase cAMP levels. As a positive control, we use forskolin, which increases cAMP levels through a mechanism that involves direct binding to adenylyl cyclase (38). As expected, forskolin doubled cAMP level compared to control (Fig. 8C), while neither (–)-EPI nor G1 increased it (Fig. 8C).

3.10 Effects of siRNA on (–)-EPI- and G1-induced ERK 1/2 activation

In order to provide additional support for our findings, we employed three pooled siRNAs targeting GPER to reduce its expression and evaluate the effects of (–)-EPI and G1 on ERK 1/2 activation. Our results show decreased GPER protein levels (67 % vs. transfection reagent) with the use of siRNA against GPER, no effect was observed with the use of non-targeting siRNA (Fig. 9A). Moreover, the effects of (–)-EPI and G1 on ERK 1/2 were attenuated by the use of siRNA targeting GPER (Fig.9B).

3.11 Effects of (–)-EPI and G1 on pre-contracted rat arteries

As a relevant physiological model to evaluate GPER activation, we employed phenylephrine pre-contracted arteries to study the vascular effects of GPER ligands as used by others (39–41). Using isolated aortic rings we evidence concentration-dependent vasodilatory effects of (–)-EPI that are comparable to those generated by the agonist G1 (Fig. 10A). Pretreatment with the GPER antagonist G15 [1μM] significantly attenuated the (–)-EPI response (Fig. 10B). Similarly, the effect of (–)-EPI on the activation of eNOS (Fig. 10C) and NO production (Fig. 10D) in BCAEC was also attenuated with G15 [1μM].

4. Discussion

In this study, we evaluated by *in silico* analysis, the binding mode of (–)-EPI on GPER. The interaction of (–)-EPI on GPER yields a favorable thermodynamic interaction similar to that described for the GPER ligands, G1 and G15. Moreover, (–)-EPI reaches the same binding site and shares similar molecular recognition properties to those observed for G1 at either 14 and 70 ns GPER conformers under focused docking. GPER is present in endothelial cells as it was localized at the cell-surface and also along cytoskeletal structures (F-actin filaments). (–)-EPI-mediated ERK 1/2 and CaMKII activation was reliant on GPER/c-SRC/EGFR and independent of $G\alpha_{i/o}$ and $G\alpha_s$. Moreover, (–)-EPI mediates vasodilation and eNOS activation through GPER. Altogether, our results indicate that GPER is a receptor, which has the capacity to mediate (–)-EPI effects on endothelial cells.

Over the past years, we have reported on the capacity of (–)-EPI to activate upstream intracellular signaling pathways that are known to be associated to the activation of GPCRs or tyrosine kinase receptors (8, 11). However, the identification of such entities remained lacking. Gathering of evidence for receptor activation by flavonoids can serve to dispel the notion that this class of natural compounds simply exerts their effects by acting as antioxidants. In this regard, there is precedent for the involvement of other GPCRs (such as GPR120) in mediating the beneficial effects of natural molecules such as omega-3 fatty acids (42).

Given the observation that the intracellular signaling pathways linked to GPER are very similar to those activated by (–)-EPI and its structural similarity to s-equal (Fig. 1B) we performed a series of experiments evaluating the participation of this receptor in mediating its effects. *In silico* studies (i.e. docking and MD simulations) have greatly aided in drug discovery by predicting the binding of molecules with existing or recently discovered orphan receptors (43). For example, the virtual and biomolecular screening of putative GPER ligands led to the identification of the first non-steroidal, high-affinity and highly selective GPER ligand, G1 (16) and recent docking studies have evidenced the favorable binding mode of synthetic compounds to GPER (23, 44–46), which clearly indicates that *in silico* docking simulations greatly contribute to the discovery of GPER ligands (47). Therefore, as an initial strategy, we employed docking studies using a GPER 3D model (previously validated by our group) that was further refined by MD simulations (21). In this study, we used two conformers retrieved from MD simulations and validated by docking. It is worth noting that we incorporated protein motion into our 3D model of GPER by using MD simulations, which provides a more physiological receptor environment as described

elsewhere (48). The use of MD simulations in conjunction with docking studies is proposed to predict in a more reliable manner protein-ligand complexes in comparison to rigid docking (49, 50). Based on our previous study (21), we focused on two GPER conformers derived from MD simulations (14 and 70 ns) that suggest a possible state of activation/inactivation switch of the receptor by the accommodation pattern of G1 and G15 into the binding site (21). Moreover, as mentioned above we have focused on the residue F208, which has been suggested as the GPER protein active site centre (21, 23, 51). Our virtual simulations suggest that the interaction between (–)-EPI and GPER is energetically favorable and similar to the GPER agonist G1. Interactions with the 14 ns conformer are mainly through aromatic π - π interaction with (–)-EPI sharing the same GPER binding site with G1, whereas G15 is located outside the mentioned binding site. The interaction between (–)-EPI and G1 with the receptor only differed by two amino acid residues (F223, W272 for G1 and E275, N276 for (–)-EPI, see Supplemental Table 1), while G15 interacted altogether with different amino acids residues. In contrast, using the retrieved 70 ns conformer from MD we denote that the binding mode of all ligands is very similar. However, G15 increased its binding affinity (ΔG becomes more negative) towards the receptor.

The first report of GPER as an orphan receptor was recorded from human umbilical vein endothelial cells (HUVEC) subjected to fluid shear stress (52). However, the presence of this receptor has been noted throughout other cellular components of the cardiovascular system such as smooth muscle cells (20). Interestingly, the majority of studies evaluating the effects of GPER on endothelial cells have focused on the use of HUVECs (53–56). Here, we evidenced for the first time the presence of GPER in coronary artery endothelial cells.

The subcellular localization of GPER has been amply debated. Studies report its presence on the Golgi apparatus (18), endoplasmic reticulum (18, 57), cell-surface (27, 58, 59) and nuclei (54, 60, 61). To address this issue in BCAECs, we used confocal imaging z-stack analysis. Using double staining, we evidence the presence of GPER at the cell-surface and in co-localization with cytoskeletal structures, F-actin filaments. The localization of GPER on cytoskeletal-like cellular structures has been previously demonstrated, and appears associated to receptor trafficking (58). However, the biological significance of GPER presence on F-actin filaments is beyond the scope of this study. Interestingly, using a cell-surface impermeable (–)-EPI dextran conjugate, we noted that its effect on intracellular signaling was comparable to that generated by (–)-EPI (11). Given these results, it appears that a cell-surface localization of GPER is likely responsible for (–)-EPI-mediated activation of intracellular pathways in endothelial cells.

Our results indicate that (–)-EPI increases the activity of ERK 1/2 and CaMKII through GPER activation, which can be effectively blocked by G15. Similarly, the use of siRNA confirms the role that GPER has on mediating ERK1/2 activation by (–)-EPI. Critical to the activation of those kinases, is the crosstalk with EGFR as evidenced by the blockade of effects elicited by the EGFR blocker AG1478. These results are in agreement with those of Filardo et al. who suggests that E_2 -mediated ERK 1/2 activation is dependent upon trans-activation of the EGFR via GPER-mediated increase in c-SRC kinase activity through a mechanism that involves the $G\beta\gamma$ -subunit protein (17). We also examined the role that c-

SRC has on (–)-EPI activation of ERK 1/2 by using the selective c-SRC antagonist PP2, which fully blocked its effects. By its mimicry of G1 effects, we also provide further evidence on the reliance of (–)-EPI actions on the GPER/c-SRC/EGFR system.

Depending on the cell type under investigation, the subtype of Gα protein associated to GPER has been ascribed so far, to the Gα_{i/o} or Gα_s subgroups (17). Our results suggest that the Gα protein coupled to GPER in BCAEC is neither Gα_s nor Gα_{i/o} due to the inability of PT to block (–)-EPI or G1 activation of ERK 1/2 and lack of changes in cAMP levels seen after ligand stimulation of endothelial cells. Interestingly, the activation of GPER has been linked to a rapid and transient intracellular Ca²⁺ increase, which is still poorly understood as to whether its directly mediated by Gα_q or EGFR (62). Thus, we do not rule the plausible participation of a Gα_q in GPER mediated activation of intracellular signaling in BCAEC.

In the vasculature, GPER activation by G1 has been associated to vasodilation in part by activation of eNOS and NO production (35, 41). We therefore, employed phenylephrine pre-contracted aortic rings as an *ex vivo* model to evaluate the role that GPER has on mediating (–)-EPI vasodilatory effects. Our results indicate that (–)-EPI causes vasodilation in a concentration-dependent manner that is not different from that observed with G1 and is mediated by GPER activation. Moreover, to assess the role that eNOS has on mediating the (–)-EPI vasodilatory effects through GPER; we evaluated its phosphorylation (Ser1179) and NO production in BCAEC. Our data suggest that (–)-EPI activates eNOS and stimulates NO production through GPER activation, as evidenced by the partial blockade elicited by G15.

Overall, using *in silico* and *in vitro* studies we evidenced on the capacity of (–)-EPI to activate intracellular pathways similar to those observed by the GPER agonist, G1, which are mediated by GPER cross-talk with the EGFR, via the kinase c-SRC. These results have important implications as they provide rigorous evidence as to a plausible mechanism of action of (–)-EPI in triggering effects in normal endothelial cells, which may partly account for the protective actions of flavanols on the cardiovascular system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Armando Cid, Alejandro Vargas, Mohammad Sedarat, Jaime Daniel Reyes Sanchez, Enrique Martinez, Alonso Rodriguez, Claudia Varela, Sarahi Picos, Nicole Rubin and Luis Miguel Roman Pintos for their valuable technical assistance.

This work was supported by NIH R24 DK092154 and R01 DK098717 to F.V. A.M.U. is a doctoral candidate supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico, fellowship #388585). This work was submitted in partial fulfillment of the requirements for the Ph.D. degree for A.M.U. at the Programa de Doctorado en Investigación en Medicina, Instituto Politécnico Nacional. JCB thanks to EDI(SIP),SIBE(COFAA)/IPN, CYTED and CONACyT grants.

Non standard abbreviations

GPER

G protein-coupled estrogen receptor

EGFR	epidermal growth factor receptor
(-)-EPI	(-)-epicatechin
BCAEC	bovine coronary artery endothelial cells
GPCR	G protein-coupled receptor
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
PBS	phosphate-buffered saline
G15	GP130 antagonist
G1	GP130 agonist
Tyrphostin AG 1478	EGFR antagonist
PP2	c-SRC antagonist
PT	pertussis toxin
ERK 1/2	extracellular signal-regulated kinase 1 and 2

References

1. Buitrago-Lopez A, Sanderson J, Johnson L, Warnakula S, Wood A, Di Angelantonio E, Franco OH. Chocolate consumption and cardiometabolic disorders: systematic review and meta-analysis. *BMJ*. 2011; 343:d4488. [PubMed: 21875885]
2. Mostofsky E, Levitan EB, Wolk A, Mittleman MA. Chocolate intake and incidence of heart failure: a population-based prospective study of middle-aged and elderly women. *Circulation Heart failure*. 2010; 3(5):612–616. [PubMed: 20713904]
3. Buijsse B, Feskens EJ, Kok FJ, Kromhout D. Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study. *Archives of internal medicine*. 2006; 166(4):411–417. [PubMed: 16505260]
4. Buijsse B, Weikert C, Drogan D, Bergmann M, Boeing H. Chocolate consumption in relation to blood pressure and risk of cardiovascular disease in German adults. *European heart journal*. 2010; 31(13):1616–1623. [PubMed: 20354055]
5. Ellinger S, Reusch A, Stehle P, Helfrich HP. Epicatechin ingested via cocoa products reduces blood pressure in humans: a nonlinear regression model with a Bayesian approach. *The American journal of clinical nutrition*. 2012; 95(6):1365–1377. [PubMed: 22552030]
6. Loke WM, Hodgson JM, Proudfoot JM, McKinley AJ, Puddey IB, Croft KD. Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *The American journal of clinical nutrition*. 2008; 88(4):1018–1025. [PubMed: 18842789]
7. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Urbe C, Schmitz HH, Kelm M. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci U S A*. 2006; 103(4):1024–1029. [PubMed: 16418281]
8. Ramirez-Sanchez I, Maya L, Ceballos G, Villarreal F. (-)-epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. *Hypertension*. 2010; 55(6):1398–1405. [PubMed: 20404222]
9. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007; 129(7):1261–1274. [PubMed: 17604717]

10. Patel TB. Single transmembrane spanning heterotrimeric g protein-coupled receptors and their signaling cascades. *Pharmacological reviews*. 2004; 56(3):371–385. [PubMed: 15317909]
11. Moreno-Ulloa A, Romero-Perez D, Villarreal F, Ceballos G, Ramirez-Sanchez I. Cell membrane mediated (–)-epicatechin effects on upstream endothelial cell signaling: evidence for a surface receptor. *Bioorganic & medicinal chemistry letters*. 2014; 24(12):2749–2752. [PubMed: 24794111]
12. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*. 1998; 139(10):4252–4263. [PubMed: 9751507]
13. Wang TT, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis*. 1996; 17(2):271–275. [PubMed: 8625449]
14. Breinholt V, Larsen JC. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. *Chemical research in toxicology*. 1998; 11(6):622–629. [PubMed: 9625730]
15. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*. 2005; 146(2):624–632. [PubMed: 15539556]
16. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI, Prossnitz ER. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nature chemical biology*. 2006; 2(4):207–212. [PubMed: 16520733]
17. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol*. 2000; 14(10):1649–1660. [PubMed: 11043579]
18. Meyer MR, Fredette NC, Howard TA, Hu C, Ramesh C, Daniel C, Amann K, Arterburn JB, Barton M, Prossnitz ER. G protein-coupled estrogen receptor protects from atherosclerosis. *Scientific reports*. 2014; 4:7564. [PubMed: 25532911]
19. Rowlands DJ, Chapple S, Siow RC, Mann GE. Equol-stimulated mitochondrial reactive oxygen species activate endothelial nitric oxide synthase and redox signaling in endothelial cells: roles for F-actin and GPR30. *Hypertension*. 2011; 57(4):833–840. [PubMed: 21300668]
20. Prossnitz ER, Barton M. Estrogen biology: new insights into GPER function and clinical opportunities. *Molecular and cellular endocrinology*. 2014; 389(1–2):71–83. [PubMed: 24530924]
21. Mendez-Luna D, Martinez-Archundia M, Maroun RC, Ceballos-Reyes G, Fragos-Vazquez MJ, Gonzalez-Juarez DE, Correa-Basurto J. Deciphering the GPER/GPR30-agonist and antagonists interactions using molecular modeling studies, molecular dynamics, and docking simulations. *Journal of biomolecular structure & dynamics*. 2015:1–12.
22. Zhang J, Zhang Y. GPCR3D: G protein-coupled receptor spatial restraint database for 3D structure modeling and function annotation. *Bioinformatics*. 2010; 26(23):3004–3005. [PubMed: 20926423]
23. Lappano R, Santolla MF, Pupo M, Sinicropi MS, Caruso A, Rosano C, Maggiolini M. MIBE acts as antagonist ligand of both estrogen receptor alpha and GPER in breast cancer cells. *Breast cancer research : BCR*. 2012; 14(1):R12. [PubMed: 22251451]
24. Moreno-Ulloa A, Cid A, Rubio-Gayosso I, Ceballos G, Villarreal F, Ramirez-Sanchez I. Effects of (–)-epicatechin and derivatives on nitric oxide mediated induction of mitochondrial proteins. *Bioorganic & medicinal chemistry letters*. 2013; 23(15):4441–4446. [PubMed: 23791569]
25. Dana L, Felice JS, Liu Rui Hai. A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods*. 2009; 1(1):109–118.
26. del Castillo-Hernandez MC, Meraz-Cruz N, Guevara-Balcazar G, Lopez-Canales J, Lopez-Canales O, Galindo N, Castillo-Henkel C. Age-related differences in the beta-adrenergic vasodilator response in rat aortic rings. *Proc West Pharmacol Soc*. 2010; 53:29–32. [PubMed: 22128447]
27. Cheng SB, Quinn JA, Graeber CT, Filardo EJ. Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. *The Journal of biological chemistry*. 2011; 286(25):22441–22455. [PubMed: 21540189]

28. Gros R, Ding Q, Liu B, Chorazyczewski J, Feldman RD. Aldosterone mediates its rapid effects in vascular endothelial cells through GPER activation. *American journal of physiology Cell physiology*. 2013; 304(6):C532–540. [PubMed: 23283935]
29. Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologna CG, Oprea TI, Yamaguchi Y, Hayashi S, Sklar LA, Hathaway HJ, Arterburn JB, Prossnitz ER. Identification of a GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *The Journal of steroid biochemistry and molecular biology*. 2011; 127(3–5):358–366. [PubMed: 21782022]
30. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologna CG, Leitao A, Brailoiu E, Deliu E, Dun NJ, Sklar LA, Hathaway HJ, Arterburn JB, Oprea TI, Prossnitz ER. In vivo effects of a GPR30 antagonist. *Nature chemical biology*. 2009; 5(6):421–427. [PubMed: 19430488]
31. Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science*. 1995; 267(5205):1782–1788. [PubMed: 7892601]
32. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *The Journal of biological chemistry*. 1996; 271(2):695–701. [PubMed: 8557675]
33. Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, Luttrell LM. Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *The Journal of biological chemistry*. 1999; 274(20):13978–13984. [PubMed: 10318809]
34. Ding Q, Gros R, Limbird LE, Chorazyczewski J, Feldman RD. Estradiol-mediated ERK phosphorylation and apoptosis in vascular smooth muscle cells requires GPR 30. *American journal of physiology Cell physiology*. 2009; 297(5):C1178–1187. [PubMed: 19741198]
35. Lindsey SH, Liu L, Chappell MC. Vasodilation by GPER in mesenteric arteries involves both endothelial nitric oxide and smooth muscle cAMP signaling. *Steroids*. 2014; 81:99–102. [PubMed: 24246735]
36. Yu X, Li F, Klusmann E, Stallone JN, Han G. G protein-coupled estrogen receptor 1 mediates relaxation of coronary arteries via cAMP/PKA-dependent activation of MLCP. *American journal of physiology Endocrinology and metabolism*. 2014; 307(4):E398–407. [PubMed: 25005496]
37. Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI. 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*. 2002; 16(1):70–84. [PubMed: 11773440]
38. Insel PA, Ostrom RS. Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling. *Cellular and molecular neurobiology*. 2003; 23(3):305–314. [PubMed: 12825829]
39. Lindsey SH, Carver KA, Prossnitz ER, Chappell MC. Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2.Lewis female rat. *Journal of cardiovascular pharmacology*. 2011; 57(5):598–603. [PubMed: 21326105]
40. Arefin S, Simoncini T, Wieland R, Hammarqvist F, Spina S, Goglia L, Kublickiene K. Vasodilatory effects of the selective GPER agonist G-1 is maximal in arteries of postmenopausal women. *Maturitas*. 2014; 78(2):123–130. [PubMed: 24796498]
41. Li ZL, Liu JC, Liu SB, Li XQ, Yi DH, Zhao MG. Improvement of vascular function by acute and chronic treatment with the GPR30 agonist G1 in experimental diabetes mellitus. *PloS one*. 2012; 7(6):e38787. [PubMed: 22679517]
42. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell*. 2010; 142(5):687–698. [PubMed: 20813258]
43. Haupt VJ, Schroeder M. Old friends in new guise: repositioning of known drugs with structural bioinformatics. *Briefings in bioinformatics*. 2011; 12(4):312–326. [PubMed: 21441562]
44. Maggiolini M, Santolla MF, Avino S, Aiello F, Rosano C, Garofalo A, Grande F. Identification of two benzopyrrolloxazines acting as selective GPER antagonists in breast cancer cells and cancer-associated fibroblasts. *Future Med Chem*. 2015; 7(4):437–448. [PubMed: 25875871]

45. Lappano R, Rosano C, De Marco P, De Francesco EM, Pezzi V, Maggiolini M. Estriol acts as a GPR30 antagonist in estrogen receptor-negative breast cancer cells. *Molecular and cellular endocrinology*. 2010; 320(1–2):162–170. [PubMed: 20138962]
46. Lappano R, Rosano C, Santolla MF, Pupo M, De Francesco EM, De Marco P, Ponassi M, Spallarossa A, Ranise A, Maggiolini M. Two novel GPER agonists induce gene expression changes and growth effects in cancer cells. *Curr Cancer Drug Targets*. 2012; 12(5):531–542. [PubMed: 22414008]
47. Rosano C, Lappano R, Santolla MF, Ponassi M, Donadini A, Maggiolini M. Recent advances in the rationale design of GPER ligands. *Curr Med Chem*. 2012; 19(36):6199–6206. [PubMed: 23116143]
48. Bello M, Martinez-Archundia M, Correa-Basurto J. Automated docking for novel drug discovery. *Expert Opin Drug Discov*. 2013; 8(7):821–834. [PubMed: 23642085]
49. Durrant JD, McCammon JA. Molecular dynamics simulations and drug discovery. *BMC biology*. 2011; 9:71. [PubMed: 22035460]
50. Alonso H, Bliznyuk AA, Gready JE. Combining docking and molecular dynamic simulations in drug design. *Medicinal research reviews*. 2006; 26(5):531–568. [PubMed: 16758486]
51. Lappano R, Rosano C, Pisano A, Santolla MF, De Francesco EM, De Marco P, Dolce V, Ponassi M, Felli L, Cafeo G, Kohnke FH, Abonante S, Maggiolini M. A calixpyrrole derivative acts as a GPER antagonist: mechanisms and models. *Dis Model Mech*. 2015
52. Takada Y, Kato C, Kondo S, Korenaga R, Ando J. Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. *Biochemical and biophysical research communications*. 1997; 240(3):737–741. [PubMed: 9398636]
53. Santolla MF, De Francesco EM, Lappano R, Rosano C, Abonante S, Maggiolini M. Niacin activates the G protein estrogen receptor (GPER)-mediated signalling. *Cellular signalling*. 2014; 26(7):1466–1475. [PubMed: 24662263]
54. Chakrabarti S, Davidge ST. G-protein coupled receptor 30 (GPR30): a novel regulator of endothelial inflammation. *PloS one*. 2012; 7(12):e52357. [PubMed: 23285008]
55. De Francesco EM, Lappano R, Santolla MF, Marsico S, Caruso A, Maggiolini M. HIF-1 α /GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). *Breast cancer research : BCR*. 2013; 15(4):R64. [PubMed: 23947803]
56. Kong BS, Cho YH, Lee EJ. G protein-coupled estrogen receptor-1 is involved in the protective effect of protocatechuic aldehyde against endothelial dysfunction. *PloS one*. 2014; 9(11):e113242. [PubMed: 25411835]
57. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science*. 2005; 307(5715):1625–1630. [PubMed: 15705806]
58. Sanden C, Broselid S, Cornmark L, Andersson K, Daszkiewicz-Nilsson J, Martensson UE, Olde B, Leeb-Lundberg LM. G protein-coupled estrogen receptor 1/G protein-coupled receptor 30 localizes in the plasma membrane and traffics intracellularly on cytokeatin intermediate filaments. *Molecular pharmacology*. 2011; 79(3):400–410. [PubMed: 21149639]
59. Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P. Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology*. 2007; 148(7):3236–3245. [PubMed: 17379646]
60. Madeo A, Maggiolini M. Nuclear alternate estrogen receptor GPR30 mediates 17 β -estradiol-induced gene expression and migration in breast cancer-associated fibroblasts. *Cancer Res*. 2010; 70(14):6036–6046. [PubMed: 20551055]
61. Pupo M, Vivacqua A, Perrotta I, Pisano A, Aquila S, Abonante S, Gasperi-Campani A, Pezzi V, Maggiolini M. The nuclear localization signal is required for nuclear GPER translocation and function in breast Cancer-Associated Fibroblasts (CAFs). *Molecular and cellular endocrinology*. 2013; 376(1–2):23–32. [PubMed: 23748028]
62. Nilsson BO, Olde B, Leeb-Lundberg LM. G protein-coupled oestrogen receptor 1 (GPER1)/GPR30: a new player in cardiovascular and metabolic oestrogenic signalling. *British journal of pharmacology*. 2011; 163(6):1131–1139. [PubMed: 21250980]

Fig. 1.
Chemical structures of (-)-epicatechin ((-)-EPI) (A), s-equol (B), G1 (C), and G15 (D).
Alphabetical letters indicates the ring position.

Fig. 2.

GPER 3D model at 14 ns docked with **A**) (-)-epicatechin ((-)-EPI), **B**) G1 and **C**) G15. **D**) (-)-EPI, G1 and G15 superimposed into the binding site (for better appreciation, the aminoacids reached by the individual ligands were omitted), purple, (-)-EPI; blue, G1; yellow, G15. GPER 3D model at 70 ns docked with **E**) (-)-EPI **F**) G1 and **G**) G15. **H**) (-)-EPI, G1 and G15 superimposed into the binding site at 70 ns (for better appreciation, the amino acids reached by the individual ligands were omitted). The amino acids reached by the ligands are presented as 3D stick structures. Hydrogen bonds are denoted as yellow dashed lines.

Fig. 3.

Expression of GPER in bovine coronary artery endothelial cells (BCAEC). Representative blot denoting the high abundance of GPER (band at ~ 38 KDa) in BCAEC (lane 1) and C2C12 (lane 3, positive control) and its relative low abundance in HEK293 cells (lane 2, negative control) by using a polyclonal antibody directed to the N-terminal domain of GPER. Arrow indicates the band corresponding to GPER. As a loading control, GAPDH was used.

Fig. 4.

Localization of GPER in bovine coronary artery endothelial cells (BCAEC). Representative confocal image derived from a Z-stack (0.3 μm step size thickness) of fixed and permeabilized BCAEC using a (A) GPER antibody directed to the N-terminal domain (red) and (B) Alexa-488 conjugated to phalloidin (green) to stain F-Actin filaments. (C) Merged image shows co-localization of GPER with F-Actin filaments (in yellow). (D) Amplification of the merged image indicates cell surface localization (white arrows indicate red staining at the cell periphery) of GPER. Images were collected using an Olympus Confocal Microscope model FV1000-IX81 at 1024 x 1024 pixel resolution with a 60X objective lens.

Fig. 5.

(-)-Epicatechin ((-)-EPI)-mediated activation of ERK 1/2 and CaMKII and the effects of GPER, EGFR and c-SRC blockade. Representative blots denoting the activation of ERK 1/2 and CaMKII by (-)-EPI (0.1 μ M, 10 min) with and without the presence of various concentrations of the inhibitors G15 (A), AG1478 (C) and PP2 (D). The activation of the kinases was evaluated by the detection of phosphorylation on their activation residues and normalized to their total protein levels. (B) Representative blot showing the presence of EGFR (band ~ at 175 KDa) in BCAEC (lane 1), HEK293 (lane 2) and C2C12 (lane 3) by using a polyclonal antibody. Arrow indicates the band corresponding to EGFR.

Fig. 6.

Quantification of the effects of G15, AG1478 and PP2 on (-)-Epicatechin ((-)-EPI)-mediated activation of ERK 1/2 and CaMKII. Graphs correspond to the inhibition of (A and B) GPER, (C and D) EGFR and (E and F) c-SRC by the lowest concentration of (30 min previous and during ligand stimulation) G15 (1 μ M), AG1478 (5 μ M) and PP2 (3 μ M) that fully blocks the activation of ERK 1/2 (left panels) and CaMKII (right panels) by (-)-EPI. Control was arbitrarily set to 1 and kinases activation is expressed as relative phosphorylation. Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 7.

Effects of the GPER agonist G1, on ERK 1/2 activation in the presence of G15, AG1478 and PP2 blockers. BCAEC were incubated with 0.1 μ M G1 for 10 min without or with (30 min previous and during ligand stimulation) (A) 1 μ M G15, (B) 5 μ M AG1478 and (C) 3 μ M PP2. Top panels show representative blots of phosphorylated ERK 1/2 and total protein levels. Bottom panels quantify the effects of G1 with or without the chemical blocker.

Control was arbitrarily set to 1 and kinase activation is expressed as relative to phosphorylation. Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 8.

cAMP levels and potential involvement of $G\alpha_{i/o}$ proteins in (-)-epicatechin ((-)-EPI)- and G1-mediated activation of ERK 1/2. (A) Activation of ERK 1/2 by (-)-EPI and (B) G1 in the presence of Pertussis Toxin (PT) (100 ng/mL). Control was arbitrarily set to 1 and kinase activation is expressed as relative to phosphorylation. (C) cAMP levels determination by ELISA after treatment of endothelial cells with 0.1 μ M of (-)-EPI and G1 for 10 min. As a positive control we used the adenylyl cyclase activator, forskolin (1 μ M, 10 min). Values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 9. Effect of GPER downregulation on (-)-epicatechin ((-)-EPI)- and G1-mediated phosphorylation of ERK. (A) Western blot analysis of BCAEC showing GPER expression in the presence of transfection reagent (ctrl), nontargeting siRNA (ctrl siRNA) and siRNA targeting GPER (GPER siRNA). (B) Western Blot analysis of ERK1/2 activation (phosphorylation) by (-)-EPI (0.1 μ M) and G1 (0.1 μ M) in the presence (+siRNA) or absence (-siRNA) of 40 nM siRNA targeting GPER. All conditions using -siRNA include Lipofectamine reagent. Values are means $\pm$ SEM. * $P < 0.05$

Fig. 10.

Aortic relaxation in response to the GPER agonist G1 and (-)-Epicatechin ((-)-EPI). (A) Concentration response curves of (-)-EPI (n=4) and G1 (n=5) on phenylephrine pre-contracted aortic rings. (B) Concentration response curve of (-)-EPI on the arterial dilation with (n=3) and without (n=4) 1 μ M G15. *P<0.05 vs (-)-EPI+G15 [1 μ M]. (C) eNOS activation (phosphorylation) by 0.1 μ M (-)-EPI (10 min) with or without 1 μ M G15. ** P<0.01 vs control. (D) NO production by 0.1 μ M (-)-EPI with or without 1 μ M G15. *** P<0.001 vs control. NO levels were normalized by protein content. All values are means $\pm$ SEM.

Fig. 11. Proposed role for GPER and EGFR in the mediation of (-)-epicatechin ((-)-EPI) effects on Bovine Coronary Artery Endothelial cells (BACEC) leading to the activation of ERK 1/2 and CaMKII.