Aldosterone mediates its rapid effects in vascular endothelial cells through GPER activation

Robert Gros, Qingming Ding, Bonan Liu, Jozef Chorazyzewski, and Ross D. Feldman

Department of Medicine, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada; Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada; and Vascular Biology Research Group, Robarts Research Institute, London, Ontario, Canada

Submitted 20 June 2012; accepted in final form 28 December 2012


Aldosterone has been increasingly appreciated as an important physiological and pathophysiological regulator of cardiovascular functions. This hormone was conventionally thought to act solely as a transcriptional regulator exclusively in the kidney. However, it is now known that aldosterone mediates its actions via “classic” transcriptional mechanisms, as well as “rapid” (previously termed “nongenomic”) mechanisms (8, 10, 14).

In vascular tissues, the rapid effects of aldosterone have been linked to regulation of vascular contractility (vasodilator and vasoconstrictor) via regulation of endothelial nitric oxide synthase (NOS) (16, 20, 22, 24) and vascular smooth muscle cell (VSMC) myosin light chain phosphorylation (13), regulation of MAP kinase (22), and regulation of apoptosis (14). These effects occurred over a time course (<15 min) not consistent with transcriptional mechanisms (8). Furthermore, these actions have been associated with a number of rapid cellular effects, including increased calcium mobilization (4), effects not explainable by “traditional” mechanisms of steroid-mediated responses. Notably, in these studies, the existence of a nonmineralocorticoid aldosterone receptor was postulated, but that receptor was never identified.

In VSMCs, we previously showed that rapid aldosterone effects are mediated in parallel via the classic mineralocorticoid receptor (MR), as well as the G protein-coupled estrogen receptor (GPER) (14). GPER [previously known as G protein-coupled receptor 30 (GPR30)] is a recently recognized GPR receptor (GPER) (14). GPER has been linked to regulation of vascular contractility (vasodilator and vasoconstrictor) (8, 10, 14). These effects occurred over a time course (<15 min) not consistent with transcriptional mechanisms (8). Furthermore, these actions have been associated with a number of rapid cellular effects, including increased calcium mobilization (4), effects not explainable by “traditional” mechanisms of steroid-mediated responses. Notably, in these studies, the existence of a nonmineralocorticoid aldosterone receptor was postulated, but that receptor was never identified.

The objective of the current studies was to clarify the role of GPER and aldosterone-mediated activation of GPER in vascular endothelial cell function. In vivo, GPER activation has been shown to mediate hypotensive effects (15, 21), predominantly mediating endothelin-dependent vasorelaxation (2, 23), although there is one report of endothelin-independent relaxation (31) and one report of GPER-mediated vasoconstriction (1). Our initial studies demonstrated aldosterone’s rapid effects on endothelin-dependent vasodilator mechanisms and endothelial cell MAP kinase regulation. However, the receptor mechanism underlying these effects was not determined. Therefore, we have sought to determine the role of GPER in mediating aldosterone’s endothelin-dependent actions. Our data demonstrate that aldosterone stimulates rapid ERK phosphorylation and mediates proapoptotic effects in cultured endothelial cells via GPER activation. Furthermore, we demon-
strate that, in isolated vascular ring segments, aldosterone mediates vasodilatory effects via GPER activation. These data support the hypothesis that, in these vascular endothelial cells, the rapid actions of aldosterone are predominantly mediated via GPER.

**METHODS**

**Aortic endothelial cell primary cultures.** Rat aortic endothelial cells were cultured according to the manufacturer’s instructions (Cell Applications, San Diego, CA) and utilized between passages 4 and 8.

**Generation of adenosinivector for GPER knockdown.** The adenosinivector used in these studies were generated with the AdMax adenosinivector creation kit according to the manufacturer’s instructions (Microbix BioSystems, Toronto, ON, Canada). For construction of short-hairpin RNA (shRNA) adenosinivector (adeno-shRNA), the shRNA sequences specific for green fluorescent protein (GFP, bp 418–438) and GPER (bp 356–376) were cloned into the modified PDC312 vector. For all adenosinivector constructs, the resultant shRNA and cDNAs were subcloned into shuttle vector PDC316 and purified. The recombinant adenosinivector was harvested by lysis of transfected HEK-293 cells using three freeze-thaw cycles. For gene transfer, cells were infected with adenosinivector constructs for 16 h at 37°C; then media were replaced with fresh culture media. Cells were utilized for experimentation 72 h after gene transfer. The duration of serum deprivation was 16–48 h.

**Assessment of endogenous receptor expression in rat aortic endothelial cells.** Isolation of total RNA was performed utilizing the one-step RNA isolation reagent TRIzol (Invitrogen, Carlsbad, CA). For analysis of GPER, MR, estrogen receptor (ER)-α, ERβ, androgen receptor (AR), and GAPDH (control) mRNA expression, 1 μg of RNA from cultured endothelial cells was utilized, along with Superscript one-step RT-PCR kits (Invitrogen) with primers specific to these receptors: 5'-GCACGTCTTTCTCTCCACC-3' (forward) and 5'-ACAGCCTGAGCTTGTCCCTG-3' (reverse) for GPER30, 5'-CCAAAGGCTACCACAGTCTC-3' (forward) and 5'-TCCGAGACGCTATGTGCT-3' (reverse) for MR, 5'-TCTTACCTGCTTGTGAG-3' (forward) and 5'-ATCTTCTCCAGACCTGTG-3' (reverse) for ERα, 5'-GTCTTCCTGTTGATGACTA-3' (forward) and 5'-GCCAGGCACATTCATAGAGG-3' (reverse) for ERβ, 5'-CGAGCCGAGGCCAGCAGGTC-3' (forward) and 5'-GCCGAGCCGAGAATTGTGAGTGT-3' (reverse) for AR, and 5'-TGAAGCAGAGAAGTCACGTC-3' (forward) and 5'-TCCGAGACGCTATGTGCT-3' (reverse) for GAPDH.

**Assessment of ERK content and EKR1/2 phosphorylation.** The effects of aldosterone or the GPER agonist G1 on ERK1/2 phosphorylation were assessed by immunoblotting, as recently described (14). Cultured endothelial cells were treated for 15 min with aldosterone or G1 and then washed twice with ice-cold PBS. Where receptor antagonists were utilized, cells were pretreated with these agents for 15 min prior to addition of the agonists noted above. Cells were then lysed in ice-cold buffer containing 20 mM Tris, pH 8.0, 1% NP-40, 0.1% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Resultant whole cell lysates were resolved by SDS-PAGE and transferred electrochemically onto Immob-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked overnight at 4°C with 5% skim milk powder in a buffer containing 20 mM Tris (pH 7.4), 0.5 M NaCl, and 0.1% Tween 20 and then incubated with anti-phosphorylated ERK1/2 or anti-ERK1/2 antibody (diluted 1:1,000). Blots were washed in Tris-buffered saline for 1 h and then incubated in anti-mouse (1:1,000 dilution) or anti-rabbit (1:5,000 dilution) antibody for 1 h at room temperature. Proteins were detected by chemiluminescence, as described by the manufacturer’s protocol (DuPont NEN, Boston, MA). Phosphorylated ERK expression was determined by densitometric analysis of 42- and 44-kDa bands and normalized to total ERK content. Total ERK content (see Figs. 2–4) was not significantly altered under any of the conditions.

**Assessment of apoptosis by annexin V labeling.** After 48 h of serum starvation, cells were treated with G1 (1 μM) or aldosterone (10 pM) for 24 h in the absence or presence of eplerenone or the GPER antagonist G15, detached with trypsin, and washed in PBS. Pooled intact cells were suspended in annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4) containing FITC-conjugated annexin V (0.25 μg/ml) and propidium iodide (5 μg/ml) and incubated in darkness for 15 min. Annexin V binding was assessed using a flow cytometer (FacsCalibur, BD Biosciences, Mississauga, ON, Canada). A total of 20,000 events were analyzed for double-stained positive cells for each sample with FlowJo software (Tree Star, Ashland, OR) by a blinded observer. Data were normalized relative to the control levels of annexin-positive staining determined for each experiment.

In gene transfer studies, endothelial cells were cultured 24 h before gene transfer and then infected with adenosinivector constructs expressing GPER shRNA (shGPER) or GFP shRNA (shGFP, control) for 16 h. Infection medium was then replaced with DMEM without serum. **Assessment of regulation of DNA synthesis as an index of proliferation by 5-ethyl-2’-deoxyuridine labeling.** After 24 h of serum starvation, cells were treated with increasing concentrations of aldosterone or G1 for 2 h, restimulated with 10% FBS for 18 h, and then incubated with 5-ethyl-2’-deoxyuridine (EdU, 10 μM) for 2 h. EdU incorporation was assessed by the Click-iT EdU flow cytometry assay kit (LifeTech, Carlsbad, CA). To assess the effect of G15 on aldosterone- or G1-mediated effects, cells were treated with G1 (1 μM) or aldosterone (10 pM) for 24 h in the absence or presence of G15. In gene transfer studies, endothelial cells were cultured for 24 h before gene transfer and then infected with adenosinivector constructs expressing shGPER (adeno-shGPER) or shGFP (control) for 16 h. Infection medium was then replaced with DMEM without serum.

**Assessment of vascular reactivity in aortic rings.** Rats utilized in our studies as a source of aortic ring segments were maintained at the University of Western Ontario, and experiments were performed as recently described (14).

---

**Vascular endothelial cells**

**Vascular smooth muscle cells**

Fig. 1. Expression of G protein-coupled estrogen receptor (GPER) and mineralocorticoid receptor (MR) mRNA. Top and bottom: RT-PCR analysis of mRNA expression of GPER and MR in cultured vascular endothelial cells and vascular smooth muscle cells (VSMCs). GPER is readily detectable in endothelial cells; under the same conditions, MR is barely detectable in cultured VSMCs. In contrast, MR is readily detectable in VSMCs but is not detectable in cultured endothelial cells. +RT and −RT, with and without reverse transcriptase.
following the guidelines and protocols approved by the University Council on Animal Care for animal research. Aortic ring studies were performed as previously described (11, 12, 22). Ten-week-old male Wistar rats were anesthetized with 5% isoflurane in oxygen. The chest was opened, and the thoracic aorta was located, dissected out, and placed in ice-cold K+ -enriched physiological salt solution (KPSS). Blood and connective tissue were removed, and the aorta was cut into 2- to 3-mm-wide ring segments, which were suspended in KPSS under optimal tension in individual double-wall organ baths maintained at 37°C and gassed with 5% CO2-95% O2. Optimal tension was 750 mg for 10-wk-old Wistar rats. The ring segments were equilibrated for 40–60 min with changes of KPSS every 10 min before the addition of any drug. Tension developed in the isolated ring segments was assessed using an isometric force-displacement transducer (model FT03, Grass Instrument, Quincy, MA).

Vascular reactivity was assessed in vascular ring preparations according to our previously published methods (22). After equilibration, we determined endothelial functional “integrity” of each ring by assessing methacholine-mediated vasodilation. If a ring did not demonstrate robust methacholine-mediated responses, it was replaced prior to initiation of studies. Ring segments were then incubated for 2 min with increasing concentrations of aldosterone or G1 followed by a submaximal constriction with phenylephrine [100 nM, ED80 of phenylephrine (obtained from previous phenylephrine concentration-response experiments)] for 10 min. Before addition of the next concentration of aldosterone or G1, rings were washed three times with KPSS for 20 min. Constriction responses to phenylephrine were quantified by determination of the area under the curve using the trapezoidal method of analysis, as described previously (22). In experiments using the GPER inhibitor, aortic ring segments were incubated with G15 (10 nM) for 5 min prior to addition of aldosterone. Solvent (DMSO, 1:1,000 dilution) concentrations were identically maintained in all ring preparations (i.e., in the absence of presence of GPER ligands).

![Figure 2](http://ajpcell.physiology.org/)

**Fig. 2.** G1 and aldosterone-mediated effects on ERK phosphorylation. **A**: time-dependent G1-mediated ERK phosphorylation. Endothelial cells were incubated with G1 (1 µM) for 2–20 min. Significant increases in ERK phosphorylation above control levels were evident from 5 min (n = 4). Inset: representative immunoblot demonstrating that the increase in phosphorylated ERK (phospho-ERK) content was independent of alterations in total ERK content. **B**: concentration-dependent G1-mediated ERK phosphorylation. Endothelial cells were incubated with increasing concentrations of G1 (0.01–10 µM) for 15 min. Significant increases in ERK phosphorylation were evident at >10 nM G1 (n = 6). Inset: representative immunoblot demonstrating that the increase in phosphorylated ERK content was independent of alterations in total ERK content. **C**: concentration-dependent aldosterone-mediated ERK phosphorylation. Endothelial cells were incubated with increasing concentrations of aldosterone (Aldo, 1–1,000 pM) for 15 min. Significant increases in ERK phosphorylation were evident at >1 µM aldosterone (n = 6). Inset: representative immunoblot demonstrating that the increase in phosphorylated ERK content was independent of alterations in total ERK content. *P < 0.05.
**Materials.** The GPER-selective agonist G1 (1-(4-(6-bromobenzofuro[1,3][1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone, 1-(3aS,4R,9bR-recl)-4-(6-bromol-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline) (1) and antagonist G15 (cis-4-(6-bromobenzofuro[1,3][1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline) (5) were purchased from Calbiochem-Novabiochem (San Diego, CA). All other chemical reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). All steroid ligands were dissolved in DMSO with a working dilution in assays of 1:1,000 DMSO-H2O. DMSO at a 1:1,000 dilution was also included in all “control” conditions tested. Anti-phosphorylated ERK antibodies were obtained from Cell Signaling Technology (Danvers, MA). The anti-ERK antibody was purchased from Upstate Cell Signaling (Charlottesville, VA).

**Statistical analyses.** In all cell-based assays, data were normalized to the control levels detected in vehicle-treated replicates, controls specific for each individual experiment and cell condition (adeno-shRNA studies) tested. For multiple group comparisons, initial analysis by ANOVA was followed by Dunnett’s multiple comparison tests. The significance of difference between paired groups was determined by Student’s t-test for paired data. P < 0.05 on a two-sided test was taken as a minimum level of significance.

**RESULTS**

**GPER expression in endothelial cells.** In primary cultured rat aortic endothelial cells, GPER expression was readily apparent on RT-PCR assessment (Fig. 1), consistent with findings in other vascular endothelial cell models (18, 28). In contrast, MR mRNA is not detected under the same conditions under which the MR transcript is readily detectable in VSMCs. Thus, in contrast to the VSMCs, which in culture express MR but not GPER (Fig. 1), this rat aortic endothelial cell model offered the opportunity to study aldosterone-mediated GPER actions in an environment devoid of competing effects of aldosterone-mediated MR activation.

**G1 and aldosterone stimulate ERK activation via GPER activation.** Our previous studies demonstrated that, in primary cultured VSMCs in which GPER was reintroduced via adenoviral gene transfer, G1 (the GPER agonist) and aldosterone mediated a concentration-dependent increase in ERK activation (14). Similarly, in endothelial cells, aldosterone and G1 increase ERK phosphorylation. Aldosterone increased ERK phosphorylation in rat aortic endothelial cells with a biphasic concentration-response curve (Fig. 2C), similar to the effects we previously reported in VSMCs (14) and bovine aortic endothelial cells (22). G1 mediated a time-dependent increase (Fig. 2A) in ERK phosphorylation and also demonstrated biphasic concentration-dependent increases (Fig. 2B). Whether these biphasic effects relate to effects of aldosterone and/or G1 at higher concentrations on non-MR, non-GPER receptors(s) has yet to be determined.

To further examine the receptor specificity of G1- and aldosterone-mediated GPER activation, we assessed the effect of the GPER antagonist G15 on G1- and aldosterone-mediated ERK phosphorylation. After G15 pretreatment, the effect of G1 on phosphorylated ERK activation was reduced to a level not significantly greater than control (96 ± 4% of control; Fig. 3). G15 pretreatment had no significant effect on baseline ERK phosphorylation (96 ± 4% of control, n = 3; Fig. 3). Additionally, after G15 pretreatment, aldosterone-stimulated ERK phosphorylation was also decreased to a level no greater than control (Fig. 3).

The extent of ANG II-stimulated ERK activation was not discernibly different with G15 pretreatment (Fig. 3). Additionally, with shRNA-mediated knockdown of GPER, the effects of G1 and aldosterone on ERK phosphorylation were no longer detectable (i.e., reduced to a level no greater than control; Fig. 4). Knockdown of GPER did not alter ANG II-mediated ERK activation (Fig. 4).

**G1 and aldosterone stimulate endothelial cell apoptosis via GPER activation.** We previously showed in VSMCs that the proapoptotic effects of aldosterone are ERK-dependent (14) and that GPER activation mediates an ERK-dependent proapoptotic action (6). Therefore, we assessed the apoptosis-regulatory effects of G1 and aldosterone in cultured endothelial cells. After 48 h of serum starvation, the baseline frequency of apoptotic cells (as assessed by annexin V-positive labeling) increased from 0.5 ± 0.1% to 9.7 ± 1.0% (n = 3).

G1 (1 μM) mediated a further concentration-dependent increase in apoptosis to 160 ± 19% of control (Fig. 5A). Similarly, aldosterone (1 nM) mediated a concentration-dependent increase in apoptotic frequency to a maximum of 159 ± 15% of baseline (Fig. 5B). With G15 pretreatment, the proapoptotic effects of G1 and aldosterone were reduced to a level not greater than control (Fig. 6A). GPER knockdown using our adeno-shGPER attenuated the proapoptotic effects of aldosterone and G1 to levels no greater than control (Fig. 6B). Neither G15 nor GPER knockdown significantly altered ANG II-mediated apoptosis in endothelial cells (Fig. 6).

**G1 and aldosterone inhibit DNA synthesis via GPER activation.** To further assess the role of GPER in growth regulation, we examined the effects of G1 and aldosterone on DNA synthesis determined by EdU incorporation. The GPER
agonist G1 mediated a concentration-dependent inhibition of DNA synthesis (Fig. 7A). Similarly, aldosterone inhibited DNA synthesis (Fig. 7A). Pretreatment with G15 attenuated the inhibitory effects of G1 and aldosterone to a level not significantly different from control (Fig. 7B). Furthermore, GPER inhibition by adeno-shGPER gene transfer attenuated aldosterone’s inhibitory effects to a level not significantly different from control (Fig. 7C).

**DISCUSSION**

The present studies demonstrate that, in rat endothelial cells devoid of detectable MR mRNA, aldosterone exploits GPER to mediate proapoptotic and antiproliferative effects, as well as vasodilation. In VSMCs, GPER expression declines in culture, in parallel with GPER-dependent signaling (14). In contrast, GPER expression in endothelial cells is persistently maintained in culture, offering the opportunity to examine the role of aldosterone in mediating its effects via GPER, devoid of the confounding effects of concurrent MR expression. These studies demonstrate that, in rat aortic endothelial cells, the effects of aldosterone are completely dependent on GPER-mediated mechanisms.

The importance of the endothelium as a target of the rapid effects of aldosterone and its significance in endothelial cell regulation have been an ongoing focus of research. Effects of aldosterone on a diverse range of endothelial cell functions, including NOS-dependent vasodilation (22), cell swelling and stiffness (17, 25), and growth-regulatory pathways (22), have been reported. The receptor mechanisms underlying these effects have not been determined or have been presumed to be due to activation of classic or membrane-associated MRs, on the basis of the inhibitory effects of presumed mineralocorticoid-specific or selective antagonists such as eplerenone and}

---

**Fig. 4.** Effect of GPER downregulation on G1- and aldosterone-mediated ERK phosphorylation. A: effect of adenoviral construct expressing GPER shRNA (adeno-shGPER) on GPER expression. GPER expression as assessed via RT-PCR was downregulated following gene transfer of shGPER. In contrast, GAPDH expression was not altered with shGPER gene transfer. B: effect of adeno-shGPER-mediated downregulation of GPER expression on aldosterone (10 pM), G1 (1 μM), and ANG II (100 nM)-mediated ERK phosphorylation. Values are means ± SE from 4 independent experiments. *P < 0.05 vs. control. Inset: representative immunoblot demonstrating that the increase in phosphorylated ERK content was independent of alterations in total ERK content. shGFP, green fluorescent protein shRNA.
The conclusion that, in aortic endothelial cells, the vasodilatory, growth-regulatory effects of aldosterone are due to GPER activation is based on two lines of evidence: 1) the effects of the GPER antagonist G15 to inhibit aldosterone’s actions on vascular reactivity and apoptotic and antiproliferative pathways and 2) the effects of GPER downregulation to ablate aldosterone’s ERK activating/proapoptotic/antiproliferative effects. We previously demonstrated that, in VSMCs where GPER has been reintroduced, aldosterone stimulates GPER-dependent activation of ERK phosphorylation and apoptosis (11). However, the importance of GPER in mediating aldosterone’s effects has been questioned on the basis of the lack of evidence in a cell model with persistent GPER expression (8). In the current studies, we observed that, in endothelial cells with persistent endogenous GPER expression, aldosterone stimulates ERK phosphorylation and apoptosis to an extent similar to that in VSMCs with GPER overexpression. Thus our findings suggest an important role of GPER in mediating aldosterone’s rapid effects on growth-regulatory mechanisms in cultured vascular cells with persistent expression of GPER, i.e., effects not dependent on the utilization of GPER overexpression cell models.

The current studies are also relevant to our understanding of aldosterone’s opposing rapid vasodilator vs. vasoconstrictor effects that have been reported over the years (5). Aldosterone has been reported to mediate rapid endothelium-dependent vasodilation and consequent increased limb blood flow in vivo (25) and endothelium-dependent attenuation of vasoconstrictor responses in vitro (27). The effects on vascular reactivity

---

**Fig. 5.** Effect of G1 and aldosterone on serum starvation-provoked apoptosis. A: concentration-dependent effects of G1. After 48 h of serum starvation, endothelial cells were incubated with increasing concentrations of G1 (0.01–10 µM), and effect on apoptosis was assessed by frequency of annexin V-positive cells (APCs). Annexin-positive labeling was detected in 10 ± 3% of serum-starved cells under control conditions. Values are means ± SE from 3 independent experiments. *P < 0.05 vs. control. B: concentration-dependent effects of aldosterone. Serum-starved cells were incubated with increasing concentrations of aldosterone (1–1,000 pM), and effect of aldosterone on apoptosis was assessed by frequency of annexin V-positive cells. Annexin-positive labeling was detected in 8 ± 1% of serum-starved cells under control conditions. Values are means ± SE from 6 independent experiments. *P < 0.05 vs. control.

**Fig. 6.** Effect of GPER inhibition on aldosterone-mediated apoptosis. A: effect of pharmacological inhibition of GPER with G15. After 48 h of serum starvation, endothelial cells were treated with aldosterone (10 pM), G1 (1 µM), or ANG II (100 nM) in the absence or presence of the GPER antagonist G15 (10 µM). G15 treatment inhibited aldosterone- and G1-mediated apoptosis but did not affect ANG II-mediated apoptosis. Values are means ± SE from 4 independent experiments performed under identical conditions. Annexin-positive labeling was detected in 10 ± 3% of serum-starved cells with control conditions. *P < 0.05 vs. control. B: effect of adeno-shGPER-mediated downregulation of GPER on aldosterone (10 pM)-, G1 (1 µM)-, and ANG II (100 nM)-mediated apoptosis. GPER downregulation attenuated aldosterone- and G1-mediated apoptosis but did not affect ANG II-mediated apoptosis. Annexin-positive labeling was detected in 9 ± 1% of serum-starved cells under control conditions. Values are means ± SE from 4 independent experiments. *P < 0.05 vs. control.
mediated by the GPER agonist G1 parallel those mediated by aldosterone (17, 20). In contrast, aldosterone-mediated vasoconstrictor effects have also been reported (1). Our previous studies showed aldosterone’s vasodilatory effects in endothe-

Fig. 7. Effect of G1 and aldosterone on DNA synthesis. A: concentration-dependent effects of G1 and aldosterone. After 24 h of serum starvation, endothelial cells were incubated with increasing concentrations of G1 (0.01–10 µM) or aldosterone (1–1,000 pM) and then restimulated with serum, and the effect on DNA synthesis was assessed by frequency of 5-ethynyl-2'-deoxyuridine (EdU)-positive cells. EdU-positive labeling was detected in 8 ± 2% of cells under control conditions. Values are means ± SE from 3 independent experiments. *P < 0.05 vs. control. B: effect of pharmacological inhibition of GPER using the GPER antagonist G15. After 24 h of serum starvation, endothelial cells were treated with aldosterone (10 pM), G1 (1 µM), or ANG II (100 nM) in the absence or presence of the GPER antagonist G15 (10 µM) and then restimulated with serum. G15 treatment inhibited aldosterone- and G1-mediated inhibition of DNA synthesis. EdU-positive labeling was detected in 9 ± 3% of cells under control conditions. Values are means ± SE from 3–6 independent experiments performed under identical conditions. *P < 0.05 vs. control. C: effect of GPER knockdown on aldosterone-mediated inhibition of DNA synthesis. GPER downregulation attenuated aldosterone (10 pM)- and G1 (1 µM)-mediated inhibition of DNA synthesis. EdU-positive labeling was detected in 7 ± 1% of serum-starved cells under baseline (control) conditions following green fluorescent protein shRNA (shGFP) gene transfer and 8 ± 1% of serum-starved cells following shGPER gene transfer (n = 5). Values are means ± SE from 3–5 independent experiments. *P < 0.05 vs. control.

Fig. 8. G1 and aldosterone inhibit phenylephrine (PE)-mediated constriction in aortic ring segments. A: G1-mediated relaxation. G1 mediated a concentration-dependent inhibition of phenylephrine (100 nM)-mediated constriction. Phenylephrine-mediated tension at baseline = 398 ± 33 mg. Values are means ± SE from 5 independent experiments. *P < 0.05 vs. control. B: representative trace of phenylephrine-mediated constriction of aortic ring segments following administration of vehicle [DMSO (control)] and the GPER agonist G1 (1 µM). C: aldosterone-mediated relaxation. Aldosterone mediates a concentration-dependent inhibition of phenylephrine (100 nM)-induced constriction. Phenylephrine-mediated tension at baseline = 432 ± 32 mg. Values are means ± SE from 5 independent experiments. *P < 0.05 vs. control. D: representative trace of phenylephrine-mediated constriction of aortic ring segments following administration of vehicle [DMSO (control)] and aldosterone (1 nM).
**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the editorial assistance of Dr. Eric Brass, David Geffen School of Medicine at UCLA, in development of the outline of this manuscript.

**GRANTS**

These studies were supported by grants-in-aid from the Heart and Stroke Foundation of Ontario (R. D. Feldman and R. Gros). R. Gros is supported by a New Investigator Award from the Heart and Stroke Foundation of Canada, the Canadian Foundation for Innovation, and the Ontario Research Fund.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

R.G. and R.D.F. are responsible for conception and design of the research; R.G. and R.D.F. interpreted the results of the experiments; R.G. and R.D.F. edited and revised the manuscript; R.G., Q.D., and R.D.F. approved the final version of the manuscript; Q.D., B.L., and J.C. performed the experiments; Q.D., B.L., and J.C. analyzed the data; Q.D. prepared the figures; R.D.F. drafted the manuscript.

**REFERENCES**


**Fig. 9.** Effect of GPER inhibition on aldosterone-mediated relaxation. G15 (10 μM) significantly attenuated the effect of aldosterone (10 pM) on phenylephrine (100 nM)-mediated constriction. Sodium nitroprusside (SNP, 1 nM)-mediated relaxation was not affected by G15 pretreatment. Phenylephrine-mediated tension at baseline = 408 ± 29 mg. Values are means ± SE from 5 independent experiments. *P < 0.05 vs. control.


