The GPER1 Agonist G-1 Attenuates Endothelial Cell Proliferation by Inhibiting DNA Synthesis and Accumulating Cells in the S and G2 Phases of the Cell Cycle

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Key Words

Cell cycle • DNA synthesis • Endothelial cells • Estrogen • Estrogen receptor • G-1 • GPER1

Abstract

G protein-coupled receptor 30 (GPR30) or G protein-coupled estrogen receptor 1 (GPER1) is expressed in the vasculature, but the importance of vascular GPER1 remains to be clarified. Here we investigate effects of the GPER1 agonist G-1 on endothelial cell proliferation using mouse microvascular endothelial bEnd.3 cells. The bEnd.3 cells express mRNA for GPER1. The bEnd.3 cells expressed both ER α and ER β immunoreactivities. Treatment with G-1 reduced DNA synthesis and cell number with IC₅₀ values of about 2 μ M. GPER1 siRNA prevented G-1-induced attenuation of DNA synthesis. G-1 accumulated cells in S and G2 phases of the cell cycle, suggesting that G-1 blocks transition between G2 and M. G-1 had no effect on DNA synthesis in COS-7 cells only weakly expressing GPER1 mRNA. 17 β -Estradiol had no effect on DNA synthesis in physiological concentrations (nM). The ER blocker ICI182780 reduced DNA synthesis with similar potency as G-1. Treatment with the ERK/MAP kinase inhibitor PD98059 had no effect on G-1-induced attenuation of DNA synthesis. G-1-induced antiproliferation was observed not only in bEnd.3

cells but also in human umbilical vein endothelial cells and HMEC-1 endothelial cells. We conclude that the GPER1 agonist G-1 attenuates endothelial cell proliferation via inhibition of DNA synthesis and by accumulation of cells in S and G2.

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Introduction

The female sex hormone estrogen possesses atheroprotective effects, observed in premenopausal women being less prone to cardiovascular disease than age-matched men [1, 2]. Several mechanisms have been suggested for the cardiovascular protective effect of estrogen, for example attenuation of vascular inflammation [3, 4], stimulation of NO formation [5] and prevention of vascular smooth muscle cell proliferation [6]. An intact endothelium is important for prevention of cardiovascular disease, and it is possible that the atheroprotective effects of estrogen are mediated through improved endothelial healing [7, 8].

Estrogen exerts its effect via two classical estrogen receptor subtypes named ER α and ER β [9]. Expression of ER α and ER β has been shown in endothelial and vascular smooth muscle cells on both mRNA and protein levels

[10, 11]. Estrogen has been shown to prevent vascular smooth muscle cell proliferation after vascular injury via ER α [6]. Studies in knockout mice have shown that ER α is critical for estrogen-induced improvement of re-endothelialization, while ER β is not [7]. Also estrogen-induced anti-inflammatory effects have been reported to be mediated via ER α [12]. Estrogen has been reported to induce eNOS and increase NO production within minutes and this effect was attributed to a subpopulation of ER α (ER α 46) residing in the plasma membrane [13, 14]. Thus, a lot of evidence suggests that ER α mediates estrogen-induced vasculoprotection.

G protein-coupled receptor 30 (GPR30), now named G protein-coupled estrogen receptor 1 (GPER1), has been reported to bind 17 β -estradiol with high affinity [15, 16]. GPER1 was shown to mediate proliferative effects of 17 β -estradiol in breast cancer cells lacking ER α and ER β [17]. Studies in GPER1 knockout mice have revealed that these mice show reduced amount of T lymphocytes expressing CD62L, hyperglycaemia, reduced body growth, reduced serum IGF-I levels and increased blood pressure [18–20]. Mesenteric arteries and endothelial cells have been shown to express GPER1, suggesting a functional role for GPER1 in the vascular system [18, 19].

Haas et al. [21] reported recently that the GPER1 agonist G-1, identified and characterized by Bologa et al. [22], reduces vascular smooth muscle cell proliferation when administered in micromolar concentrations, suggesting that G-1 might be used as a blocker of unwanted vascular smooth muscle cell proliferation in atherosclerosis. In order to obtain a more complete picture of how G-1 affects vascular function, not only the effects of G-1 on vascular smooth muscle cells, but also its effects on endothelial cells, have to be considered. Therefore, we investigated the effects of G-1 on endothelial cell proliferation and disclose that G-1, in the micromolar concentration range, exerts an antiproliferative effect on microvascular endothelial cells by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle.

Material and Methods

Cell Culture

The mouse brain microvascular endothelial cell line (bEnd.3) was purchased from American Type Tissue Culture Collection (Manassas, Va., USA) and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 100 U/ml, streptomycin 100 μ g/ml). The human dermal microvascular endothelial cell line HMEC-1 was cultured in Gibco MCDB 131 medium supplemented with 10%

FCS, 10 ng/ml epidermal growth factor and antibiotics. COS-7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FCS and antibiotics (penicillin 100 U/ml, streptomycin 100 μ g/ml). Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, Md., USA) and cultured in endothelial cell culture medium as recommended by the manufacturer. Cell culture dishes were placed in a water-jacketed cell incubator at 37°C in 5% CO₂ in air. Experiments were performed when cells reached 80% confluence. Before experiments, normal culture medium was replaced with phenol red-free and FCS-free culture medium for 24 h, or 3 h for HUVEC, to make cells quiescent and to remove the estrogen-like activity of phenol red. The phenol red-free culture medium was used throughout the experiment. Experiments were normally performed under growth-arrested conditions (0% FCS), but in some experiments cells were growth stimulated with (5%) dextran-coated charcoal-stripped FCS. Dextran-coated charcoal was used to remove steroids from normal FCS.

Cell Counting and Cell Viability

Number of cells was determined by cell counting in a Bürker chamber after trypsination (0.25%). Cell viability was assessed by trypan blue exclusion test. After removing culture medium cells were washed with 0.9% NaCl and incubated for 2 min with 0.4% trypan blue (Sigma Chemicals, St. Louis, Mo., USA). Then the cells were washed three times and the number of cells containing trypan blue was determined as a measure of dead/dying cells.

RT-PCR

The bEnd.3, COS-7 and RAW264.7 cells were washed carefully in PBS and then total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). cDNA was generated and mRNA for GPER1 determined as previously described by Mårtensson et al. [19]. Reverse transcriptase-negative controls were included to exclude the presence of contaminating genomic DNA. The sequences of GPER1 (GPR30) primers employed are as follows: forward, GATCGTTAGATTAACAGAGCAG; reverse, CCTGGGAGCCTGTTAGTCTCAG. This primer pair produces a GPR30-specific product [19]. One-step quantitative real-time PCR measurements were performed using QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus real-time thermal cycler from Applied Biosystems (Foster City, Calif., USA). Each sample was analyzed in duplicate. GPER1 gene expression was calculated using GAPDH as reference gene as described by Pfaffl [23]. The PCR primers (QuantiTect Primer Assays) for GPER1 (Mm_Gper_1_SG) and GAPDH (Mm_Gapdh_3_SG) were from Qiagen.

Immunocytochemistry

Cells grown on glass cover slips were fixed in 4% formaldehyde in PBS for 2 h at 4°C. After washing in PBS, the cells were incubated with polyclonal ER α (NeoMarkers, Fremont, Calif., USA; code Ab-16) or ER β (Affinity BioReagents, Golden, Colo., USA; code PA1-311) antibodies raised in rabbits at a dilution of 1:100. ER α and ER β antibodies were purchased from Thermo Scientific (Waltham, Mass., USA). For visualization of ER α and ER β immunoreactivity a fluorescein isothiocyanate-conjugated swine anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, Pa., USA) was used. For negative controls, the primary antibody was omitted.

DNA Synthesis Measurement

DNA synthesis was determined by measuring incorporation of [³H] thymidine. The isotope was included for the last hour of the 24-hour incubation. The cells were washed in PBS and then harvested using a rubber policeman. Cells were sonicated in 5 M NaOH twice for 10 s. Aliquots of the sonicate were precipitated with 5% trichloroacetic acid and centrifuged (10,621 g for 2 min at 4°C). After washing with trichloroacetic acid the pellet was dissolved in solune. Liquid scintillation cocktail was added and radioactivity measured in a liquid scintillation counter (Beckman, Fullerton, Calif., USA). Protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif., USA) based on the Lowry method [24]. Radioactivity was expressed as DPM and normalized to the concentration of total protein in each sample.

GP_{ER}1 siRNA

The bEnd.3 cells were treated with commercially available GP_{ER}1 siRNA (accession No. S104924283) or scramble control (scramble, accession No. S103650325) purchased from Qiagen. Free-floating, newly trypsinated cells were treated with the GP_{ER}1 siRNA or scramble control and HiPerFect transfection reagent (Qiagen) for 3 h in serum-free and antibiotic-free culture medium. The final siRNA and scramble control concentration was 100 nM as recommended by the manual from Qiagen. The cells were allowed to attach and equilibrate for 48 h in normal culture medium before being challenged with G-1 or DMSO vehicle. A lower GP_{ER}1 mRNA level in siRNA- versus scramble control-treated bEnd.3 cells, demonstrated by quantitative real-time PCR, confirmed downregulation of GP_{ER}1 transcript by GP_{ER}1 siRNA.

Flow Cytometry

Distribution of bEnd.3 cells in the cell cycle was determined by flow cytometry of propidium iodide-stained nuclei as described by Odenlund et al. [25]. Briefly, flow-cytometric DNA analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San José, Calif., USA) equipped with Cell Quest PRO™ program for data acquisition. DNA contents were determined in about 13,000 nuclei in each sample. Processor signals were digitized and sorted into frequency distribution, DNA histograms, with 256 units resolution. Cell cycle phase distribution was determined by using ModFit LT 3.1 (Verity Software House Inc., Topsham, Me., USA).

Drugs

G-1 was purchased from Cayman Chemical (Ann Arbor, Mich., USA). E₂ was purchased from Sigma Chemicals. ICI182780 was a kind gift from Zeneca Pharmaceuticals (Macclesfield, UK). PD98059 was purchased from Calbiochem (San Diego, Calif., USA). G-1, E₂, ICI182780 and PD98059 were dissolved in DMSO. Controls received DMSO as vehicle.

Statistics

Summarized data are presented as means ± SEM. Statistical significance was calculated using ANOVA and Student's two-tailed t test for unpaired comparison with Bonferroni correction for post hoc analysis as appropriate.

Results

Expression of GP_{ER}1, ER α and ER β in bEnd.3 Endothelial Cells

In order to characterize ER expression in the bEnd.3 cells, we determined GP_{ER}1 mRNA and ER α and ER β protein expression. The bEnd.3 cells expressed mRNA for GP_{ER}1 as demonstrated in figure 1a. For positive control we used mouse macrophage RAW264.7 cell cDNA (fig. 1a). The RAW264.7 cells are well known to express GP_{ER}1 [26]. In COS-7 cells a weak GP_{ER}1 band was detected showing that these cells only weakly express GP_{ER}1 mRNA (fig. 1a). This is in agreement with previous studies reporting that COS-7 cells lack GP_{ER}1 expression or only show weak GP_{ER} mRNA expression [27, 28]. Since there is no reliable antibody available for mouse GP_{ER}1, we could not confirm GP_{ER}1 transcript expression on the protein level. The bEnd.3 cells expressed ER α and ER β proteins as demonstrated by immunocytochemistry (fig. 1b). ER α and ER β immunoreactivities were mainly observed in the nuclei, but some ER β immunoreactivity was also observed in the cytoplasm.

Effects of G-1 on bEnd.3 Cell DNA Synthesis

Stimulation with a submaximal concentration of charcoal-stripped FCS (5%) for 24 h increased DNA synthesis by about 3-fold compared to the basal level of DNA synthesis (0% FCS) as shown in figure 2. Treatment with G-1 at a low concentration (0.03 μ M) had no effect on FCS-stimulated DNA synthesis, while a higher concentration (3 μ M) fully prevented FCS-induced DNA synthesis (fig. 2). An intermediate concentration of G-1 (0.9 μ M) also reduced FCS-induced DNA synthesis, but this effect was not statistically significant (fig. 2). G-1 caused a concentration-dependent decrease in DNA synthesis under nonstimulated (0% FCS) basal conditions with an IC₅₀ value of about 1.9 μ M (fig. 3). As seen in figure 4, stimulation with G-1 (3 μ M) for 24 h reduced basal DNA synthesis by about 80%. An equimolar concentration (3 μ M) of the endogenous estrogen 17 β -estradiol also reduced DNA synthesis (30%), but the effect of 17 β -estradiol was smaller than that of G-1. Because 3 μ M is not a physiological concentration of 17 β -estradiol, we also tested lower concentrations (0.1–30 nM) of this estrogen. As shown in figure 4, treatment with 0.1–30 nM 17 β -estradiol had no effect on DNA synthesis. Combined treatment with 3 μ M 17 β -estradiol as well as the ER α and ER β antagonist ICI182780 (1 μ M) reduced DNA synthesis by about 80% (fig. 4). Treatment with 1 μ M ICI182780 alone reduced DNA synthesis similar to the combination

Fig. 1. a The bEnd.3 cells express a strong GPER1 mRNA band (GPR30, 290 bp) as demonstrated by RT-PCR. The leftmost panel shows the DNA ladder. No band was observed in reverse transcriptase-negative (RT-) control. RAW264.7 cell cDNA (RAW) was used as positive control producing a very strong 290-bp

GPER1 band. In COS-7 cells a very weak 290-bp GPER1 band was observed. **b** The bEnd.3 cells express immunoreactivity for ERα as well as ERβ as demonstrated by immunocytochemistry. No immunoreactivity was observed after omission of the primary ERα or ERβ antibodies. Scale bars = 20 μm.

of 17β-estradiol and ICI182780 (fig. 4). To provide direct evidence for the involvement of GPER1 we treated bEnd.3 cells with GPER1 siRNA and scramble control, respectively, and then monitored the effects of 24-hour treatment with G-1 on FCS (5%)-stimulated DNA synthesis. G-1 (3 μM) reduced DNA synthesis by 60% in scramble control, while it had no effect in GPER1 siRNA-treated cells (fig. 5a). Downregulation of GPER1 mRNA level by siRNA was confirmed by quantitative real-time PCR (fig. 5b).

We tested the effects of G-1 on DNA synthesis in a human microvascular endothelial cell line as well, that is, human dermal microvascular endothelial HMEC-1 cells [29]. Treatment with G-1 (3 μM) for 24 h under nonstimulated (0% FCS) basal conditions reduced DNA synthesis by about 50% in HMEC-1 cells (fig. 6), showing that G-1 reduces DNA synthesis in microvascular endothelial cells derived from a different origin than bEnd.3 cells as well. Treatment with 3 μM G-1 for 24 h (0% FCS) reduced DNA synthesis by about 80% in HUVEC (fig. 6). We com-

pared the effects of G-1 on DNA synthesis in GPER1-positive bEnd.3 cells with those in COS-7 cells only weakly expressing GPER1 [27, 28]. As demonstrated in figure 6, treatment with G-1 (3 μM) for 24 h (0% FCS) reduced DNA synthesis by about 80% in bEnd.3 cells, but had no effect in COS-7 cells.

Effects of G-1 on bEnd.3 Cell Number and Cell Cycle Phase Distribution

Treatment with G-1 (3 μM) for 5 days reduced the cell number by about 75% under conditions representing submaximal growth stimulation, that is, 5% charcoal-stripped FCS (fig. 7a). Treatment with 3 μM G-1 for a shorter time, that is, 2 days, reduced cell number by about 65% ($70,000 \pm 4,100$ cells/ml after G-1 treatment vs. $215,000 \pm 45,000$ cells/ml in controls, $p < 0.05$, $n = 4$ in each group). Trypan blue exclusion test of cells cultured with 5% stripped FCS showed very few trypan blue-containing cells in both control cells and cells treated with 3 μM G-1, suggesting that G-1 has no effect on cell viabil-

Fig. 2. G-1 (3 μM) prevents FCS-induced DNA synthesis in bEnd.3 cells growth-stimulated with 5% stripped FCS for 24 h. Cells were pretreated with G-1 for 2 h before FCS was introduced. G-1 was then present throughout the 24-hour incubation. DNA synthesis was determined by incubation with radiolabeled thymidine during the last hour of incubation (^3H -thymidine, 5 μCi). Radioactivity was normalized to protein concentration and presented as per cent of the radioactivity in parallel controls. Values are means $\pm$ SEM of 3–11 observations in each group. * $p < 0.05$; ** $p < 0.01$ compared to cells stimulated with 5% FCS alone. NS = Not significant compared to 5% FCS.

Fig. 3. Dose-response curve showing the effects of increasing concentrations of G-1 on DNA synthesis in bEnd.3 cells. DNA synthesis in cells treated with G-1 was normalized to that observed in untreated control cells. DNA synthesis in control cells was set to 100%. The cells were treated with G-1 for 24 h under nonstimulated (0% FCS) basal conditions. The IC_{50} value was calculated to 1.9 μM . Values are means $\pm$ SEM of 5–9 observations in each group. ** $p < 0.01$ compared to controls.

Fig. 4. Effects of treatment with 17β -estradiol, ICI182780 and G-1 for 24 h on DNA synthesis in bEnd.3 cells (0% FCS). ICI182780 was introduced 2 h before 17β -estradiol and was then present throughout the 24-hour incubation. Values are means $\pm$ SEM of 4–9 observations in each group. ** $p < 0.01$; *** $p < 0.001$ compared to controls. +++ $p < 0.001$ compared to treatment with 3 μM E2 alone.

Fig. 5. a GPER1 siRNA abolishes G-1-induced attenuation of DNA synthesis in bEnd.3 cells. The cells were stimulated with G-1 (3 μ M) for 24 h under growth-stimulated conditions (5% stripped FCS). **b** Downregulation of GPER1 mRNA level by siRNA was confirmed by quantitative real-time PCR. Values are means $\pm$ SEM of 5–6 observations in each group. * $p < 0.05$; *** $p < 0.001$. NS = Not significant.

Fig. 6. Treatment with 3 μ M G-1 for 24 h reduces DNA synthesis by about 80% in both bEnd.3 and HUVEC endothelial cells and by 50% in HMEC-1 endothelial cells. G-1 (3 μ M) has no effect in COS-7 cells. The cells were treated with G-1 under non-stimulated (0% FCS) basal conditions. Values are means $\pm$ SEM of 3–9 observations in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. NS = Not significant.

ity (data not shown). Treatment with G-1 (3 μ M) for 24 h under the same culture conditions as used for measuring cell number (see above) accumulated cells in S and G2 phases of the cell cycle (fig. 7b), suggesting that G-1 prevents the endothelial cells from entering the M phase.

Effects of ERK/MAP Kinase Inhibitor PD98059 on G-1-Induced Inhibition of DNA Synthesis in bEnd.3 Cells

The ERK/MAP kinase inhibitor PD98059 was included 2 h before G-1 and then present throughout the 24-

hour incubation (0% FCS) with G-1. As seen in figure 8, combined treatment with PD98059 (1 μ M) and G-1 (3 μ M) reduced DNA synthesis similar to G-1 alone, suggesting that G-1-induced reduction of DNA synthesis does not involve attenuation of ERK/MAP kinase activity. PD98059 at 1 μ M had no effect on DNA synthesis by itself (fig. 8). PD98059 is known to block ERK/MAP kinase activity already at 1 μ M with an IC_{50} value of 2 μ M [30]. Incubation with higher concentrations (10 and 50 μ M) of PD98059 without G-1 reduced DNA synthesis in a concentration-dependent manner (fig. 8).

Fig. 7. a Treatment with 3 μM G-1 for 5 days in the presence of 5% stripped FCS reduces bEnd.3 cell number by about 75%. **b** Stimulation with G-1 (3 μM) for 24 h in the presence of 5% stripped FCS accumulates cells in the S and G2 phases of the cell cycle. DNA was stained with propidium iodide and distribution of cells in G0/G1, S and G2 phases computed based on DNA histograms and presented as percentage. Values are means ± SEM of 4–6 observations in each group. * $p < 0.05$; *** $p < 0.001$.

Fig. 8. The ERK/MAP kinase inhibitor PD98059 (1 μM) has no effect on G-1-induced (3 μM) inhibition of DNA synthesis in bEnd.3 cells. PD98059 was introduced 2 h before G-1 and was then present throughout the 24-hour incubation (0% FCS). Values are means ± SEM of 3–18 observations in each group. *** $p < 0.001$ compared to controls. NS = Not significant.

Discussion

Here we show that the GPER1 agonist G-1, in the micromolar concentration range, reduces endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle. G-1-induced cell cycle arrest in S and G2 suggests that G-1 prevents the endothelial cells from entering the M phase. Haas et al. [21] reported recently that G-1 at 1 μM reduces vascular smooth muscle cell proliferation and proposed that G-1 might be used to attenuate unwanted vascular smooth muscle cell proliferation observed in atherosclerosis. Taken together, these findings suggest that G-1 reduces both endothelial cell and vascular smooth muscle cell proliferation. Unwanted endothelial cell proliferation occurs, for example, in angiogenesis in tumor growth, and G-1 may be used to prevent/reduce this unwanted formation of new blood vessels. Treatment with G-1 has been reported to reduce urothelial cell proliferation as well [28], but in this study growth inhibition by G-1 was observed at a lower concentration (0.01–0.1 μM), suggesting that urothelial cells are more sensitive to G-1 than endothelial and vascular smooth muscle cells. The difference in G-1 sensitivity of vascular and urothelial cells suggests that G-1 inhibits proliferation through different mechanisms depending on the cell type.

In the present study we show that G-1 reduces mouse brain microvascular bEnd.3 endothelial cell proliferation by inhibition of DNA synthesis and by causing cell cycle arrest in S and G2. G-1-induced attenuation of DNA synthesis was also observed in the widely used and well-characterized human dermal microvascular endothelial cell line HMEC-1 [29, 31–33] and in HUVEC, showing that G-1-induced reduction of DNA synthesis may be general to endothelial cells independent of origin. The bEnd.3 endothelial cell line is a well-established and widely used endothelial cell line [34, 35]. Furthermore, the bEnd.3 cells are representative for native endothelial cells as they possess endothelial cell morphology and express the endothelial cell marker protein eNOS [36, 37]. In the present study we show that bEnd.3 cells express ER α , ER β and GPER1, confirming their expression patterns observed in endothelial cells in vivo [11, 18]. The bEnd.3 cells thus express several endothelial cell markers, suggesting that the G-1-induced antiproliferative mechanism of action that we observe in bEnd.3 cells is representative of native endothelial cells in vivo.

G-1 has been shown to reduce blood pressure both in normotensive Sprague-Dawley rats and in ovariectomized hypertensive MREN2.LEWIS rats [21, 38]. This agent relaxes rat mesenteric arteries precontracted with either UTP or angiotensin II, human internal mammary arteries precontracted with prostaglandin F $_2\alpha$ and mouse carotid arteries precontracted with prostaglandin F $_2\alpha$, suggesting that the G-1-induced reduction of blood pressure is achieved by reducing vascular resistance [21, 38]. Acetylcholine-induced vascular relaxation was not affected by G-1, suggesting that G-1 does not act via stimulation of eNOS activity and increased NO production [38]. Relaxation of isolated vascular preparations is observed in response to 1–3 μ M G-1, which is in the same concentration range as that reducing proliferation of endothelial and vascular muscle smooth cells. Thus, the GPER1 agonist G-1 affects, in micromolar concentrations, vascular function by reducing vascular resistance but also by attenuation of endothelial and vascular smooth muscle cell proliferation.

The reduction in DNA synthesis by G-1 was unaffected by the ERK/MAP kinase inhibitor PD98059, suggesting that G-1 reduces endothelial cell DNA synthesis via another mechanism than by reducing ERK/MAP kinase activity. We used a concentration of PD98059 known to reduce ERK/MAP kinase activity [30] without having any effects on endothelial cell proliferation, suggesting that G-1 acts via another pathway than ERK/MAP kinase, but still we cannot completely rule out the possibil-

ity that G-1, at least in part, acts via ERK/MAP kinase inhibition. The bEnd.3 endothelial cells express a strong mRNA band for GPER1, suggesting that the antiproliferative effect of G-1 is mediated via GPER1. It is very well documented that COS-7 cells express no GPER1 or only weakly express this receptor [27, 28]. Here we show that G-1 reduces DNA synthesis by about 80% in GPER1-positive bEnd.3 cells, while it has no effect in COS-7 cells only weakly expressing GPER1 mRNA, suggesting that G-1-induced endothelial cell antiproliferation is mediated by GPER1. The low GPER1 expression level in COS-7 cells is probably not sufficient to evoke antiproliferation upon stimulation by G-1. Another support for the involvement of GPER1 is that the ER antagonist ICI182780, reported to act as a GPER1 agonist [22, 39], reduces endothelial cell DNA synthesis with similar potency as G-1. GPER1 siRNA abolished the G-1-induced attenuation of DNA synthesis, providing direct evidence for the involvement of GPER1. Taken together, these data show that G-1-induced endothelial cell antiproliferation is mediated by the GPER1.

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