Cold-induced cutaneous vasoconstriction is mediated by Rho kinase in vivo in human skin

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Thompson-Torgerson CS, Holowatz LA, Flavahan NA, Kenney WL. Cold-induced cutaneous vasoconstriction is mediated by Rho kinase in vivo in human skin. Am J Physiol Heart Circ Physiol 292: H1700–H1705, 2007. First published December 15, 2006; doi:10.1152/ajpheart.01078.2006.—Cutaneous vasoconstriction (VC) is the initial thermoregulatory response to cold exposure and can be elicited through either whole body or localized skin cooling. However, the mechanisms governing local cold-induced VC are not well understood. We tested the hypothesis that Rho kinase participates in localized cutaneous VC in human skin. In seven men and women (20–27 yr of age), up to four ventral forearm skin sites were instrumented with intradermal microdialysis fibers for localized drug delivery during cooling. Skin blood flow was monitored at each site with laser-Doppler flowmetry while local skin temperature was decreased and maintained at 24°C for 40 min. Cutaneous vascular conductance (CVC; laser-Doppler flowmetry/mean arterial pressure) was expressed as percent change from 34°C baseline. During the first 5 min of cooling, CVC decreased at control sites (lactated Ringer solution) to -45 ± 6% (P < 0.001), increased at adrenergic-antagonized sites (yohimbine + propranolol) to 15 ± 14% (P = 0.002), and remained unchanged at both Rho kinase-inhibited (fasudil) and adrenergic-antagonized + Rho kinase-inhibited sites (yohimbine + propranolol + fasudil) (−9 ± 1%, P = 0.4 and −6 ± 2%, P = 0.4, respectively). During the last 5 min of cooling, CVC further decreased at all sites when compared with baseline values (control, −77 ± 4%, P < 0.001; adrenergic antagonized, −61 ± 3%, P < 0.001; Rho kinase inhibited, −34 ± 7%, P < 0.001; and adrenergic antagonized + Rho kinase inhibited sites, −35 ± 3%, P < 0.001). Rho kinase-inhibited and combined treatment sites were significantly attenuated when compared with both adrenergic-antagonized (P < 0.01) and control sites (P < 0.0001). Rho kinase mediates both early- and late-phase cold-induced VC, supporting in vitro findings and providing a putative mechanism through which both adrenergic and nonadrenergic cold-induced VC occurs in vivo human thermoregulatory model.

fasudil; local cooling; vascular function; adrenergic; norepinephrine

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guided horizontally through the skin such that entry and exit points were ~2 cm apart. The fiber, consisting of a cylindrical 10-mm membrane (320 μm OD, 20-kDa molecular mass cutoff) and connective tubing attached to either end of the membrane, was threaded through the needle. The needle was then withdrawn, leaving the membrane in the skin. After fiber insertion, subjects rested quietly for ~90 min to allow local hyperemia due to insertion trauma to subside, during which time lactated Ringer solution was perfused through all fibers at a rate of 2.0 μl/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems).

Skin blood flow was measured by using laser-Doppler flowmetry (MoorLAB, Moor Instruments) to provide an index of cutaneous red blood cell flux. Laser-Doppler probes were placed on the skin directly over each microdialysis site, and red blood cell flux data were collected continuously throughout the experiment. Arterial blood pressure was monitored periodically via brachial auscultation approximately every 20 min and during experimental interventions. Mean arterial pressure was calculated as [(1/3 systolic blood pressure) + (2/3 diastolic blood pressure)]. Skin blood flow was converted to cutaneous vascular conductance (CVC; red blood cell flux/mean arterial pressure) and expressed as percent change from baseline CVC values (%ΔCVCbase). Local skin temperature (Tloc) at each site was controlled within ±0.02°C by using Peltier elements (TecThermo Temperature Controller 1575, Menlo Park, CA) with a small aperture in the center to accommodate the laser-Doppler probe.

Protocol. Following the resolution of needle insertion trauma, microdialysis sites were randomly assigned to continuously receive combinations of yohimbine (competitively antagonizes α-adrenoceptors), propranolol (competitively antagonizes β-adrenoceptors), and fasudil (competitively binds to ATP-binding domain to inhibit Rho kinase) as follows: site 1, lactated Ringer (control); site 2, 5 mM yohimbine + 1 mM propranolol (Y + P); and site 3, Y + P + 3 mM fasudil (Y + P + fasudil). Although yohimbine is typically used as an α2-adrenoceptor-specific antagonist, it is effective as a nonspecific α-adrenoceptor antagonist in higher concentrations, including those used in this study (18, 38–40, 42). In a subset of subjects (n = 5), data were also collected at a fourth site treated only with 3 mM fasudil. In vivo human pilot studies, varying concentrations of fasudil (0.1–5 mM) were delivered at 2 μl/min for at least 1 h before and then throughout a localized cooling protocol. Although all doses of fasudil inhibited cold-induced VC to varying degrees, inhibition reached a plateau at doses ≥3 mM. Therefore, 3 mM fasudil was perfused in the present study as the minimum effective inhibitory dose during localized cooling.

After solutions perfused the sites for ~60 min, Tloc was clamped at 34°C for a 15-min baseline period. Following baseline, 1 μM norepinephrine + 1 mg/ml L-ascorbate (preservative) (20) were added to the perfusate through all fibers for 4 min to test vascular responsiveness and the integrity of Y + P adrenoceptor antagonism. After a washout period (~30 min) to allow CVC to recover to pre-norepinephrine baseline values, all sites were cooled at a rate of 3°C/min, and Tloc was clamped at 24°C for 40 min, after which sites were rewarmed back to 34°C. All drugs were obtained from Sigma-Aldrich (St. Louis, MO), except fasudil, which was obtained from Tocris Bioscience (Ellisville, MO). All drug solutions were mixed just before usage, dissolved in lactated Ringer solution, and sterilized by using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI).

Data collection and analysis. Data were recorded and stored as 1-min averages by using computer software (LabView) and a data acquisition system (National Instruments, Austin, TX). Baseline values for normalization were determined by averaging the last 5 min of baseline data. Representative time course data are expressed as 5-min averages throughout the entire 40-min cooling protocol. Mean summary cooling data are expressed as averages of the first (early phase) or last (late phase) 5 min of cooling. These time points were chosen to clearly illustrate discrete early- and late-phase mechanisms of VC, unobscured by transition-phase mechanisms (~10–25 min). Data were analyzed by using repeated-measures, two-way analysis of variance, with planned comparison post hoc tests when significant differences were detected. Statistical significance was set at α = 0.05. Values are expressed at means ± SE, unless otherwise noted.

RESULTS

The seven subjects who participated in the study were young, healthy, normotensive, and nonobese (Table 1). No subjects were excluded due to a failure of Y + P to fully antagonize adrenoceptors. No sex differences in CVC responses were detected, so data from men and women were pooled for analysis.

In a representative time course tracing of cold-induced VC from a single subject (Fig. 1), all pharmacological treatments blocked at least a portion of cold-induced VC. Adrenergic antagonism alone (Y + P) mildly attenuated VC compared with control throughout the cooling protocol, whereas both Rho kinase inhibition (fasudil) and combined adrenoceptor antagonism and Rho kinase inhibition (Y + P + fasudil) blocked VC to a greater degree throughout the entire cooling protocol.

During the first 5 min of skin cooling, mean early-phase VC in all subjects (n = 7; fasudil, n = 5) was abolished by adrenoceptor antagonism and Rho kinase inhibition, administered both alone and in combination (Fig. 2A). CVC at control sites decreased to −45 ± 6% ΔCVCbase (P < 0.0001 vs. 34°C baseline). CVC at Y + P sites increased to 15 ± 14% ΔCVCbase (P = 0.002 vs. 34°C baseline), whereas CVC at fasudil and Y + P + fasudil sites remained unchanged (fasudil, −9 ± 1% ΔCVCbase, P = 0.4 vs. 34°C baseline; Y + P + fasudil, −6 ± 3% ΔCVCbase, P = 0.4 vs. 34°C baseline).

During the last 5 min of cooling, all sites exhibited greater VC when compared with the early phase of cooling (Fig. 2B). CVC at control sites further decreased to −77 ± 4% ΔCVCbase (P < 0.0001 vs. 34°C baseline). CVC at Y + P sites decreased to −61 ± 3% ΔCVCbase (P < 0.0001 vs. 34°C baseline), but the late-phase VC response at this site was significantly attenuated when compared with control (P = 0.03). CVC at fasudil and Y + P + fasudil sites decreased to −34 ± 7% ΔCVCbase and −35 ± 3% ΔCVCbase (both P < 0.0001 vs. 34°C baseline) and exhibited significantly less VC compared with the Y + P and control sites (P < 0.001).

In response to 4 min of 1 μM norepinephrine infusion under noncooled conditions (Tloc = 34°C), control and Rho kinase-inhibited sites exhibited significant VC, whereas CVC at adrenoceptor-antagonized sites remained unchanged (Fig. 3). CVC at control and fasudil sites significantly decreased from baseline (control, −33 ± 6% ΔCVCbase, P < 0.0001 vs. 34°C baseline; fasudil, −22 ± 8% ΔCVCbase, P = 0.001 vs. 34°C baseline).

Table 1. Subject characteristics

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Values are means ± SE for 4 men (M) and 3 women (F). BMI, body mass index; MAP, mean arterial pressure.
with the latter effect predominating. Thus Rho kinase activation in the vasculature seems to be predominantly associated with either physiological (including cooling) or pathophysiological stressors; it is likely that superimposing these two types of stressors would elicit even greater Rho kinase activation, although this speculation requires further validation. Conversely, Rho kinase participation in vascular regulation may be less pronounced in healthy, resting states (25, 28, 32, 33).

This study tested the hypothesis that Rho kinase also contributes to cold-induced VC in vivo during localized skin cooling. During the first 10 min of local cooling, an immediate and pronounced VC occurs that can be inhibited by either presynaptic sympatholytics or adrenoceptor antagonists (specifically α2) but not with proximal nerve blockade, suggesting a localized (i.e., nonreflex) mechanism dependent on norepinephrine of sympathetic origin binding to α2-adrenoceptors (9, 12, 14, 19, 24, 27, 34). The results from the present study help to further clarify the mechanisms underlying this early-phase response to cooling. Adrenergic antagonism abolished VC, whereas both Rho kinase inhibition and simultaneous adrenergic antagonism + Rho kinase inhibition blocked 85–90% of the VC response. These results indicate that VC during the first

DISCUSSION

The primary findings of the present study were that 1) early-phase cold-induced VC is inhibited by yohimbine + propranolol (adrenoceptor antagonism) or fasudil (inhibition of Rho kinase), 2) late-phase cold-induced VC is minimally attenuated by adrenergic antagonists and is significantly attenuated by a Rho kinase inhibitor, and 3) Rho kinase inhibition more effectively attenuates VC at cold, compared with thermoneutral, local skin temperatures.

Rho kinase is a key intracellular mediator directly involved in VC, operating through inhibition of myosin light chain phosphatase, ultimately resulting in an increase in vascular smooth muscle sensitivity to extant intracellular Ca2+ (2, 4, 15, 17, 36, 37). In healthy resting vessels, tonically active RhoA/Rho kinase downregulates the endothelial NOS (eNOS) pathway (13, 29, 30, 41), whereas the nitric oxide pathway conversely downregulates RhoA/Rho kinase function (6, 7, 35). This mutual antagonism effectively maintains a healthy and necessary balance between dilator and constrictor influences in the vasculature. However, Rho kinase activity is disproportionately increased when vessels are exposed to either a particular physiological stress, such as stretch or a change in redox status (3, 11), or a specific disease state, such as hypertension, hypercholesterolemia, or diabetes (6, 16, 21, 23, 28, 31, 43). Recently, Bailey and colleagues (2, 3) established that RhoA/Rho kinase is also activated by direct cooling of cutaneous arteriolar smooth muscle cells and mediates cold-induced VC in vitro. Specifically, cold-induced Rho kinase activation mediates VC via two distinct pathways: Ca2+ sensitization and the characteristic α2C-adrenoceptor translocation observed with the onset of cutaneous tissue cooling (significantly augmenting the adrenoceptor population on vascular smooth muscle cells),
few minutes of cooling is very sensitive to both adrenergic blockade and Rho kinase inhibition, suggesting that both pathways are integrally involved in early-phase cold-induced VC and most likely interact, as has been shown in vitro (2, 3).

As localized skin cooling progresses past 10–15 min, CVC continues to decrease, albeit at a slower rate than during the first 10 min. During this prolonged phase of VC, pre- and postsynaptic sympathetic inhibitors only block ~20% of the VC response, suggesting that nonadrenergic mechanisms help mediate late-phase cold-induced VC (19, 24, 34, 44). The results from the present study confirm that prolonged cooling is predominantly mediated by nonadrenergic mechanisms and provide direct evidence identifying cellular mechanisms centrally involved in this response. Whereas adrenergic antagonism only blocked 20% of late-phase VC in this study, Rho kinase inhibition or concurrent administration of both adrenergic antagonists and a Rho kinase inhibitor blocked ~60% of late-phase VC. Attenuation with Rho kinase inhibition indicates that Rho kinase participates in late-phase cold-induced VC, and similar attenuation with combined adrenergic antagonism + Rho kinase inhibition suggests that, although Rho kinase and norepinephrine are both involved in late-phase VC, their effects are not additive. Rather, these data cumulatively indicate that a smaller portion of late-phase VC is mediated by an interaction between adrenergic and Rho kinase pathways, whereas the majority of the response is mediated directly by Rho kinase, possibly through Ca2+ sensitization.

To establish whether the Rho kinase contribution to cold-induced VC was specifically elicited by cooling per se, norepinephrine was infused at all treatment sites when Tloc = 34°C (thermoneutral). CVC at adrenergic antagonist sites did not significantly change from preinfusion baseline, verifying the integrity of the adrenergic antagonist at those sites. However, control and Rho kinase-inhibited sites both exhibited significant VC in response to thermoneutral norepinephrine administration. Specifically, Rho kinase inhibition only attenuated norepinephrine-mediated VC by ~33% (indicating only modest Rho kinase activity at rest), compared with 85–90% reduction of cold-induced VC. These data preclude the argument that fasudil nonspecifically suppresses VC by indicating that Rho kinase inhibition much more effectively blocked VC during cooling, suggesting that Rho kinase is significantly more involved in mediating VC under cold (compared with thermoneutral) conditions. This conclusion is consistent with the cold-induced increase in Rho kinase activity that has been observed in vitro (2, 3). This conclusion also supports data in the literature indicating that vessels that have been exposed to a specific physiological and/or pathophysiological stressor tend to exhibit greater Rho kinase-mediated VC when compared with healthy, resting vessels in vivo (25, 28, 32, 33).

Cumulatively, the data from this study indicate that the interaction of norepinephrine and Rho kinase plays an integral role in both early- and late-phase cold-induced VC. During early-phase cooling, inhibition of either the adrenergic or the Rho kinase pathway fully abolished cold-induced VC, suggesting that these two pathways interactively mediated early-phase VC. During late-phase cooling, this interaction accounted for a smaller, but still significant, portion of the VC response, ~20%. Although we cannot conclusively articulate the point(s) of interaction between the two pathways based on the current data, there at least two possible explanations. It is feasible that norepinephrine-mediated VC is relatively Rho kinase independent until the onset of tissue cooling, whereupon the cold-induced increase in Rho kinase activity provides an additional intracellular mechanism through which norepinephrine can elicit VC. Alternatively, it is possible that cold-induced Rho kinase activity augments norepinephrine-mediated VC through its actions on α2C-adrenoceptors. Taking into consideration 1) the results of the present study suggesting a cold-specific Ca2+-dependent element in Rho kinase-mediated VC, 2) the established role of Rho kinase in mediating cold-induced α2C-adrenoceptor translocation in vitro (2, 3, 10, 22), and 3) the preponderance of in vivo human literature suggesting that early-phase cold-induced VC is mediated almost exclusively by α2C-, but not α1-, adrenoceptors (despite the presence of functional α1-adrenoceptors in skin), it is plausible that Rho kinase may mediate VC, at least in part, through stimulation of α2C-adrenoceptor translocation. However, this speculation requires further testing to verify its validity in an in vivo model.

In addition to its reliance on Rho kinase putatively working through/interacting with adrenergic mechanisms, late-phase VC also comprises at least two other mechanisms. A Rho kinase-dependent mechanism that was independent of adrenoceptors (i.e., a direct VC effect of Rho kinase) accounted for an additional ~40% of the overall VC response. In view of the long-established role of Rho kinase in Ca2+-independent VC via inhibition of myosin light chain phosphatase (15, 36, 37), it is likely that this portion of late-phase VC was mediated by Ca2+- sensitization. This conclusion is further supported by the observation that Rho kinase-mediated Ca2+- sensitization can contribute to the maintenance of prolonged VC (36, 37). These results also suggest that there is at least one additional mechanism contributing to late-phase cold-induced VC that is not sensitive to either adrenoceptor antagonist or Rho kinase inhibition.

Rho kinase activation during localized skin cooling also supports a recently articulated role of the eNOS system in cold-induced VC, suggesting a putative third mechanism.

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Fig. 3. Average maximal cutaneous vasoconstriction in response to 34°C 
1 μM norepinephrine infusion at control, adrenoceptor-antagonized (Y + P), Rho kinase-inhibited (fasudil), and combined adrenoceptor-antagonized + Rho kinase-inhibited (Y + P + fasudil) sites. Control, Y + P, and Y + P + fasudil, n = 7; fasudil, n = 5. *P < 0.05 vs. baseline; †P < 0.05 vs. control site.
through which Rho kinase may mediate cold-induced VC. Hodges and colleagues (19) found that full expression of cold-induced VC involves a decrease in eNOS activity and/or nitric oxide production but did not speculate as to the mechanism(s) through which localized skin cooling might regulate the eNOS pathway. Just as nitric oxide (through cyclic GMP-dependent protein kinase) counters Rho kinase-mediated VC by activating myosin light chain phosphatase (7) and inhibiting activation of upstream RhoA (35), RhoA and Rho kinase similarly downregulates several steps in the eNOS pathway: decreased eNOS gene expression (30), decreased eNOS mRNA expression (13), eNOS mRNA destabilization (26, 41), decreased eNOS protein expression (6, 13, 41), decreased activation of eNOS (6, 8, 30), and increased arginase activity, which decreases nitric oxide bioavailability (5, 29). With a consideration for the interaction of these two systems under other conditions and in other vascular beds, it is plausible that they may interact in the cutaneous circulation during cooling, as well. Thus the combined findings of the present study and Hodges et al. (19) may illustrate one of two possible regulatory mechanisms through which localized skin cooling might regulate the eNOS pathway: decreased eNOS activity or increased Rho kinase activity or both. 

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**References.**


