GPER-1 and Estrogen Receptor-β Ligands Modulate Aldosterone Synthesis

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Fertile women have lower blood pressure and cardiovascular risk than age-matched men, which suggests that estrogens exert cardiovascular protective effects. However, whether 17 β-estradiol (E2) blunts aldosterone secretion, and thereby affects the gender dimorphism of blood pressure, is unknown. We therefore sought for the estrogen receptor (ER) subtypes in human adrenocortical tissues ex vivo by performing gene and protein expression studies. We also investigated the effect of E2 on aldosterone synthesis and the involved receptors through in vitro functional experiments in the adrenocortical cells HAC15. We found that in the human adrenal cortex and aldosterone-producing adenoma cells, the most expressed ERs were the ERβ and the G protein-coupled receptor-1 (GPER-1), respectively. After selective ERβ blockade, E2 (10 nmol/L) markedly increased both the expression of aldosterone synthase and the production of aldosterone (+5- to 7-fold vs baseline, $P < .001$). Under the same condition, the GPER-1 receptor agonist 1-[4-(6-bromo-benzo (1, 3)dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone (G-1) (10 nmol/L) mimicked this effect, which was abrogated by cotreatment with either the GPER-1 receptor antagonist (3aS*,4R*,9bR*)-4-(6-Bro-mo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline (G-15), or a selective protein kinase A inhibitor 8-Bromo-2-monobutyryladenosine-3,5-cyclic monophosphorothioate, Rp-isomer. Silencing of the ERβ significantly raised aldosterone synthase expression and aldosterone production. Conversely, silencing of the GPER-1 lowered aldosterone synthase gene and protein expression. Moreover, it blunted the stimulatory effect of E2 on aldosterone synthase that was seen during ERβ blockade. These results support the conclusion that in humans, E2 inhibits aldosterone synthesis by acting via ERβ. Pharmacologic disinhibition of ERβ unmasks a potent secretagogue effect of E2 that involves GPER-1 and protein kinase A signaling. **(*Endocrinology* 155: 4296–4304, 2014)**

Fertile women are at lower risk of cardiovascular (CV) events and have lower blood pressure (BP) values than age-matched men (1, 2); for example, among hypertensive patients recruited in the ONTARGET trial, women had a 22% lower risk for myocardial infarction than men (3). Therefore, estrogens can decrease BP and thereby CV risk,

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Abbreviations: APA, aldosterone-producing adenoma; BP, blood pressure; $[Ca^{2+}]_i$, intracellular calcium; CCS, cosmic calf serum; Ct, threshold cycle; CV, cardiovascular; CYP11B2, aldosterone synthase gene; DCC-CCS, dextran-coated charcoal-stripped CCS; E2, 17 β-estradiol; ER, estrogen receptor; ESR2, estrogen receptor beta gene; G-1, 1-[4-(6-bromo-benzo (1, 3)dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G-15, (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline; GPER-1, G protein-coupled receptor-1; H89, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; IHC, immunohistochemistry; MPP, 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; NAC, normal human adrenal cortex; PA, primary aldosteronism; PBGD, porphobilinogen deaminase; PKA, protein kinase A; Rp-8-Br-MB-cAMPS, 8-Bromo-2-monobutyryladenosine-3,5-cyclic monophosphorothioate, Rp-isomer; siRNA, small interfering RNA; THC, (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; ZG, zona glomerulosa.

possibly by affecting the renin-angiotensin-aldosterone system and the sympathetic nervous system, which feature major gender-related differences (4).

Estrogens affect their target tissues via activation of the classical estrogen receptors (ERs) ERα and ERβ and also via a more recently identified G protein-coupled receptor (GPER)-1 called GPER-1, also known as GPR30 (5, 6), whose potential role in CV and endocrine regulation is scarcely known (7).

Aldosterone plays a key role in the regulation of BP by affecting sodium and volume homeostasis and by increasing vascular tone and inducing vascular remodeling (8). Accordingly, hyperaldosteronism is held to be instrumental in raising BP in patients with primary aldosteronism (PA), the most common form of endocrine hypertension, as well as in those with the metabolic syndrome (9–12). Notwithstanding the importance of hyperaldosteronism in arterial hypertension and also in heart failure and left ventricular dysfunction (13, 14), the mechanisms resulting in chronic hyperaldosteronism are poorly known. A role for an aberrant expression of ectopic receptors, including the ERs, and/or for a malfunctioning of eutopic receptors has, however, been suggested in PA, at least in a subset of the patients (15, 16). Moreover, even though a clear-cut gender dimorphism was identified in the prevalence of the recently discovered Kir3.4 mutations (17, 18), it is unclear whether estrogens play any pathophysiologic role in PA. This is not surprising, given that information on the expression and functional role of ER subtypes in the human adult adrenal cortex and in PA is scarce.

In the human fetal adrenal gland, the levels of expression of ERα and ERβ were found to be low and high, respectively (19, 20). In the postadrenarche adrenal gland, ERα was barely detectable, the ERβ was detected in the zona reticularis, and the GPER-1 was found in the zona glomerulosa (ZG) and the medulla (21). Moreover, even though it was reported that in a human adrenocortical carcinoma cell line (H295R) estrogens affect cell proliferation acting via ERβ (22), information on the expression and cortical zonation of the ERs in the adult human adrenal gland and in aldosterone-producing adenoma (APA) was scant. Furthermore, whether estrogens play any role in regulating aldosterone secretion remains unknown. If proven, this could shed new mechanistic light on the gender dimorphism of BP and CV risk. Therefore, this study had 2 major ends: 1) to determine whether 17 β-estradiol (E2) affects aldosterone synthesis and 2) to identify the receptor subtype(s) that can be involved in this action in humans.

Materials and Methods

Patients and tissues

We obtained APA tissue from 30 patients (16 women and 14 men). The diagnosis of APA was confirmed by the "4 corner criteria," which comprised biochemical features of PA, lateralization of aldosterone secretion, finding of an adenoma at surgery and pathology, and demonstration of normokalemia and cure or improvement of hypertension at follow-up after adrenalectomy (10). Clinical and biochemical features at baseline and follow-up in adrenalectomized APA patients divided according to gender are reported in Supplemental Table 1. As control/reference tissue, we used 5 histologically normal human adrenal cortex (NAC) specimen obtained at surgery from renal cancer patients undergoing unilateral nephrectomy and ipsilateral adrenalectomy. All tissues were obtained under sterile conditions in the operating room immediately after excision, frozen in liquid nitrogen, and stored at −80°C. The creation of a biobank of human adrenal tissues was approved by the Ethics Committee; informed consent was obtained from each patient.

Immunohistochemistry (IHC)

From paraffin blocks, 4-μm-thick serial sections were prepared for IHC. Details of the technique used are given in the Supplemental Methods. As negative controls, normal rabbit serum or normal mouse serum was used instead of primary antibodies. HAC15 cells were obtained and used for immunocytochemistry staining as described (23).

Immunoblotting

Immunoblotting for ERβ, GPER-1, and aldosterone synthase gene (CYP11B2) was performed following standard protocol. Protein samples were obtained from APA tissues (n = 6) and the adjacent normal adrenal gland (n = 6). Details of the methods used are given in the Supplemental Methods. Images were analyzed by Molecular imager VersaDoc system (Bio-Rad). Bands for ERβ, GPER-1, and CYP11B2 were normalized to glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling).

Membrane protein isolation

HAC15 cells at confluence (80%) were labeled with Sulfo-NHS-SS-biotin. The biotin-labeled membrane proteins were isolated from membrane fraction using NeutrAvidin Agarose (Thermo Scientific) as described in the Supplemental Methods. By centrifugation (10 000*g* for 2 min at 4°C), they were divided in 2 cellular fractions: a membrane fraction (total membrane proteins) and an intracellular fraction (intracellular proteins) (24).

APA primary cell cultures

Primary cultures of APA cells were obtained from APAs (n = 3) as detailed in the Supplemental Methods. Briefly, we used an immunoseparation technique based on CD56 precoated magnetic beads to obtain aldosterone-producing cells, as described (25).

HAC15 adrenocortical cell line

HAC15 adrenocortical cells (a gift of Professor W. E. Rainey, University of Michigan, Ann Arbor, MI) were originally isolated

from an 11-month-old girl with hypertension and hirsutism due to an adrenocortical carcinoma (26). They were grown in DMEM/F12 medium supplemented with 10% cosmic calf serum (CCS) (Thermo Scientific), 1% insulin/transferrin/selenium Premix (ITS plus; BD Biosciences), and 1% antibiotic/antimycotic mixture. For the experiments, they were seeded in 12-well plates at 5×10^5 cells per well and grown to subconfluence. Before treatment, they were synchronized by incubation for 48 hours in phenol red-free DMEM/F12 with 3% dextran-coated charcoal-stripped CCS (DCC-CCS) and then treated with 10 nmol/L E2 for 12 hours in 3% DCC-CCS supplemented medium alone or with a selective a selective ERα antagonist (10 μmol/L 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride [MPP]; Sigma-Aldrich), a selective ERβ antagonist (10 μmol/L (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol [THC]; Tocris Biosciences), a selective ERα and ERβ antagonist (10 μmol/L MPP; 10 μmol/L THC), and a selective GPER-1 receptor antagonist (10 μmol/L (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta [c]quinoline [G-15]; Sigma-Aldrich). The cells were also exposed to the selective GPER-1 agonist 1-[4-(6-bromobenzo (1, 3)dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone (G-1) (10 nmol/L; Sigma-Aldrich), alone or in the presence of MPP, or THC, or MPP and THC, or G-15. E2 and G-1 were used at concentrations that provided the maximal response based on determination of the concentration-response curves. To warrant complete receptor blockade, the antagonists MPP, THC, and G-15 were used at doses 10^3-fold higher than those of the agonists.

RNA extraction and quantitative real-time PCR

The integrity and quality of total RNA, extracted with the RNeasy Mini kit (QIAGEN), was systematically checked with a laboratory-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA 6000 Nano Assay (Agilent Technologies). One microgram of total RNA was reverse transcribed with Iscript (Bio-Rad) in a final volume of 20 μL.

The ERα, its variant ERα36, ERβ, and GPER-1 mRNAs were measured in HAC15 cells, in NAC, and in APA by real-time RT-PCR with Universal ProbeLibrary Probes (Roche) in the LightCycler 480 Instrument (Roche). The sequences of primers used in real-time PCR are reported in Supplemental Table 2. The ERα, ERβ, and GPER-1 mRNAs were also measured in APA CD56+ cells. The reaction efficiency was optimized by preliminarily performed standard curves that were thereafter used for quantification of the ER genes (ERα, ERα36, ERβ, and GPER-1) using the ratio of target gene and the housekeeping gene porphobilinogen deaminase (PBGD) threshold cycle (Ct).

To assess the effects of E2 in HAC15 cells, the expression of aldosterone synthase (CYP11B2) mRNA was examined using real-time RT-PCR. After treatment, CYP11B2 gene expression was calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method: each sample was quantified against its PBGD transcript content and normalized to the control group. Each experiment was repeated 4 times in duplicate, and the results are presented as mean fold increase ± SEM.

Aldosterone measurement

Aldosterone levels were quantified with the Aldosterone Elisa kit (Alpha Diagnostic International); cells were treated with 10 nmol/L E2 or G-1 for 24 hours alone, or with a selective ERα antagonist (10 μmol/L; MPP) and a selective ERβ antagonist (10 μmol/L; THC), or with a selective GPER-1 receptor antagonist (10 μmol/L; G-15) plus MPP and THC.

The cells were also exposed to the selective GPER-1 agonist G-1, alone or in the presence of MPP and THC, or MPP and THC and G-15. When receptor antagonists were used, they were added in fresh media 1 hour before E2 or G-1 stimulation. Cell culture medium was collected after 24 hours of stimulation. A total of 50 μL of cell medium was added to aldosterone-coated wells following producer's protocol, and the signal was detected in an ELISA Reader (PerkinElmer). Aldosterone levels were normalized to the amount of cell RNA content.

Small interfering RNA (siRNA) and transfection

The expression of ERβ and GPER-1 was silenced in HAC15 cells by RNA interference using the Nucleofector technology (Amaxa Biosystems) following the manufacturer's protocol. To silence ERβ, cells were transfected with 50nM ON-TARGET plus estrogen receptor beta gene (ESR2) siRNA (ERβ siRNA; Dharmacon). To silence GPER-1, they were transfected with 50nM ON-TARGET plus GPER-1 siRNA (GPER-1 siRNA; Dharmacon). An ON-TARGET plus Nontargeting Pool (Dharmacon) transfection was used as control in mock-transfected cells. All cells were seeded in 6-well culture plates at the density of 1×10^6 cells per well. After 24 hours of transfection, total RNA isolation and real-time quantitative RT-PCR for ERβ, GPER-1, and CYP11B2 were performed. After 48 hours, protein extraction was performed followed by immunoblotting analysis for ERβ, GPER-1, and CYP11B2.

E2-activated GPER-1-mediated signaling pathway

We examined some putative intracellular pathways to elucidate the mechanisms of E2-activated GPER-1-mediated effects on aldosterone production. Intracellular Ca^{2+} changes induced by E2 stimulation were investigated in Fura 2-AM-loaded HAC15 cells. Measurement of intracellular calcium ($[Ca^{2+}]_i$) was determined in cultured HAC15 by fluorescence spectrophotometry (27). The baseline fluorescence was obtained by alternating rapidly the excitation wavelength between 340 and 380 nm and recording the 510-nm emission intensity. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio recordings according to the standard formula: $[Ca^{2+}]_i = Kd\ [(R - Rmin)/(Rmax - R)](Sf2/Sb2)$. Dissociation constant (Kd) was taken as 224 nmol/L; Rmax (340/380 ratio under Ca^{2+}-saturating conditions), Rmin (340/380 ratio under Ca^{2+}-free conditions;), and Sf2/Sb2 (ratio of baseline fluorescence) were calculated by a calibration curve with buffers containing different Ca^{2+} concentrations; 10 nmol/L E2 was added when baseline fluorescence was stable; and 100 nmol/L Angiotensin II (AngII) was used as positive control.

We also explored the potential involvement of protein kinase A (PKA) and cAMP in the increase of CYP11B2 induced by GPER-1 stimulation. To this aim, HAC15 cells were treated with E2 (10 nmol/L) or G-1 (10 nmol/L) in addition to MPP (10 μmol/L) and THC (10 μmol/L) in the presence of 8-Bromo-2-monobutyryladenosine-3,5-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-MB-cAMPS) (10 μmol/L; Sigma-Aldrich), a selective inhibitor of PKA or in the presence of N-[2-(p-Bromo-

cinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) (10 μmol/L), an inhibitor of PKA.

Statistical analysis

Results were expressed as mean ± SEM. Differences between groups were analyzed for significance by *t* test; multiple groups were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Statistical significance was set at $P < .05$.

Results

Expression of ER subtypes in the human adrenal cortex and in HAC15 cells

All known ERs resulted to be expressed in the NAC (n = 5), in APA tissues (n = 30), and in APA CD56+ cells (n = 3), albeit in varying amounts. When considering ER gene expression within each tissue type, abundant ERβ mRNA levels were seen in NAC and APA tissue (Figure 1, A and B). GPER-1 transcript showed the highest expression levels in the NAC tissue and in the APA CD56+ cells, a purified population of aldosterone-producing cells (Figure 1C).

The transcript of ERα and its variant ERα36 were low in all adrenocortical specimen, including NAC, APA, and APA CD56+ cells. Thus, in the normal human adult adrenal cortex, the rank of expression of ERs was ERβ > GPER-1 > ERα > ERα36 (Figure 1A). At variance, in APA and in APA CD56+ cells, it was GPER-1 > ERβ > ERα > ERα36 (Figure 1, B and C).

ER proteins were immunochemically detected in the NAC (n = 3) and in APA tissues (n = 3). At IHC, the ERα was barely seen at the nuclear level in the ZG and in APA cells (Supplemental Figure 1, A and D). The ERβ was detectable in the nucleus and cytoplasm of both NAC and APA cells (Supplemental Figure 1, B and E). The immunostaining was much stronger for GPER-1 than for ERβ in the ZG; unlike ERα and ERβ that localized mainly in the nucleus, in both NAC and APA cells, GPER-1 predominated on the plasma membrane and cytoplasm (Supplemental Figure 1, C and F).

Immunoblotting (Figure 2) confirmed the aforementioned hierarchy of ER genes expression: ERβ and GPER-1 were the predominant subtypes in NAC (n = 6) and APA tissues (n = 6), respectively, the ERα was scantly detectable in all the examined tissues (data not shown).

The transcripts of all ER subtypes were detectable in HAC15 cells. ERα immunostaining was weak in the nuclei (Supplemental Figure 2A), ERβ immunostaining was strong in both cytoplasm and nucleus (Supplemental Figure 2A), and GPER-1 staining predominated in the plasma membrane and the cytoplasm (Supplemental Figure 2A).

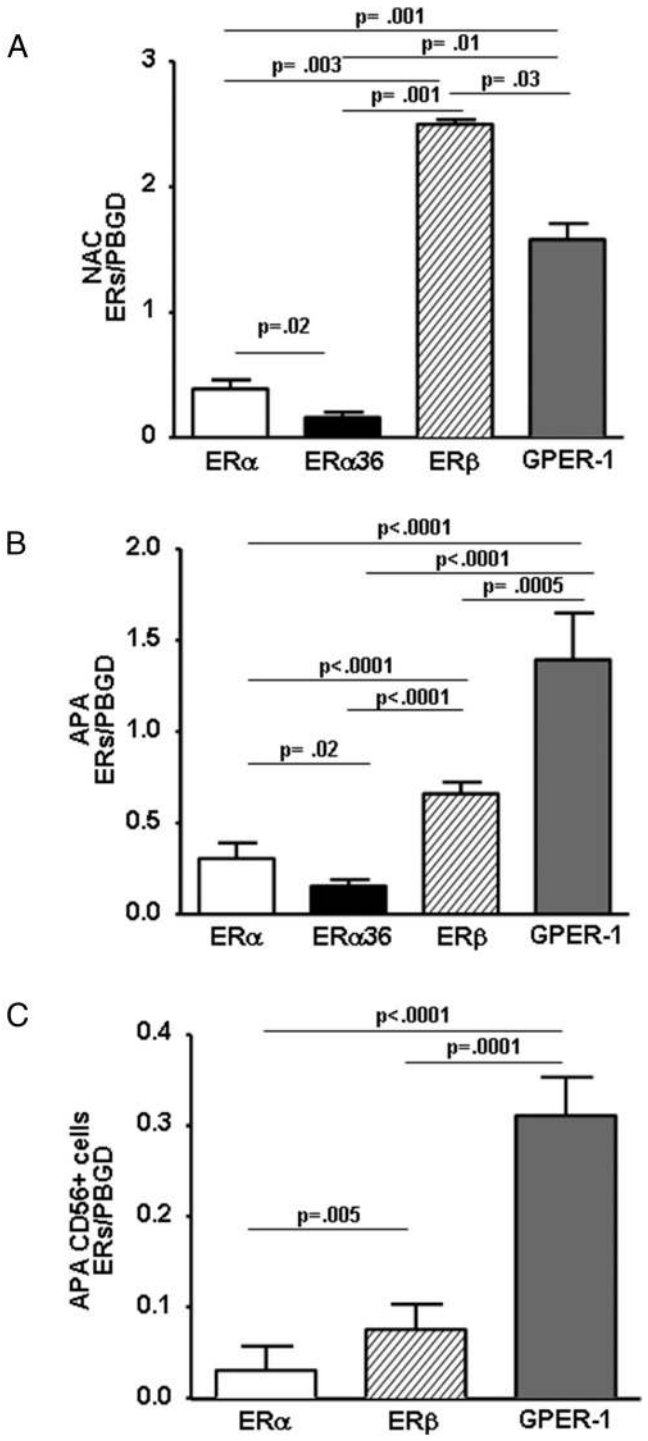

Figure 1. Relative amount of the different ERs subtypes ERα, ERα36, ERβ, and GPER-1 by real-time RT PCR in the normal adrenal cortex (NAC) (n = 5) (A), in aldosterone-producing adenoma (APA) (n = 30) (B) and in CD56+ cells from different APA (n = 3) (C). In the normal adrenal cortex, ERβ was the most expressed ER subtype, whereas in APA, GPER-1 was significantly higher than the ERβ. Expression is calculated as the ratio of Ct of target gene (ERs) and housekeeping gene (PBGD) Ct.

To sublocalize GPER-1 in HAC15 cells, we biotinylated the plasma membrane and isolated the biotin-labeled membrane proteins from the membrane fraction using

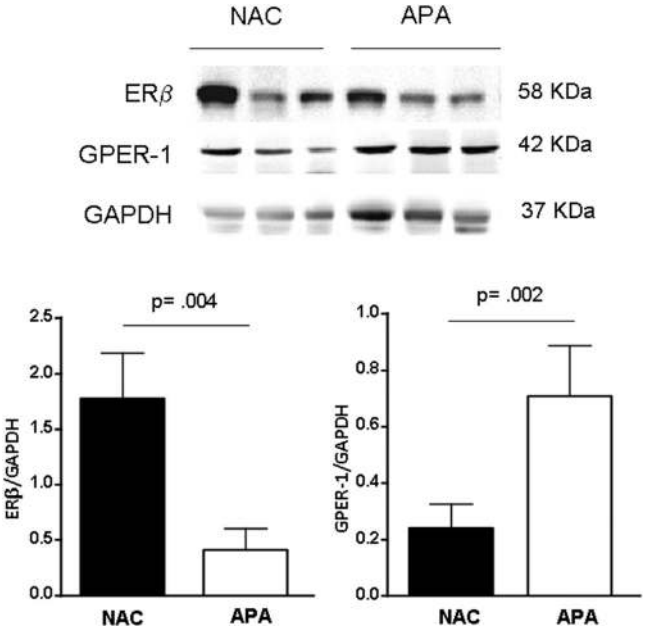

Figure 2. Immunoblotting of ERβ and GPER-1 protein in APA (n = 6) and in the normal adjacent adrenal cortex (n = 6) (upper panel). Relative quantification is shown in the bar plots (lower panel).

streptavidin. This technique allowed detection of GPER-1 in both the membrane (M proteins) and the intracellular proteins (I protein) fraction (Supplemental Figure 2B).

E2 effects on CYP11B2

Because the HAC15 cells were found to express all E2 receptor subtypes, we could use them as a model for investigating the modulation of CYP11B2 expression by E2. After establishing the concentration-response curve for the various agonists, we used the antagonist concentration that provided the maximal response. Cells were exposed to E2 (10 nmol/L) alone, or after pretreatment with the selective ERα antagonist MPP (10 μmol/L), or the selective ERβ antagonist THC (10 μmol/L), or the ERα antagonist MPP (10 μmol/L) combined with the ERβ antagonist THC (10 μmol/L) (Figure 3A).

Incubation of HAC15 cells with E2 alone, or after ERα antagonism with MPP, did not alter CYP11B2 expression. By contrast, E2, when given in addition to ERβ antagonism (with THC), caused a 2.5-fold increase in CYP11B2 gene expression (Figure 3A). The increase of CYP11B2 was even more prominent when cells were treated with both MPP and THC in the presence of E2 (Figure 3A). At variance, the cells treated with MPP alone, or with THC alone, did not increase CYP11B2 gene expression (data not shown). Therefore, E2 activates CYP11B2 transcription during antagonism of either the ERβ, or of both the ERα and the ERβ. Hence, collectively, these results evidence 1) a tonic ERβ subtype-mediated inhibitory effect of E2 on CYP11B2 transcription; 2) a pharmacologic disihibition of this effect of E2 when the

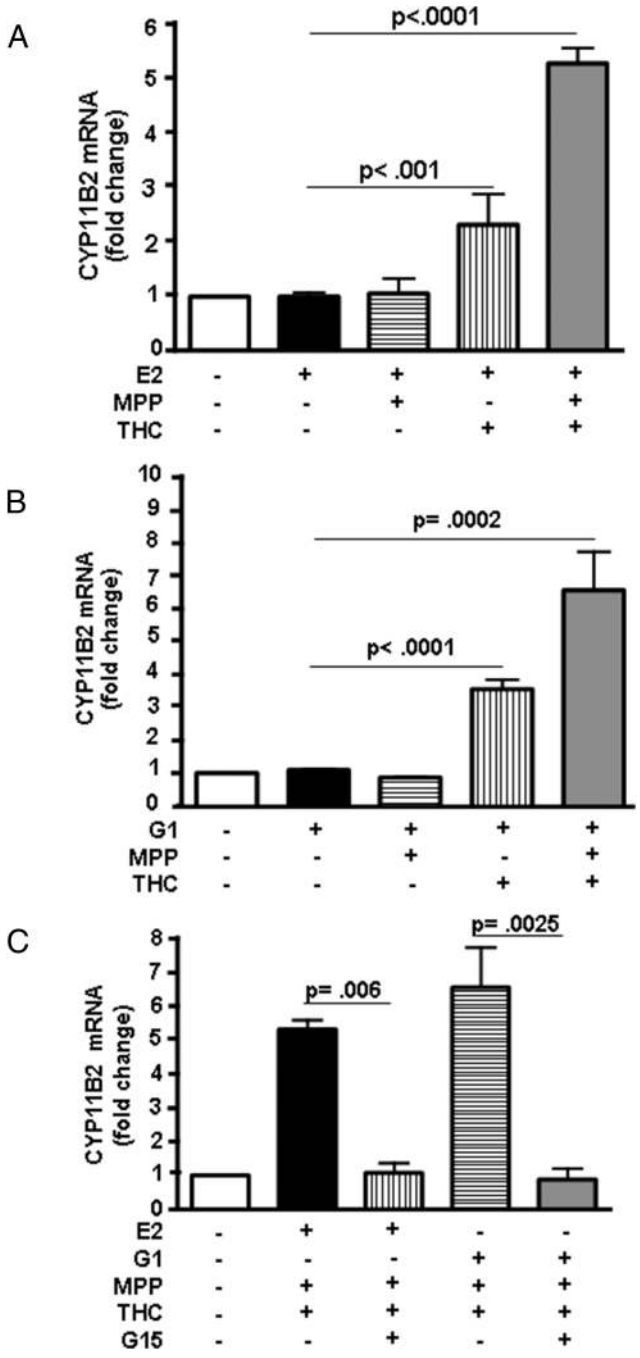

Figure 3. A, Aldosterone synthase (CYP11B2) gene expression after exposure of HAC15 cells to 10 nmol/L estradiol (E2) alone or in addition to a selective ERα antagonist (MPP) (10 μmol/L), a selective ERβ antagonist (THC) (10 μmol/L), a selective ERα and ERβ antagonist (MPP 10 μmol/L + THC 10 μmol/L) (mean ± SEM, n = 5 in duplicate). B, CYP11B2 expression after treatment with a selective GPER-1 agonist 10 nmol/L G-1 alone or on top MPP, or THC, or MPP and THC (mean ± SEM, n = 5 in duplicate). C, E2- or G-1-induced CYP11B2 gene expression in HAC15 cells after pretreatment with a selective ERα antagonist (MPP) and a selective ERβ antagonist (THC), and/or the selective GPER-1 antagonist G-15 (10 μmol/L) (mean ± SEM, n = 5 in duplicate). Abbreviations: G-1, 1-[4-(6-bromo-benzo (1, 3)dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone; G-15, (3aS*,4R*,9bR*)-4-(6-Bro-mo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline; MPP, 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; THC, (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol.

ERβ is effectively antagonized; and 3) a secretagogue effect of E2 on aldosterone acting via GPER-1 receptor after this pharmacologic disinhibition.

To confirm this hypothesis, we incubated HAC15 cells with the selective GPER-1 agonist G-1 (10 nmol/L), alone or with MPP (10 μmol/L), or THC (10 μmol/L), or MPP (10 μmol/L) plus THC (10 μmol/L) (Figure 3B). When given alone, G-1 left CYP11B2 gene expression unaffected. However, when given in addition to ERβ (THC), or combined ERα and ERβ blockade (MPP + THC), it markedly increased CYP11B2 gene expression (Figure 3B). These results evidence a role of GPER-1 in mediating the E2-induced stimulation of aldosterone synthase expression after ERβ blockade.

To further demonstrate that E2 activated CYP11B2 expression via GPER-1, HAC15 cells were pretreated for 1 hour with the selective GPER-1 antagonist G-15 (10 μmol/L) alone, or with MPP (10 μmol/L) and THC (10 μmol/L). Although G-15 alone did not affect CYP11B2 gene expression (data not shown), when ERβ was antagonized alone or along ERα (with MPP and THC), G-15 abrogated the increase of CYP11B2 expression induced by either E2 (10 nmol/L) or G-1 (10 nmol/L) (Figure 3C).

Aldosterone production

We measured aldosterone concentration in the supernatant of HAC15 cells at baseline and after 24 hours of stimulation with E2 (10 nmol/L) or G-1(10 nmol/L) alone, or in addition to MPP (10 μmol/L) and THC (10 μmol/L), or MPP (10 μmol/L), THC (10 μmol/L), and G-15 (10 μmol/L). When given alone, neither agents affected aldosterone secretion (Figure 4). By contrast, when administered in the presence of MPP and THC, both E2 and G-1 increased aldosterone levels (Figure 4). Pretreatment with G-15 abolished E2-induced aldosterone secretion under ERα and ERβ blockade with MPP and THC (Figure 4).

Effects of ERβ and GPER-1 knock-down

We used siRNA technology to further prove the functional role of ERβ and GPER-1 in modulating aldosterone synthase expression and aldosterone production. HAC15 cells mock transfected (with non targeting siRNA) were used as control. Silencing of HAC15 cells with ERβ siRNA significantly reduced the mRNA and protein expression of ERβ by 67% and 42%, respectively (Figure 5A). At same time, it increased the CYP11B2 mRNA and protein expression by 62% and 93%, respectively (Figure 5C), and aldosterone production by 17% as compared with mock-transfected cells (data not shown).

Silencing GPER-1 in HAC15 cells significantly reduced the mRNA and protein expression of GPER-1 by 81% and 40%, respectively (Figure 5B); it also lowered the

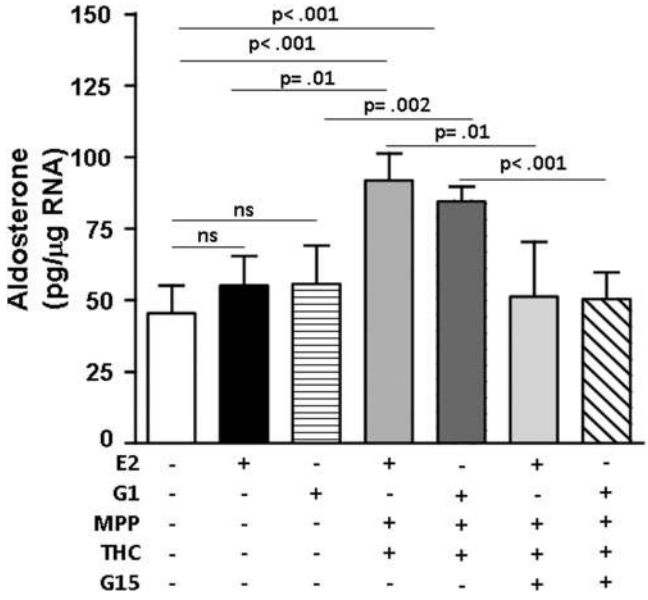

Figure 4. Aldosterone secretion of HAC15 cells after 24 hours of exposure to 10 nmol/L E2 or 10 nmol/L G-1 alone or in addition to a selective ERα antagonist (MPP) (10 μmol/L) and a selective ERβ antagonist (THC) (10 μmol/L) with or without the selective GPER-1 antagonist G-15 (10 μmol/L) (mean ± SEM, n = 3 in triplicate).

CYP11B2 mRNA and protein expression by 53% and 72%, respectively, as compared with mock-transfected cells (Figure 5D).

HAC15 cells silenced for GPER-1 were incubated with E2 (10 nmol/L), alone or in addition to ERβ blockade (THC 10 μmol/L). After GPER-1 silencing, E2 either alone or in addition to ERβ antagonist, did not increase CYP11B2 gene expression (Figure 5E), thus conclusively proving that E2-activated CYP11B2 under ERβ antagonism requires GPER-1.

E2-induced GPER-1-mediated signaling pathway

Exposure of HAC15 cells to E2 alone, or in the presence of the different antagonists (THC or MPP + THC), elicited no discernible change of $[Ca^{2+}]_I$ levels (Supplemental Figure 3A), thus indicating that neither the ERβ inhibitory effect nor the GPER-1-mediated stimulatory effect of E2 on aldosterone involves increased $[Ca^{2+}]_i$.

The results were altogether different when the specific PKA inhibitor Rp-8-Br-MB-cAMPS was used. Pretreatment with this agent (10 μmol/L), which by itself had no effect, abrogated the increase of CYP11B2 expression induced by either E2 (10 nmol/L) or G-1 (10 nmol/L) during combined ERα and ERβ blockade (with MPP and THC) (Figure 6). Similar results were found using H89, another, possibly less specific, PKA inhibitor (Supplemental Figure 3B). Hence, collectively, these results suggest that the GPER-1-mediated secretagogue effect on aldosterone is PKA-cAMP dependent.

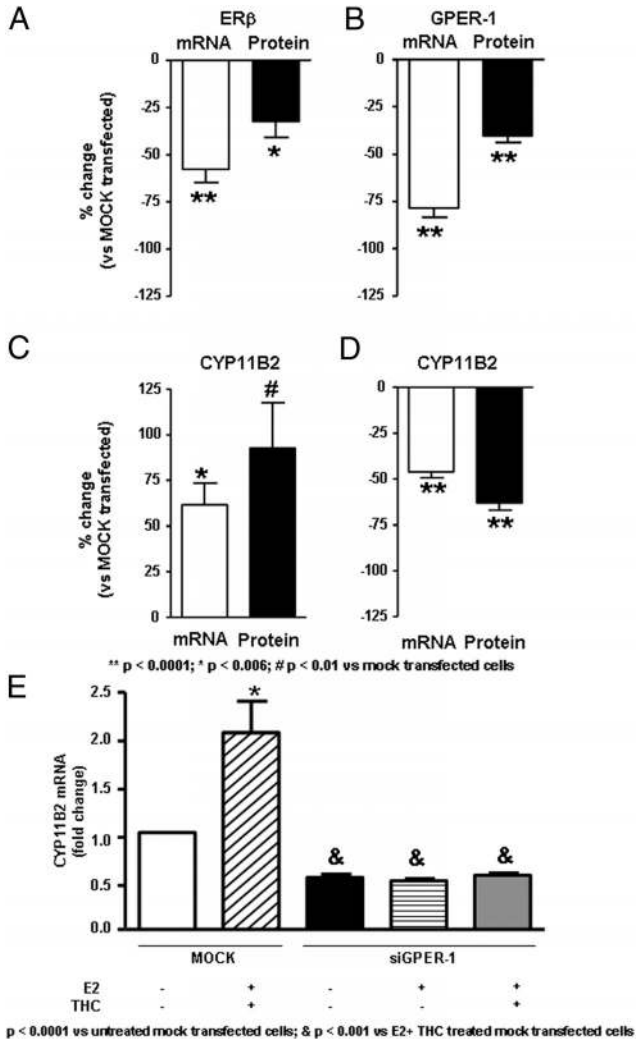

Figure 5. ERβ and CYP11b2 mRNA and protein levels in HAC15 cells after transfection with ERβ siRNA compared with mock-transfected cells (mean ± SEM, n = 2 in triplicate) (A and C). GPER-1 and CYP11b2 mRNA and protein levels in HAC15 cells after transfection with ERβ siRNA compared with mock-transfected cells (mean ± SEM, n = 2 in triplicate) (B and D). CYP11B2 gene expression after exposure of mock transfected HAC15 cells to 10 nmol/L E2 in addition to a selective ERβ antagonist (THC) (10 μmol/L) and of silenced GPER-1 cells to 10 nmol/L E2 alone or in addition to a selective ERβ antagonist (THC) (10 μmol/L) (mean ± SEM, n = 2 in triplicate) (E).

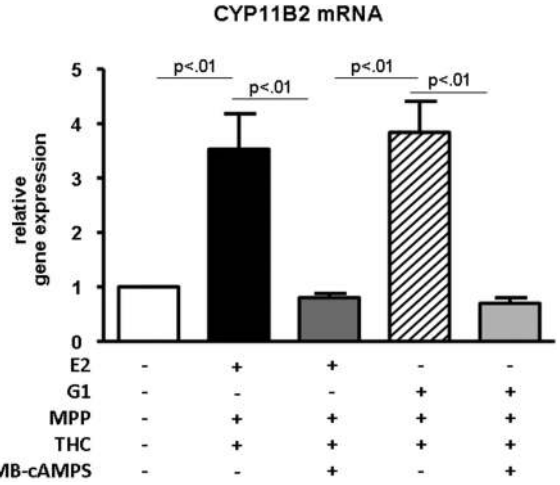

Figure 6. E2- or G-1-induced CYP11B2 gene expression in HAC15 cells after pretreatment with Rp-8-Br-MB-cAMPS (10 μmol/L), a selective inhibitor of cAMP-dependent protein kinase (PKA) in addition to a selective ERα antagonist (MPP) (10 μmol/L) and a selective ERβ antagonist (THC) (10 μmol/L) (mean ± SEM, n = 3 in triplicate). Abbreviations: Rp-8-Br-MB-cAMPS, 8-Bromo-2-monobutyryladenosine-3,5-cyclic mono-phosphorothioate, Rp-isomer.

Discussion

The investigation of ERs and their role in regulating aldosterone synthesis in humans has been focused only on the classical nuclear ERs thus far. Using ex vivo and in vitro studies in the normal human adult adrenal cortex and in APA tissues, we here showed that, consistently with previous findings (19), the ERβ mRNA is far more abundant than the ERα and its variant ERα36. Moreover, we demonstrated that the GPER-1 is detectable also in the zona fasciculata and reticularis but is highly expressed in the normal human adrenocortical ZG. These findings are novel in that they evidenced the expression of GPER-1 in the portion of the adult human adrenal cortex that produces aldosterone. They also established the hierarchy of ERs expression in the normal adult human adrenal cortex.

Of much interest for human hypertension, only 2 previous reports of the ERβ expression in APA exist, but none reported the expression of GPER-1 in these tumors (21, 28–30). Hence, this study evidences for the first time not only the expression of GPER-1 in APA but also the fact that GPER-1 is expressed at a higher amount than ERβ, a finding that is potentially relevant for the pathophysiology of PA, a common cause of human high BP (10).

Based on this evidence, we sought to establish the functional role of the different ERs in the regulation of aldosterone synthesis in vitro. To this aim, we could use HAC15 adrenocortical cells, because we found that they are endowed with GPER-1, ERα, and also ERβ. Using functional studies in this cell model, we found that E2 inhibits aldosterone synthesis by acting via ERβ, because after ERβ blockade and also after the molecular knockdown of the ERβ with a siRNA strategy E2 or G-1 stimulated aldosterone synthesis. Consistently with a tonic inhibitory role of ERβ, we found that both the CYP11B2 mRNA and protein amount and the aldosterone production were enhanced in ERβ knock-down cells.

After blockade or knock-down of ERβ, E2 potently stimulated aldosterone synthase expression and the release of aldosterone through a mechanism involving GPER-1 stimulation. This was proven by 3 lines of evidence. First, during ERβ blockade, the secretagogue effect of E2 was replicated by the selective GPER-1 agonist G-1. Second, the effect of both E2 and G-1 on aldosterone syn-

thesis and secretion during ERα and ERβ blockade (with MPP and THC) was abrogated by G-15, a GPER-1 antagonist. Third, the increase of CYP11B2 induced by E2 after ERβ blockade was abolished after GPER-1 silencing. Therefore, altogether, these results indicate that 1) the ERβ functionally interacts with GPER-1 in modulating aldosterone production and 2) the blockade of ERβ unmasks a potent GPER-1-mediated secretagogue effect of E2 on aldosterone secretion.

Of further interest, we could provide evidence that the GPER-1-mediated secretagogue effect on aldosterone involved activation of PKA and cAMP, because a specific PKA inhibitor, Rp-8-Br-MB-cAMPS, was shown to abolish this effect. At variance, we could find no evidence that the secretagogues effect of E2 after ERα and ERβ blockade or ERβ blockade involves changes of $[Ca^{2+}]_i$.

In summary, the following conclusions can be drawn: 1) under physiological conditions, estrogens inhibit aldosterone synthesis by acting on ERβ, which explains why they do not elicit any appreciable effect on aldosterone secretion when given in vitro (31); and 2) removal of this tonic ERβ-mediated inhibition of estrogens unmasks a potent secretagogue effect on aldosterone that occurs via GPER-1 receptor subtype. Of note, the latter is the predominant ER subtype in APA. Because PA exhibits a milder phenotype in women than in men (32), unless the APA carry a Kir3.4 mutation (17), we would like to contend that an interaction between ERβ and GPER-1 activation determines the degree of hyperaldosteronism in these common human tumors. This hypothesis obviously deserves further specific research.

Limitations and strengths

Because estradiol alone does not alter CYP11B2 expression in the absence of a second ERβ ligand or antagonist, probably due to a combination of inhibitory and stimulatory actions, we cannot exclude the possibility that the observed results are primarily a pharmacologic effect. Nevertheless, the consistency of our results with pharmacologic manipulation and also with molecular silencing of ERβ and GPER-1 argues against this interpretation. Moreover, these results were obtained using an in vitro strategy in HAC15, a tumor cell line from a female patient. Therefore, they need to be confirmed in vivo using different approaches, because BP is regulated in a highly complex and integrated manner. Moreover, the effect of estrogens on aldosterone should be considered in the context of their action on other components of the renin-angiotensin system, as angiotensinogen, and related signaling pathways (33, 34).

Nonetheless, we would like to point out that our findings might be relevant not only for mechanistically explaining the gender dimorphism of BP, CV risk, and PA but also for the regulation of BP in fertile women. Their cyclically high estrogens level can inhibit aldosterone secretion and, moreover, can explain why their aldosterone-renin ratio varies along the menstrual cycle (35) and also why they have lower BP than both postmenopausal women and age-matched men (1, 36). Finally, these results could have implications for the vast population of ER-positive breast cancer women, who are being treated with ER modulators with no knowledge of what happens to their aldosterone and BP values (37).

Conclusions

We here showed that besides the classical ERα and ERβ receptors, both the normal adult human adrenal cortex and APA cells express the GPER-1, a receptor that mediates a secretagogues effect of estrogens on aldosterone upon pharmacologic disinhibition of ERβ-mediated actions.

These results might be relevant for clarifying the gender dimorphism of BP, CV risk, and PA and for better understanding the pressor and CV effects of treatment with ER modulators.

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