Am J Physiol Heart Circ Physiol 293: H1955–H1961, 2007.
First published July 20, 2007; doi:10.1152/ajpheart.00306.2007.

Estrogen increases smooth muscle expression of α_{2C}-adrenoceptors and cold-induced constriction of cutaneous arteries

A. H. Eid,[1] K. Maiti,[1] S. Mitra,[1] M. A. Chotani,[1] S. Flavahan,[2] S. R. Bailey,[3] C. S. Thompson-Torgerson,[2] and N. A. Flavahan[2]

[1]*Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio;* [2]*Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University, Baltimore, Maryland; and* [3]*Faculty of Veterinary Science, University of Melbourne, Parkville, Victoria, Australia*

Submitted 12 March 2007; accepted in final form 17 July 2007

Eid AH, Maiti K, Mitra S, Chotani MA, Flavahan S, Bailey SR, Thompson-Torgerson CS, Flavahan NA. Estrogen increases smooth muscle expression of α_{2C}-adrenoceptors and cold-induced constriction of cutaneous arteries. *Am J Physiol Heart Circ Physiol* 293: H1955–H1961, 2007. First published July 20, 2007; doi:10.1152/ajpheart.00306.2007.—Raynaud's phenomenon, which is characterized by intense cold-induced constriction of cutaneous arteries, is more common in women compared with men. Cold-induced constriction is mediated in part by enhanced activity of α_{2C}-adrenoceptors (α_{2C}-ARs) located on vascular smooth muscle cells (VSMs). Experiments were therefore performed to determine whether 17β-estradiol regulates α_{2C}-AR expression and function in cutaneous VSMs. 17β-Estradiol (0.01–10 nmol/l) increased expression of the α_{2C}-AR protein and the activity of the α_{2C}-AR gene promoter in human cultured dermal VSMs, which was assessed following transient transfection of the cells with a promoter-reporter construct. The effect of 17β-estradiol was associated with increased accumulation of cAMP and activation of the cAMP-responsive Rap2 GTP-binding protein. Transient transfection of VSMs with a dominant-negative mutant of Rap2 inhibited the 17β-estradiol-induced activation of the α_{2C}-AR gene promoter, whereas a constitutively active mutant of Rap2 increased α_{2C}-AR promoter activity. The effects of 17β-estradiol were inhibited by the estrogen receptor (ER) antagonist, ICI-182780 (1 μmol/l), and were mimicked by a cell-impermeable form of the hormone (estrogen:BSA) or by the selective ER-α receptor agonist 4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)tris-phenol (PPT; 10 nmol/l) or the selective ER-β receptor agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; 10 nmol/l). Therefore, 17β-estradiol increased expression of α_{2C}-ARs by interacting with cell surface receptors to cause a cAMP/Rap2-dependent increase in α_{2C}-AR transcription. In mouse tail arteries, 17β-estradiol (10 nmol/l) increased α_{2C}-AR expression and selectively increased the cold-induced amplification of α_2-AR constriction, which is mediated by α_{2C}-ARs. An estrogen-dependent increase in expression of cold-sensitive α_{2C}-ARs may contribute to the increased activity of cold-induced vasoconstriction under estrogen-replete conditions.

Raynaud's phenomenon; Rap1; Rap2; adenosine 3′,5′-cyclic monophosphate; estrogen receptors

COLD-INDUCED VASOCONSTRICTION in the cutaneous circulation is a protective physiological response that acts to reduce heat loss (49). The constriction results from a reflex increase in sympathetic activity and a direct local effect of cold to increase vasoconstriction to nerve-released norepinephrine (49). This latter effect is mediated by a cold-induced, selective increase in α_2-adrenoceptor (α_2-AR) reactivity on vascular smooth muscle cells (VSMs) (19, 20, 23). Although α_2-ARs comprise three subtypes (α_{2A}, α_{2B}, and α_{2C}) (41), only α_{2C}-ARs appear to be regulated by cold (11, 30). Moderate cooling causes redistribution of α_{2C}-ARs from the *trans*-Golgi, where they are normally retained, to the cell surface where they can respond to agonist stimulation (2, 3, 30). The local cold sensitivity of the cutaneous vascular system is increased in individuals with Raynaud's phenomenon and scleroderma, resulting in cold-induced peripheral vasospasm, which can be prevented by α_2-AR blockade (25).

Primary Raynaud's phenomenon is more common in women compared with men, and in women the severity of complaints is most pronounced in the period between menarche and menopause (6, 50). In postmenopausal women, the prevalence of Raynaud's phenomenon increased significantly in individuals receiving estrogen replacement therapy (24). These studies suggest that female sex hormones, in particular estrogen, may increase susceptibility to cold-induced vasopasm. In healthy individuals, cutaneous vasoconstriction evoked by local cooling of the hand is more intense and more prolonged in women compared with men (5, 8, 14). Furthermore, the increased cold-induced vasoconstriction in women is most prominent under estrogen-replete conditions and is prevented by α_2-AR but not by α_1-AR blockade (5, 8, 26). Indeed, the mRNA level for α_{2C}-ARs, but not α_{2B}-ARs or α_{2A}-ARs, is higher in VSMs of female compared with male cutaneous arteries (38). The present experiments were therefore performed to determine whether estrogen might increase expression of α_{2C}-ARs in cutaneous VSMs and, if so, to identify the signaling pathway responsible for this effect.

METHODS

Materials. Actinomycin D, 17β-estradiol, β-estradiol 6-(O-carboxy-methyl)oxime:BSA, 3-isobutyl-1-methyxanthine (IBMX), SQ-22536, and UK-14304 were purchased from Sigma (St. Louis, MO). 2,3-Bis(4-hydroxyphenyl)-propionitrile (DPN; ICI-182780) and 4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl)tris-phenol (PPT) were purchased from Tocris (Ellisville, MO).

Cell culture. Cutaneous VSMs were cultured from human dermal arterioles (male and female donors) and used between *passages 9–12*, as previously described (2, 12, 13). VSMs were grown in Ham's growth medium (DMEM: F12, 50:50) supplemented with 10% heat inactivated FBS, L-glutamine, and an antibiotic/antimycotic cocktail (at 37°C). At least 3 days before the addition of 17β-estradiol, cells

Address for reprint requests and other correspondence: N. A. Flavahan, Dept. of Anesthesiology and Critical Care Medicine, Johns Hopkins Univ., Ross Research Bldg., R 370/372, 720 Rutland Ave., Baltimore, MD 21205 (e-mail: nflavah1@jhmi.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

were washed (3 times) and made quiescent in phenol red-free medium (DMEM: F12, 50:50, supplemented with L-glutamine and an antibiotic/antimycotic cocktail) without serum or containing 0.5% of charcoal-stripped serum. Responses to 17β-estradiol were similar in cultured VSMs derived from male and female donors.

Western blot analysis. Cells were rinsed twice with phosphate-buffered saline (PBS) and scraped into lysis buffer (2% SDS and 60 mM Tris, pH 6.8). After sonication, the lysate was centrifuged at 5,000 *g* for 10 min, and the supernatant was analyzed for protein concentration (bicinchoninic acid, Pierce; Rockford, IL). Equal protein amounts of cell lysates (25 μg) were separated using 10% SDS-PAGE, transferred to polyvinyldifluoride membrane (Millipore), and incubated with a specific α_{2C}-AR affinity-purified polyclonal antibody (1:1,000 dilution, 1 h, room temperature), which specifically recognizes the α_{2C}-AR on Western blots (2, 12, 13). α_{2C}-ARs have a molecular mass of ~70 kDa (2, 12, 13, 30). After being extensively washed, the membrane was incubated with a secondary anti-rabbit antibody (1:2,000 dilution, 1 h, room temperature). Blots were then developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified by densitometry (Personal Densitometer, Molecular Dynamics).

Measurement of intracellular cAMP levels. Cells were treated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.225 mM) for 30 min before and during exposure to estrogen or BSA-conjugated estrogen (12, 30). At selected times, cells were placed on ice, washed with ice-cold PBS, and then lysed with 10 mM HCl in absolute ethanol (30 min at −20°C). Cells were scraped into the acidified ethanol and centrifuged at 5,000 *g* for 10 min at 4°C, and cAMP levels in the supernatant were measured using an ^{125}I radioimmunoassay kit (Biomedical Technologies, Stoughton, MA), as previously described (12, 30).

Plasmids. The constitutively active mutant of Rap2GTPase (Rap2V12; Rap2-CA) and the corresponding empty murine stem cell virus vector (pMSCV) were a generous gift from Dr. Michael Gold (University of British Columbia, Vancouver, Canada) (37). The dominant-negative mutant of Rap2 GTPase (Rap-DN) and the corresponding empty (pRK5) plasmids were kindly provided by Dr. Martina Schmidt (Institute für Pharmakologie, Universitätsklinikum Essen, Germany) (44). The α_{2C}-AR promoter/reporter plasmid spanning the full-length promoter sequence (−1,915/+892, relative to the transcription start site +1) was a kind gift from Dr. Herve Paris and is engineered as described by Schaak et al. (43). The α_{2A}-AR full-length (−1,066/+928) promoter/reporter construct has been described previously (13). *Renilla* luciferase plasmid (pRL)-cytomegalovirus (CMV) (*Renilla* luciferase gene driven by CMV promoter/enhancer) was purchased from Promega (Madison, WI).

Transient transfections. Cells were transiently transfected by nucleofection with the Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Transfection was optimized to achieve an ~80% transfection efficiency, with minimal toxicity, achieved with nucleofection of 400,000 cells with 4 μg of nucleic acids. The total amount of transfected DNA was kept constant throughout the study by using the appropriate empty plasmids. When we used reporter constructs, pRL-CMV was used as an internal control to normalize the firefly luciferase units (12, 13). After transfection, cells were allowed to recover overnight in Ham's growth medium. After aspiration of the growth medium, cells were washed thoroughly and maintained in serum-free phenol red-free media for 72 h before the addition of estrogen. For luciferase analysis, cells were washed, lysed in luciferase lysis buffer (Promega), snap frozen, and then thawed at room temperature. Cell lysates were centrifuged at 9,300 *g* for 10 min, and luciferase activity in the supernatant was determined.

Rap pull-down assay. Activation of RapGTPases was assessed using a pull-down assay (48), with an activation-specific probe corresponding to 97 amino acids of human Ral GDS-rap-binding domain, according to the manufacturer's instructions (Upstate, Lake Placid, NY). The pull-down assay was performed using 200 μg of VSM lysate, whereas total Rap expression was determined using 30 μg of the same cell lysate. Western blot analysis for activated, as well as total, Rap GTPases was assessed using antibodies specific for Rap1 (1:500 dilution, Upstate) or Rap2 (1:2,500 dilution, BD Transduction, Lexington, KY) for 1 h at room temperature.

Vasomotor activity of isolated arterioles. Male and female mice (C57BL6) were euthanized by CO_2 asphyxiation, and segments of tail artery were rapidly removed and placed in cold Krebs-Ringer bicarbonate solution containing (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 (control solution). In some experiments, the responses of tail arteries from male and female mice were analyzed immediately. In others, paired arterial segments were incubated in the absence and presence of 17β-estradiol (10 nmol/l, 24 h) in serum-free, phenol red-free DMEM (37°C, 5% CO_2-balance air). The arteries were then rinsed in control solution and prepared for Western blot (see *Western blot analysis*) or vasomotor analyses.

For vasomotor analysis, arteries were cannulated at both ends with glass micropipettes in a microvascular chamber (Living Systems, Burlington, VT). The arteries were maintained at a constant transmural pressure of 60 mmHg in the absence of flow, and vasoconstrictor responses were analyzed as previously described (2, 11). The chamber was superfused with control solution and maintained at 37°C (pH 7.4) and gassed with 16% O_2-5% CO_2-balance N_2. The chamber was placed on the stage of an inverted microscope (×20, Nikon TMS-F) connected to a video camera (Panasonic, CCTV camera). The vessel image was projected onto a video monitor, and the internal diameter was continuously determined by a video dimension analyzer (Living Systems Instrumentation) and monitored using a Biopac (Santa Barbara, CA) data acquisition system. Concentration-effect curves to the selective α_1-AR agonist, phenylephrine, or the selective α_2-AR agonist, UK-14304, were generated by increasing the concentration of the agonists in half-log increments, once the constriction to the previous concentration had stabilized (2, 11). After the completion of the concentration-effect curve, the influence of the agonists was terminated by repeatedly exchanging the buffer solution and allowing the artery to return to its stable baseline level. When the influence of cold was analyzed, the temperature of the superfusate was decreased to 28°C for 30 min before assessing α_2-AR vasoconstriction (2, 11). Concentration-effect curves were analyzed by comparing the agonist concentration causing 20% constriction (CC_{20}), determined by regression analysis.

Statistical analyses. Statistical evaluation of the data was performed by Student's *t*-test for either paired or unpaired observations. When more than two means were compared, ANOVA was used: either a one-way ANOVA with Dunnett's post hoc test or a two-way ANOVA with Tukey-Kramer's post hoc test (GraphPad Software, San Diego, CA). Data are presented as means ± SE, where *n* equals the number of different cell culture experiments or the number of animals from which arteries were studied.

RESULTS

Estrogen increases expression of α_{2C}-ARs. 17β-Estradiol (0.01–10 nmol/l, 24 to 48 h) caused a concentration-dependent increase in α_{2C}-AR protein expression, which was maximal at 0.1 nmol/l with a 4.4 ± 0.7-fold increase (Fig. 1, $n = 6$, $P < 0.01$).

Estrogen increases α_{2C}-AR transcription. Actinomycin D (1.6 μmol/l), an inhibitor of DNA-dependent RNA synthesis, prevented the increase in α_{2C}-AR expression in response to 17β-estradiol (0.1 nmol/l) [1.2 ± 0.1-fold increase in α_{2C}-ARs, $n = 4$, P = not significant (NS)]. To directly assess the effect of the hormone on the promoter activity of α_2-ARs, VSMs were transiently transfected with α_{2C}-AR or α_{2A}-AR promoter:

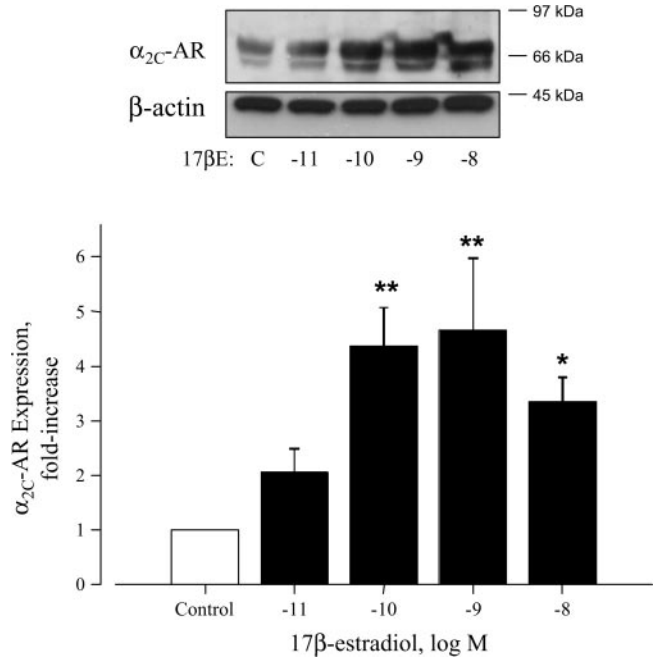

Fig. 1. The effect of 17β-estradiol (17βE; 0.01–10 nmol/l) on protein expression of α_{2C}-adrenceptors (α_{2C}-ARs) in human cutaneous vascular smooth muscle cells (VSMs). Similar results were obtained after 24 or 48 h of incubation with the hormone, and the results were therefore combined. α_{2C}-AR expression was quantified by SDS-PAGE/Western blot analysis of cell lysates, and the effect of 17βE is presented as the fold increase in the basal, unstimulated level of α_{2C}-ARs. *Top*: data from a representative experiment. The molecular mass of α_{2C}-ARs is ~70 kDa (see Refs. 2, 12, 13, and 30). Data are presented as means ± SE for $n = 6$. *$P < 0.05$ and **$P < 0.01$ compared with control (C).

luciferase reporter constructs. 17β-Estradiol (0.1 nmol/l) increased the transcriptional activity of the α_{2C}-AR reporter by 3.1 ± 0.3-fold ($n = 3$, $P < 0.05$) but did not affect the activity of the α_{2A}-AR reporter (0.97 ± 0.02-fold, $n = 3$, P = NS).

Estrogen increases cAMP accumulation. In a number of cell types, estrogen regulates the activity of adenylyl cyclase and the intracellular levels of cAMP (21). In human cutaneous VSMs, 17β-estradiol (0.01–1 nmol/l) increased cAMP accumulation, reaching a maximal effect at 0.1 nmol/l, which increased cAMP from 0.31 ± 0.04 to 1.05 ± 0.09 pmol/10^5 cells ($n = 4$, $P < 0.005$) (Fig. 2*A*). At this concentration, the increase in cAMP peaked within 15 min of exposure to the hormone (Fig. 2*B*). The increase in cAMP evoked by 17β-estradiol (0.1 nmol/l) was significantly inhibited by the adenylyl cyclase inhibitor SQ-22536 (400 μmol/l). Under control conditions, 17β-estradiol (0.1 nmol/l, 15 min) increased cAMP accumulation from 0.38 ± 0.09 to 0.94 ± 0.14 pmol/10^5 cells ($n = 3$, $P < 0.01$). SQ-22536 (400 μmol/l) did not significantly affect the basal level of cAMP (0.24 ± 0.02 pmol/10^5 cells, $n = 3$, P = NS) but markedly reduced the cAMP levels attained after 17β-estradiol (0.1 nmol/l) (to 0.50 ± 0.07 pmol/10^5 cells, $n = 3$, $P < 0.01$). Indeed, after SQ-22536, 17β-estradiol no longer significantly increased cAMP accumulation above basal levels.

17β-Estradiol modulates the activity of Rap GTPases. Elevations in cAMP can increase α_{2C}-AR expression in cutaneous VSMs by activating a Rap GTPase pathway (12). Experiments were therefore performed to determine whether 17β-estradiol altered Rap GTPase activity in these cells. Indeed, 17β-estradiol (0.1 nmol/l) increased the activity of Rap2 GTPase (2.7 ± 0.3-fold, $n = 3$, $P < 0.05$) but decreased the activity of Rap1 GTPase (0.4 ± 0.1-fold, $n = 3$, $P < 0.05$) (Fig. 3*A*). After inhibition of adenylyl cyclase with SQ-22536 (400 μmol/l), 17β-estradiol (0.1 nmol/l) failed to increase the activity of Rap2 GTPase (Fig. 3*B*).

Activation of Rap GTPase modulates α_{2C}-AR expression. To determine whether activation of Rap2 GTPase was responsible for the increased α_{2C}-AR expression in response to 17β-estradiol, transient transfections were employed in cutaneous VSMs. Transfection with a constitutively active mutant of Rap2 GTPase [Rap2-CA (37)] caused a significant increase in the promoter activity of the cotransfected α_{2C}-AR promoter/reporter construct (3.8 ± 0.6-fold increase; $n = 4$; $P < 0.05$). Furthermore, transfection of microvascular cells with a dominant-negative mutant of Rap2 [Rap2-DN (44)] abolished the effect of 17β-estradiol (0.1 nmol/l) to increase the activity of the α_{2C}-AR promoter. Under control conditions (after transfection with control vector), 17β-estradiol (0.1 nmol/l) increased the α_{2C}-AR promoter activity by 3.9 ± 0.5-fold (n =

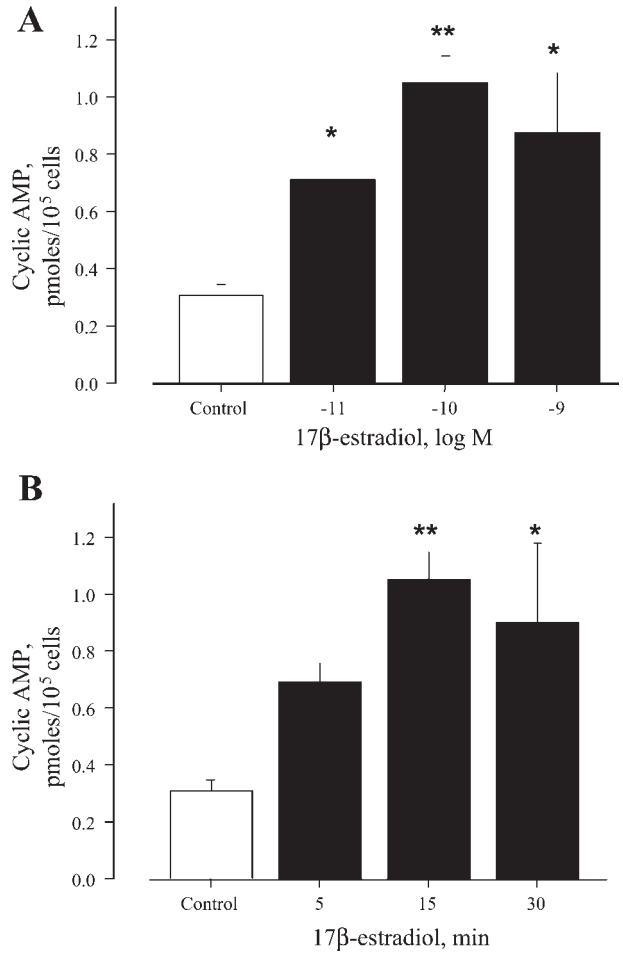

Fig. 2. The effect of 17βE on cAMP accumulation in human cutaneous VSMs. The influence of increasing concentrations (*A*: 0.01–1 nmol/l, 15 min) and increasing times of incubation (*B*: 5–30 min, 0.1 nmol/l) with the hormone was determined. Intracellular cAMP is presented as picomoles of cAMP per 10^5 cells, and data are presented as means ± SE for $n = 3$ to 4. *$P < 0.05$ and **$P < 0.01$ compared with control.

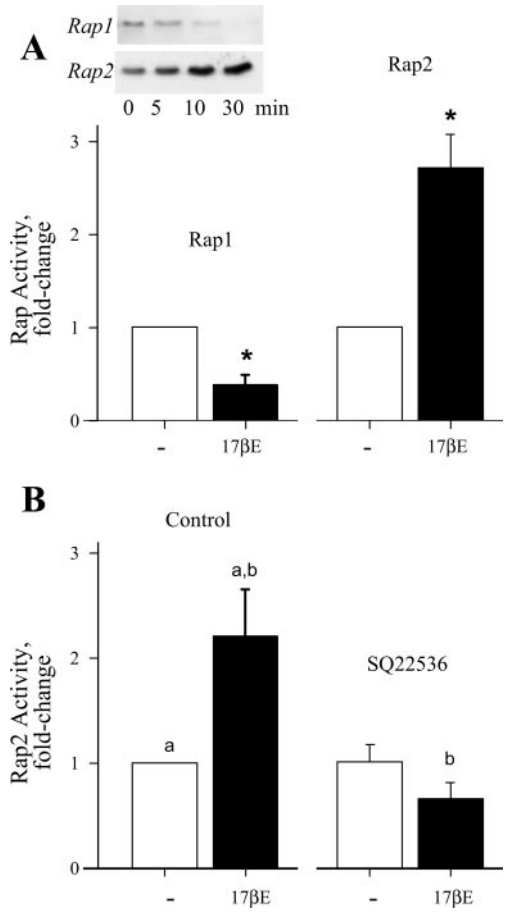

Fig. 3. The effect of 17βE on the activity of Rap GTPases in cutaneous VSMs. Rap activity was determined by a pull-down assay, as described in METHODS. Active Rap was normalized to total Rap in the sample and then expressed as a fold increase in the basal, unstimulated activity. *A*: influence of 17βE (0.1 nmol/l, 30 min) on the activity of Rap1 and Rap2. Data are presented as means ± SE for $n = 3$. *$P < 0.05$ compared with untreated cells (−). *Inset*: time course for the effect of 17βE on Rap1 and Rap2 activity. *B*: effects of the adenylyl cyclase inhibitor SQ-22536 (400 μmol/l) on the 17βE-induced stimulation of Rap2 GTPase. Cells were incubated with SQ-22536 for 60 min before and during administration of 17βE (0.1 nmol/l, 30 min). Cell lysates were then analyzed for Rap2 activity. Data are presented as means ± SE for $n = 4$. $^{a,b}P < 0.05$, values with the same letter are significantly different.

4, $P < 0.01$). Rap2-DN did not significantly affect the basal activity of the α_{2C}-AR promoter (1.5 ± 0.2-fold, $n = 4$, P = NS) but abolished the increased activity following 17β-estradiol (1.3 ± 0.2-fold, $n = 4$; P = NS compared with basal activity, and $P < 0.001$ compared with the control response to the hormone).

Role of estrogen receptors. The estrogen receptor (ER) antagonist ICI-182780 (1 μmol/l) abolished the increase in α_{2C}-AR expression in response to 17β-estradiol (0.1 nmol/l) (1.2 ± 0.3-fold increase in α_{2C}-ARs, $n = 3$, P = NS), suggesting that the effects of 17β-estradiol were mediated by binding to its receptors, ER-α and/or ER-β. To test whether activation of ER-α or ER-β could mimic the observed effects of estrogen, we used the following subtype-selective agonists: PPT for ER-α and DPN for ER-β. PPT (10 nmol/l) increased α_{2C}-AR expression by 3.9 ± 1.0-fold ($n = 4$; $P < 0.005$), and DPN (10 nmol/l) increased expression by 2.9 ± 0.5-fold ($n = 7$, $P < 0.05$). Although nuclear ER-α and/or ER-β can activate gene transcription, the increase in cAMP, which mediated the increased expression of α_{2C}-ARs to 17β-estradiol, was too rapid to involve transcriptional regulation. In addition to nuclear localization, estrogen receptors are also present at the plasma membrane. To assess the role of a plasma membrane receptor, we analyzed the response of cutaneous VSMs to a cell-impermeable form of estrogen, 17β-estradiol 6-(*O*-carboxy-methyl)oxime:BSA (17β-estradiol:BSA). As observed with 17β-estradiol, 17β-estradiol:BSA (0.1 to 10 nmol/l) increased cAMP accumulation in a concentration-dependent manner [from a basal level of 0.34 ± 0.07 pmol/10^5 cells to 0.72 ± 0.12 at 0.1 nmol/l ($P < 0.01$), 0.94 ± 0.09 at 1 nmol/l ($P < 0.01$), and 0.98 ± 0.08 pmol/10^5 cells at 10 nmol/l 17β-estradiol:BSA ($P < 0.01$), $n = 3$]. According to the manufacturer, ~3% of 17β-estradiol:BSA may be present in the unbound form. However, conjugated 17β-estradiol was only 10-fold less potent than free 17β-estradiol (Fig. 3), which is consistent with the activity of the conjugated form of the hormone (47).

Cold-induced vasoconstriction. In mouse tail arteries, α_2-AR constriction at 37°C is mediated by α_{2A}-ARs, whereas the heightened constriction observed during cooling (e.g., to 28°C) is mediated by α_{2C}-ARs (11). At 37°C, constriction to the α_2-AR agonist UK-14304 was similar between tail arteries of male and female mice (at 1 nmol/l, UK-14304 caused constriction of 9.0 ± 1.2% and 8.7 ± 0.8% in male and female arteries, respectively, $n = 6$ or 7, P = NS). However, during exposure to 28°C, the increased constriction to α_2-AR activation by UK-14304 was further increased in female compared with male arteries (at 1 nmol/l, UK-14304 caused constriction of 19.1 ± 2.2% and 26.5 ± 1.6% in male and female arteries, respectively, $n = 6$ or 7, $P \leq 0.02$).

To determine the effects of 17β-estradiol, tail arteries from male arteries were incubated with the hormone for 24 h (at 37°C). 17β-Estradiol (10 nmol/l, 24 h) increased the expression of α_{2C}-ARs in tail arteries by 4.1 ± 0.8-fold ($n = 4$, $P \leq 0.001$, Fig. 4). When subsequently assessed at 37°C, 17β-estradiol (10 nmol/l, 24 h) did not significantly affect constriction evoked by activation of α_1-ARs with phenylephrine (0.01 to 3 μmol/l) (data not shown) or activation of α_2-ARs with UK-14304 (Fig. 4). However, cooling to 28°C augmented constriction to the α_2-AR agonist, which was significantly further increased in 17β-estradiol-treated arteries (Fig. 4). Therefore, although the hormone did not significantly affect constriction to UK-14304 at 37°C, it increased constriction to the α_2-AR agonist during cold exposure [log CC_{20} values of −8.36 ± 0.12 and −8.91 ± 0.07 for control and 17β-estradiol-treated arteries, respectively; log shift of 0.55 ± 0.08 (3.5-fold shift), $n = 5$, $P < 0.005$, Fig. 4].

DISCUSSION

In the present study, 17β-estradiol increased the expression of α_{2C}-ARs in cutaneous VSMs by activating the α_{2C}-AR gene promoter. The effect of 17β-estradiol was mediated by a novel signaling cascade, namely activation of plasma membrane estrogen receptors, which resulted in a rapid increase in cAMP levels and a subsequent activation of the Rap2 GTP-binding protein. Indeed, 17β-estradiol-induced activation of the α_{2C}-AR gene promoter was inhibited by transient transfection with a Rap2-DN and mimicked by transfection with a constitutively

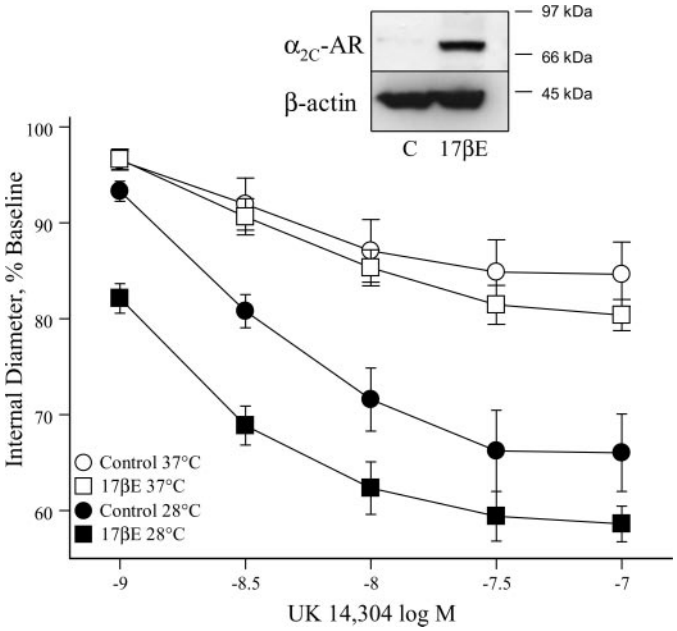

Fig. 4. Effect of 17βE (10 nmol/l, 24 h) on the expression and function of α_2-ARs in mouse isolated tail arteries. *Inset*: representative Western blot demonstrating the effect of 17βE (10 nmol/l, 24 h) to increase the expression of α_{2C}-ARs (by 4.1 ± 0.8-fold, $n = 4$, $P \leq 0.001$). The molecular mass of α_{2C}-ARs is ~70 kDa (2, 12, 13, 30). Vasoconstriction to the α_2-AR agonist, UK-14304 (1–100 nmol/l) was assessed at 37° and 28°C. Responses to the agonist were expressed as a percentage of the stable baseline diameter and are presented as means ± SE ($n = 5$).

active mutant of the GTP binding protein. Therefore, activation of Rap2 was necessary and sufficient for the transcriptional activation of the α_{2C}-AR gene. In isolated cutaneous arteries, 17β-estradiol did not alter α_2-AR vasoconstriction at warm temperatures, which is mediated by α_{2A}-ARs, but increased cold-induced amplification of the α_2-AR response, which is mediated by α_{2C}-ARs. Therefore, increased expression of α_{2C}-ARs in response to 17β-estradiol might contribute to the increased activity of cold-induced vasoconstriction occurring in estrogen-replete conditions.

Cutaneous arterial VSMs express α_{2A}- and α_{2C}- but not α_{2B}-ARs (13, 38). In the present study, physiological levels of 17β-estradiol increased the activity of the α_{2C}-AR full-length promoter, resulting in increased expression of α_{2C}-ARs in cutaneous VSMs. The hormone-induced increase in protein expression was also abolished by actinomycin D, confirming that the effect was mediated by transcriptional activation of the α_{2C}-AR gene rather than by posttranscriptional regulation. The effect of 17β-estradiol was selective for α_{2C}-ARs, and the hormone did not increase activity of the α_{2A}-AR promoter. A previous report demonstrated that VSM expression of α_{2C}-ARs, but not α_{2A}-ARs, was increased in cutaneous tail arteries of female compared with male rats (38). The results of the present study suggest that this sex-dependent difference in α_2-AR subtype expression in cutaneous VSMs is mediated by estrogen-dependent activation of α_{2C}-AR transcription.

α_{2C}-AR expression in cutaneous VSMs is regulated in a complex fashion by cAMP signaling pathways (12). In addition to the prototypic A-kinase signaling cascade, cAMP can activate other effectors including exchange protein activated by cAMP (EPAC), a guanine nucleotide exchange factor for the Ras-like GTPases Rap1 and Rap2 (18, 33). cAMP has positive and negative effects on the α_{2C}-AR promoter activity mediated through these distinct pathways: A-kinase-mediated inhibition and an EPAC/Rap-mediated activation (12). cAMP elevation increases the expression of α_{2C}-AR in human cutaneous VSMs, indicating that the positive cAMP/EPAC/Rap pathway dominates the inhibitory cAMP/A-kinase cascade (12). Physiological concentrations of estrogen activate adenylyl cyclase and increase cAMP levels in different cell types, including rat VSMs (21). We therefore investigated the effect of estrogen on cAMP/Rap signaling in human cutaneous VSMs. Indeed, 17β-estradiol, at physiological concentrations, increased intracellular accumulation of cAMP in these cells. 17β-Estradiol also increased the activity of Rap2 but inhibited the activity of Rap1. Inhibition of the increase in cAMP by SQ-22536, an adenylyl cyclase inhibitor, prevented the 17β-estradiol-induced activation of Rap2, indicating that it reflected cAMP/EPAC signaling. Transient transfection of cutaneous VSMs with a Rap2-DN abolished the hormone-induced activation of the α_{2C}-AR promoter/reporter, indicating that this signaling pathway was essential for the induction of α_{2C}-ARs in response to the hormone. Furthermore, transient transfection using a Rap2-CA demonstrated that Rap2 activity was sufficient for the 17β-estradiol-mediated activation of the α_{2C}-AR promoter. Taken together, these data indicate that Rap2 is a crucial mediator for estrogen to increase expression of α_{2C}-ARs in human cutaneous VSMs.

The 17β-estradiol-induced activation of the cAMP/Rap2 signaling pathway was rapid (<30 min), which is not consistent with a genomic mechanism involving classical cytoplasmic/nuclear estrogen receptors. Plasma membrane ER-α and/or ER-β receptors have been demonstrated in a number of cell types (21, 40). Other reports have demonstrated the presence of a distinct membrane receptor, which is resistant to inhibition by the ER-α/ER-β antagonist ICI-182780 (16, 22, 27, 28, 31). In the present study, the effect of 17β-estradiol to increase α_{2C}-AR expression was inhibited by ICI-182780 and mimicked by the selective ER-α agonist PPT or the selective ER-β agonist DPN. Likewise, the rapid increase in cAMP levels in cutaneous VSMs evoked by 17β-estradiol was mimicked by a cell-impermeable analog of the hormone, 17β-estradiol:BSA (9, 47). Therefore, these results suggest that the effects of 17β-estradiol were mediated by ER-α and/or ER-β located in the plasma membrane.

This is the first demonstration that 17β-estradiol can regulate Rap activity. Previous studies have proposed that estrogen can inhibit EPAC-dependent signaling in dorsal root ganglia neurons (29) but increase EPAC-dependent signaling in neuroendocrine cells (46). The present results indicate that the divergence may reside at the level of the Rap GTPases, with estrogen causing stimulation of Rap2 but inhibition of Rap1. Interestingly, adenylyl cyclase, EPAC, Rap GTPases, and estrogen receptors have been reported to reside in caveolae microdomains (1, 7, 17). Restriction of cAMP signaling within caveolae has been shown to play a role in the selective activation of certain signaling pathways (15, 45). Therefore, compartmentalization and interaction between these signaling components might account for a selective activation of Rap2, but decreased Rap1 activity, in response to 17β-estradiol. Indeed, stimulation of G_S-coupled receptors has been reported to activate extracellular-signal regulated kinase (ERK)1/2 in a

cAMP- and Rap2-dependent, but Rap1-independent, mechanism (34). Interestingly, ERK1/2 can activate cytoplasmic/nuclear estrogen receptors by phosphorylating a critical Ser^{118} needed for activation (32). Therefore, although increased expression of α_{2C}-ARs appears to be initiated by activation of membrane estrogen receptors and cAMP/Rap2 signaling, this pathway may act in part by enhancing activation of cytoplasmic/nuclear estrogen receptors. Indeed, the α_{2C}-AR promoter encompasses many SP1 sites, one AP1 site, and a half estrogen response element site, transcription from all of which can be modulated by estrogen receptors (4, 39, 42). Further studies are needed to define whether these additional mechanisms may contribute to the increased expression of α_{2C}-ARs by 17β-estradiol.

Cold-induced vasoconstriction of cutaneous blood vessels is mediated by a reflex increase in sympathetic activity and a direct sensitizing effect of cold to increase constriction to the sympathetic neurotransmitter norepinephrine. This latter effect results from a selective increase in α_{2C}-AR constrictor activity, which is mediated by cold-induced mobilization of α_{2C}-ARs from the Golgi compartment, where they are normally retained, to the plasma membrane (2, 3, 11, 30). Indeed, α_2-AR constriction at 37°C is mediated predominantly by α_{2A}-ARs, whereas the heightened cold-induced constriction is mediated by α_{2C}-ARs (11). As was observed in cultured VSMs, 17β-estradiol also increased expression of α_{2C}-ARs in cutaneous arteries. Interestingly, 17β-estradiol did not alter constriction to α_2-AR activation at warm temperatures but significantly increased α_2-AR constriction during cold exposure. These results suggest that increased expression of α_{2C}-ARs augments the pool of receptors available for cold-induced mobilization to the cell surface, resulting in a selective increase in cold-induced amplification of sympathetic constriction.

The present study describes a novel signaling pathway whereby estrogen can increase the expression of α_{2C}-ARs, which may contribute to increased cold-induced vasoconstriction and to increased prevalence of Raynaud's phenomenon in estrogen-replete women (6, 24, 50). In fact, the increased cold-induced constriction in healthy women and in those suffering from Raynaud's phenomenon is prevented by α_2-AR blockade (5, 8, 26). In addition to increasing expression of constrictor adrenergic receptors, estrogen also increases the expression of endothelial nitric oxide (NO) synthase and endothelial production of NO (36). Indeed, the elevated estrogen level occurring in the late follicular phase of the menstrual cycle is associated with increased vasodilatation to the endothelium-dependent agonist, bradykinin, as well as increased constriction to cold exposure or to norepinephrine (10, 26). Furthermore, cutaneous vasodilatation evoked by local warming, which is mediated in part by NO (35), is increased in women compared with men (5, 14). Therefore, although estrogen appears to have antagonistic effects on the cutaneous vascular system, it appears to generate a system that is especially sensitive to changes in temperature.

GRANTS

This work was funded by National Institutes of Health Grants HL-080119 and OH-008531 (to N. A. Flavahan) and a grant from the Scleroderma Research Foundation (to N. A. Flavahan).

REFERENCES

1. **Acconcia F, Bocedi A, Ascenzi P, Marino M.** Does palmitoylation target estrogen receptors to plasma membrane caveolae? *IUBMB Life* 55: 33–35, 2003.
2. **Bailey SR, Eid AH, Mitra S, Flavahan S, Flavahan NA.** Rho kinase mediates cold-induced constriction of cutaneous arteries: role of alpha2C-adrenoceptor translocation. *Circ Res* 94: 1367–1374, 2004.
3. **Bailey SR, Mitra S, Flavahan S, Flavahan NA.** Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. *Am J Physiol Heart Circ Physiol* 289: H243–H250, 2005.
4. **Barkhem T, Haldosen LA, Gustafsson JA, Nilsson S.** pS2 Gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor alpha, an estrogen-responsive element, and the activator protein 1 response element. *Mol Pharmacol* 61: 1273–1283, 2002.
5. **Bartelink ML, De Wit A, Wollersheim H, Theeuwes A, Thien T.** Skin vascular reactivity in healthy subjects: influence of hormonal status. *J Appl Physiol* 74: 727–732, 1993.
6. **Block JA, Sequeira W.** Raynaud's phenomenon. *Lancet* 357: 2042–2048, 2001.
7. **Bundey RA, Insel PA.** Discrete intracellular signaling domains of soluble adenylyl cyclase: camps of cAMP? *Sci STKE* 2004: pe19, 2004.
8. **Cankar K, Finderle Z, Strucl M.** The role of alpha1- and alpha2-adrenoceptors in gender differences in cutaneous LD flux response to local cooling. *Microvasc Res* 68: 126–131, 2004.
9. **Chaban VV, Lakhter AJ, Micevych P.** A membrane estrogen receptor mediates intracellular calcium release in astrocytes. *Endocrinology* 145: 3788–3795, 2004.
10. **Chan NN, MacAllister RJ, Colhoun HM, Vallance P, Hingorani AD.** Changes in endothelium-dependent vasodilatation and alpha-adrenergic responses in resistance vessels during the menstrual cycle in healthy women. *J Clin Endocrinol Metab* 86: 2499–2504, 2001.
11. **Chotani MA, Flavahan S, Mitra S, Daunt D, Flavahan NA.** Silent α_{2C}-adrenergic receptors enable cold-induced vasoconstriction in cutaneous arteries. *Am J Physiol Heart Circ Physiol* 278: H1075–H1083, 2000.
12. **Chotani MA, Mitra S, Eid AH, Han SA, Flavahan NA.** Distinct cAMP signaling pathways differentially regulate α_{2C}-adrenoceptor expression: role in serum induction in human arteriolar smooth muscle cells. *Am J Physiol Heart Circ Physiol* 288: H69–H76, 2005.
13. **Chotani MA, Mitra S, Su BY, Flavahan S, Eid AH, Clark KR, Montague CR, Paris H, Handy DE, Flavahan NA.** Regulation of α_2-adrenoceptors in human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 286: H59–H67, 2004.
14. **Cooke JP, Creager MA, Osmundson PJ, Shepherd JT.** Sex differences in control of cutaneous blood flow. *Circulation* 82: 1607–1615, 1990.
15. **Cooper DM.** Molecular and cellular requirements for the regulation of adenylate cyclases by calcium. *Biochem Soc Trans* 31: 912–915, 2003.
16. **Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB.** Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. *Proc Natl Acad Sci USA* 94: 12786–12791, 1997.
17. **De Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL.** Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *J Biol Chem* 275: 20829–20836, 2000.
18. **De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL.** Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396: 474–477, 1998.
19. **Ekenvall L, Lindblad LE, Norbeck O, Etzell BM.** α-Adrenoceptors and cold-induced vasoconstriction in human finger skin. *Am J Physiol Heart Circ Physiol* 255: H1000–H1003, 1988.
20. **Faber JE.** Effect of local tissue cooling on microvascular smooth muscle and postjunctional α_2-adrenoceptors. *Am J Physiol Heart Circ Physiol* 255: H121–H130, 1988.
21. **Farhat MY, Abi-Younes S, Ramwell PW.** Non-genomic effects of estrogen and the vessel wall. *Biochem Pharmacol* 51: 571–576, 1996.
22. **Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI.** Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 16: 70–84, 2002.
23. **Flavahan NA, Lindblad LE, Verbeuren TJ, Shepherd JT, Vanhoutte PM.** Cooling and α_1- and α_2-adrenergic responses in cutaneous veins: role

of receptor reserve. *Am J Physiol Heart Circ Physiol* 249: H950–H955, 1985.
24. **Fraenkel L, Zhang Y, Chaisson CE, Evans SR, Wilson PW, Felson DT.** The association of estrogen replacement therapy and the Raynaud phenomenon in postmenopausal women. *Ann Intern Med* 129: 208–211, 1998.
25. **Freedman RR, Baer RP, Mayes MD.** Blockade of vasospastic attacks by alpha 2-adrenergic but not alpha 1-adrenergic antagonists in idiopathic Raynaud's disease. *Circulation* 92: 1448–1451, 1995.
26. **Greenstein D, Jeffcote N, Ilsley D, Kester RC.** The menstrual cycle and Raynaud's phenomenon. *Angiology* 47: 427–436, 1996.
27. **Gu Q, Korach KS, Moss RL.** Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* 140: 660–666, 1999.
28. **Hewitt SC, Deroo BJ, Korach KS.** Signal transduction. A new mediator for an old hormone? *Science* 307: 1572–1573, 2005.
29. **Hucho TB, Dina OA, Kuhn J, Levine JD.** Estrogen controls PKCepsilon-dependent mechanical hyperalgesia through direct action on nociceptive neurons. *Eur J Neurosci* 24: 527–534, 2006.
30. **Jeyaraj SC, Chotani MA, Mitra S, Gregg HE, Flavahan NA, Morrison KJ.** Cooling evokes redistribution of α_{2C}-adrenoceptors from golgi to plasma membrane in transfected HEK293 cells. *Mol Pharmacol* 60: 1195–1200, 2001.
31. **Jones SE.** Fulvestrant: an estrogen receptor antagonist that downregulates the estrogen receptor. *Semin Oncol* 30: 14–20, 2003.
32. **Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P.** Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270: 1491–1494, 1995.
33. **Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM.** A family of cAMP-binding proteins that directly activate Rap1. *Science* 282: 2275–2279, 1998.
34. **Keiper M, Stope MB, Szatkowski D, Bohm A, Tysack K, Vom Dorp F, Saur O, Oude Weernink PA, Evellin S, Jakobs KH, Schmidt M.** Epac- and Ca^{2+}-controlled activation of Ras and extracellular signal-regulated kinases by Gs-coupled receptors. *J Biol Chem* 279: 46497–46508, 2004.
35. **Kellogg DL Jr, Liu Y, Kosiba IF, O'Donnell D.** Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol* 86: 1185–1190, 1999.
36. **Kleinert H, Wallerath T, Euchenhofer C, Ihrig-Biedert I, Li H, Forstermann U.** Estrogens increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. *Hypertension* 31: 582–588, 1998.
37. **McLeod SJ, Shum AJ, Lee RL, Takei F, Gold MR.** The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J Biol Chem* 279: 12009–12019, 2004.
38. **McNeill AM, Leslie FM, Krause DN, Duckles SP.** Gender difference in levels of alpha2-adrenoceptor mRNA in the rat tail artery. *Eur J Pharmacol* 366: 233–236, 1999.
39. **Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS.** Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 277: 1508–1510, 1997.
40. **Pedram A, Razandi M, Levin ER.** Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* 20: 1996–2009, 2006.
41. **Philipp M, Brede M, Hein L.** Physiological significance of α_2-adrenergic receptor subtype diversity: one receptor is not enough. *Am J Physiol Regul Integr Comp Physiol* 283: R287–R295, 2002.
42. **Safe S.** Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* 62: 231–252, 2001.
43. **Schaak S, Devedjian JC, Cayla C, Sender Y, Paris H.** Molecular cloning, sequencing and functional study of the promoter region of the human alpha2C4-adrenergic receptor gene. *Biochem J* 328: 431–438, 1997.
44. **Schmidt M, Evellin S, Oude Weernink PA, Dorp FV, Rehmann H, Lomasney JW, Jakobs KH.** A new phospholipase-C-calcium signaling pathway mediated by cyclic AMP and Rap GTPase. *Nat Cell Biol* 3: 1020–1024, 2001.
45. **Schwencke C, Yamamoto M, Okumura S, Toya Y, Kim SJ, Ishikawa Y.** Compartmentation of cyclic adenosine 3′,5′-monophosphate signaling in caveolae. *Mol Endocrinol* 13: 1061–1070, 1999.
46. **Sedej S, Rose T, Rupnik M.** cAMP increases Ca^{2+}-dependent exocytosis through both PKA and Epac2 in mouse melanotrophs from pituitary tissue slices. *J Physiol* 567: 799–813, 2005.
47. **Taguchi Y, Koslowski M, Bodenner DL.** Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). *Nucl Recept* 2: 5, 2004.
48. **Van Triest M, de Rooij J, Bos JL.** Measurement of GTP-bound Ras-like GTPases by activation-specific probes. *Methods Enzymol* 333: 343–348, 2001.
49. **Vanhoutte PM.** Physical factors of regulation. In: *Handbook of Physiology*, edited by Bohr DF, Somlyo AP, and Sparks HV. Bethesda, MD: Am. Physiol. Soc., 1980, p. 443–474.
50. **Wigley FM, Flavahan NA.** Raynaud's phenomenon. *Rheum Dis Clin North Am* 22: 765–781, 1996.