G PROTEIN-COUPLED ESTROGEN RECEPTOR (GPER)-MEDIATED RELAXATION OF CORONARY ARTERIES IS MITIGATED BY PHOSPHORYLATION OF ERK1/2

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ABSTRACT

G Protein-Coupled Estrogen Receptor (GPER)-Mediated Relaxation of Coronary Arteries is Mitigated by Phosphorylation of ERK1/2

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GPER is a membrane-bound estrogen receptor, distinct from ERα or ERβ, and exerts genomic and non-genomic effects. GPER's effect on the cardiovascular system has been controversial; evidence indicates it relaxes arteries, whereas other findings suggest it contracts arteries. Our objective is to better understand the dual nature of GPER. Previously, our work demonstrated that G-1 stimulates cAMP production. I hypothesize GPER mediates relaxation response through cAMP and constriction via ERK1/2. Isometric tension studies were used to measure GPER-mediated coronary tone response in porcine coronary arteries. Western blots were applied to detect pERK1/2 in primary cell culture of smooth muscle cells. The identity of smooth muscle cells was validated by immunohistochemistry techniques using α actin as a marker. G-1 inhibited phosphorylation; however, under adenylyl cyclase inhibition by SQ22536, G-1 stimulated phosphorylation of ERK1/2. The effect of G-1 was blocked by G36, a GPER inhibitor. A time course of E2 (100 nM) demonstrated E2 acutely stimulated phosphorylation of ERK1/2. Tension studies demonstrated that G-1 caused concentration-dependent relaxation of PGF2α (1 μM) precontracted, endothelium denuded, coronary arteries. PD98059, a MEK inhibitor that blocks the phosphorylation of ERK1/2, led to further relaxation than G-1 alone. I

conclude that phosphorylation of ERK1/2 lessens the coronary artery relaxation caused by GPER.

DEDICATION

To my teachers throughout the years. Thank you for affirming my quirky love of learning, answering my never-ending stream of questions, and showing me kindness and friendship. I want to be you when I grow up.

And to Susan Harlow, who has taught me everything I know about compassion. Mom, you are my biggest supporter, closest confidant, dearest role model, silliest vacation buddy, and simply my best friend. My best days are those spent with you. I love you to the moon and back, and back again.

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NOMENCLATURE

CASMC	Coronary artery smooth muscle cells
CHD	Coronary heart disease
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
E2	Estrogen, estradiol
GPER	G-protein coupled estrogen receptor
HRT	Hormone replacement therapy
pERK1/2	Phosphorylated ERK1/2
PGF2α	Prostaglandin F2α

CHAPTER I

INTRODUCTION

Heart disease is the number one killer of women in the United States, according to the CDC [1]. Contrary to popular belief, the gender gap of cardiovascular disease decreases with age, even affecting more women than men in the elderly population [2]. By redefining the male-normalized standards for diagnosis [3], clinicians have identified "atypical" pattern of heart attacks and stroke and now recognize the epidemic, spurring the scientific community to investigate underlying risk factors and prevention, pathogenesis and therapy.

Hormone replacement therapy

Because women typically do not develop coronary heart disease (CHD) until after menopause [4], the estrogen present before menopause was considered to be a protective vasodilator. Dilation in coronary arteries allows better blood supply to the cardiac muscle itself, lowering the risk of cardiac hypoxia and possible heart attack [5]. The theory led to the reasonable assumption that simple replacement via hormone replacement therapy (HRT) would continue this protection [6, 7]. And in some ways, estrogen replacement therapy exhibits marked benefits, such as slowing progression of osteoporosis by limiting bone demineralization [8], treating depression associated with the hormonal change of menopause [9] and preventing eye disease [10].

However, risks of estrogen HRT were noticed as early as 1991, including higher incidence of gallbladder disease [11] and breast cancer [12, 13]. Save its beneficial reduction of LDL levels [14], postmenopausal estrogen HRT has been shown to not only fail to maintain the anticipated

protective effect [15, 16], but in many cases increase the risk of cardiovascular disease: increased plasma levels of inflammatory C Reactive Protein (CRP) [17] and increased incidence of cardiovascular events such as venous thromboembolism [18], heart attack and stroke [19]. In fact, the massive randomized trial by Women's Health Initiative studying postmenopausal estrogen hormone therapy was terminated due to emerging preliminary data of these risks, particularly in patients receiving combination therapy of estrogen and progestin [8]. Respectively, postmenopausal estrogen HRT is presently not standard clinical practice but used cautiously and under extenuating circumstances [20].

Estrogen

It is near universal knowledge that estrogen is the female sex hormone. Indeed, estrogen is pivotal in female reproductive health as it is synergistic in luteinization and ovulation in the ovaries and permissive to progesterone's effect on the uterus. Yet with receptors present in a variety of tissue types, estrogen exerts a wide array of cellular effects outside the reproductive realm, such as inhibiting proliferation and stimulating maturation of chondrocytes [21] and stimulating endothelial nitric oxide synthase (eNOS) in endothelium via transactivation of EGFR [22]. To this end, estrogen explains the disparity addressed by gender-specific medicine. Perhaps a better label of estrogen would be simply "the female hormone."

The idea that estrogen is a vasodilator began in 1884 when John Mackenzie amusingly linked sinus irritation with menstruation (conversely, the estrogen present at ovulation dilates the nasal passages) [23]. Modern research has greatly expounded its investigation of effects of estrogen on vascular cells: Raddino et al. found that blood flow in the heart was improved by acute administration of estrogen via decreasing resistance in the coronary vasculature walls [24],

a mechanism later delineated to involve relaxation of vascular smooth muscle cells, both directly and indirectly via stimulation of vasodilator nitric oxide release from endothelium [25] . However, in 2005, White et al. noted a vasoconstrictor nature of estrogen of coronary arteries *in vitro* [26]. Such seemingly contradictory results have brought estrogen to the forefront of scientific debate: is estrogen a pure vasodilator or a pure vasoconstriction? Alternatively, I posit that evidence supports a dual role of estrogen in the regulation of arterial tone, mediating *both* relaxation and contraction of vascular smooth muscle cells.

GPER

Estrogen has two classical receptors, ERα and ERβ. Both of these nuclear receptors trigger genomic responses via endothelium growth factor receptor (EGFR activation), causing long-term vasodilatory effects, likely via nitric oxide production [22]. Yet several discrepancies led to the suspicion of an estrogen receptor mechanistically different from ERα/ERβ [27]. For instance, estrogen been linked to the cAMP, a G-protein associated pathway [28], and nongenomic activation of eNOS [22], as well as non-nuclear subcellular localizations such as the plasma membrane [29].

Before its ultimate renaming to G protein-coupled estrogen receptor (GPER) by Prossnitz and Arterburn in 2007 [30], various instances of simultaneous discovery prompted several notable aliases: Owman et al. discovered the cDNA, "CMKRL2" [31] which was isolated and cloned by Carmeci et al. who coined the cloned protein "GPR30" (as it was an orphaned G protein-coupled receptor without its cognate ligand) [32]; Hawkins et al. identified it in 2000 as an estrogen receptor, naming it ERλ, [33], triggering the piecing together of the various identities [34, 35].

Though this 7-transmembrane α helical protein is primarily associated with the plasma membrane, other subcellular localizations such as the endoplasmic reticulum have been controversially reported [34, 36]. GPER has been found in most human tissues [37]: endothelium [38], lung, heart, lymphoid tissue [39], brain and peripheral nervous system [31], bone [40], renal tubules [41] as well as cancers of the breast [32], ovarian [42], endometrium [43], and some thyroid [44].

It is unusual for one ligand to target both nuclear receptors (classic steroid model) as well as plasma membrane receptors (such as GPCRs), which are typically reserved for hydrophilic ligands. Yet lipophilic estrogen binds to GPER and exerts effects via adenylyl cyclase and cAMP cascade, likely by MLCP activation [45]. Unlike other GPCRs, GPER also exerts genomic effects, likely via transactivation of EGFR [27] and ERK1/2 [46]. Mediating both rapid non-genomic and slow lingering non-genomic effects might explain the complexity and seeming incongruity among studies.

Objective

In my study, I investigated GPER as the possible underlining mechanism by which HRT causes adverse effects, e.g. higher incidence of heart attack and stroke [8]. Researchers have well-established GPER-mediated vasodilation, yet further investigation of GPER-mediated *vasoconstriction* has been largely overlooked. My objective is to further elucidate the dual nature of GPER by studying vascular tone in porcine coronary arteries. I hypothesize that GPER mediates relaxation via cAMP and constriction via ERK1/2.

CHAPTER II

MATERIALS AND METHODS

Experimental Rationale

First, I aim to link GPER activation with the phosphorylation of ERK1/2, accomplished through western blot techniques. Second, I aim to use isometric tension studies to prove a causal relationship between ERK1/2 phosphorylation (under various conditions of inhibition) and changes of vascular tone in coronary arteries. I propose that though often masked by GPER-mediated vasodilation, GPER mediates vasoconstriction via ERK1/2 pathway.

Ligand/agent	Receptor	Notes and Significance
Estrogen	ERα/ERβ agonist GPER agonist	Estradiol (E2) selectivity
CI182,780 (fulvestrant),	ERα/ERβ "pure" antagonist (eliminating constitutive activity) GPER agonist	Vasoconstrictor via ERα/ERβ and a vasodilator via GPER
G-1	ERα/ERβ: insignificant binding GPER: agonist	GPER-selective agonist
G36	GPER antagonist	Reverses effects seen by GPER. When paired with E2, isolates ERα/ERβ-specific effects
SQ22536	Inhibits adenylyl cyclase	Inhibits cAMP effects mediated by GPER (effects seen attributed to ERK1/2 pathway)
PD98059	MEK inhibitor	Upstream blockade of ERK1/2 activation
EGF	EGFR	Positive control

Table 1. Estrogen Receptor Ligands. All estrogen receptors bind estrogen with estriadiol (E2) selectivity. G-1 binding to ERα and ERβ is insignificant, thus G-1 is regarded as GPER-specific,

and is used to distinguish GPER activity from ERα/ERβ activity. ICI182,780 (fulvestrant), a "pure" ERα/ERβ antagonist binds with extraordinary high affinity, distorting the receptor shape and disrupting its constitutive activity [47] (unlike general antagonists), yet acts as an *agonist* to GPER [48, 49]. According to Han et al., this divergence of effects among the estrogen receptors explains how estrogen can be both a vasoconstrictor via ERα/ERβ and a vasodilator via GPER [50]. SQ22536 inhibits adenylyl cyclase, thus blocking cAMP-mediated effects (used in this study to block the vasodilation pathway). PD98059 inhibits MEK, the kinase directly responsible for activating ERK1/2.

Isolation of porcine coronary arteries

Freshly collected porcine hearts were obtained from a local slaughterhouse, K&C meat processing, and transported in 4°C Krebs solution. Coronary arteries were grossly dissected in 4°C Krebs solution.

Culture of porcine coronary artery smooth muscle cells

Grossly dissected coronary arteries were sliced open and mechanically endothelium-denuded by a cotton swab. Smooth muscle cells were enzymatically dispersed from vessel wall with collagenase, soybean trypsin inhibitor, and dissociation buffer mixture and shaken for 2 hours in a 37°C water bath, then washed repeatedly with 4°C PBS buffer. Primary cultured porcine coronary artery smooth muscle cells were treated with penicillin antibiotic (100 μg/ml) and streptomycin antifungals (100 μg/ml) then allowed to grow in Medium 231 with Smooth Muscle growth Supplement (GIBCO USA) in a 37°C humidified incubator under 5% CO_2-95% O_2. Coronary artery smooth muscle cells were cultured to 80% confluence and underwent 8-9 passages of culture.

Validation of smooth muscle cells

The identity of smooth muscle cells was validated by immunohistochemistry using α actin as a marker of smooth muscle cells, and DAPI (4', 6-diamidino-2-phenylindole) stain of nuclei.

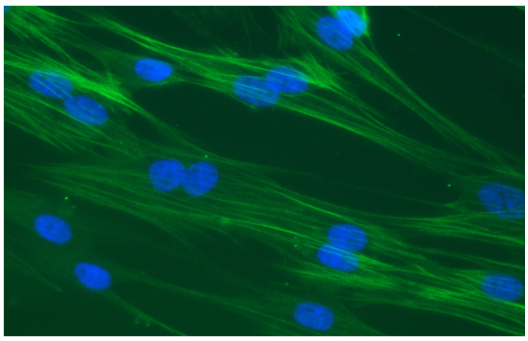

Figure 1. Immunohistochemistry slide of smooth muscle cells. Nuclei are stained blue, α actin in green, thus validating the cells' identity as smooth muscle cells.

Western blot

Cell cultures were serum deprived for 48 hours, then set in vehicle in phenol red free MEMα medium and treated for 1-2 days with various combinations of agonists and antagonists: pretreated with adenylyl cyclase inhibitor SQ22536, selective GPER agonist G-1 (1 μM) and GPER antagonist G36 (10 μM), and EGF (10 ng/ml) as positive control. Similar experiment was done with E2 (100 nM) at various times of administration (2, 5, 15, 30 min), without E2 as negative control, and EGF (10 ng/ml) as positive control, however under no adenylyl cyclase inhibition.

In order to stop the drug action, cells were washed with cold PBS, placed on ice, lysed for 5 min with RIPA lysis buffer with protease and phosphatase inhibitor. Next, cells were lifted from plates and centrifuged at 4°C for 20 min and stored in -20°C overnight. Loading buffer was

added to samples before boiling for 5 min. 15% gel with 4-12 uM size specificity was loaded and run at 200 volts for 40 min, then proteins were transferred onto blotting membrane using transfer buffer before being run at 100 volts for 90 min. Membrane was set in nonfat 5% milk and probed with 1:1000 rabbit pERK1/2 (primary antibody), rocked in fridge overnight. The next day, membrane was washed with cold PBS, incubated with 4% BSA-PBS for 1 hour, before administration of 1:5000 anti-rabbit IgG (secondary antibody) conjugated with FITC (PA1-29388 Thermo Scientific Pierce) for 1 hour at room temperature in the dark. After three washes with PBS, the coverslips were mounted for imaging.

Isometric tension studies

Porcine left anterior descending (LAD) artery was dissected, endothelium-denuded, and cut into 3mm rings which were then mounted on isometric myographs (DMT) filled with modified Krebs-Henseleit buffer bubbled with 95% O2-5% CO2 (pH = 7.4) at 37°C. Optimal resting tension was determined then used to equilibrate rings for 90 min before checking rings' contractility by administration of PGF2α, a vasoconstrictor prostaglandin. To block phosphorylation of ERK1/2, MEK inhibitor PD98059 was added to the treatment group 30 min prior to the measurement of a complete G-1 (selective GPER agonist) relaxation response (1 to 3000 nM).

Fig 2. Wire myograph system (DMT). Photograph of equipment used in large vessel isometric tension studies.

Statistical analysis

Data are presented as means ± standard deviation (SD). For statistical analysis, Prism program (GraphPad Software Inc., San Diego, CA) performed one-way or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test paired with repeated measures. The level of statistically significance was set as $P < .05$.

CHAPTER III

RESULTS

G-1 and pERK1/2

Western blot technique was employed to detect the pERK1/2 in porcine coronary artery smooth muscle cells treated with GPER agonist G-1, with and without adenylyl cyclase inhibition by SQ22536.

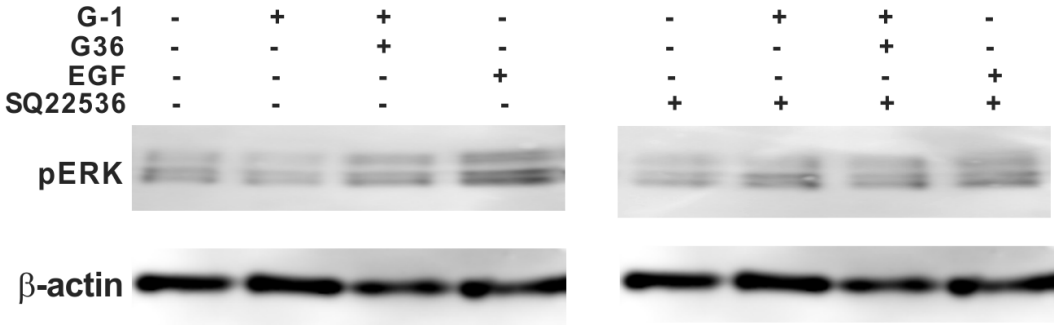

Figure 3. GPER activation by G-1 alone inhibits phosphorylation of ERK1/2. Under adenylyl cyclase inhibition by SQ22536, G-1 stimulates phosphorylation of ERK1/2. Western blot detection of phosphorylated ERK1/2 (pERK1/2). Porcine coronary artery smooth muscle cells were pretreated with or without adenylyl cyclase inhibitor SQ22526, then incubated with DMSO (solvent control), G-1 (1 μM), G36 (10 μM) + G-1 (1 μM), and EGF (10 ng/ml, positive control).

GPER activation with by G-1 alone inhibits phosphorylation or ERK1/2; an effect blocked by the GPER antagonist G36. However, under adenylyl cyclase inhibition by SQ22536, G-1 *stimulated* phosphorylation of ERK1/2. These results are consistent with my theory that GPER is a dual regulator of two converse pathways: blocking cAMP activation enhances ERK1/2 activation.

E2 and pERK1/2

Unlike G-1, which is GPER specific, E2 binds to all three of the estrogen receptors (ERα, ERβ and GPER) thus convoluting findings and variables. Therefore, I seek to elucidate a small piece of the puzzle: estrogen's relationship with phosphorylating ERK1/2.

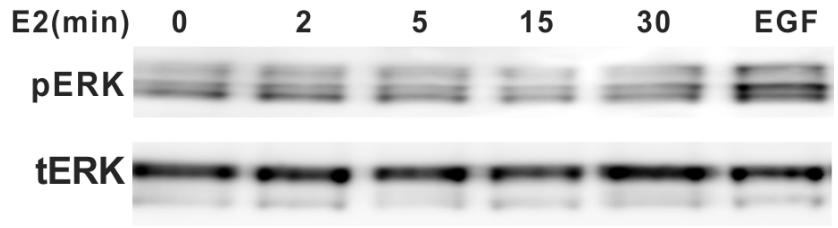

Figure 4. GPER activation by E2 stimulates phosphorylation of ERK1/2. Western blot detection of ERK1/2 of coronary artery smooth muscle cells incubated either without E2 (negative control, first column), with E2 (100 nM) administered at 2, 5, 15, or 30 min, or with EGF (10 ng/ml, positive control). Top row is phosphorylated ERK1/2, bottom row in total ERK1/2.

Contrary to G-1 treatment alone (see Fig. 3), treatment of E2 (100 nM) acutely *stimulates* phosphorylation of ERK1/2.

G-1's effect on vascular tone via ERK1/2

Performing an isometric tension study, I tested the effects of G-1-induced GPER activation on vascular tone under two conditions: G-1 alone, and G-1 with MEK inhibitor PD98059. Since MEK is the kinase directly responsible for phosphorylation (and thus activation) of ERK1/2, addition of PD98059 should reduce or reverse the actions of ERK1/2; if vascular tone is partially mediated by phosphorylation of ERK1/2, then the two condition groups should yield varying effects on vascular tone.

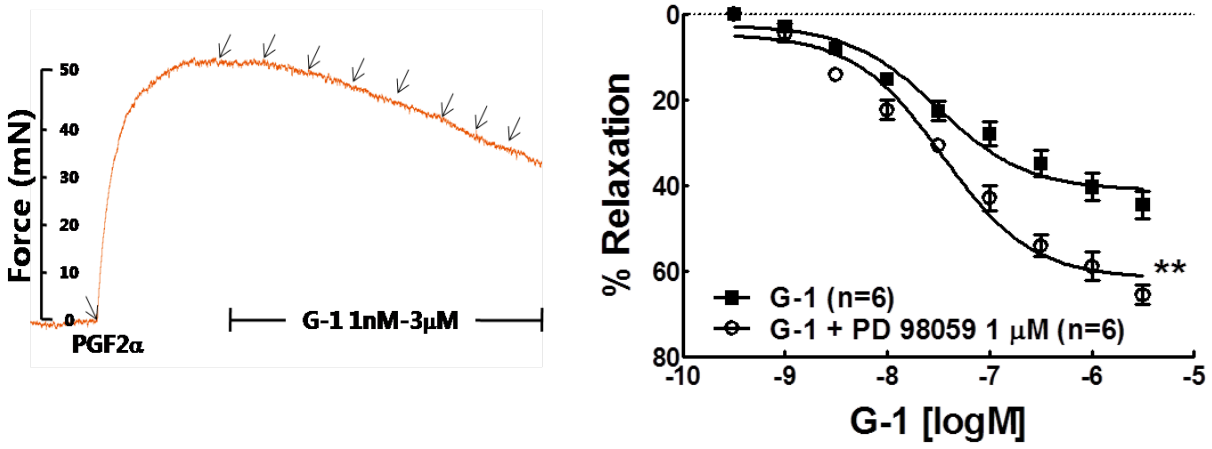

Figure 5. G-1 treatment induces porcine coronary artery smooth muscle cell relaxation, and PD98059 further increases G-1 –induced relaxation. Left: Typical trace of an isometric tension study. Initial basal vascular tone of coronary artery cross-sectional ring was defined as 0 mN (with force as a measure of constriction), before vessel was precontracted by PGF2α (1 μM). Right: Percent relaxation effect by administration of G-1, with and without addition of PD98059 (1 μM), a MEK inhibitor. The percent decrease of contraction caused by G-1 administration, as compared to the maximal contraction induced by PGF2α relaxation, was calculating for each data point using the formula:

$$\% \text{ Relaxation} = \frac{(F_{\text{maximal contraction induced by PGF2}\alpha}) - (F_{\text{contraction after G-1 added}})}{(F_{\text{maximal contraction induced by PGF2}\alpha})} \times 100$$

Results are expressed as mean relaxation effect ± SE of 6 experiments. **P < 0.01, compared with the G-1 only group by two-way ANOVA analysis.

G-1-alone treatment induced relaxation of precontracted coronary arteries in a concentration-dependent manner, whereas blocking phosphorylation of ERK1/2 by the administration of PD98059 increased the relaxation effect of G-1. Thus, it is reasonable to infer that the physiological converse is true: permitting phosphorylation of ERK1/2 mitigates GPER-mediated vasorelaxation. Taken together, these data support a vasoconstrictor component of GPER, and suggests this action to be mediated in part by phosphorylation of ERK1/2.

Summary of results

1. GPER activation by G-1 (1 μM) alone inhibits phosphorylation of ERK1/2. However, under adenylyl cyclase inhibition by SQ22536, G-1 stimulates phosphorylation of ERK1/2.
2. GPER activation by E2 (100 nM) acutely stimulates phosphorylation of ERK1/2, an opposite effect of G-1 treatment alone.
3. G-1 treatment induces coronary artery relaxation in a concentration-dependent manner. However, upstream inhibition increases the relaxation effect of G-1, indicating a vasoconstriction component of GPER activated by G-1.

CHAPTER IV

DISCUSSION

Phosphorylation of ERK1/2 lessens the coronary artery relaxation caused by GPER. Such mitigation implies a vasoconstrictive component of GPER that is mediated by ERK1/2 – a conclusion bolstering the support of the dual-regulator model of GPER. By mediating both vasorelaxation via cAMP and vasoconstriction via ERK1/2 cascade, GPER explains the paradoxical nature of estrogen on vascular tone. In these studies, vasodilation appears to be a more prominent pathway, possibly masking the vasoconstrictive component.

It is interesting to note that G-1 and E2 had opposing effects on the phosphorylation of ERK1/2: inhibition by G-1, stimulation by E2, or G-1 under adenylyl cyclase inhibition. This contrast might be explained by the difference of target receptors between G-1 and E2, an idea seemingly congruent with research area of selective estrogen receptor modulators (SERM).

A deeper understanding of GPER can lead to groundbreaking pharmacological therapies to inhibit estrogen's deleterious effect of vasoconstriction, while still permitting its many beneficial effects for the rest of the body. For instance, combination therapy with a GPER-selective antagonist to block the vasoconstrictive component of GPER activation might enable the safe resurgence of estrogen replacement therapy. Women seeking relief from hot flashes and protection from osteoporosis could resupply estrogen without risk of heart disease. Women requiring hysterectomies could have smoother hormonal transitions.

With more research of the scope, mechanism, and regulation of GPER's effects in the body, the full power of estrogen may be harnessed.

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