Reflex vasoconstriction in aged human skin increasingly relies on Rho kinase-dependent mechanisms during whole body cooling

James A. Lang,1 John D. Jennings,1 Lacy A. Holowatz,1 and W. Larry Kenney1,2

1Department of Kinesiology, Noll Laboratory, and 2Graduate Physiology Program, The Pennsylvania State University, University Park, Pennsylvania

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Lang JA, Jennings JD, Holowatz LA, Kenney WL. Reflex vasoconstriction in aged human skin increasingly relies on Rho kinase-dependent mechanisms during whole body cooling. Am J Physiol Heart Circ Physiol 297: H1792–H1797, 2009. First published August 28, 2009; doi:10.1152/ajpheart.00509.2009.—Primary human aging may be associated with augmented Rho kinase (ROCK)-mediated contraction of vascular smooth muscle and ROCK-mediated inhibition of nitric oxide synthase (NOS). We hypothesized that the contribution of ROCK to reflex vasoconstriction (VC) is greater in aged skin. Cutaneous VC was elicited by 1) whole body cooling [mean skin temperature (Tsk) = 30.5°C] and 2) local norepinephrine (NE) infusion (1 × 10−6 M). Four microdialysis fibers were placed in the forearm skin of young (Y) and eight older (O) subjects for infusion of 1) Ringer solution (control), 2) 3 mM fasudil (ROCK inhibition), 3) 20 mM Nω-nitro-arginine methyl ester (NOS inhibition), and 4) both ROCK + NOS inhibitors. Red cell flux was measured by laser-Doppler flowmetry over each site. Cutaneous vascular conductance (CVC) was calculated as flux/mean arterial pressure and normalized to baseline CVC (%ΔCVCbaseline). VC was reduced at the control site in O during cooling (Y, −34 ± 3; and O, −18 ± 3 %ΔCVCbaseline; P < 0.001) and NE infusion (Y, −53 ± 4, and O, −41 ± 9 %ΔCVCbaseline; P = 0.006). Fasudil attenuated VC in both age groups during mild cooling; however, this reduction remained only in O but not in Y skin during moderate cooling (Y, −30 ± 5; and O, −7 ± 1 %ΔCVCbaseline; P = 0.016) and was not altered by NOS inhibition. Fasudil blunted NE-mediated VC in both age groups (Y, −23 ± 4; and O, −7 ± 3%ΔCVCbaseline; P < 0.01). Cumulatively, these data indicate that reflex VC is more reliant on ROCK in aged skin such that approximately half of the total VC response to whole body cooling is ROCK dependent.

In aged skin (>60 yr), the reflex VC response is not only blunted but relies entirely on an already compromised adrenergic mechanism (18, 32–34). Furthermore, the vascular signaling pathways that couple adrenoceptor activation to VC may be altered (20, 31). During localized cooling in aged skin, the magnitude of the VC response is not diminished but relies more on ROCK and less on other adrenegically stimulated protein kinase signaling cascades (31–33). ROCK can be stimulated by NE or mitochondrial superoxide generated in response to localized cooling (2, 28). Activated ROCK elicits VC through two distinct mechanisms: 1) inhibition of myosin light chain (MLC) phosphatase, thereby maintaining MLC phosphorylation without Ca2+ influx (i.e., Ca2+ sensitization) and 2) inducing the translocation of α2c-receptors from the Golgi apparatus to the cell membrane (1, 5, 14). Although ROCK has a clear role in VC during localized cooling, the extent with which ROCK mediates reflex VC remains unclear.

A greater dependence on ROCK in primary aging may parallel that observed in other age-associated vascular pathologies such as atherosclerosis, hypertension, erectile dysfunction, and diabetes (3, 4, 8, 13, 19, 23, 35). From a thermoregulatory standpoint, ROCK may serve as an important mechanism in aged skin to sustain cutaneous VC and prevent excessive heat loss during cold exposure; however, this may occur at the expense of microvascular function since ROCK has a mutually inhibitory influence on endothelial nitric oxide synthase (eNOS) (21–23). In vitro, ROCK decreases eNOS expression and activity and increases arginase activity, thereby reducing NO bioavailability, whereas cGMP-dependent protein kinase, a product of NO metabolism, inhibits Rho activation (21–23). Thus ROCK inhibition may have a vasoprotective effect due in large part to its putative effects on NO bioavailability (23). Collectively, augmented ROCK activity appears to have a deleterious effect on vascular function; whether or not upregulated ROCK during whole body cooling predates the onset of other vascular pathologies has yet to be determined.

The purpose of this study was to determine the extent with which ROCK participates in reflex VC during whole body cooling. We hypothesized that ROCK-dependent mechanisms would contribute to the reflex VC response to a greater extent in aged skin. We further hypothesized that ROCK inhibition with local fasudil supplementation would attenuate the VC response to 1) gradual whole body cooling and 2) localized NE perfusion, and that this reduction would be larger in aged than in young skin, which would suggest that ROCK is upregulated or unmasked due to the reduced activity of other adrenergically stimulated protein kinase signaling mechanisms. Finally, we sought to determine whether nitric oxide synthase (NOS) inhibition, which would putatively function to disinhibit ROCK, would have a differential effect on the cutaneous VC response between age groups.

METHODS

Subjects. With Pennsylvania State University Institutional Review Board approval and after verbal and written informed consent, eight...
young (20 ± 1 yr; 3 men and 5 women) and eight older (73 ± 2 yr; 5 men and 3 women) subjects participated in the study. Young women were tested in the early follicular phase (days 1–7) of the menstrual cycle, and older women were postmenopausal and not taking hormone replacement therapy. All subjects were healthy, nonobese, normotensive, normal cholesterolemic, nonsmokers, and not taking any medications that would otherwise alter cardiovascular or thermoregulatory function. All procedures conformed to the standards set by the Declaration of Helsinki.

**Instrumentation.** On the morning of an experiment, between 8:00 AM and 10:00 AM, subjects arrived at the laboratory and were instrumented with four microdialysis (MD) fibers (10 mm, 20-kDa cutoff membrane, MD 2000 Bioanalytical Systems, West Lafayette, IN) placed intradermally in the ventral forearm using aseptic technique. Before fiber placement, ice packs were applied to MD sites for 5 min to temporarily anesthetize the skin. For each fiber, a 25-gauge needle guide was inserted horizontally into the dermis such that the entry and exit points were ~2.5 cm apart. MD fibers were threaded through the needle. The needle was then withdrawn leaving the membrane in place. After all fibers were taped in place, lactated Ringer solution was perfused at 2 μl/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) for 60–90 min to allow for local hyperemia due to needle insertion trauma to subside.

To control skin temperature, subjects wore a water-perfused suit that covered the entire body except for the face, feet, hands, and forearms. Copper-constantan thermocouples were placed on the surface of the skin at six sites: calf, thigh, abdomen, chest, back, and upper arm. The unweighted mean of these sites provided an index of mean skin temperature (Tsk).

To obtain an index of mean skin blood flow, red cell flux was continuously measured with laser-Doppler flowmetry (LDF) probes ( Moor-LAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). LDF probes were placed in the center of local heaters and positioned directly over each MD fiber site. To specifically isolate reflex mechanisms, local skin temperature was clamped at 33°C throughout the experiment. Arterial blood pressure was measured every 5 min.

**Protocol.** After the instrumentation of MD fibers and the resolution of local hyperemia, pharmacological agents were perfused for 45–60 min. MD fiber sites were randomly assigned with respect to the position on the forearm and were perfused with J) lactated Ringer solution serving as control), 2) 3 mM fasudil (ROCK inhibitor), 3) 20 mM N\(^{\text{6}}\)-nitro-l-arginine methyl ester (l-NAME), and 4) fasudil + l-NAME. All drugs were mixed just before use, dissolved in lactated Ringer solution, and sterilized using syringe microfilters (Acrodisc, Pall, Ann Arbor, MI). Drug dosages were determined from previous studies where the concentrations of fasudil and l-NAME used maximally inhibited ROCK and NOS, respectively (12, 30, 31). Throughout the baseline period, mean Tsk was held constant at 34°C by perfusing thermonuclear water through the suit.

After baseline measurements, cold water was circulated through the suit to induce reflex VC. Mean Tsk decreased gradually from 34°C to 30.5°C over 30 min and was then clamped for an additional 10 min at 30.5°C (i.e., above the threshold for shivering). Rewarming for ~30 min followed to return Tsk back to 34°C, after which a 1 × 10\(^{-6}\) M dose of NE was perfused at all sites for 15 min to verify that VC responsiveness was preserved postcooling. The CVC established after rewarming was used as a baseline to assess NE-mediated VC. Lastly, 28 mM sodium nitroprusside (SNP) was perfused until a plateau in the vasodilatation response was achieved (~30 min) at each MD site to ensure that vascular function remained intact postcooling. Because of the VC stimuli used in the protocol, the SNP-induced vasodilation was likely submaximal (Table 1).

**RESULTS**

Age groups were well matched with regard to height [young (Y), 170 ± 3; and older (O), 171 ± 3 cm], weight (Y, 67 ± 3; and O, 71 ± 3 kg), body mass index (Y, 23.3 ± 1.0; and O, 24.5 ± 0.5 kg/m\(^2\)], resting MAP (Y, 85.6 ± 2.4; and O, 85.7 ±

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Table 1. Group means ± SE for absolute cutaneous vascular conductance

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Control</th>
<th>Fasudil</th>
<th>Fasudil + l-NAME</th>
<th>l-NAME</th>
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<tr>
<td><strong>Baseline</strong></td>
<td></td>
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<tr>
<td>Young</td>
<td>0.29 ± 0.06</td>
<td>1.60 ± 0.24*</td>
<td>1.28 ± 0.24*</td>
<td>0.22 ± 0.04</td>
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<tr>
<td>Older</td>
<td>0.30 ± 0.09</td>
<td>1.71 ± 0.41*</td>
<td>1.16 ± 0.29*</td>
<td>0.19 ± 0.02</td>
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<td><strong>Cooling</strong></td>
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<td>Young</td>
<td>0.19 ± 0.05 (−34 ± 3)</td>
<td>1.16 ± 0.20* (−30 ± 5)</td>
<td>0.89 ± 0.16* (−28 ± 4)</td>
<td>0.14 ± 0.02 (−38 ± 4)</td>
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<tr>
<td>Older</td>
<td>0.23 ± 0.06 (−18 ± 3)</td>
<td>1.59 ± 0.38*† (−7 ± 1)</td>
<td>1.01 ± 0.25* (−11 ± 4)</td>
<td>0.15 ± 0.02 (−23 ± 4)</td>
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<td><strong>Rewarming</strong></td>
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<tr>
<td>Young</td>
<td>0.30 ± 0.06</td>
<td>1.31 ± 0.27*</td>
<td>0.95 ± 0.17*</td>
<td>0.20 ± 0.04</td>
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<tr>
<td>Older</td>
<td>0.35 ± 0.07</td>
<td>1.64 ± 0.37†</td>
<td>1.07 ± 0.32*</td>
<td>0.20 ± 0.02</td>
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<td><strong>NE</strong></td>
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<tr>
<td>Young</td>
<td>0.14 ± 0.03 (−53 ± 4)</td>
<td>1.06 ± 0.24* (−23 ± 4)</td>
<td>0.76 ± 0.15* (−21 ± 4)</td>
<td>0.12 ± 0.02 (−34 ± 3)</td>
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<tr>
<td>Older</td>
<td>0.18 ± 0.03 (−41 ± 9)</td>
<td>1.52 ± 0.36*† (−7 ± 3)</td>
<td>0.87 ± 0.29* (−19 ± 5)</td>
<td>0.15 ± 0.01 (−24 ± 4)</td>
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<td><strong>SNP</strong></td>
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<tr>
<td>Young</td>
<td>2.17 ± 0.22</td>
<td>2.51 ± 0.33*</td>
<td>2.03 ± 0.23</td>
<td>2.53 ± 0.39*</td>
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<tr>
<td>Older</td>
<td>2.86 ± 0.43†</td>
<td>2.49 ± 0.47*</td>
<td>2.31 ± 0.44*</td>
<td>2.65 ± 0.36</td>
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Absolute cutaneous vascular conductance (CVC) values (laser-Doppler flux × mmHg\(^{-1}\)) at baseline, maximal cooling (i.e., mean skin temperature = 30.5°C), precooling baseline following rewarming, norepinephrine (NE) perfusion, and sodium nitroprusside (SNP) perfusion are expressed as means ± SE for young (n = 8) and older (n = 8) subjects. Beside each CVC value for cooling and NE (in parentheses) are the corresponding relative values expressed as a percent change from baseline CVC. *P < 0.05 vs. control; †P < 0.05 vs. young.
3.0 mmHg), and cholesterol ratio (total cholesterol to HDL cholesterol) (Y, 3.1 ± 0.2; and O, 3.1 ± 0.3).

The absolute CVC values, calculated as laser-Doppler flux, $\times$ mmHg$^{-1}$ for each MD fiber site are illustrated in Table 1. At the fasudil-treated sites, CVC values differed from the control site at baseline, during cooling, and during NE perfusion ($P < 0.01$). CVC values at the fasudil site during both cooling and NE perfusion were higher in aged skin ($P < 0.01$), as was the CVC during SNP perfusion at the control site ($P < 0.01$). When compared with the control site, CVC during SNP was greater in both fasudil ($P = 0.01$)- and l-NAME ($P < 0.01$)-treated sites in young skin, whereas in aged skin CVC was lower in the fasudil ($P < 0.01$)- and fasudil + l-NAME ($P < 0.01$)-treated sites.

In Fig. 1, the CVC response at every 0.5°C drop in mean $T_{sk}$ during whole body cooling is illustrated. In young subjects (Fig. 1A), fasudil attenuated the VC response to mild cooling (mean $T_{sk} \geq 31.0°C; P < 0.05$) but VC was unaffected at lower $T_{sk}$. In contrast, older subjects exhibited blunted VC at the fasudil site as cooling became more severe (mean $T_{sk} \leq 33.0°C; P < 0.05$) (Fig. 1B).

The effect of ROCK inhibition with fasudil on VC function during whole body cooling ($T_{sk} = 30.5°C$) is illustrated in Fig. 2A. When compared with young subjects, older subjects exhibited a blunted VC response at the control site (Y, $-34 \pm 3$; and O, $-18 \pm 3\%$ CVCbaseline; $P < 0.01$). The local administration of fasudil significantly attenuated VC in older subjects ($-7 \pm 1\%$ CVCbaseline; $P = 0.02$) but had no effect in young subjects ($-30 \pm 5\%$ CVCbaseline; $P = 0.33$). Similarly, when compared with l-NAME alone (Y, $-38 \pm 4$; and O, $-23 \pm 4\%$ CVCbaseline), the combined administration of fasudil and l-NAME (Y, $-29 \pm 4$; and O, $-11 \pm 4\%$ CVCbaseline) blunted the VC response in older ($P = 0.01$) and young subjects ($P = 0.03$).

During NE (1 $\times$ 10$^{-6}$ M) perfusion (Fig. 2B), VC at the control site was significantly lower in aged skin (Y, $-53 \pm 4$; and O, $-41 \pm 9\%$ CVCbaseline; $P < 0.01$). When compared with control, fasudil attenuated the VC response in both young and older subjects (Y, $-23 \pm 4$; and O, $-7 \pm 3\%$ CVCbaseline; $P < 0.01$). Moreover, NE-mediated VC at l-NAME (Y, $-34 \pm 3$; and O, $-24 \pm 4\%$ CVCbaseline; $P < 0.01$) and fasudil + l-NAME (Y, $-21 \pm 4$; and O, $-19 \pm 5\%$ CVCbaseline; $P < 0.01$) sites were also reduced relative to the VC at the control.
site. When compared with L-NAME alone, adding fasudil to L-NAME blunted the VC response in young ($P < 0.01$) but not older subjects ($P = 0.35$).

Figure 3 illustrates the ROCK contribution to the VC response, expressed as a percentage of the total VC elicited by moderate whole body cooling (mean $T_{sk} = 30.5^\circ C$) or by NE (1 $\times$ $10^{-6}$ M), and calculated as the percentage of VC mediated by ROCK = [($\% \Delta \text{CVC}_{\text{baseline}}$ at control site $-$ $\% \Delta \text{CVC}_{\text{baseline}}$ at fasudil site)/$\% \Delta \text{CVC}_{\text{baseline}}$ at control site]. During whole body cooling, ROCK contributed to reflex VC to a greater extent in aged (52 $\pm$ 9%; $P < 0.01$) than in young skin (13 $\pm$ 13%). However, the ROCK contribution to NE-mediated VC was not different between age groups (Y, 56 $\pm$ 7%; and O, 77 $\pm$ 17%).

**DISCUSSION**

The primary finding from this study was that ROCK inhibition diminished the VC response to mild cooling in both age groups, and this reduction remained during more severe cooling in aged but not young skin. In fact, fasudil attenuated $\sim$50% of the VC response to more severe whole body cooling (mean $T_{sk} = 30.5^\circ C$) in aged skin. Similar reductions in the VC response due to ROCK inhibition were observed at NOS-inhibited sites. In contrast, the VC response to an exogenous physiological dose of NE was blunted by fasudil in both age groups. Cumulatively, these data suggest that ROCK mediates approximately half of the reflex VC response to whole body cooling in aged skin.

The data from the present investigation are consistent with previous studies demonstrating that reflex VC to whole body cooling is not only attenuated in aged skin but relies almost entirely on a compromised adrenergic mechanism (7, 10, 18, 32–34). In addition, the second messenger responses coupling adrenoreceptor activation to reflex VC are altered in aged skin such that VC relies more on ROCK (31–34). This may be due in part to how Ca$^{2+}$ is handled in vascular smooth muscle. NE induces VC through Ca$^{2+}$-dependent and Ca$^{2+}$-independent mechanisms; the latter is stimulated by ROCK and sensitizes vascular smooth muscle to extant intracellular Ca$^{2+}$ by deactivating MLC phosphatase. In aged rats, small vessels demonstrate a reduced sensitivity of contractile proteins to Ca$^{2+}$ as well as a greater NE-evoked VC when placed in a Ca$^{2+}$-free medium. Thus it is plausible that compromised Ca$^{2+}$-induced signaling explains the augmented ROCK component of cutaneous VC in older humans.

In addition to altered Ca$^{2+}$ handling in vascular smooth muscle, ROCK may be unmasked during reflex VC in light of absent cotransmitter function in aged skin. At milder skin temperatures (>31.0°C), ROCK inhibition attenuated reflex VC in both young and older skin, which suggests not only that ROCK contributes to this response but that the ROCK-mediated component of reflex VC is not different between age groups at these temperatures. However, with more severe cooling (<31.0°C), ROCK had no appreciable affect on the VC response in younger skin but remained a considerable component in older skin. Interestingly, it is in this skin temperature range that cotransmitter-mediated VC significantly contributes to the reflex VC response in younger skin (29, 34). Because cotransmitter and noradrenergic-mediated VC are absent or reduced, respectively, in aged skin, it is plausible that the ROCK component is what functionally remains to contribute to reflex VC. In contrast, sympathetic cotransmitters may be inhibiting the ROCK pathway in young skin; thus the VC differences with fasudil may actually be a consequence of absent cotransmitter function, or disinhibited ROCK, in aged skin rather than differences in the ROCK-mediated component.

However, the interaction between ROCK and the cotransmitter function during whole body cooling requires further investigation.

Much of what is known about how ROCK implements cold-induced VC comes from in vivo localized cooling studies in humans (30, 31) and in vitro work using mouse tail arteries. The in vitro studies revealed that localized cooling of vessels increases the generation of mitochondrial superoxide which directly stimulates ROCK (2). Elevated ROCK activity augments VC through two distinct mechanisms: 1) Ca$^{2+}$ sensitization by inhibiting MLC phosphatase and maintaining MLC phosphorylation in the absence of Ca$^{2+}$ influx and 2) translocation of $\alpha_2C$-receptors from the Golgi apparatus to the plasma membrane, thereby augmenting adrenoreceptor binding sites for NE by as much as fivefold (1, 5, 14). In these in vivo studies the VC response to a locally applied cold stimulus, demonstrating that $\sim$60% of the VC response is ROCK mediated in young subjects, and the VC becomes more dependent on ROCK with age (30, 31). In contrast to local cooling, our data demonstrate that during moderate whole body cooling ($T_{sk} = 30.5^\circ C$), young subjects exhibited little ROCK-mediated VC ($\sim$10%), whereas over half of the VC response in older skin was ROCK dependent. The collective results from both localized and whole body cooling in humans suggest that cutaneous VC is more reliant on ROCK with primary human aging.

The mechanism of how ROCK participates in localized versus reflex VC may differ. In contrast to whole body cooling, the VC to local cooling in aged skin 1) does not differ in magnitude from the VC response observed in young skin (31,
33), 2) is independent of efferent sympathetic reflex activity (9, 24), and 3) is only modestly attenuated by adrenergoreceptor blockade during more prolonged (i.e., “late phase”) cooling (16, 31). However, the VC to whole body cooling in aged skin is completely abolished in response to bretylium tosylate and to adrenergoreceptor blockade, indicating that reflex VC is entirely dependent on axonal release of NE from sympathetic adrenergic nerves (24, 34). Moreover, we found that fasudil substantially reduced the VC in response to exogenous NE (10^{-6} M) in older subjects (i.e., ROCK contributed to ~80% of the NE-mediated VC response). Cumulatively, these data suggest that NE relies primarily on ROCK to elicit VC in aged skin.

Oxidative stress may also play a role in NE-induced stimulation of ROCK. In rat renal arteries, phenylephrine (α-adrenergoreceptor agonist) infusion can rapidly stimulate superoxide production (17). Subsequently, superoxide induces VC through ROCK-mediated pathways (15). Perhaps the globalized increase in reactive oxygen species that occurs with primary aging tonically augments ROCK activity and increases the gain of the ROCK response under adrenergoreceptor stimulation. However, this mechanism requires further study.

In addition to its effects on vascular smooth muscle, ROCK also reciprocally inhibits NOS (21–23). ROCK can decrease NO bioavailability by inhibiting eNOS transcription and activity (22) and by augmenting arginase activity (13, 21). As a result, increased ROCK activity may precede more serious age-related clinical pathologies such as atherosclerosis (19), diabetes (8), endothelial dysfunction (3, 4), cerebral and coronary vasospasm (25), and hypertension (13, 35). As such, inhibition of ROCK may be beneficial in mitigating these vascular pathologies (23). Much of the underlying protective effects of ROCK inhibition are mediated by the upregulation of eNOS (3, 4, 21–23).

We examined the interactive role of NOS and ROCK during whole body cooling and found that the reduction in VC associated with fasudil was similar at NOS-inhibited and NOS-intact sites. Thus NOS inhibition does not appear to affect the ROCK contribution to VC during whole body cooling in aged skin. However, in young skin, the VC response was reduced when comparing the effect of fasudil across the NOS-blocked sites but was unaffected when comparing fasudil with control. This would suggest that NOS inhibition is required to fully express the ROCK-mediated component of reflex VC. In contrast, L-NAME blunted the VC response to NE in both young and older subjects. This was counter to what we hypothesized based on previous evidence that NOS inhibition augments cutaneous VC (26, 27). Possible explanations for the discrepancy with L-NAME may be that it is dose related or that there is an order effect of perfusing NE following cooling. Additionally, baseline CVC at the L-NAME site tended to be lower than other sites (Table 1), which may limit the signal gain during a VC stimulus, resulting in an artificially blunted response. Nevertheless, ROCK inhibition clearly attenuated NE-mediated VC in both young and older subjects, suggesting that ROCK has an important role in NE-mediated VC.

Limitations. The baseline vasodilation observed in the fasudil sites may be a source of concern. Although fasudil is a selective inhibitor of ROCK, it may be acting on other signaling pathways in addition to ROCK. It is apparent that at least a portion of the dilatory response to fasudil was NO-mediated. However, more selective inhibitors could not be substituted as they are not available for in vivo administration in humans. As a result, it is difficult to determine the influence of ROCK on resting tone. Because normalization occurred at higher baseline CVCs, it is possible that the prior dilation to fasudil may have blunted the subsequent VC to cooling or NE. This is refuted in part by the observation that the VC to whole body cooling at the fasudil site did not differ from the control site in young skin.

In summary, the present study suggests that VC is more dependent on ROCK during more severe whole body cooling in aged but not young skin, which was largely unaffected by NOS inhibition. Furthermore, much of the VC to exogenous NE relies on ROCK, particularly in aged skin. Thus reflex VC in aged skin relies entirely on noradrenergic function, and approximately half of this response is ROCK mediated. Greater dependence on ROCK to elicit VC in primary aging may predate more serious clinical pathologies due primarily to its inhibitory effects on NOS.

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GRANTS

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RHO KINASE AND REFLEX VASOCONSTRICTION


