

# GPER Acts Through the cAMP/Epac/JNK/AP-1 Pathway to Induce Transcription of Alpha 2C Adrenoceptor in Human Microvascular Smooth Muscle Cells

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**Abstract:** Raynaud's phenomenon, which results from exaggerated cold-induced vasoconstriction, is more prevalent in females than males. We previously showed that estrogen increases the expression of alpha 2C-adrenoceptors ( $\alpha_{2C}$ -AR), the sole mediator of cold-induced vasoconstriction. This effect of estrogen is reproduced by the cell-impermeable form of the hormone ( $E_2$ :bovine serum albumin [BSA]), suggesting a role of the membrane estrogen receptor, G-protein-coupled estrogen receptor [GPER], in  $E_2$ -induced  $\alpha_{2C}$ -AR expression. We also previously reported that  $E_2$  upregulates  $\alpha_{2C}$ -AR in microvascular smooth muscle cells (VSMCs) via the cAMP/Epac/Rap/JNK/AP-1 pathway, and that  $E_2$ :BSA elevates cAMP levels. We, therefore, hypothesized that  $E_2$  uses GPER to upregulate  $\alpha_{2C}$ -AR through the cAMP/Epac/JNK/AP-1 pathway. Our results show that G15, a selective GPER antagonist, attenuates the  $E_2$ -induced increase in  $\alpha_{2C}$ -AR transcription. G-1, a selective GPER agonist, induced  $\alpha_{2C}$ -AR transcription, which was concomitant with elevated cAMP levels and JNK activation. Pretreatment with ESI09, an Epac inhibitor, abolished G-1-induced  $\alpha_{2C}$ -AR upregulation and JNK activation. Moreover, pretreatment with SP600125, a JNK-specific inhibitor, but not H89, a PKA-specific inhibitor, abolished G-1-induced  $\alpha_{2C}$ -AR upregulation. In addition, transient transfection of an Epac dominant negative mutant (Epac-DN) attenuated G-1-induced activation of the  $\alpha_{2C}$ -AR promoter. This inhibitory effect of Epac-DN on the  $\alpha_{2C}$ -AR promoter was overridden by the cotransfection of constitutively active JNK mutant. Furthermore, mutation of AP-1 site in the  $\alpha_{2C}$ -AR promoter abrogated G1-induced expression. Collectively, these results indicate that GPER upregulates  $\alpha_{2C}$ -

AR through the cAMP/EPAC/JNK/AP-1 pathway. These findings unravel GPER as a new mediator of cold-induced vasoconstriction, and present it as a potential target for treating Raynaud's phenomenon in estrogen-replete females.

**Key Words:** Raynaud's phenomenon, estrogen, vascular smooth muscle, alpha 2C adrenoceptor, G-protein estrogen receptor, peripheral vascular disease

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## INTRODUCTION

Raynaud's phenomenon (RP) is a vasospastic and ischemic condition mainly triggered by exposure to cold temperatures. It is manifested as triple color change (pallor, cyanosis, and erythema) at the level of the digits.<sup>1,2</sup> Severe RP may cause puffiness, ulcerations, and necrosis of the affected area.<sup>3</sup> Mechanistically, RP results from exaggerated reflex reaction mediated by neuronal (norepinephrine) and local effectors ( $\alpha_{2C}$  adrenergic receptors;  $\alpha_{2C}$ -AR) that increases vascular sensitization to cold.<sup>4–6</sup>

RP affects 3%–5% of the general population, with a remarkably higher incidence in females.<sup>7,8</sup> This may reach up to 77% in females versus 22% in males in some studies.<sup>9</sup> This gender-based distribution indicates that being a female is a risk factor for RP.<sup>7,8</sup> Interestingly, the most affected age group among females is the premenopausal group,<sup>9,10</sup> suggesting a role of estrogen in RP. This suggestion was further reinforced by other observations such as the association of unopposed estrogen replacement therapy with RP,<sup>11</sup> and the increased vascular responsiveness in estrogen-replete females.<sup>12,13</sup> In addition, estrogen plays a role in regulating body temperature.<sup>14</sup> As such, RP is considered a vascular thermoregulatory control disorder, and its positive association with estrogen becomes obvious.<sup>15</sup> Indeed, estrogen contributes to onset or pathogenesis of RP by potentiating cold-induced vasoconstriction.

Estrogen elicits its vasomotor effects via the cytoplasmic estrogen receptors  $\alpha$  and  $\beta$  (ER- $\alpha$  and ER- $\beta$ ) or the membrane bound G-protein-coupled estrogen receptor (GPER). These three subclasses of receptors are expressed in the vasculature.<sup>16,17</sup> The cytoplasmic ERs mediate the classical genomic pathway, which ultimately regulates the transcription of target genes.<sup>18,19</sup> However, GPER initiate the rapid nongenomic signaling in response to estrogen.<sup>18,19</sup> Although this pathway is known to activate protein kinases via a cascade of phosphorylation reactions,<sup>20</sup> substantial amount of evidence support its involvement in rapid gene

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transcription.<sup>21–23</sup> Thus, GPER has now become recognized not only as a major mediator of estrogen's rapid cellular effects, but also as a regulator of gene expression.<sup>24</sup> Collectively, the multitude of these signaling pathways initiated by estrogen, allows for the fine-tuning of estrogen-induced regulation of gene expression.<sup>24</sup>

We have previously reported that estrogen induces upregulation of  $\alpha_2C$ -AR, the sole mediator of cold-induced vasoconstriction.<sup>5</sup> Whether this effect is mediated classically via cytoplasmic estrogen receptors or rapidly via GPER is still unclear. However, several observations suggest the implication of GPER in estrogen-induced  $\alpha_2C$ -AR expression. Bovine serum albumin-conjugated  $E_2$  ( $E_2$ : BSA), which is a cell impermeable form of  $E_2$ , was able to induce  $\alpha_2C$ -AR expression.<sup>25</sup> In addition, this estrogen-induced  $\alpha_2C$ -AR expression is mediated via a cAMP/EPAC-mediated JNK/AP-1-dependent mechanism.<sup>26</sup> The stimulation of early downstream players of this signaling pathway in response to estrogen was rapid.<sup>25,26</sup> Furthermore, the activation of GPER leads to increased cAMP levels in many cell types, including VSMCs.<sup>27,28</sup> Thus, it becomes only reasonable to hypothesize that GPER mediates  $\alpha_2C$ -AR expression via cAMP-dependent signaling pathway. However, whether estrogen uses GPER to mediate the upregulation of  $\alpha_2C$ -AR expression is still unknown. In this study, we delineate the role of GPER in estrogen-induced  $\alpha_2C$ -AR expression and dissect the underlying signaling pathway.

## MATERIALS AND METHODS

### Reagents

DMEM:F12, phenol red-free DMEM, L-glutamine, fetal bovine serum, phosphate buffer saline,  $17\beta$ -estradiol,  $\beta$ -estradiol 6-(O-carboxy-methyl) oxime:BSA, 3-isobutyl-1-methylxanthine (IBMX), phosphate buffer saline, H89 and Epac inhibitor ESI09 were obtained from Sigma-Aldrich (Schnellendorf, Germany). Penicillin/streptomycin, trypsin, and Amaxa Nucleofector were purchased from Lonza (Basel, Switzerland).  $17\beta$  estradiol and JNK inhibitor SP600125 were purchased from Abcam (Cambridge, MA). DC Protein Assay kit and Clarity Western ECL Substrate were purchased from Biorad (CA). Insulin-transferin-selenium was obtained from ThermoFisher Scientific. Luciferase assay kit and Renilla luciferase plasmid (pRL)-cytomegalovirus (CMV) (Renilla luciferase gene driven by CMV promoter/enhancer) were purchased from Promega (Madison, WI). The activator protein (AP)-1-luciferase reporter plasmid was obtained from Stratagene (La Jolla, CA). The  $\alpha_2C$ -AR promoter-reporter plasmid (−1915/+5, relative to the transcription start site +1) was a kind gift from the late Dr. Herve' Paris.<sup>29,30</sup> G-1 and G15 were obtained from Tocris. In-Cell ELISA for JNK was purchased from ThermoFisher.

### Cell Culture

Human arteriolar SMCs were extracted by a nonenzymatic sprouting method from dermal arterioles of a postcircumcision tissue of a newborn boy. No IRB approval is needed because this tissue is considered clinical waste;

however, ethical approval was obtained. Cells were cultured in Ham's Growth medium (DMEM: F12; 50:50) supplemented with 10% FBS and 1% penicillin/streptomycin. Only cells between passages 6 and 11 were used in the experiments as the expression and regulation of  $\alpha_2C$ -ARs is similar among these passages.<sup>30</sup> For all the experiments, cells were starved for 48 hours in a phenol red-free, serum-free medium supplemented with insulin-transferin-selenium and L-glutamine, before commencing with the treatment. Cells were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> atmosphere. Each experiment was performed with its own control (vehicle; DMSO), the amount of which was that of the highest volume of DMSO in the treatment groups. All experiments were performed in triplicates and repeated 3 times (n = 3).

### cAMP Assay

cAMP assay was measured using a bioluminescent assay as per the manufacturer's protocol (cAMP-Glo; Promega) with 1 modification, where IBMX was added before commencing with any treatment.

### Site-Directed Mutagenesis

Site-directed mutagenesis of AP-1 site was performed using the QuickChange™ XL-site-directed mutagenesis kit (Stratagene) as previously reported.<sup>20</sup> Wild-type AP-1 site (ATGATTCAT, −346/−338, relative to the transcription start site) in the  $\alpha_2C$ -AR promoter-luciferase reporter construct was mutated to ACTGTTTGT using mutant oligonucleotide primers and double stranded DNA.

### Transient Transfection

Cells were transiently transfected by nucleofection with the Amaxa Nucleofector (Lonza) according to the manufacturer's instructions as we previously described.<sup>29</sup> Optimal transfection (80% transfection efficiency, with minimal toxicity) was achieved with nucleofection of 400,000 cells with 4 g of nucleic acids. The total amount of transfected DNA was kept constant throughout the study using the appropriate empty plasmids. When reporter constructs were used, pRL-CMV was used as an internal control to normalize the firefly luciferase units. After transfection, cells were allowed to recover overnight.

### Luciferase Assay

Cells were washed with PBS, lysed in luciferase lysis buffer (Promega), snap frozen, and then thawed at room temperature. Cell lysates were centrifuged at 9300 g for 10 minutes, and luciferase activity in the supernatant was determined.

### Statistical Analysis

Statistical analyses were performed using the Student's *t* test for paired or unpaired observations. For the comparison of more than 2 means, ANOVA was used— one-way ANOVA (with Dunnett's post hoc test) or 2-way ANOVA (with Tukey–Kramer's post hoc test). Experiments were performed at least three times, and each time was made of triplicate wells. The average of the triplicate from each

experiment (individual mean) was calculated, and these means were then averaged. Data were presented as mean  $\pm$  standard error of the mean (SEM). A *P*-value of  $<0.05$  was considered as significant.

## RESULTS

### GPER Mediates Estrogen-Induced Activation of $\alpha_{2C}$ -AR Promoter

As expected,  $17\beta$ -Estradiol ( $10^{-11}$ – $10^{-7}$  M) caused a concentration-dependent increase of the transcriptional activity of  $\alpha_{2C}$ -AR promoter-reporter construct, which reached a maximal level at  $10^{-9}$  M ( $P < 0.01$ ; Fig. 1A). This estrogen-induced increase in the  $\alpha_{2C}$ -AR promoter activity was abrogated when cells were pretreated with G15, a selective GPER antagonist (1  $\mu$ M; Fig. 1A). These results indicate that GPER mediates estrogen-induced transcription of  $\alpha_{2C}$ -AR.

To further validate our results, we tested whether G-1, a selective GPER agonist, induces  $\alpha_{2C}$ -AR promoter activity. Indeed, our results show that G-1 ( $10^{-7}$  to  $10^{-5}$  M) caused a significant increase in  $\alpha_{2C}$ -AR promoter-reporter construct activity in a concentration-dependent manner (Fig. 1B). This confirms that GPER is necessary and sufficient for  $\alpha_{2C}$ -AR upregulation.

### GPER Induction Increases cAMP Level and Activates JNK

Activation of membrane estrogen receptor, GPER, was reported to increase cAMP levels in many cell types, including VSMCs.<sup>27,28</sup> We sought to determine whether GPER modulates cAMP levels in microVSMCs. As expected, estrogen caused a significant and concentration-dependent increase in cAMP levels, with a logEC50 of  $-9.36 \pm 0.18$  M (Fig. 2A). Interestingly, in the presence of G15 (1  $\mu$ M), there was a right shift in the estrogen-induced cAMP levels, with a logEC50 of  $-8.47 \pm 0.15$  M. Moreover, G1, a GPER agonist ( $10^{-7}$  to  $10^{-5}$  M) increased

cAMP levels in a concentration-dependent manner with a logEC50 of  $-6.232 \pm 0.060$  M (Fig. 2B).

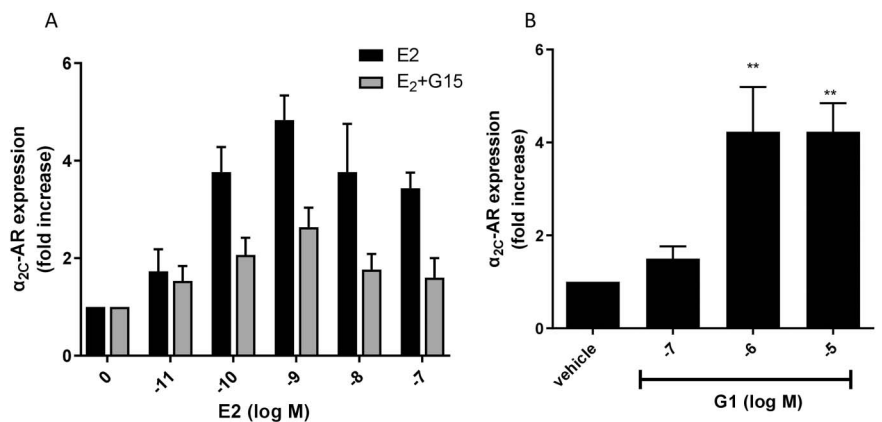
We have previously reported that  $17\beta$ -estradiol causes a time-dependent activation of JNK, which then drives  $\alpha_{2C}$ -AR expression.<sup>26</sup> Having established that G-1 induces  $\alpha_{2C}$ -AR upregulation, we wanted to check the effect of G-1 on JNK. Using in-cell ELISA, we show that G-1 causes a concentration- ( $10^{-7}$  to  $10^{-5}$  M) and time-dependent (5, 10, 30 minutes) activation of JNK (Figs. 2C, D respectively). The highest activation was attained 30 minutes after  $10^{-6}$  M of G-1 ( $5.4 \pm 1.2$ -fold increase;  $P < 0.01$ ).

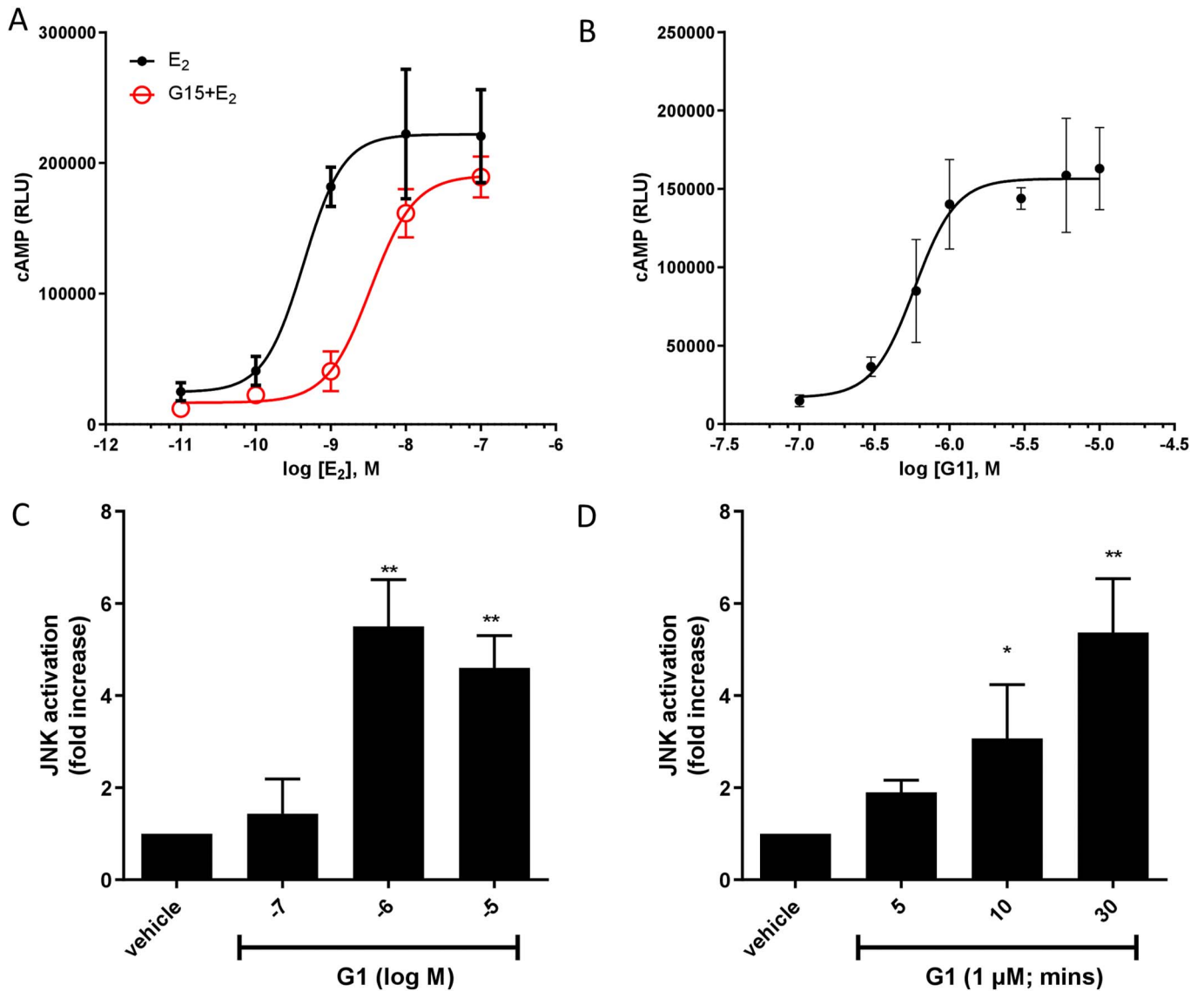
### Epac and JNK, but Not PKA Mediate G1-Induced $\alpha_{2C}$ -AR Upregulation

We have previously demonstrated that Epac acts downstream of cAMP to mediate estrogen-induced  $\alpha_{2C}$ -AR expression.<sup>26,29</sup> Therefore, we next sought to determine whether Epac mediates the G1-induced  $\alpha_{2C}$ -AR expression shown in Figure 1B. Indeed, pretreatment with ESI 09, an Epac inhibitor (10  $\mu$ M), abrogated G1-induced expression of  $\alpha_{2C}$ -AR (Fig. 3A). In the absence or presence of ESI09, G1 caused a  $4.9 \pm 0.6$  or  $2.1 \pm 0.3$ -fold increase in expression ( $P < 0.05$ ), indicative of Epac mediation of this G1-induced effect on  $\alpha_{2C}$ -AR promoter activity.

In addition to Epac, another downstream target of cAMP is the protein kinase A (PKA). We have previously reported that pharmacologic inhibition of PKA does not inhibit cAMP-induced increase in  $\alpha_{2C}$ -AR expression,<sup>29</sup> indicating that this expression is PKA-independent. However, given the potential cAMP microdomains that could be associated with G-protein-coupled signaling, it is critical to conclusively determine whether the GPER-induced cAMP could, nonetheless, use PKA to elicit its effects on  $\alpha_{2C}$ -AR expression. To that end, we used H89, a PKA selective inhibitor (2  $\mu$ M). Our results show that H89 does not significantly reduce G1-induced  $\alpha_{2C}$ -AR transcription (Fig. 3B). In the absence or presence of H89, G1 caused a  $4.50 \pm 0.56$ - or  $5.37 \pm 1.35$ -fold increase in expression ( $P > 0.05$ ). This finding confirms that PKA is not involved in G1-induced  $\alpha_{2C}$ -AR upregulation. Therefore, GPER mediates  $\alpha_{2C}$ -AR

**FIGURE 1.** GPER mediates estrogen-induced activation of  $\alpha_{2C}$ -AR promoter in VSMCs. Cells were transiently transfected with the  $\alpha_{2C}$ -AR promoter-reporter construct and internal *Renilla* control construct. A, After recovery, cells were starved for 48 hours, then treated with increasing concentrations of estrogen ( $10^{-11}$  to  $10^{-7}$  M), in the presence or absence of G15 (1  $\mu$ M; a GPER antagonist). G15 was administered 30 minutes before and during exposure of the cells to estrogen. B, Cells were treated with increasing concentrations of G-1 ( $10^{-7}$  to  $10^{-5}$  M; a GPER agonist), and promoter activity was measured. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means  $\pm$  SD. \*\* $P < 0.01$ .





**FIGURE 2.** GPER activation increases cAMP and activates JNK in VSMCs. A, Cells were treated with increasing concentrations of estrogen in the absence or presence of G15 (1 μM). Intracellular concentration of cAMP was then measured using a bioluminescent assay. G15 was added 30 minutes before estrogen. B, Cells were treated with increasing concentrations of G1 (10<sup>-7</sup> to 10<sup>-5</sup> M) and intracellular levels of cAMP were then measured using a bioluminescent assay. C, Using in-cell ELISA, cells were treated with increasing concentration of G1 (10<sup>-7</sup> to 10<sup>-5</sup> M) for 30 minutes and JNK activity was assessed. D, Cells were treated with 1 μM of G1 for various time points and JNK activity was measured using in-cell ELISA. Data are presented as means ± SD. \**P* < 0.05 and \*\**P* < 0.01.

transcription in a cAMP/Epac-dependent, but PKA-independent signaling mechanism.

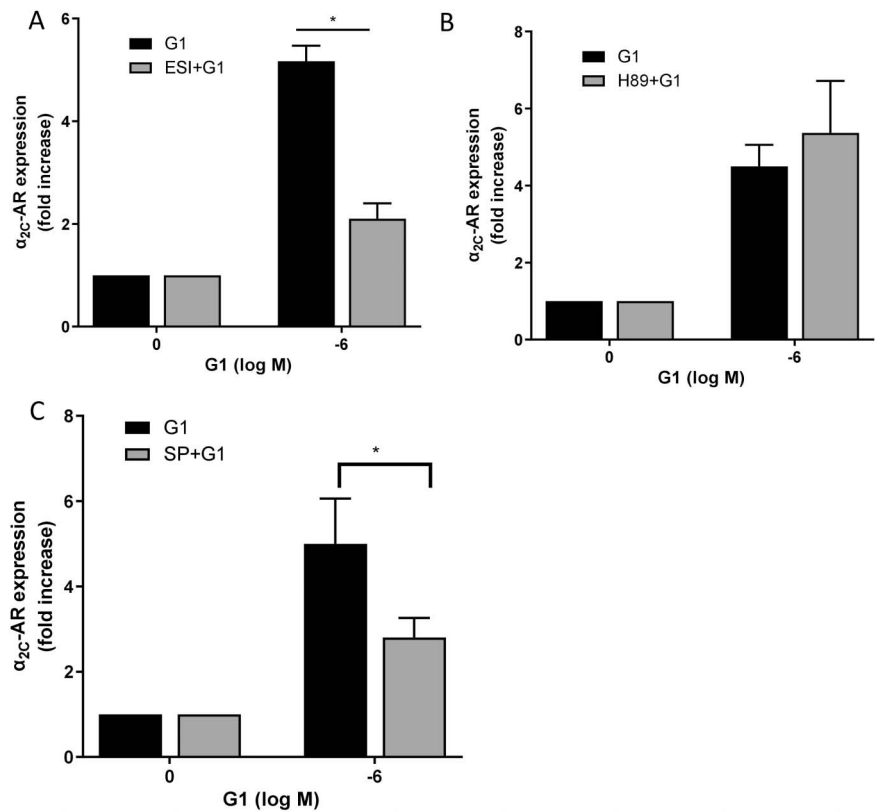
We have previously reported that JNK mediates estrogen-induced expression of α<sub>2C</sub>-AR.<sup>26</sup> Having established that G-1 activates JNK, we next wished to assess the role of JNK in G-1-induced transcription of α<sub>2C</sub>-AR. As Figure 3C shows, G-1-induced increase in α<sub>2C</sub>-AR transcription was significantly diminished by SP600125, a JNK inhibitor (3 μM; *P* < 0.05). This indicates that JNK mediates G-1-induced transcription of α<sub>2C</sub>-AR. Importantly, JNK inhibition did not completely abolish G-1-induced expression, evident by the significant difference between vehicle-treated or SP600125 + G-1-treated cells. This

may suggest that pathways other than JNK mediated the G-1-cAMP-Epac effect on α<sub>2C</sub>-AR expression.

### Epac Mediates G-1-Induced JNK Activation

We have previously established that estrogen-induced JNK phosphorylation in human microvascular smooth muscle cells is exerted via a critical signaling through Epac.<sup>26</sup> Having established that Epac and JNK mediate G-1-induced α<sub>2C</sub>-AR upregulation., we sought to evaluate the role of Epac in G-1-induced JNK activation. In the absence or presence of ESI09 (10 μM; a specific Epac inhibitor), G1 caused a 5.6 ± 1.2- or 3.2 ± 0.8-fold increase in JNK activation, respectively (*P* <

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**FIGURE 3.** G-1-induced JNK activation is mediated by Epac in VSMCs. Cells were transfected with  $\alpha_{2C}$ -AR promoter-reporter construct. After recovery, they were treated with G-1 (1  $\mu$ M) in the presence or absence of (A) ESI09 (ESI; 10  $\mu$ M; a specific Epac inhibitor), (B) H89 (a PKA inhibitor; 2  $\mu$ M), or (C) SP600125 (a JNK inhibitor; 3  $\mu$ M). The inhibitor was added 30 minutes before and during exposure of the cells to G1 (1  $\mu$ M). Reporter activity was then measured using luciferase assay. Results are expressed as fold increase from the control and are presented as means  $\pm$  SD. \* $P < 0.05$ .

0.01; Fig. 4). This indicates that JNK activation in response to G-1 is mediated via Epac.

### JNK Acts Downstream of Epac to Mediate G1-Induced $\alpha_{2C}$ -AR Transcription

To better characterize the mechanism of G1-induced  $\alpha_{2C}$ -AR transcription, we wanted to assess whether JNK acts downstream of Epac in this signaling pathway. Transient transfection of dominant negative mutant form of Epac (Epac DN) was used and the activity of  $\alpha_{2C}$ -AR promoter-reporter was determined. Figure 5A shows that the expression of the dominant negative mutant of Epac significantly attenuated G-1 induction of  $\alpha_{2C}$ -AR. G1 caused a  $5.2 \pm 0.9$ - versus  $2.6 \pm 0.6$ -fold increase in expression ( $P < 0.05$ ). This molecular inhibition by Epac-DN was overridden when EPAC-DN transfected cells were cotransfected with the constitutively active form of JNK (JNK CA; Fig. 5B;  $2.4 \pm 0.5$  vs.  $5.7 \pm 0.8$ -fold;  $P < 0.01$ ). This suggests that JNK acts downstream of Epac, and that G-1 activates Epac, which then stimulates JNK to drive  $\alpha_{2C}$ -AR upregulation.

### AP-1 Site is Necessary for G-1-Induced $\alpha_{2C}$ -AR Transcription (via GPER)

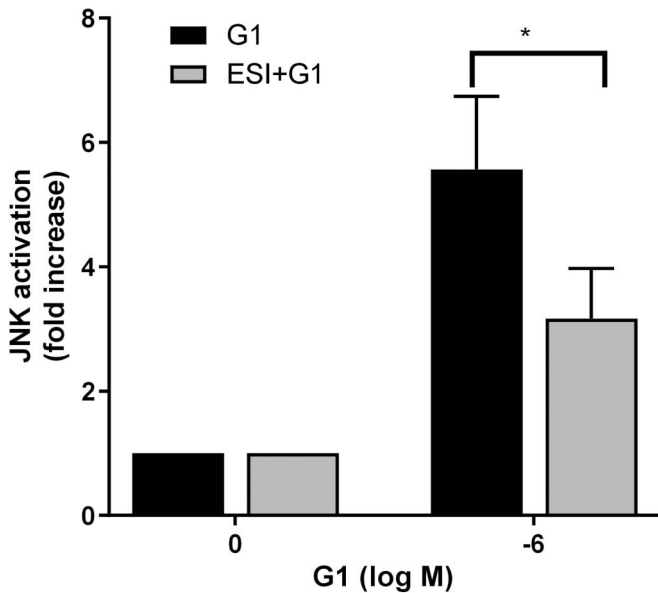
The promoter region of  $\alpha_{2C}$ -AR harbors an AP-1 consensus binding site located at  $-346/-338$  (relative to the transcription start site), known to be activated by JNK.<sup>31</sup> Having established that G-1 activates JNK, we next wanted to evaluate whether this AP-1 site mediates G1 effects.

Indeed, G1 (1  $\mu$ M) caused a  $4.7 \pm 1.2$  versus  $2.4 \pm 0.6$  ( $P < 0.05$ ) increase in  $\alpha_{2C}$ -AR expression in wild-type versus mutant AP-1-transfected cells, respectively (Fig. 6). These results show that the AP-1 site is necessary for G-1-induced  $\alpha_{2C}$ -AR upregulation. Importantly, G1 still elicited a significant increase in mutant-AP1 transfectants, suggesting that sites other than AP-1 are also involved.

## DISCUSSION

RP is a vasospastic disorder characterized by exaggerated cold-induced constriction of peripheral blood vessels.<sup>32</sup> It is classified into primary or secondary, depending on the disease origin.<sup>33</sup> Whereas secondary RP is caused by an underlying pathologic condition, mainly scleroderma,<sup>34</sup> primary RP is idiopathic and has no known reason.<sup>35</sup> However, recent evidence supports a multitiered theory of RP.<sup>10</sup> This theory states that the RP is the result of local, hormonal, and neuronal mediators.<sup>6</sup> The impaired function of any of these mediators may lead to exaggerated vasoconstriction.<sup>36</sup> Furthermore, the high incidence of RP in premenopausal estrogen-replete females along with other previously discussed observations supported a positive correlation between estrogen and RP.<sup>13</sup>

Studies from our laboratory reported that estrogen potentiates cold-induced vasoconstriction by upregulating  $\alpha_{2C}$ -AR expression. We also showed that this estrogen-induced upregulation is mediated via an EPAC-mediated JNK/AP-1- dependent mechanism. Furthermore, we showed



**FIGURE 4.** Epac mediates G-1-induced JNK activation. Cells were treated with G-1 (1  $\mu$ M) for 30 minutes, in the presence or absence of ESI09 (ESI; 10  $\mu$ M; a specific Epac inhibitor). The inhibitor was added 30 minutes before adding G1. JNK activity was then assessed as in the Methods section.

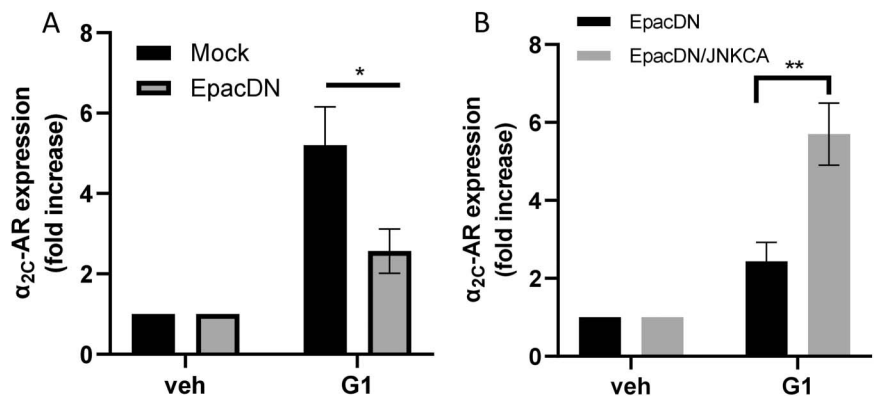
that this estrogenic effect is mimicked by a cell-impermeable form of the hormone (estrogen:BSA), suggesting the implication of estrogen membrane receptor, GPER, in estrogen-induced  $\alpha_{2C}$ -AR upregulation. However, the role of GPER in this upregulation remains to be conclusively established. In this study, we delineated the role of GPER in  $\alpha_{2C}$ -AR expression. Indeed, we showed that GPER-mediated estrogen-induced  $\alpha_{2C}$ -AR upregulation in a cAMP/Epac/JNK/AP-1 signaling pathway. The pharmacologic inhibition of any of these players abrogated  $\alpha_{2C}$ -AR upregulation in response to GPER activation.

To our knowledge, this is the first study to irrefutably report that estrogen activates its membrane receptor to induce  $\alpha_{2C}$ -AR upregulation in microVSMCs. This is of utmost

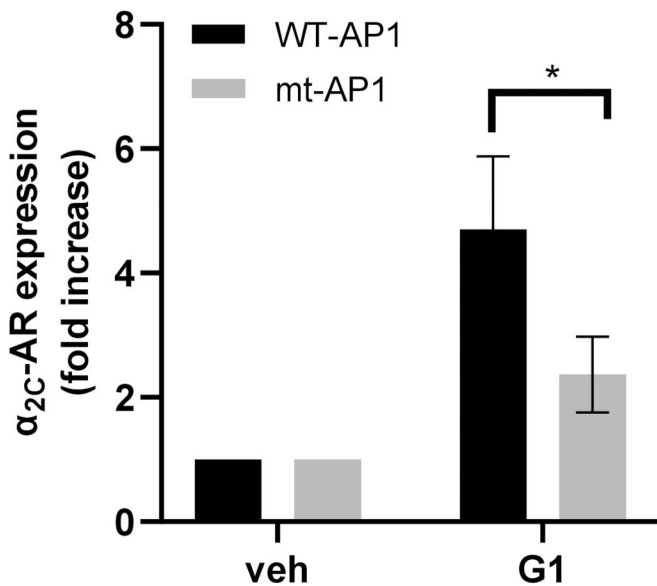
significance for at least 2 reasons. First, this study presents GPER as a therapeutic target for RP in estrogen-replete females. Second, this study is performed in VSMCs extracted from human cutaneous arterioles. Given the absence of an appropriate animal model for RP, which is a major challenge for RP-related studies, these cells are considered the best mimicry of RP pathophysiology. It is worth-mentioning that the vascular bed from which VSMCs are extracted greatly affects their response to estrogen.<sup>37</sup> Thus, estrogen-induced signaling pathways identified in macroVSMCs may not necessarily be valid in microVSMCs. Furthermore, in compliance with the cross-over approach, we used VSMCs of male origin to test the effect of estrogen or GPER agonist, G-1. The aim of this approach is to ease the background noise by minimizing the carryover of the tested hormone from the donor.<sup>38</sup> Yet, a drawback to this approach is that cells extracted from a given sex, a male in our case, have not been exposed to high concentration of the hormone of the other sex. Therefore, GPER has not been activated by high concentrations of estrogen or GPER agonist. Another limitation is the reliance on transcriptional studies to infer conclusions herewith. That said, it is important to mention here that in all our previous studies, cAMP-increased or E2-induced transcription of  $\alpha_{2C}$ -AR have always been reflected in increased protein levels as well.

The protective role of GPER in vasculature has been extensively studied. Female GPER<sup>-/-</sup> mice showed elevated blood pressure, increased vascular resistance, and enhanced progression of atherosclerosis.<sup>39</sup> Treatment with GPER agonist reduced postmenopausal atherosclerosis in mice, suggesting an atheroprotective function of GPER.<sup>40</sup> Intravenous infusion of GPER agonist into normotensive rats Sprague-Dawley rats decreased blood pressure and dilated precontracted resistance arteries.<sup>41</sup> These findings highlight the role of GPER in regulating blood pressure and vascular tone.<sup>41</sup> In accordance, genetic linkage analysis in humans showed that the GPER gene maps to chromosome 7p22.3, a region implicated in arterial hypertension.<sup>42,43</sup> In addition to this blood pressure-lowering and antiatherogenic role, GPER plays a vital role in maintaining cardiac function and structure. Activation of GPER enhanced myocardial relaxation in the

**FIGURE 5.** JNK acts downstream of Epac to mediate G1-induced  $\alpha_{2C}$ -AR transcription. A, Cells were transiently cotransfected with  $\alpha_{2C}$ -AR promoter-reporter along with mock or expression plasmids for Epac DN. After recovery, cells were treated with G1 (1  $\mu$ M), and reporter activity was measured. B, Cells were transiently cotransfected with  $\alpha_{2C}$ -AR promoter-reporter and expression plasmid for Epac DN with (black bars) or without (gray bars) an expression plasmid for constitutive active JNK (JNKCA). After recovery, cells were treated with G1 (1  $\mu$ M), and reporter activity was measured. Results (for A and B) are expressed as fold increase in the firefly (FF)/*Renilla* luminescence and are presented as means  $\pm$  SD. \* $P$  < 0.05 and \*\* $P$  < 0.01.



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**FIGURE 6.** AP-1 site is necessary for G-1-induced  $\alpha_{2C}$ -AR expression. Cells were transiently transfected with  $\alpha_{2C}$ -AR promoter-reporter construct (wild type, WT, or the AP-1 site mutant, mtAP-1). After recovery, cells were treated with or without G-1 (1  $\mu$ M) and promoter activity was measured. Data were normalized for transfection efficiency using an internal control (*Renilla*) and results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means  $\pm$  SEM. \* $P < 0.05$ .

hypertensive female mRen2.Lewis rat and attenuated cardiac hypertrophy and wall thickness in high-salt diet-fed ones.<sup>44</sup> The deletion of GPER in mice lead to left ventricular dysfunction, impaired systolic and diastolic functions, and adverse remodeling.<sup>40,45,46</sup>

In this study, we showed that pharmacologic inhibition of GPER attenuates estrogen-induced  $\alpha_{2C}$ -AR transcription, whereas GPER activation upregulates  $\alpha_{2C}$ -AR. This indicates that GPER mediates estrogen-induced transcription of  $\alpha_{2C}$ -AR. Knowing that  $\alpha_{2C}$ -AR is the sole mediator of cold-induced vasoconstriction, it becomes evident that GPER is necessary and sufficient for estrogen-potentiated cold-induced vasoconstriction. In accordance, previous study showed that the activation of GPER by its agonist or by estrogen induced vasoconstriction in basal renal perfusion pressure.<sup>47</sup> Another study reported that intravenous application of GPER agonist, G15, abolished the facilitatory effect of estrogen on cooling-induced reduction of PSBF.<sup>48</sup> Whereas we delineated the mechanism by which GPER mediates estrogen-evoked upregulation of  $\alpha_{2C}$ -AR, this aforementioned study did not elucidate the role of GPER in  $\alpha_{2C}$ -AR regulation. Other studies showed that the activation of GPER by its agonist, G-1, induced vasodilatory effects in small arteries,<sup>49</sup> rat aorta and carotid artery,<sup>50,51</sup> porcine coronary arteries,<sup>52</sup> and human internal mammary arteries.<sup>53</sup> Similar vasodilatory effect was observed in mesenteric resistance arteries.<sup>28,54</sup> The presumed vasoconstrictor effect in our study suggests that GPER expectedly mediates vasoconstriction at low temperature. This may explain inconsistency with the previously reported vasodilatory effect in mesenteric arteries.

Activation of GPER initiates a transduction signaling pathway whose players are often cAMP, MAPK, and phosphatidylinositol 3-kinase (PI3K), and intracellular calcium.<sup>55</sup> Here, the activation of GPER by its agonist elevates cAMP level. This is in accordance with a 2 previous studies showing that GPER induction increased cAMP levels in rat mesenteric smooth muscle cells<sup>28</sup> and in rat aortic smooth muscle (A7r5) cell line.<sup>56</sup> Notably, these groups reported that cAMP signaling contributed to GPER-mediated vasorelaxation<sup>28</sup> and suppressed angiotensin (Ang) II-induced hypertension of mesenteric arteries, respectively. In discordance, our results indicate that GPER-induced elevation of cAMP mediates  $\alpha_{2C}$ -AR upregulation, and thus potentiates cold-induced vasoconstriction. It is worth mentioning that previous reports from our laboratory showed that estrogen increased cAMP accumulation in microVSMCs, and that this increment activated a signaling cascade leading to  $\alpha_{2C}$ -AR upregulation.<sup>25</sup> Here, we showed that GPER activation mediated  $\alpha_{2C}$ -AR upregulation via a cAMP-dependent signaling pathway. Thus, it is only logical to conclude that this estrogenic effect is mediated via GPER.

An elevation in cAMP level is sensed by 2 intracellular cAMP receptors, the extensively studied protein kinase A and the later-discovered cAMP-regulated guanine nucleotide exchange factor Epac.<sup>57</sup> These effectors, PKA and Epac, regulate cAMP signaling pathways by working synergistically or by opposing each other.<sup>57</sup> This provides a delicate control over cellular responses. We previously demonstrated that in microVSMCs, cAMP plays a dual effect on  $\alpha_{2C}$ -ARs, upregulation via Epac, and downregulation via PKA.<sup>29</sup> However, the excitatory role of Epac is predominant and masks the suppressive effect of PKA.<sup>29</sup> We also showed that estrogen elevates cAMP levels leading to Epac-mediated increase in  $\alpha_{2C}$ -AR expression.<sup>25</sup> Here, we showed that the activation of GPER by its agonist induced an elevation in cAMP, which was responsible for Epac-dependent, but PKA-independent transcriptional activation of  $\alpha_{2C}$ -ARs. Therefore, it is GPER that mediates estrogen-induced  $\alpha_{2C}$ -AR upregulation via cAMP/Epac signaling.

cAMP signaling pathways are determined by caveolae microdomains, where adenylyl cyclase, Epac, PKA, and estrogen receptors reside.<sup>58–60</sup> Here, we showed that Epac, but not PKA mediates the activation of JNK in response to GPER induction. This result is in discordance with a previous study reporting that Epac synergizes with PKA to inhibit the JNK activation in macroVSMCs.<sup>32</sup> This discrepancy could be explained by the difference in the used model, micro- versus macro-VSMC or by the signaling components compartmentalized in the involved caveolae. In this study, we can suggest that GPER, adenylyl cyclase, Epac, and JNK are compartmentalized in the same microdomain.

Pharmacologic inhibition of JNK inhibited the upregulation of  $\alpha_{2C}$ -AR in response to GPER activation. In addition, molecular inhibition of Epac abolished GPER-mediated  $\alpha_{2C}$ -AR upregulation. Cotransfection of VSMCs with Epac-DN and constitutively active JNK restored  $\alpha_{2C}$ -AR upregulation. These results show that JNK activation is necessary and sufficient for  $\alpha_{2C}$ -AR transcription in response to GPER activation. Knowing that  $\alpha_{2C}$ -AR is the sole mediator of cold-

induced vasoconstriction, it becomes evident that JNK is necessary for this vasoconstriction. This result is in line with previous studies highlighting the role of JNK in regulating vascular tone. For instance, JNK inhibition induced relaxation of norepinephrine-precontracted aortic rings and reduced angiotensin II-induced increase in systolic blood pressure.<sup>61</sup> Notably, in these studies, JNK contributed to vascular relaxation rather than vasoconstriction.

In the context of  $\alpha_2C$ -AR expression in response to estrogen, we previously showed that JNK activation was achieved within minutes post-treatment,<sup>26</sup> suggesting a rapid nongenomic effect of estrogen. We also showed that JNK mediated estrogen-potentiated cold-induced  $\alpha_2C$ -AR translocation.<sup>26</sup> This suggests that JNK-facilitated  $\alpha_2C$ -AR translocation is also a rapid nongenomic estrogenic response. Here, we showed that JNK induction in response to GPER activation occurs rapidly (also within minutes). This further reinforces that estrogen activates JNK via GPER in a rapid nongenomic response. It also suggests that estrogen-potentiated cold-induced  $\alpha_2C$ -AR translocation occurs via GPER-activated JNK-mediated signaling. Notably, in isolated perfused rat kidney, GPER vasoconstrictor response to G1 or estrogen takes place through a plethora of signaling pathways including PKC, p38, and ERK1/2, but not JNK.<sup>47</sup>

It is well-established that estrogen receptors regulate gene expression not only through the classical estrogen response element (ERE), but also via AP-1 site. Whereas ER $\alpha$  and ER $\beta$  bind to ERE in the promoter region, thus inducing the expression of target gene, GPER mediates this expression by increasing the activity of Jun/Fos and subsequently activating the AP-1 site in its promoter.<sup>62</sup> For example, in SkBr3 breast cancer cells and cancer-associated fibroblasts obtained from breast cancer patients, GPER mediated the expression of SIRT-1 in response to estrogen via a c-fos/AP-1-dependent pathway.<sup>63</sup> Furthermore, in MCF-10A cells, GPER mediated estrogen-induced upregulation and secretion of matrix metalloproteinase 3 (MMP-3) and interleukin-1 $\beta$  (IL-1 $\beta$ ) via JNK/AP-1 signaling pathway.<sup>64</sup> In VSMCs, the promoter region of  $\alpha_2C$ -AR harbors an AP-1 binding site at -346/-338 relative to transcription start site.<sup>31</sup> Estrogen increased the transcriptional activity of the  $\alpha_2C$ -AR promoter through JNK/AP-1 signaling.<sup>26,65</sup> However, the estrogenic receptor mediating this signaling pathway was not identified. Here, we show that the activation of GPER increased the transcriptional activity of the  $\alpha_2C$ -AR promoter via the same AP-1/JNK signaling pathway. It is worth mentioning that, GPER mediates estrogen-induced recruitment of the AP-1 to different nucleosome in the promoter of target genes, thus inducing their expression.<sup>66</sup> Notably, mutating the AP-1 site did not completely abrogate GPER-induced  $\alpha_2C$ -AR expression, suggestive of the involvement of other sites in the promoter region of  $\alpha_2C$ -AR. Indeed, preliminary results from our laboratory support the role of SP-1, SP-3, and half ERE sites in the induced activation of  $\alpha_2C$ -AR promoter.

Enormous efforts in clinical and basic research have been undertaken to unravel the mechanism of RP onset, pathogenesis, and treatment. However, the aforementioned mosaic theory of the disease seems to be strongly supported,

thus adding another level of complication to this condition, which then makes this disease rather challenging to treat.<sup>10</sup> Another challenge is the expression of  $\alpha_2C$ -AR in many brain regions and its implication in the presynaptic regulation of the heart. Thus, the attempt to treat RP by targeting  $\alpha_2C$ -AR may impede its function in these vital organs. Till now, no definitive drug for RP has been yet approved by the American Food and Drug Administration.<sup>67</sup> However, some pharmacologic drugs are believed to lessen the symptoms of the disease.<sup>13</sup> Indeed, dissecting the mechanisms underlying RP provides insight to the pathogenesis of the disease and importantly presents the downstream players as potential therapeutic targets of RP. Previous reports from our laboratory identified Epac, Rap, and JNK as potential therapeutic targets for RP.<sup>13,25,26,48</sup> Here, we present GPER as a therapeutic target that may alleviate deleterious effects of RP. Interestingly, targeting GPER has been tested for the treatment of other vascular diseases. Indeed, treatment of postmenopausal mice with the synthetic small molecule GPER-selective agonist G-1 attenuated atherosclerosis.<sup>40</sup> In addition, intravenous infusion of G-1 resulted in dilation of precontracted resistance arteries of Sprague-Dawley rats.<sup>41</sup>

## CONCLUSIONS

Collectively, these studies introduce selective GPER targeting as a novel therapeutic approach to attenuate vascular diseases such as postmenopausal atherosclerosis, hypertension, and importantly RP. Further studies are warranted to ensure efficiency and safety of this approach.

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