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Estrogen increases expression of vascular alpha 2C adrenoceptor through the cAMP/Epac/JNK/AP-1 pathway and potentiates cold-induced vasoconstriction

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ABSTRACT

Cutaneous cold-induced vasoconstriction is a normal physiological reaction mediated by alpha 2C-adrenergic receptors (α_{2C}-ARs) expressed in vascular smooth muscle cells (VSMCs). When this reaction is exaggerated, Raynaud's phenomenon (RP) ensues. RP is more prevalent in females compared to age-matched men. We previously established that 17-β estradiol (estrogen) upregulates α_{2C}-ARs in human VSMCs via a cAMP/Epac/Rap pathway. We also showed that cAMP acts through JNK to increase α_{2C}-AR expression. However, whether estrogen employs JNK to regulate α_{2C}-AR is not investigated. Knowing that the α_{2C}-AR promoter harbors an activator protein-1 (AP-1) binding site that can be potentially activated by JNK, we hypothesized that estrogen regulates α_{2C}-AR expression through an Epac/JNK/AP-1 pathway. Our results show that estrogen (10^{-10} M) activated JNK in human VSMCs extracted from cutaneous arterioles. Pretreatment with ESI09 (10 μM; an Epac inhibitor), abolished estrogen-induced JNK activation. In addition, pre-treatment with SP600125 (3 μM; a JNK specific inhibitor) abolished estrogen-induced expression of α_{2C}-AR. Importantly, estrogen-induced activation of α_{2C}-AR promoter was attenuated with SP600125. Moreover, transient transfection of VSMCs with an Epac dominant negative mutant (Epac-DN) abolished estrogen-induced activation of α_{2C}-AR promoter. However, co-transfection of constitutively active JNK mutant overrode the inhibitory effect of Epac-DN on α_{2C}-AR promoter. Moreover, estrogen caused a concentration-dependent increase in the activity of AP-1-driven reporter construct. Mutation of AP-1 site in the α_{2C}-AR promoter abolished its activation by estrogen. This in vitro estrogen-increased α_{2C}-AR expression was mirrored by an increase in the ex vivo functional responsiveness of arterioles. Indeed, estrogen potentiated α_{2C}-AR-mediated cold-induced vasoconstriction, which was abolished by SP600125. Collectively, these results indicate that estrogen upregulates α_{2C}-AR expression via an EPAC-mediated JNK/AP-1- dependent mechanism. These results provide an insight into the mechanism by which exaggerated cold-induced vasoconstriction occurs in estrogen-replete females and identify Epac and JNK as potential targets for the treatment of RP.

1. Introduction

Cold-induced vasoconstriction at the level of the extremities is a normal physiological reaction [1]. It aims at reducing heat loss and redirecting blood towards internal, more vital body organs [1]. Exaggerated cold-induced vasoconstriction leads to a pathological condition termed Raynaud's phenomenon (RP) [2]. Due to increased cold sensitivity, affected individuals suffer from cold-induced vasospastic attacks and triple color change at the level of the digits [2].

Epidemiological studies show a significantly greater incidence of RP in pre-menopausal females compared to age-matched males [3,4]. In addition, post-menopausal women receiving unopposed estrogen replacement therapy (ERT) are at a higher risk of RP than those not receiving ERT [5]. Moreover, noradrenaline-mediated vasoconstriction is higher in premenopausal females at their mid-menstrual cycle [6]. This stage is characterized by higher estrogen level as compared to other

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stages of the cycle [6]. Furthermore, evidence supports a direct role of estrogen in vasoreactivity. For instance vascular responsiveness is higher in human and rat females in their reproductive age as compared to their counter males [7]. Supplying males with estrogen enhances their vascular responsiveness [7]. In addition to its role in vasoreactivity, estrogen plays a role in regulating body temperature [8]. Since RP is a vascular thermoregulatory control disorder, the implication of estrogen in the disease becomes obvious. Taken together, these observations imply that there is a positive association between the female hormone, 17β-estradiol (estrogen) and RP [43].

Mechanistically, cold-induced vasoconstriction results from a reflex reaction mediated by neuronal (norepinephrine) and local effectors that increase vascular sensitization to cold [1,9]. The latter effect is mediated by adrenergic receptors located on the surface of vascular smooth muscle cells (VSMCs) of cutaneous arterioles [1,9]. VSMCs express α_1, α_2, and β_2 adrenergic receptors [10]. While β_2-ARs are involved in vasodilation [11], α_1-ARs and α_2 -ARs are known to exert a vasoconstrictive effect [12]. In arterioles, α_1-ARs do not play a role in cold-induced vasoconstriction evident by the lack of any effect of their blockers on this constriction [13]. α_{2A}-ARs and α_{2C}-ARs, but not α_{2B}-ARs, are expressed in human cutaneous VSMCs. Interestingly, inhibition of α_{2C}-ARs, but not α_{2A}-ARs, abolished cold-induced vasoconstriction [13]. This clearly shows that α_{2C}-AR is the sole mediator of the entirety of cold-induced vasoconstriction.

A distinctive characteristic of α_{2C}-ARs is their intra-cellular localization in the ER-Golgi apparatus [14]. These entrapped α_{2C}-ARs are functionally competent, as they successfully bind to radioligands [15,16]. Interestingly, α_{2C}-ARs were observed on the cell membrane of PC12 and At-T20 neuroendocrine cells, indicating that their mobilization to the cell surface could be regulated [16]. Relevantly, certain stimuli such as cold induces the mobilization of α_{2C}-ARs to the cell surface [17], where they become available to their ligand [14,17]. This spatial translocation of α_{2C}-AR is prerequisite to unmask their functional ability to induce vasoconstriction [13,18].

We have previously reported that estrogen increases α_{2C}-AR expression in VSMCs via a cAMP-mediated pathway [19]. Moreover, elevated cAMP levels in VSMCs increases the expression of α_{2C}-AR by activating JNK [20]. Activated JNK is known to initiate the transcription of many AP-1-dependent genes [21]. Interestingly, the promoter region of α_{2C}-AR harbors an AP-1 binding site [22]. However, whether estrogen employs JNK and AP-1, or whether Epac acts through AP-1, to regulate α_{2C}-AR expression is still unknown. In this study, we investigated the role of JNK and AP-1 in estrogen-induced or Epac-mediated α_{2C}-AR expression.

2. Materials and methods

2.1. Reagents

DMEM:F12 (D8437), phenol red free DMEM (D6434), L-Glutamine (G7513), Fetal Bovine Serum (F9665), Phosphate Buffer Saline (D1408), L-Glutamine (G7513), and Epac inhibitor ESI09 (SML0814) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Penicillin/Streptomycin (17-602E), Trypsin (BE02-007E) and Amaxa Nucleofector (VPC-1001) were obtained from Lonza (Basel, Switzerland). Anti-JNK1 + JNK2 + JNK3 (phospho T183 + T183 + T221) (ab124956), Anti-JNK1 + JNK2 + JNK3 antibody (ab179461), anti-alpha 2C adrenergic receptor (ab123368), anti-β-actin antibody (ab119716), HRP-conjugated Goat Anti-Mouse antibody (ab97040), HRP-conjugated Goat Anti-Rabbit antibody (ab150080), 17-β estradiol (ab120657), JNK inhibitor SP600125 (ab120065) and UK 14,304 (ab120773) were purchased from Abcam. *DC*™ Protein Assay kit and Clarity™ Western ECL Substrate were purchased from Biorad (CA, USA). Insulin-Transferin-Selenium (41400045) was obtained from Thermofischer Scientific (USA). Luciferase assay kit (E1500) and pRL-CMV [*Renilla* luciferase gene driven by cytomegalovirus (CMV) promoter/enhancer] were purchased from Promega (Wisconsin, USA). The activator protein (AP)-1-luciferase reporter plasmid was obtained from Stratagene (California, United States). The α_{2C}-AR promoter-reporter plasmid (−1915/+5, relative to the transcription start site +1) was a kind gift from Dr. Herve Paris [23,24].

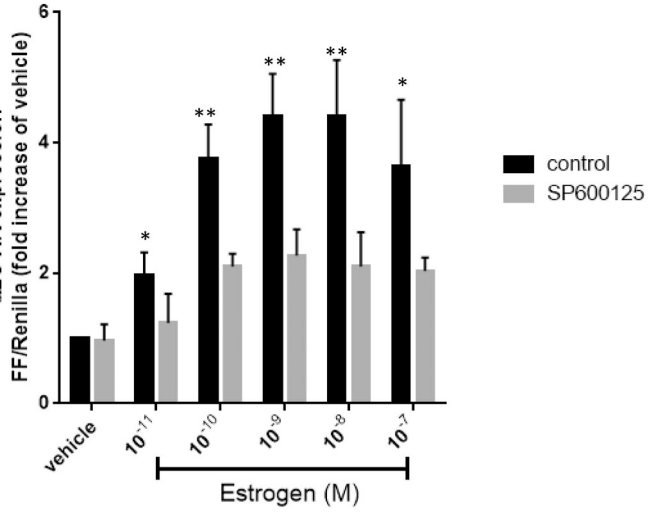

Fig. 1. JNK mediates estrogen-induced activation of α_{2C}-AR promoter in VSMCs.
Cells were transiently transfected with the α_{2C}-AR promoter-reporter construct and internal *Renilla* control construct. After recovery, cells were starved for 48 h then treated with increasing concentrations of estrogen (10^{-11} to 10^{-7} M, 24 h), in the presence or absence of SP600125 (3 μM; a JNK specific inhibitor). SP600125 was administered 30 min before and during exposure of the cells to estrogen. Promoter activity was assessed by luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means ± SEM. $n = 3$, $^* p < .05$

2.2. Cell culture

Human arteriolar SMCs were extracted by non-enzymatic sprouting method from dermal arterioles of a post-circumcision tissue of a newborn boy. No IRB approval is needed as this source is considered clinical waste; however, ethical approval was obtained. Cells were used between passages 6 and 11, during which the expression and regulation of α_{2C}-ARs is similar [24]. VSMCs were maintained in Ham's Growth medium (DMEM: F12; 50:50) supplemented with 10% FBS and 1% penicillin/streptomycin. Prior to treatment, cells were made quiescent for 48 h in phenol red free DMEM supplemented with L-glutamine, insulin-transferrin-selenium, and 1% penicillin/streptomycin. Cells were grown in a 5% CO_2 incubator at 37 °C.

2.3. Western blotting

Cells were washed with PBS and then lysed using lysis buffer (2% SDS, 60 mM Tris (pH 6.8)), as previously described [20]. Proteins were quantified using *DC*™ Protein Assay and equal amounts of protein (20-30 μg) were loaded on SDS-PAGE. After running, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Biorad). The membrane was blocked with fat-free milk (5% in TBS-T) for 1 h at room temperature, then incubated overnight with primary antibody at 4 °C. Then, the membrane was washed with TBS-T, 3 times for 10 min each, and incubated with the relevant HRP-conjugated secondary antibody for 1 h at room temperature. The membrane was washed again, developed using enhanced chemiluminescence (ECL clarity, Biorad), and quantified using Chemidoc MP Imaging system. Each experiment was repeated at least 3 different times, and western blot quantitated.

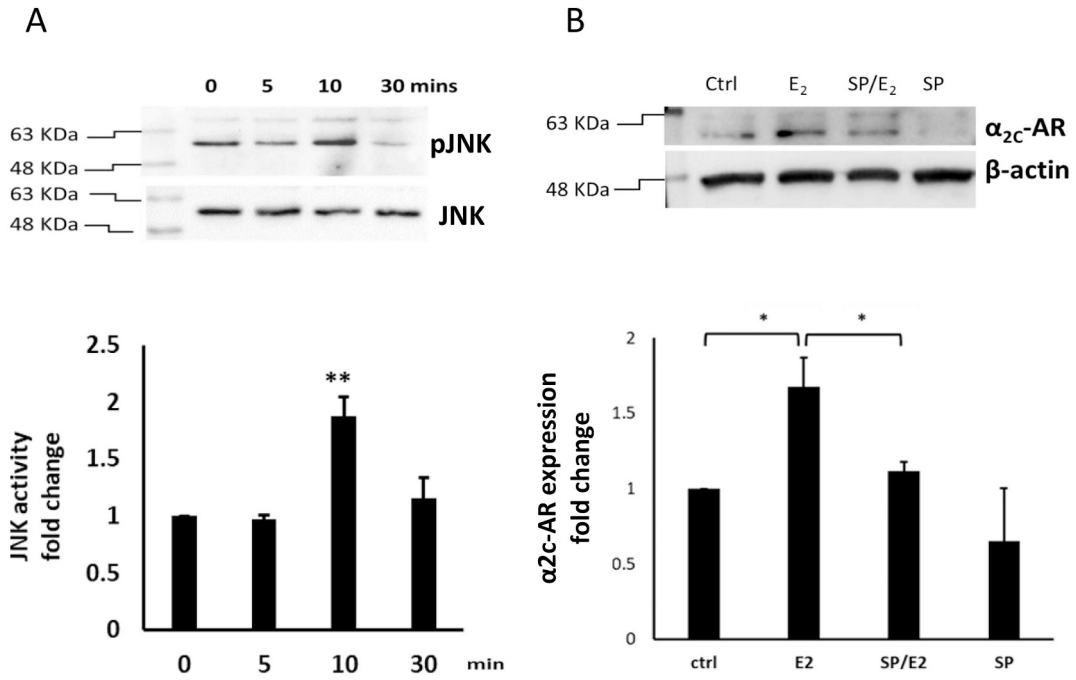

Fig. 2. JNK mediates estrogen-induced expression of α_{2C}-AR in VSMCs.
A. Cells were treated with estrogen (10^{-10} M) for 5, 10 and 30 min. Total and phosphorylated JNK were assessed using Western blotting. Top: blot representing the effect of estrogen (10^{-10} M) on phospho-JNK levels. The results are expressed as fold increase from the control at time 0 and are presented as means ± SEM. $n = 3$, ** $p < .01$. B. Cells were treated with estrogen (10^{-10} M) for 24 h, in the presence or absence of SP600125 (3 μM; a JNK specific inhibitor), and cell lysates were assessed for α_{2C}-AR expression using Western blotting. SP600125 was administered 30 min before and during exposure of the cells to estrogen. Top: blot representing the effect of estrogen (10^{-10} M) on α_{2C}-AR expression in the absence or presence of SP600125. The results are expressed as fold increase from the control and are presented as means ± SEM. $n = 3$, *$p < .05$.

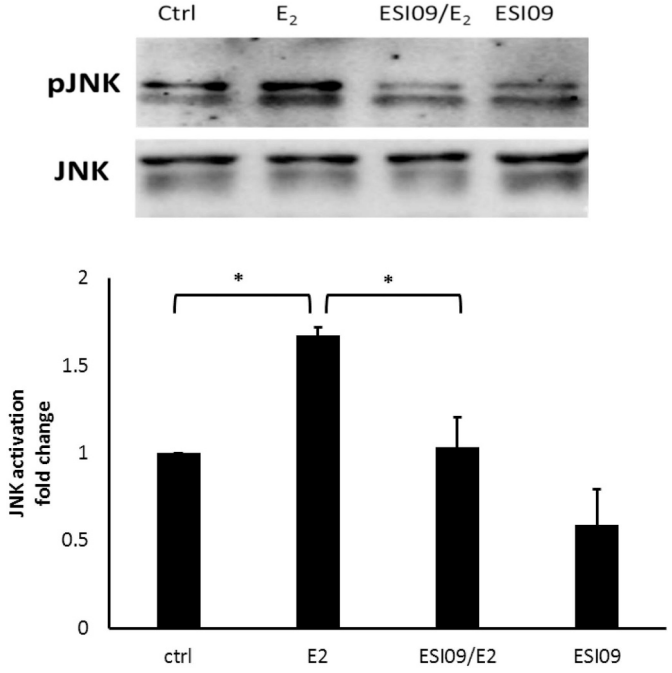

Fig. 3. Epac mediates estrogen-induced JNK activation.
Cells were treated with estrogen (10^{-10} M) for 10 min, in the presence or absence of ESI09 (10 μM; an Epac specific inhibitor). ESI09 was administered 30 min before and during exposure of the cells to estrogen. Cell lysates were assessed for JNK phosphorylation using Western blotting. Top: blot representing the effect of estrogen on JNK phosphorylation, in the presence or absence of ESI09. The results are expressed as fold increase from the control and are presented as means ± SEM. $n = 3$, *$p < .05$.

2.4. Site directed mutagenesis

As we previously reported [20], site-directed mutagenesis of AP-1 site was performed using the QuickChangeTM XL-site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutant oligonucleotide primers and double stranded DNA were employed to mutate wild-type AP-1 site (ATGATTCAT, −346/−338, relative to the transcription start site) in the α_{2C}-AR promoter-luciferase reporter construct to ACTGTTTGT.

2.5. Transient transfections

VSMCs were transiently transfected by nucleofection through the Nucleofector device according to the manufacturer's instructions (Lonza) as we previously described [23]. Optimum transfection level (80% transfection efficiency, minimal toxicity) was attained with nucleofection of 400 × 10^3 cells with 4 μg of nucleic acid. Total amount of nucleic acid was kept constant for all conditions. When needed, appropriate empty plasmid was used for adjustment. After transfection, cells were allowed to recover overnight in complete media. *Renilla* luciferase CMV immediate early enhancer/promoter (pRL-CMV) was used as an internal control to normalize the firefly luciferase units.

For luciferase analysis, cells were washed with PBS and lysed with luciferase lysis buffer, snap frozen, and then thawed at room temperature water bath. Cell lysates are centrifuged at 10,000*g* for 10 min and the supernatant was used to determine luciferase activity.

2.6. Vasomotor activity of isolated arterioles

Male mice (C57BL6) aged 10 to 12 weeks were sacrificed by CO_2 asphyxiation, and the tail artery was isolated and placed in cold Krebs buffer (in mM: 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 glucose). Arterial segments were mounted in a microvascular chamber, and maintained at constant temperature

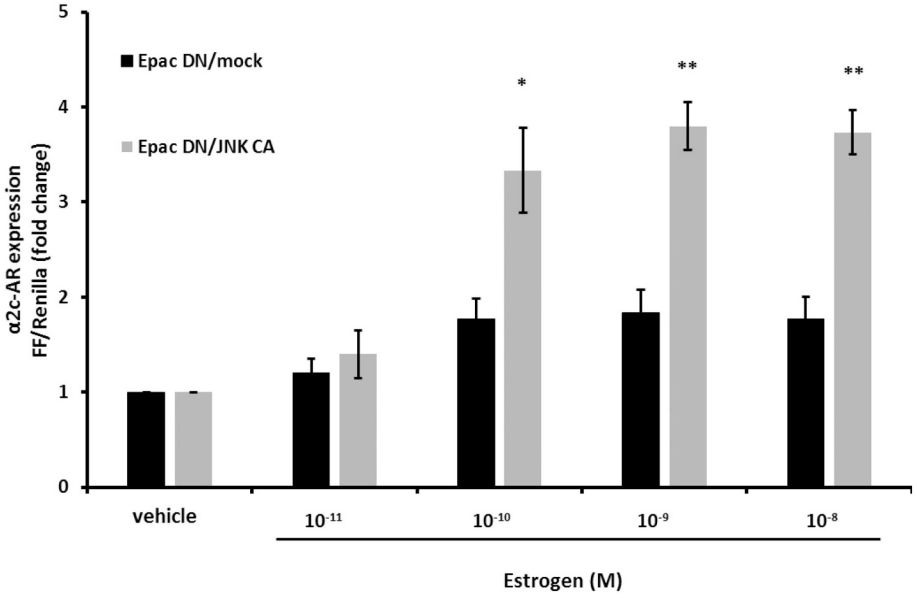

Fig. 4. JNK acts downstream of Epac to mediate estrogen-induced activation of α2c-AR promoter.
Cells were transiently co-transfected with α_{2C}-AR promoter: reporter along with expression plasmids for Epac DN, without or with another expression plasmid for a constitutively active mutant of JNK (JNK CA). Internal *Renilla* plasmid was used as an internal control. After recovery, cells were starved for 48 h then treated with increasing concentrations of estrogen (10^{-11}–10^{-8} M, 48 h). Promoter activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescence and are presented as means ± SEM. $n = 3$, * denotes $p < .05$ and ** denotes $p < .01$.

(37 °C) and pressure (60 mmHg), with continuous perfusion in Krebs solution. Live vessel images were acquired through a video camera connected to an inverted microscope, focused on the vessel chamber. The vessel diameter was directly determined using acquisition system (LabChart, ADInstruments, UK). Vessel stabilization was done using increasing concentrations of α_2-AR selective agonist (UK-14304). The effect of the agonist was stopped by successive washes of the artery, allowing it to return to its baseline level. For cold temperature studies, the temperature of the chamber medium was decreased to 28 °C for 30 min before assessing α_2-AR vasoconstriction. Concentration-effect curves were analyzed by comparing the agonist concentration causing 20% constriction (CC_{20}), determined by regression analysis.

The use of male mice (C57BL6) was approved by American University of Beirut animal care and use committees in compliance with the national institute of health guide for the care and use of laboratory animals.

2.7. Immunofluorescence-confocal microscopy

Cells were washed with PBS then fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were then washed with PBS and blocked with 3% normal goat serum (NGS) for 1 h at room temperature. Next, cells were washed and probed overnight at 4 °C using primary antibody in 1% NGS. After washing, cells are incubated with secondary antibody prepared in 1% NGS for 1 h at room temperature. Nuclear staining was done using Hoechst dye for 15 min at room temperature RT. T Cells were then mounted on a slide using anti-fade medium. Cells were visualized by laser scanning confocal microscopy (×40 oil objective, 512 × 512 pixels, LSM510, Zeiss, Germany). Quantitation of mean fluorescence intensity of cell surface α_{2C}-ARs was assessed at the cell boundary of each cell using the Image J software.

2.8. Statistical analysis

Data were statistically evaluated using student's *t*-test using GraphPad Prism version 5.0 and InStat3 Software (GraphPad software, Inc. San Diego, CA). For the luciferase assay, 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 ± SEM, where *n* is equal to the number times an experiment was repeated for in vitro experiments, and number of subjects for ex vivo experiments. For the comparison of more than two means, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). It is worth mentioning here that statistical analysis of non-normalized values yielded similar results to normalized values.

3. Results

3.1. JNK mediates estrogen-induced activation of α_{2C}-AR promoter

We sought to assess the role of JNK in estrogen-induced activation of full length α_{2C}-AR promoter. As expected, treatment of cells with increasing concentrations of 17-β estradiol (10^{-11}- 10^{-7} M) increased the transcriptional activity of α_{2C}-AR promoter-reporter construct in a concentration-dependent manner (Fig. 1). The highest activation was attained at an estrogen concentration of 10^{-9} M (4.2 ± 0.2; $p < .01$) (Fig. 1). This estrogen-induced increase in the α_{2C}-AR promoter activity was attenuated by pretreatment with SP600125, a JNK inhibitor (3 μM) (Fig. 1). These results indicate that estrogen-induced transcription of α_{2C}-AR is mediated by JNK.

3.2. Estrogen induces α_{2C}-AR expression via JNK activation

We next sought to determine whether estrogen activates JNK. Indeed, 17β-estradiol (10^{-10} M) caused a time-dependent activation of JNK (Fig. 2A). The maximal JNK phosphorylation was attained 10 min post-estrogen treatment.

We further wanted to assess the role of JNK activation in estrogen-induced expression of α_{2C}-AR. Whereas estrogen increased α_{2C}-AR expression ($p < .05$), pretreatment with SP600125 (3 μM) abolished this estrogen-induced increase ($p < .05$) (Fig. 2B). This indicates that JNK mediates estrogen-induced expression of α_{2C}-AR.

3.3. Epac mediates estrogen-induced JNK activation

It has been reported that estrogen-induced α_{2C}-AR expression is mediated through the activation of Epac/Rap signaling [19]. Having established that α_{2C}-AR expression in response to estrogen is also dependent on JNK, we wanted to evaluate the role of Epac in estrogen-induced JNK activation. Estrogen-induced increase in JNK activity was inhibited by pretreatment with ESI09 (10 μM), a specific Epac inhibitor ($p < .05$) (Fig. 3). This indicates that estrogen-induced JNK phosphorylation in mediated through Epac signaling.

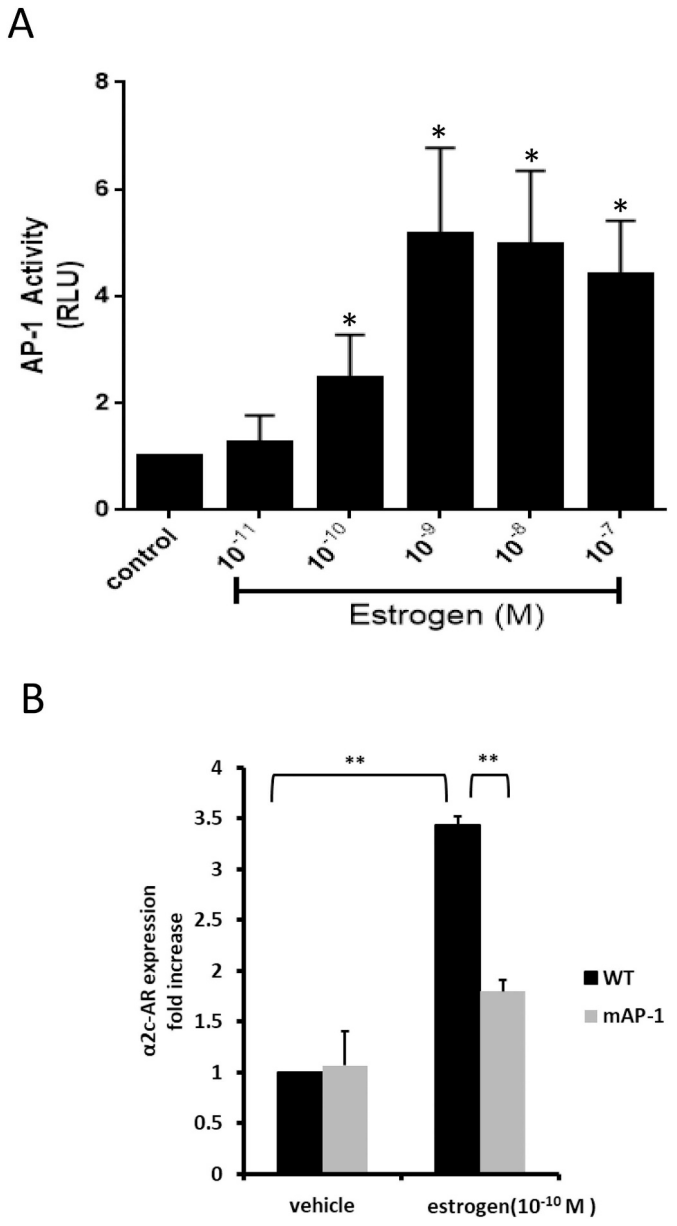

Fig. 5. AP-1 site is necessary for estrogen-induced α2C-AR expression.
A. Cells were transiently transfected with the AP-1 site-reporter construct and internal *Renilla* control construct. After recovery, cells starved for 48 h then treated with increasing concentrations of estrogen (10^{-11} to 10^{-7} M, 48 h). AP-1 site activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescencel and are presented as means ± SEM. $n = 3$, $p < .05$.
B. Cells were transiently transfected with α_{2C}-AR promoter-reporter construct (wild type, WT, or the AP-1 site mutant, mAP-1) and internal *Renilla* control construct. After recovery, cells starved for 48 h then treated estrogen (10^{-10} M, 48 h). Promoter activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means ± SEM .n = 3, ** $p < .01$.

3.4. JNK acts downstream of Epac to mediate estrogen-induced activation of α_{2c}-AR promoter

To determine whether JNK acts downstream of Epac in estrogen-induced α_{2C}-AR signaling, transient transfection were used and the activity of α_{2C}-AR promoter: reporter was assessed. Transfection with a dominant negative mutant form of Epac (Epac DN) completely abrogated estrogen-induced activation of the promoter (Fig. 4). However, co-transfection of Epac DN along with the constitutively active form of JNK (JNK CA) overweighed the inhibitory effect of Epac DN (3.8 ± 0.25 fold v/s 1.83 ± 0.24 fold for estrogen concentration of 10^{-9} M, $p < .01$). These findings suggest that estrogen elicits its effects on the α_{2C}-AR promoter by increasing the activity of Epac and JNK successively. This means that JNK acts downstream of Epac to mediate estrogen-induced α_{2C}-AR expression.

3.5. AP-1 site is necessary for estrogen-induced α_{2C}-AR expression

Estrogen induces α_{2C}-AR promoter activation through JNK, which in turn is known to activate AP-1 dependent genes [23]. Knowing that the promoter region of α_{2C}-AR harbors an AP-1 consensus binding site located at −346/−338 (relative to the transcription start site) [22], we wanted to assess the effect of estrogen on the AP-1 site activity. Treatment with increasing concentrations of 17β-estradiol (10^{-11} - 10^{-7} M, 1 h) caused a concentration-dependent increase in AP-1 activity (Fig. 5A). This increase was attenuated by mutation of the AP-1 site in the α_{2C}-AR promoter (3.43 ± 0.33 fold v/s 1.8 ± 0.11 fold, $p < 0.01$) (Fig. 5B). These results show that the AP-1 site is necessary for estrogen-induced α_{2C}-AR expression.

3.6. Effect of JNK on estrogen potentiated cold-induced vasoconstriction

We then sought to determine whether JNK-mediated α_{2C}-AR expression is associated with functional rescuing. In accordance with our previously published results, at 37 °C, 17β-estradiol (10^{-10} M, 24 h) did not significantly affect the constriction induced by activation of α_{2C}-ARs with UK-14304 (10^{-9} -10^{-5} M) (Fig. 6A) [19]. However, estrogen significantly increased vasoconstriction to UK-14304 at 28 °C. This cold-induced estrogen-potentiated vasoconstriction was abolished when JNK was inhibited by SP600125 (Fig. 6B). No effect of SP00125 alone on vasoreactivity was noted (data not shown). It is worth mentioning that we have already established that the entirety of cold-induced vasoconstriction is solely mediated by α_{2C}-ARs [13].

3.7. JNK mediates estrogen-augmented α_{2C}-AR translocation at cold temperature

Having established that JNK mediates estrogen-induced α_{2C}-AR expression, we wanted to test whether JNK affects α_{2C}-AR translocation in human microVSMCs. Cells were treated with estrogen in the presence or absence of SP600125 (3 μM), and the localization of α_{2C}-AR was determined. Indeed, our results show that estrogen induced cell-surface localization of α_{2C}-AR (Fig. 7). This estrogen-potentiated cold-induced translocation of α_{2C}-AR was inhibited by SP600125 (Fig. 7). This means that JNK mediated estrogen-induced spatial rescue of α_{2C}-AR.

4. Discussion

In this study, we showed that estrogen acts through the Epac/JNK/AP-1 signaling pathway to induce α_{2C}-AR expression (Fig. 8). We also showed that pharmacological inhibition of JNK attenuated estrogen-induced spatial and functional rescue of α_{2C}-AR at cold temperature (Fig. 8). These findings present an insight on a signaling cascade that may explain exaggerated cold-induced vasoconstriction in estrogen-replete females.

In the vasculature, cAMP regulates α_{2C}-AR expression, positively via Epac or negatively via PKA [19,23], with the former effect being more dominant in microVSMCs [23,24]. Notably, estrogen induces an increase in cAMP levels, leading to Epac-mediated Rap-2 dependent increase in α_{2C}-AR expression, or Rap-1 dependent change in cell adhesion to the substratum [44]. These selective and rather divergent activation of Epac could be explained by caveolae microdomains, where adenylate cyclase, Epac, and Rap are compartmentalized [25,26]. Here we show that this Epac mediates estrogen-induced activation of JNK in

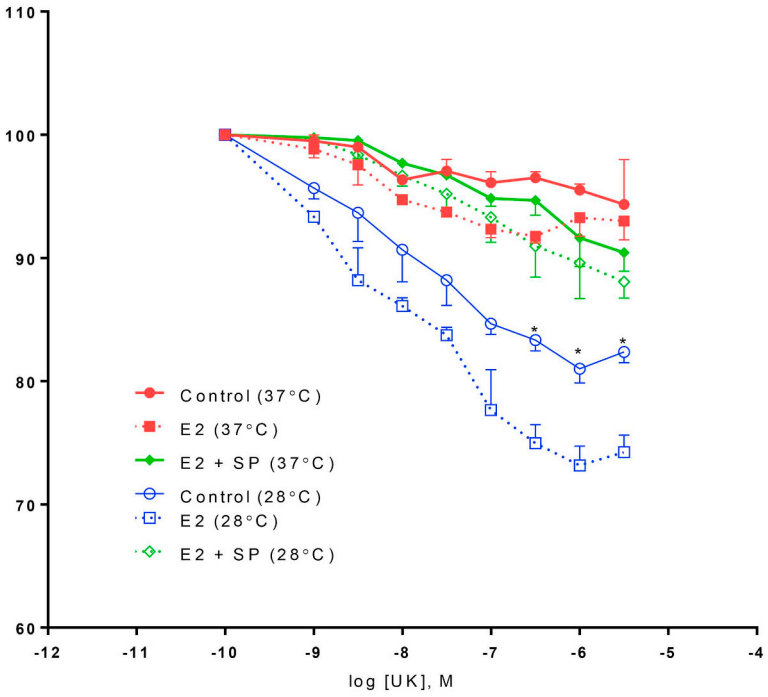

Fig. 6. Effect of estrogen (10^{-8} M, 24 h) on the function of α_{2C}-ARs in the presence or absence of SP600125, a JNK inhibitor, in mouse isolated tail arteries. SP600125 (50 μM) was administered 30 min before and during exposure of the vascular segments to estrogen. Vasoconstriction to the α_2-AR agonist, UK-14304 (10^{-9}–10^{-5} M), was assessed at 37 °C and 28 °C. Responses to the agonist were expressed as a percentage of the stable baseline diameter and are presented as means ± SEM ($n \geq 5$). Significant differences ($p < .05$) are denoted as *. Comparison here was made between Control (28 °C) and either E2 (28 °C) or E2 + SP (28 °C). Significant differences between other curves were not made here as we have already published them (e.g. between Control (37 °C) and control (28 °C), or between E2(37 °C) and E2(28 °C).

microVSMCs. We speculate that JNK is present in the vicinity of Epac, probably in the same microdomain. On the other hand, our results appear to disagree with a previous study reporting that Epac synergizes with PKA to inhibit the JNK activation in macroVSMCs [27]. Knowing that our experiments are carried in microVSMCs, the discrepancy in Epac effect may be due to the difference in the vascular bed from which VSMCs were isolated. Thus, it is not safe to extrapolate results between micro and macro VSMCs.

In the present study, we show that pharmacological inhibition of JNK attenuated estrogen-induced transcription and expression of α_{2C}-AR. In cells transfected with Epac-DN, estrogen failed to induce α_{2C}-AR expression. However, this inhibitory effect was overridden by co-transfection of VSMCs with Epac-DN and constitutively active JNK. These results show that JNK activation is necessary and sufficient for estrogen-induced α_{2C}-AR expression. Furthermore, we showed that JNK spatially and functionally rescued α_{2C}-AR in estrogen-replete environment. Indeed, while estrogen potentiated cold-induced vasoconstriction [19], inhibiting JNK abolished this effect, probably by reducing α_{2C}-ARs mobilization to the cell membrane. This mobilization is likely facilitated via Rho A/Rho-mediated actin rearrangement as we and other recently reported [17,28] . Here, we propose that JNK is a mediator of estrogen-induced α_{2C}-AR translocation. Importantly, this would not be the first report to incriminate JNK in cytoskeleton-dependent events. Indeed, in Ang-II-induced VSMC migration, the effect of Rho/Rock signaling on cytoskeletal rearrangement was mediated by JNK [29]. Thus, it may be postulated that a similar cross talk between estrogen-activated Rho/ROCK and JNK lead to α_{2C}-AR translocation in VSMCs. Further analysis is needed to validate this hypothesis.

To the best of our knowledge, this is the first study to show that estrogen increases the expression and translocation of α_{2C}-AR via JNK. In fact, JNK plays a significant role in regulating the vascular tone. Inhibiting JNK leads to a dose-dependent relaxation of norepinephrine-pre-constricted aortic rings and abolishes their norepinephrine-induced vasoconstriction [30]. In addition, JNK inhibition reduced Ang II-induced increase in systolic blood pressure [30]. Contextually, this blood pressure regulation is most critically regulated at the levels of small rather than large vessels. Knowing that Ang II has the potential to activate JNK [31], we may suggest that the hypotensive effect resulting from JNK inhibition may be due to decreased α_{2C}-AR expression and/or translocation. Alarmingly, the aforementioned experiments were done on aortic rings, which is a major limitation. The role of JNK in vascular contractility was further determined by genetic inhibition of JNK. Whereas vascular contraction was not affected in single JNK knock-out mice, it was significantly increased in JNK2 + 3 double knock-outs [32]. This implies that there is a gene-dosage effect and that JNK1 is responsible for enhanced vascular contractility, especially that JNK1-deficient mice have lower blood pressure than wild type mice [33], and that JNK-1 is an Epac target [34]. This finding also supports our notion that JNK is implicated in vasoconstriction. Further studies are warranted to test whether JNK1 particularly mediates α_{2C}-AR expression/mobilization.

Several previous studies assessed the interplay between JNK and adreneroceptors. For example, activation of $\alpha_{2_}$adrenoceptor lead to JNK phosphorylation in rat aortic SMCs [35,36] and enhanced contraction in rat aortic rings [36]. In addition, stimulation of α_1-adrenergic receptor activated JNK in rat myocytes [37]. Whether this adrenoceptor/JNK interplay is translated to α_{2C}-AR is yet to be determined. If this hypothesis is validated, then it will reveal a positive feedback loop between JNK and α_{2C}-AR, contributing to α_{2C}-AR re-sensitization.

Estrogen elicits its effects through the classical genomic pathway, involving intracellular estrogen receptors α and β (ERα and ERβ), or by rapid non-genomic response mediated by membranous G-protein estrogen receptor (GPER) [38]. In the context of α_{2C}-AR expression, we report that estrogen induced JNK activation as early as 10 min. Thus, it is only reasonable to assume that this activation is due to a rapid non-genomic effect. Notably, ERα and ERβ agonists were able to increase the expression of α_{2C}-AR in cutaneous VSMCs [19]. Likewise, 17β-estradiol:BSA, a cell-impermeable form of estrogen, mimicked estrogen effect [19]. Therefore, estrogen may act through a cell membrane receptor to activate JNK.

JNK acts through c-Jun, which dimerizes with c-Fos to form AP-1, a transcription factor that drives AP-1 mediated expression [39]. Interestingly, the promoter region of α_{2C}-AR harbors an AP-1 binding site at −346/−338 relative to transcription start site [22]. Previous findings showed that cAMP increased the transcriptional activity of the α_{2C}-AR promoter through JNK/AP-1 signaling [20]. However, in that study, cAMP levels were elevated by forskolin, a non-physiological agonist of

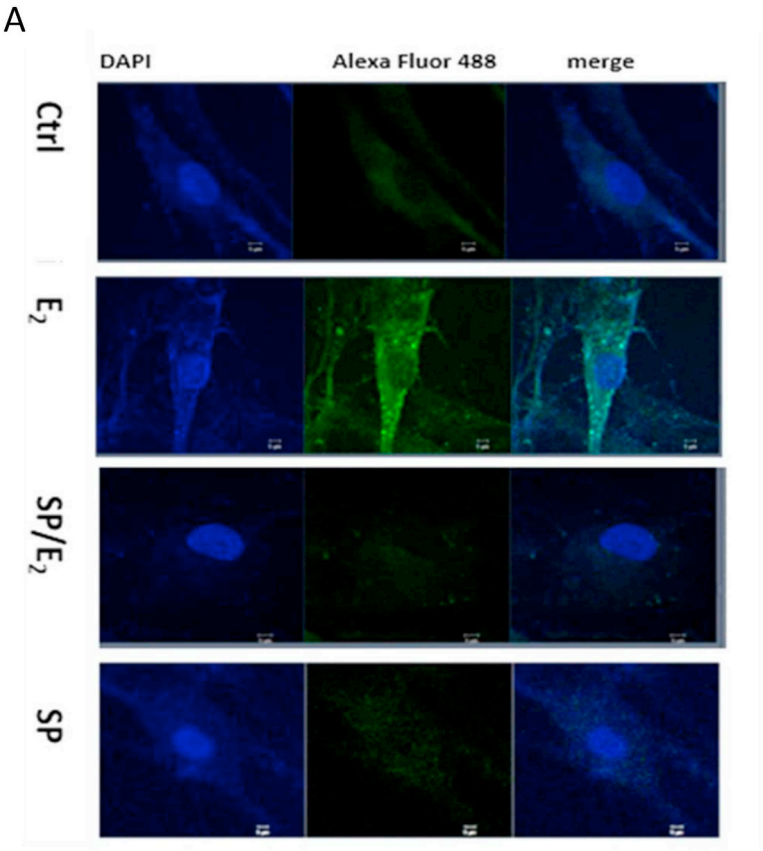

Fig. 7. JNK mediates cold-induced α_{2C}-AR localization in human micro VSMCs.

A. Localization of α_{2C}-ARs in micro VSMCs (green, Alexa Fluor 488) by indirect immunofluorescence-confocal microscopy at cold (28 °C) conditions. Nuclei were stained with Hoechst dye (blue). Following 48 h starvation, cells were treated with estrogen (10^{-10} M) for 1 h, in the presence or absence of SP600125 (3 μM; a JNK specific inhibitor), and incubated for 1 h at cold (28 °C) conditions. Cells were then fixed and stained for membrane α_{2C}-ARs. SP600125 was added 30 min prior to treatment. Scale bars, 20 μm. The optical slices obtained by this approach allowed spatial visualization of cell surface α_{2C}-ARs. For fluorescence comparisons, the highest intensity observed was used as reference for each set of experiments and identical settings and conditions were used to capture and process all images. Nuclei were visualized with Hoechst stain dye (blue). B. Quantitation of the mean fluorescence intensity of α_{2C}-AR on cell boundary. Data presented as relative mean intensity of fluorescence from 3 replicates of the experiment are shown. $*p < .05$, $p < .01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adenylate cycles. Here, we showed that estrogen, a physiologic agonist, activates AP-1 via JNK, leading to increased transcriptional activation of the α_{2C}-AR promoter. Therefore, the JNK/AP-1 interaction may represent a therapeutic target in treating RP. Certainly, this approach proved to be successful in other CVDs. This may be due to the fact that AP-1 plays an important role in regulating many phenotypic aspects of VSMC biology, including proliferation [6]. For example, interrupting JNK/AP-1 interaction decreased VSMC hypertrophy [40]. This JNK/AP-1 blockage did not affect other mitogen-activated protein kinases (MAPKs) such as ERK1/2 and p38. Thus, other cellular pathways mediated by these MAPKs may not be affected. Another endeavor could be utilizing JNK inhibition, which has indeed been used in the management and treatment diabetes. Indeed, intraperitoneal injection of cell-permeable JNK inhibitory peptide improved insulin resistance and glucose tolerance in mice. Since JNK is a key mediator in estrogen-induced α_{2C}-AR regulation, such an inhibitory peptide might have a potential for the treatment of RP [33]. It is tempting to envision such drugs for RP treatment. However, further studies must be carried on to ensure their efficiency and safety.

Notably, estrogen-induced α_{2C}-AR upregulation is likely independent of a functional rescue of the receptor. We have previously established that cold mobilizes α_{2C}-AR to the cell membrane of VSMCs [1]. Here, we showed that estrogen potentiated this cold-induced translocation via JNK activation. Thus, the two pathways employed by

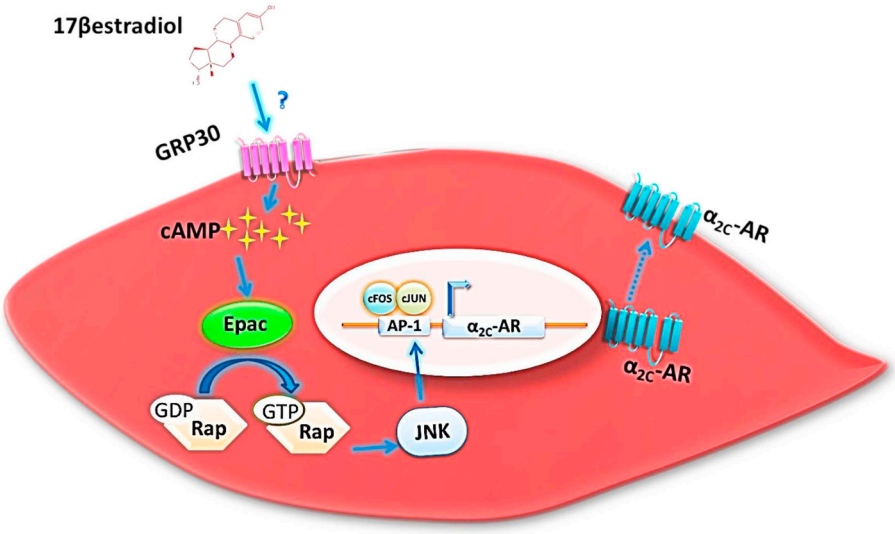

Fig. 8. Schematic representation of the proposed mechanism for α_{2C}-AR expression and translocation in VSMCs.
Estrogen induces an increase in cAMP level, which activates its downstream target Epac. In turn, activated Epac catalyzes the exchange of GDP to GTP on Rap, thus activating it. The now active Rap induces JNK phosphorylation, leading to the dimerization of the c-Fos and c-Jun to form the AP-1 transcription factor. AP-1 binds to AP-1 site in the α_{2C}-AR promoter, initiating its transcription. Furthermore, estrogen-activated JNK mediates α_{2C}-AR trafficking to the cell membrane.

estrogen to regulate α_{2C}-AR expression and functional rescue converge at the level of JNK. This JNK activation is achieved within minutes after adding estrogen. Since estrogen-induced cold-augmented α2C-AR translocation was abrogated by JNK inhibition, we can then deduce that this JNK involvement in translocation is also a result of rapid non-genomic effect of estrogen.

It is worth mentioning that we used VSMCs and tail arteries of male origin to test the effect of estrogen. This is called a cross-over approach. Knowing that hormones have a relatively long half-life, the aim of this approach is to decrease the carryover of the tested hormone from the donors, in an attempt to decrease the background noise . However, a limitation to this approach is that cells/arteries extracted from a given sex are not exposed to the high concentrations of the hormone of the other sex. Notably, the estrogen level in female mice varies during estrous cycle. Knowing that estrogen increases α_{2C}-AR expression in VSMCs [19], using female mice will affect our results, as the exact phase of the estrous cycle cannot be maintained during all experiments.

Our study presents new insights on the signaling pathway by which estrogen regulates α_{2C}-ARs expression and activity, and may thus explain the prevalence of RP in premenopausal females. Initially, α_2-AR blockers were used to alleviate RP symptoms [41]. However, no definitive drug for RP has been yet approved by the US Food and Drug association [42]. The challenge in finding a specific treatment arises from the fact that α_{2C}-AR is expressed in many brain regions and is implicated in the presynaptic regulation of the heart. Therefore, targeting α_{2C}-AR in an attempt to cure RP might interfere with α_{2C}-AR function in other organs, thus causing deleterious side effects. It would be tempting to speculate that RP management may involve targeted therapies against Epac/Rap/JNK pathway especially that Epac-induced activation of Rap occurs in microVSMCs but not human aortic smooth muscle cells (unpublished observations). Indeed, although aortic SMCs express Rap, its activation in these cells appears to be Epac-independent. Therefore, targeting microVSMC-specific Epac/Rap/JNK pathway may be successful in the realm of RP therapy.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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