Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries

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Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. Am J Physiol Heart Circ Physiol 289: H243–H250, 2005. First published March 11, 2005; doi:10.1152/ajpheart.01305.2004.—Cold constricts cutaneous blood vessels by selectively increasing the activity of smooth muscle α2-adrenoceptors (α2-ARs). In mouse tail arteries, α2-AR constriction is mediated by α2A-ARs at 37°C, whereas the cold-induced augmentation in α2-AR activity is mediated entirely by α2C-ARs. Cold causes translocation of α2C-ARs from the trans-Golgi to the plasma membrane, mediated by cold-induced activation of RhoA and Rho kinase. The present experiments analyzed the mechanisms underlying these responses. Mouse tail arteries were studied in a pressure myograph. Cooling the arteries (28°C) caused a rapid increase in reactive oxygen species (ROS) in smooth muscle cells, determined by confocal microscopy of arteries loaded with the ROS-sensitive probes, dichlorodihydrofluorescein or reduced Mitotracker Red. The inhibitor of mitochondrial complex I rotenone (10 μmol/l) or MnTMPyP (50 μmol/l) did not affect vasoconstriction to α2-AR stimulation (UK-14304) at 37°C but dramatically inhibited the response at 28°C. Indeed, these ROS inhibitors abolished the cold-induced increase in α2-AR constrictor activity. NAC (20 mmol/l) or MnTMPyP (50 μmol/l) also abolished the cold-induced activation of RhoA in human cultured vascular smooth muscle cells and the cold-induced mobilization of α2C-ARs to the cell surface in human embryonic kidney 293 cells transfected with the receptor. The combined results suggest that cold-induced constriction is mediated by redox signaling in smooth muscle cells, initiated by mitochondrial generation of ROS, which stimulate RhoA/Rho kinase signal and the subsequent mobilization of α2C-ARs to the cell surface. Altered activity of ROS may contribute to cold-induced vasospasm occurring in Raynaud’s phenomenon.

Raynaud’s phenomenon; thermoregulation; α2C-adrenoceptors; mouse tail artery

COLD-INDUCED VASOCONSTRICTION in the cutaneous circulation is a protective physiological response that acts to reduce heat loss (36). The constriction results from a reflex increase in sympathetic output and a direct local effect of cold to increase the activity of nerve-released norepinephrine (36). This local effect is mediated by a cold-induced, selective augmentation of α2-adrenoceptor (α2-AR) reactivity on vascular smooth muscle cells (14, 16, 17). Local cold sensitivity is increased in Raynaud’s phenomenon and scleroderma resulting in cold-induced peripheral vasospasm, which can be prevented by α2-AR blockade (18).

Although α2-ARs comprise three subtypes (α2A, α2B, α2C) (31), only α2C-ARs appear to be regulated by cold (8, 24). In cutaneous arteries of the mouse tail, α2-AR constriction at 37°C is mediated by α2A-ARs, with no apparent contribution from α2C-ARs (8). However, after moderate cooling, α2C-ARs are no longer silent and mediate the cold-induced augmentation of α2-AR reactivity (8). Following transfection in human embryonic kidney (HEK) 293 cells, α2A-ARs are expressed on the cell surface and respond to activation by regulating adenylyl cyclase activity. Moderate cooling does not influence α2A-AR location or function (24). In contrast, α2C-ARs are not functional at 37°C and are localized by subcellular fractionation and immunofluorescent analysis to the Golgi compartment (24). A similar mislocalization of α2C-ARs is also observed in transfected COS-7, NRK, Madin-Darby canine kidney, and rat1 fibroblast cells (11, 23, 31). Moderate cooling caused redistribution of α2C-ARs to the cell surface and rescued the α2C-AR functional response, demonstrated by agonist-dependent inhibition of adenylyl cyclase (24).

Although α2C-ARs are important effectors in the cutaneous vascular response to cooling, they do not respond directly to cold and therefore cannot be defined as “thermosensors” (1). The mobilization of α2C-ARs is mediated by cold-induced activation of RhoA and Rho kinase (1). Inhibition of Rho kinase using pharmacological or molecular targeting abolished the mobilization of α2C-ARs to the cell surface and cold-induced vasoconstriction in cutaneous arteries (1). The temperature-sensitive mechanism responsible for activating the RhoA signaling pathway and initiating cold-induced vasoconstriction has not yet been defined. Because heat stress has been associated with altered activity of reactive oxygen species (ROS) (26, 39), the present study was performed to assess the role of ROS in the cutaneous vascular response to cold.

METHODS

Experiments were performed using mouse isolated tail arteries in a pressure myograph system (1, 8), human vascular smooth muscle cells cultured from small dermal arteries and arterioles (1, 9, 10), and HEK 293 cells transiently transfected with α2C-ARs (1, 24). Mouse tail arteries were used for analyses of cold-induced amplification of α2-AR constriction and for functional imaging (1, 8). Although responses of tail arteries are consistent with cold-induced activation of RhoA and Rho kinase, their small size precludes biochemical analysis of RhoA activity (1). Analysis of cold-induced activation of RhoA was therefore performed in human cultured dermal vascular smooth muscle cells (1). Similarly, although cold-induced modulation of α2C-ARs in tail arteries is consistent with their mobilization to the

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surface of smooth muscle cells (1, 8), this process is most easily analyzed and quantified using HEK 293 cells transiently transfection with α2C-ARs (1, 24). Our previous studies suggest that cold-induced signaling mechanisms are similar in each of these cell types (1, 8, 24).

**Blood vessel preparation.** Male mice (C57BL/6), from The Jackson Laboratory, were killed by CO2 asphyxiation. Segments of tail artery were then quickly removed and placed in cold Krebs-Ringer bicarbonate solution containing (in mmol/l) NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, and 11.1 glucose (control solution). The small arteries (120–200 μm internal diameter) were cannulated at both ends with glass micropipettes, secured using 12–0 nylon monofilament suture, and placed in a microvascular chamber (Living Systems, Burlington, VT) (1, 8). The arteries were maintained at a constant transmural pressure (PtM) of 90 mmHg in the absence of flow (1, 8). The chamber was superfused with Krebs solution, maintained at 37°C, pH 7.4, and gassed with 16% O2-5% CO2, balance N2. The chamber was placed on the stage of an inverted microscope (Nikon TMS-F), which was connected to a video camera (CCTV camera; Panasonic). Arteries were allowed to equilibrate under these conditions for 50–60 min before experiments were commenced (8). The vessel image was projected on a video monitor, and the internal diameter was continuously determined by a video dimension analyzer (Living Systems Instrumentation) and monitored using the Acqknowledge data-acquisition system (Biopac Systems, Santa Barbara, CA).

Concentration-effect curves to the α2-AR agonist UK-14304 were generated by increasing agonist concentration in half-log increments, once constriction to the previous concentration had stabilized. After the concentration-effect curve was completed, the agonist was removed from the superfusate and the artery was allowed to return to its baseline level. Concentration-effect curves to UK-14304 were determined under control conditions and in the presence of the mitochondrial complex I inhibitor rotenone (10 μmol/l), the antioxidant N-acetylcysteine (NAC; 20 mmol/l), the cell-permeable superoxide dismutase mimic MnTMPyP (50 μmol/l, SODm), and/or the inhibitor of nitric oxide (NO) synthase N3-nitro-l-arginine methyl ester (l-NAME; 100 μmol/l). When these inhibitors were used, the preparations were incubated for 30 min with the drugs before and during exposure of the arteries to the agonist. When analyzing the influence of cold on α2-AR responsiveness, the temperature of the superfusate was decreased to 28°C for 30 min before commencing a concentration-effect curve to UK-14304 (1, 8). The arteries were incubated in the absence and presence of rotenone (10 μmol/l), NAC (20 mmol/l), MnTMPyP (50 μmol/l, SODm), and/or l-NAME (100 μmol/l) for 30 min before cooling and remaining present during the subsequent exposure to cold temperature and UK-14304. For each experiment, an adjacent artery from the same animal that was not exposed to inhibitors served as a time control (1, 8).

**Rhotekin binding assay for RhoA-GTP.** To assess RhoA activation, a rhotekin binding domain affinity precipitation assay for RhoA-GTP was performed (Upstate Biotechnology, Lake Placid, NY) (1). Human microvascular (dermal arteriolar) smooth muscle cells were grown to 80% confluence in Ham’s serum-free medium for 72 h, as previously described (1, 10). Cells were then incubated in the absence or presence of SODm (50 μmol/l) or NAC (20 mmol/l) for 30 min at 37°C. The medium was then replaced with precooled medium at 28°C or washed with medium at 37°C in the continued presence and absence of the ROS inhibitors, and the cells were maintained at these temperatures for 60 min (1).

Cells were then lysed in ice-cold lysis/wash buffer as directed by the manufacturer and the lysates were cleared by centrifugation (13,400 g, 4°C, 1 min). Total protein content was determined (BCA/bicinchoninic acid method, Pierce Biotechnology, Rockford, IL) and an aliquot of each sample was analyzed by Western blotting (normalized for total protein) to compare total RhoA content between samples. The remainder of the cell lysates (500 μl; adjusted to ensure equal protein) was added to 50 μl of glutathione agarose slurry containing the rhotekin Rho binding domain (Upstate Biotechnology) and incubated at 4°C for 45 min. The beads were washed thoroughly with buffer and retained proteins were resolved by SDS-polyacrylamide gel electrophoresis (12%) and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). RhoA was detected with anti-RhoA primary antibody (1:500 dilution, 60 min at room temperature) and horseradish peroxidase-conjugated sheep anti-mouse IgG. Bands were detected with enhanced chemiluminescence (ECL Super Signal; Pierce) and quantified by densitometry (Molecular Dynamics, Personal Densitometer, Sunnyvale, CA).

**Quantitation of cell-surface α2C-ARs.** HEK 293 cells (American Type Culture Collection, Manassas, VA) were transiently transfected in 100-mm dishes with either pCDNA3 expression vector (mock) or pCDDN3-α2C-AR with hemaglutinin (HA) tagged to the NH2-terminal domain of the receptor, as previously described (1, 24). Forty-eight hours after transfection, cells were incubated in the absence or presence of SODm (50 μmol/l) or NAC (20 mmol/l) for 30 min at 37°C. The medium was then replaced with precooled medium at 28°C or washed with medium at 37°C in the continued presence and absence of the ROS inhibitors, and the cells were maintained at these temperatures for 60 min (1). The cells were then washed with ice-cold PBS and incubated with anti-HA monoclonal antibody (1:200 dilution) for 1 h at 4°C to label cell-surface α2C-ARs (1). If the receptor is expressed at the cell surface, then the NH2-terminal HA tag will be accessible for labeling (1). The cells were then washed twice with ice-cold PBS and lysed in 300 μl of lysis buffer (PBS containing 1% digitonin, 0.5% deoxycholate and proteinase inhibitors: 15.7 μg/ml each of chymostatin, antipain, pepstatin; 57.7 μg/ml leupeptin and 250 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride) for 1 h at 4°C. After centrifugation (13,400 g, 20 min, 4°C), supernatant containing 250 μg of total protein (in 250 μl of buffer) was incubated with 20 μl of protein A/G-agarose beads for 1 h at 4°C to precipitate antibody-bound surface receptors. The immunoprecipitates were then washed twice with PBS containing 0.1% digitonin and eluted with 50 μl of Laemmli’s sample buffer. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (8%) and gels were transferred to Immobilon-P membranes (Millipore). The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with an affinity-purified rabbit polyclonal antibody directed against the α2C-AR (1:1,000 dilution; 60 min at room temperature) (1, 10, 24) and horseradish peroxidase-conjugated sheep anti-rabbit IgG. Proteins were visualized using an ECL kit (Amersham Pharmacia Biotech) and quantified by densitometry (Molecular Dynamics, Personal Densitometer).

**Fluorescent imaging of ROS.** Mouse small tail arteries were de-endothelized by gently pulling them onto a 70-μm-diameter wire and then cannulated in a pressure myograph chamber with an integral glass coverslip for use with a confocal microscope (chamber model CH/1, Living Systems) (1). To analyze ROS activity in smooth muscle cells, arteries were incubated with the H2O2-sensitive fluorescent probe 5-(& 6)-chloromethyl-2’-7’-dichlorodihydrofluorescein diacetate (H2DCFH) or with the reduced form of MitoTracker Red (MitoTracker Red CM-H2XROS), which associates with mitochondria and becomes fluorescent after exposure to ROS (30, 34). H2DCFH or MitoTracker Red was perfused through the lumen of the arteries, for 30 min [37°C, PtM of 15 mmHg (30)], at concentrations of 5 μg/ml or 5 μM, respectively, in 0.05% dimethylsulfoxide and 0.001% pluronic acid. The probes were then removed by perfusion with fresh Krebs solution, the PtM was increased to 60 mmHg, and the arteries were allowed to stabilize for 30 min before commencing imaging (in the absence of flow). Arteries were imaged using a confocal laser-scanning microscope (Zeiss model LSM 510) and a ×20 air objective (numerical aperture 0.75). H2DCFH was excited using the Argon/2 488 laser line, and the emission was processed through a LP 505-nm filter. MitoTracker Red was imaged using the HeNe 534 line and a LP 560 filter.
In an initial series of experiments, images of the smooth muscle cells were acquired from the front surface of the arteries before and 10 min after cooling to 28°C using the following settings: a 1,024 × 1,024 pixel frame and microscope zoom setting of 2.5 to generate x-y pixel dimensions of 0.18 × 0.18 μm, a resident pixel time of 3.2 μs, line averaging of 4 scans, and pinhole size of 1.25 Airy units. When experiments were performed to determine the time course of ROS activity, images were captured at ~1.9-s intervals through the side wall of the arteries. For these images, a 512 × 512 pixel frame and microscope zoom setting of 3.5 were used to generate x-y pixel dimensions of 0.25 × 0.25 μm, resident pixel time of 6.4 μs, line averaging of 2 scans, and pinhole size of 1.25 Airy units. The changes in fluorescence in defined regions of interest (ROIs) were determined using image analysis software (LSM 510 software, Zeiss).

**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 two-way ANOVA followed by Bonferroni’s post hoc test (GraphPad Software, San Diego, CA). Data are presented as means ± SE, where n equals the number of animals from which blood vessels were taken or the number of cell culture experiments.

**Reagents and antibodies.** The following reagents were used: MnTMPyP (Axxora/Alexis, San Diego, CA); L-NAME, rotenone, and UK-14304 (Sigma, St. Louis, MO); DCDHF and MitoTracker Red (Molecular Probes, Portland, OR). The following antibodies were used: MHA.11 antibody against the HA epitope (Berkley Antibody, Berkley, CA), mouse monoclonal RhoA antibody (BD Transduction Laboratories, Lexington, KY), an affinity-purified rabbit polyclonal antibody against the α2c-AR COOH terminus was produced by Bethyl Laboratories (Houston, TX) (1, 10). All secondary antibodies were purchased from Amersham.

**RESULTS**

**Cold causes a rapid increase in ROS activity in smooth muscle cells.** Cooling to 28°C caused a rapid increase in ROS activity within smooth muscle cells of mouse tail arteries, detected by LSM analyses of reduced Mitotracker Red or DCDHF fluorescence (Figs. 1 and 2). Mitotracker Red fluorescence began to increase immediately on exposure to cold and attained a maximal increase in fluorescence (46.0 ± 6.2% of baseline) 148.1 ± 21.0 s after the temperature change (n = 5; Fig. 1). With DCDHF, the increase in fluorescence was significantly later, starting 57.7 ± 3.2 s after the decrease in temperature and reaching peak intensity (35.5 ± 4.6% of baseline) 193.7 ± 5.3 s after the temperature change (n = 4; Fig. 2). When temperature was maintained at 37°C, there was no significant change in fluorescence for either probe (Figs. 1 and 2). Although DCDHF fluorescence was visible throughout the cytoplasm, Mitotracker Red fluorescence was localized to discrete regions of smooth muscle cells, consistent with its mitochondrial localization (Figs. 1 and 2).

![Fig. 1. Cold increased reactive oxygen species (ROS) activity in smooth muscle cells of mouse tail arteries, detected using laser scanning microscope (LSM) analysis of Mitotracker Red fluorescence. LSM images were obtained and quantified as described in the text. Top: images present Mitotracker Red fluorescence in an LSM section through vascular smooth muscle cells of a tail artery before and 10 min after cooling to 28°C. Fluorescence is expressed as a percentage of the initial starting value (time 0) and is presented as means ± SE (at selected points) for n = 5.](http://ajpheart.physiology.org/ by 10.220.32.246 on July 1, 2017)
ROS inhibitors selectively inhibit cold-induced vasoconstriction in cutaneous arteries. Moderate cooling (to 28°C) increased constriction evoked by the \( \alpha_2 \)-adrenoceptor (\( \alpha_2 \)-AR) agonist UK-14304 in mouse tail arteries, increasing the maximal response from 23.1 ± 2.1 to 45.2 ± 2.3% constriction \((n = 16, P < 0.001;\) Figs. 3, 4, 5). The mitochondrial complex I inhibitor rotenone (10 \( \mu \)mol/l) did not affect the response to UK-14304 at 37°C [maximal constriction of 24.1 ± 3.9 and 22.6 ± 3.9% for control and treated arteries, respectively, \( n = 4, P = \) not significant (NS)] but significantly reduced the agonist response at 28°C [maximal constrictions of 41.7 ± 3.8 and 23.8 ± 5.6% for control and treated arteries, respectively, \( n = 4, P < 0.05;\) Fig. 3]. Similarly, the antioxidant NAC (20 mmol/l) did not affect constriction to UK-14304 at 37°C (maximal constriction...
Cold, ROS, and RhoA activation. To directly assess the effects of ROS inhibition on cold-induced Rho activation, experiments were performed using smooth muscle cells cultured from human cutaneous arteries (1). RhoA activity was assessed using a RhoA-GTP pull-down assay. Moderate cooling (28°C) caused a 2.7 ± 0.4-fold increase in RhoA activity (n = 7, P < 0.005). NAC (20 mmol/l) or SOD (50 μmol/l) did not significantly affect RhoA activity at 37°C but abolished the cold-induced activation of the GTP-binding protein (Fig. 6).

ROS and cold-induced translocation of α2C-ARs. Cold-induced activation of RhoA/Rho kinase is essential for translocation of α2C-ARs to the cell surface (1). To investigate whether ROS are involved in receptor mobilization, experiments were performed in HEK 293 cells transiently transfected with HA-tagged α2C-ARs. The HA tag is at the NH2 terminus, which is an extracellular domain of cell-surface receptors (1). By using a live cell labeling technique directed against the HA tag, we confirmed that moderate cooling (to 28°C) increased localization of α2C-ARs to the cell surface (3.3 ± 0.6-fold increase; n = 4, P < 0.05), without affecting cellular expression of the receptor (Fig. 7). NAC (20 mmol/l) or SODm (50 μmol/l) abolished the cold-induced translocation of the α2C-ARs to the cell surface, without affecting the low-level, cell-surface expression of α2C-ARs present at 37°C or the total expression of the receptor (Fig. 7).

DISCUSSION

Cold-induced constriction of cutaneous blood vessels is mediated by a selective increase in the constrictor activity of control and treated arteries, respectively, (28°C) caused a 2.7 ± 0.4-fold increase in RhoA activity at 37°C. Moderate cooling (28°C) increased RhoA activity compared with control arteries (1). RhoA activity was assessed using a RhoA-GTP pull-down assay. Moderate cooling (28°C) caused a 2.7 ± 0.4-fold increase in RhoA activity (n = 7, P < 0.005; Fig. 4). The cell-permeable mimic of SOD (SODm, 50 μmol/l) also had no effect on responses to the α2-AR agonist at 37°C (maximal constriction of 23.8 ± 2.0% for L-NAME and L-NAME (50 μmol/l) still did not affect constriction to agonist at 37°C (maximal constriction of 21.3 ± 2.8 and 26.7 ± 4.0% for control and treated arteries, respectively, n = 5, P = NS) but continued to inhibit the response to the agonist at 28°C (maximal constractions of 50.7 ± 2.0% for control and treated arteries, respectively, n = 5, P < 0.005; Fig. 5). Indeed, in the presence of rotenone, NAC, or SODm, cooling no longer increased the constriction response to α2-AR stimulation (Figs. 3, 4, 5).

The effect of SODm was also evaluated after inhibition of NO synthase with l-NAME (100 μmol/l). In the presence of l-NAME, SODm (50 μmol/l) still did not affect constriction to UK-14304 at 37°C (maximal constriction of 19.3 ± 1.5 and 17.0 ± 3.0% for control and treated arteries, respectively, n = 5, P = NS) but reduced the response to 28°C (maximal constractions of 44.7 ± 2.0 and 29.3 ± 0.7% for l-NAME and l-NAME + SODm-treated arteries, respectively, n = 3, P < 0.01).

Cold, ROS, and RhoA activation. To directly assess the effects of ROS inhibition on cold-induced Rho activation, experiments were performed using smooth muscle cells cultured from human cutaneous arteries (1). RhoA activity was assessed using a RhoA-GTP pull-down assay. Moderate cooling (28°C) caused a 2.7 ± 0.4-fold increase in RhoA activity (n = 7, P < 0.005). NAC (20 mmol/l) or SOD (50 μmol/l) did not significantly affect RhoA activity at 37°C but abolished the cold-induced activation of the GTP-binding protein (Fig. 6).

Fig. 6. Effect of the antioxidant NAC (20 mmol/l; A) or the cell-permeable mimic of SODm MnTMPyP (50 μmol/l; B) on the cold-induced activation of RhoA in human cultured dermal arteriolar smooth muscle cells. Cells were cooled to 28°C (60 min) and Rho activity was determined by a pull-down assay, as described in METHODS. Active Rho was normalized to total Rho in the sample and expressed as a fold-change in the basal activity at 37°C (set as 1). Insets: results of a typical experiment. The bar graph presents the means ± SE for n = 3 (NAC) or 5 (SODm).
ROS activity played an essential role in the subsequent functional changes in cutaneous smooth muscle cells. In contrast, moderate cooling caused an immediate increase in smooth muscle constriction. Indeed, the antioxidant NAC or a cell-permeable SOD mimic prevented cold-induced amplification of α2-AR constriction. These agents did not affect constriction to α2-AR activation at 37°C, which is mediated by α2A-ARs, but abolished the cold-induced augmentation of the α2-AR response, which is mediated solely by α2C-ARs. These results are therefore consistent with selective inhibition of the α2C-AR component of constriction, which is observed only during cold exposure. Indeed, NAC and SOD each prevented the cold-induced translocation of α2C-ARs to the cell surface, determined in HEK 293 cells transfected with α2C-ARs. We previously demonstrated that translocation of α2C-ARs is mediated by cold-induced activation of RhoA and Rho kinase (1). Although ROS can inhibit RhoA activity by activating the RhoA GTPase activating protein p190Rho-GAP (29), exogenous ROS activate RhoA in the smooth muscle of rat aorta (25). Indeed, in the present study, cold-induced activation of RhoA in cutaneous smooth muscle cells was abolished by NAC or SODm. Therefore, the results suggest that a cold-induced increase in ROS was responsible for activating RhoA, which leads to the subsequent activation of Rho kinase, the translocation of α2C-ARs to the cell surface, and increased vasoconstriction to α2-AR activation.

ROS, in particular superoxide, can contribute to vasoconstriction by inactivating endothelium-derived NO (19, 33). However, cold-induced augmentation of α2-AR constrictor activity is still present in endothelium-denuded tail arteries (1), indicating that endothelium-derived NO does not contribute to the response. This was confirmed in the present study by the observation that cold-induced augmentation of α2-AR constriction was unaffected by the NO synthase inhibitor L-NAME. Furthermore, the SOD mimic still inhibited cold-induced amplification of α2-AR constriction in arteries treated with L-NAME. Therefore, the cold-induced increase in ROS is not acting indirectly through modulation of NO activity. These results are consistent with our previous observations suggesting that the signal transduction process activated by cold is selectively targeted to regulation of smooth muscle α2C-ARs (1).

After exposure to cold, the increase in DCDHF fluorescence was delayed compared with Mitotracker Red fluorescence. Furthermore, DCDHF fluorescence had a diffuse distribution throughout the cytoplasm of smooth muscle cells, whereas Mitotracker Red fluorescence was localized to discrete punctuate structures. This is consistent with localization of Mitotracker Red to mitochondria and with a predominant cytosolic distribution for DCDHF (12, 27, 30). Indeed, DCDHF is thought to respond to mitochondrial generation of ROS after their movement to the cytoplasm (12). The difference in time course between these ROS probes would therefore be consistent with a mitochondrial source for cold-induced generation of ROS. Superoxide can be produced as a result of the one-electron reduction system within the mitochondrial electron transport chain (4, 35). The most important pathway for physiological ROS production appears to be reverse electron flow to complex I of the electron transport chain, which is inhibited by the complex I inhibitor rotenone (3, 28). Indeed, rotenone abolished the cold-induced augmentation of α2-AR constriction in the mouse tail artery. As occurred with NAC and SODm, rotenone did not affect the response to α2-AR activation occurring at 37°C but selectively inhibited the augmented response during cold exposure. Therefore, these results suggest that the mitochondria of smooth muscle cells function exclusively by α2C-ARs to the cell surface, determined in HEK 293 cells transfected with α2C-ARs. The HA tag is at the NH2 terminus and is therefore in the extracellular domain of cell-surface receptors. Cells were incubated in the absence or presence of NAC or SODm for 30 min at 37°C. The medium was then replaced with precooled medium at 28°C or washed with medium at 37°C in the continued presence and absence of the ROS inhibitors, and the cells were maintained at these temperatures for 60 min. The cells were then labeled with anti-HA antibody and the cell-surface α2C-ARs were subsequently immunoprecipitated from cell lysates with protein-A/G sepharose beads. Inset: images were obtained from a representative experiment and show the immunoprecipitated cell-surface receptors, immunoblotted using anti-α2C-AR antibody. The bar graph presents means ± SE for n = 4.

In the mouse tail artery, α2-AR constriction at 37°C is mediated by α2A-ARs, whereas the cold-induced augmentation in α2-AR reactivity is mediated exclusively by α2C-ARs (8). Unlike other α2-AR subtypes, α2C-ARs are largely associated with the trans-Golgi compartment at 37°C but are translocated to the plasma membrane in response to cooling (24). Although α2C-ARs are an important effector for cold-induced vasoconstriction in cutaneous arteries, they are not directly responsive to cold and are therefore not thermosensitive (1). The mobilization of α2C-ARs to the cell surface is mediated by cold-induced activation of RhoA and Rho kinase signaling (1). The results of the present study suggest that the initial stimulus for cold-induced signaling in cutaneous arteries is an increase in ROS activity from smooth muscle mitochondria, which results in RhoA activation and the subsequent translocation of α2C-ARs to the cell surface, enabling enhanced α2-AR reactivity and cold-induced vasoconstriction.

Cold-induced activation of RhoA and the subsequent functional changes in cutaneous smooth muscle cells are relatively slow events, occurring over the course of 5 to 30 min (1, 17). In contrast, moderate cooling caused an immediate increase in ROS activity within the smooth muscle cells of cutaneous arteries, as detected using two ROS-sensitive fluorescent probes DCDHF and reduced Mitotracker Red. The increased ROS activity played an essential role in the subsequent functional responses to cold. Indeed, the antioxidant NAC or a...
as a cold thermosensor and initiate cold-induced vasoconstrictive
process through the generation of ROS and the initiation of redox
signaling. Moderate cooling also increased the mitochondrial
production of ROS in isolated, perfused hearts of guinea pigs
(5). Cold-induced generation of mitochondrial ROS may reflect
increased electron leak from the ETC to molecular O2, combined with a decreased efficiency of ROS scavenging
systems at lower temperatures (5).

The concept of mitochondria as a cold thermosensor is
intriguing. Thermogenesis, the process of heat generation, is
mediated by uncoupling of mitochondrial oxidative phosphory-
lization, which dissipates the energy of substrate oxidation as
heat (3, 28, 32). This is mediated by uncoupling proteins
(UCPs) that decrease the efficiency of coupling between res-
piration and ATP production and decrease the proton gradient
generated by complexes I, III, and IV across the inner mito-
chondrial membrane (3, 28, 32). Indeed, deletion of the UCP1
gene renders mice cold sensitive because of a deficiency in
brown fat thermogenesis (15). The uncoupling activity of
UCPs is stimulated by ROS (3, 28). Furthermore, because ROS
production is dependent on the magnitude of the proton gra-
dient, UCP activity reduces ROS generation by mitochondria
(UCPs) that decrease the efficiency of coupling between res-
piration and ATP production and decrease the proton gradient
mediated by uncoupling of mitochondrial oxidative phosphor-
lation and ATP production and decrease the proton gradient.

Cold-induced vascular constriction is increased in
individuals with Raynaud’s phenomenon resulting in cold-
induced vasospasm in the affected extremities (2). This condi-
tion is frequently associated with rheumatic diseases, in partic-
ular systemic sclerosis or scleroderma, where 95% of these
patients initially present with Raynaud’s phenomenon (2, 38).
It can also occur following treatment with antineoplastic che-
motherapeutic agents (e.g., bleomycin) (2, 7, 37). Increased cold sensitivity in scleroderma can be prevented by P2-adreno-
ceptor blockade (18). Therefore, oxidative stress associated with these conditions (6, 13, 20–22) may contribute to abnormal cold-
induced redox signaling and enhanced P2-adrenoceptor activity
in vascular smooth muscle cells, precipitating cold-induced vaso-
spasm in the cutaneous circulation.

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