



Rho-Kinase Activity And Cutaneous Vasoconstriction Is Upregulated In Essential Hypertensive Humans

By: **Caroline J. Smith**, Lakshmi Santhanam, and Lacy M. Alexander

Abstract

Essential hypertension (HT) is associated with endothelial dysfunction augmented vasoconstriction (VC) which may be secondary to increased Rho/Rho-Kinase (ROCK)-dependent mechanisms. Our aim was to assess the in vivo magnitude of cutaneous VC to local cooling as a ROCK specific stimulus, and in vitro evaluate ROCK activity in skin from HT humans. Four microdialysis fibers were placed in the forearm of 9 pre- to stage I hypertensive (MAP: 106 ± 3 mmHg) and 11 normotensive (NT; 86 ± 1 mmHg) men and women: Ringers (control), 3 mM fasudil (ROCK inhibited), 5 mM yohimbine + 1 mM propranolol (α - and β -adrenoceptor inhibited; Y + P), Y + P + 3 mM fasudil (ROCK and adrenoceptor inhibited). Skin blood flow was measured during local cooling (Tskl 24°C) and ROCK activity in skin biopsy samples was determined with western blot. In vitro phosphorylated myosin phosphatase target subunit 1 (pMYPT-1)/ROCK was increased in HT skin samples ($p=0.0018$). Functionally, no difference in basal vasomotor tone (Tskl 34°C) was observed between groups (HT: 0.36 ± 0.07 vs. NT: 0.31 ± 0.07 CVC), nor at the control site during local cooling. Pre- to stage I hypertensives show greater ROCK-mediated vasoconstriction at early (1–5 min; HT: -0.8 ± 0.2 versus NT: -0.3 ± 0.2 ΔCVC baseline 1; $P < 0.0001$) and late (36–40 min; HT: -0.9 ± 0.1 versus NT: -0.5 ± 0.2 ΔCVC baseline 1; $P < 0.0001$) phases of local cooling. These data suggest that the magnitude of cutaneous vasoconstriction to local cooling does not differ in normotensive and pre- to stage I essential hypertensive humans; however, ROCK activity is increased and functional vasoconstriction is increasingly dependent upon Rho/ROCK mechanisms with essential hypertension.

Rho-Kinase activity and cutaneous vasoconstriction is upregulated in essential hypertensive humans

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Abstract

Essential hypertension (HT) is associated with endothelial dysfunction augmented vasoconstriction (VC) which may be secondary to increased Rho/Rho-Kinase (ROCK)-dependent mechanisms. Our aim was to assess the *in vivo* magnitude of cutaneous VC to local cooling as a ROCK specific stimulus, and *in vitro* evaluate ROCK activity in skin from HT humans. Four microdialysis fibers were placed in the forearm of 9 pre- to stage I hypertensive (MAP: 106 ± 3 mmHg) and 11 normotensive (NT; 86 ± 1 mmHg) men and women: Ringers (control), 3 mM fasudil (ROCK inhibited), 5 mM yohimbine + 1 mM propranolol (α - and β -adrenoceptor inhibited; Y + P), Y + P + 3 mM fasudil (ROCK and adrenoceptor inhibited). Skin blood flow was measured during local cooling (T_{skl} 24°C) and ROCK activity in skin biopsy samples was determined with western blot. *In vitro* phosphorylated myosin phosphatase target subunit 1 (pMYPT-1)/ROCK was increased in HT skin samples ($p=0.0018$). Functionally, no difference in basal vasomotor tone (T_{skl} 34°C) was observed between groups (HT: 0.36 ± 0.07 vs. NT: 0.31 ± 0.07 CVC), nor at the control site during local cooling. Pre- to stage I hypertensives show greater ROCK-mediated vasoconstriction at early (1–5 min; HT: -0.8 ± 0.2 versus NT: -0.3 ± 0.2 Δ CVC baseline 1; $P < 0.0001$) and late (36–40 min; HT: -0.9 ± 0.1 versus NT: -0.5 ± 0.2 Δ CVC baseline 1; $P < 0.0001$) phases of local cooling. These data suggest that the magnitude of cutaneous vasoconstriction to local cooling does not differ in normotensive and pre- to stage I essential hypertensive humans; however, ROCK activity is increased and functional vasoconstriction is increasingly dependent upon Rho/ROCK mechanisms with essential hypertension.

Keywords

Rho-Kinase; cutaneous vasoconstriction; pre-hypertension; hypertension; microvascular dysfunction; superoxide; nitric oxide

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Disclosures

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Introduction

Abnormal RhoA/Rho Kinase (ROCK) signaling in vascular smooth muscle cells (VSMC) has been implicated in the pathogenesis of cardiovascular disease. In hypertensive animal models ROCK activity is upregulated and contributes to endothelial dysfunction, and vascular remodeling. The pathogenesis of hypertension is multifaceted, involving inflammation, endothelial dysfunction, and upregulation of pro-constrictor pathways. Microvascular dysfunction is evident in essential hypertensive humans (Holowatz and Kenney, 2007c; Serne et al., 2001; Smith et al., 2011) and evolves in parallel in multiple vascular beds (Abularrage et al., 2005; Coulon et al., 2011). In concert with endothelial dysfunction altered ROCK-dependent vasoconstriction (VC) in the cutaneous circulation may precede impairments in conduit vessel function (Cohuet and Struijker-Boudier, 2006; Holowatz and Kenney, 2007a; Holowatz and Kenney, 2007c; Lauer et al., 2005; Levy et al., 2001; Rizzoni and Agabiti-Rosei, 2001; Rizzoni et al., 2003), making the skin a minimally invasive, easily accessible, generalizable vascular bed for assessing *in vivo* vascular function and dysfunction in pre-clinical and cardiovascular disease groups (Abularrage et al., 2005; Holowatz et al., 2008; Rossi et al., 2006; Stewart et al., 2004).

Accumulating evidence supports the involvement of ROCK in hypertension, yet its role in cutaneous microvascular dysfunction has not been elucidated in a hypertensive human model. Animal and *in vitro* studies have demonstrated the upregulation of ROCK by inflammatory stimuli, including superoxide anion ($O_2^{\cdot-}$), acting to increase VSMC contractility via two mechanisms including: 1) a Ca^{2+} independent increase in the translocation of α_2 -adrenoceptors from the Golgi apparatus to the plasma membrane, and 2) inhibition of myosin light chain phosphatase (MLCP) increasing intracellular Ca^{2+} sensitivity (Bailey et al., 2004; Chotani et al., 2000; Jeyaraj et al., 2001). Furthermore, ROCK negatively regulates the nitric oxide (NO) pathway via inhibition of endothelial nitric oxide synthase (eNOS) gene expression, eNOS activation, and NOS uncoupling resulting from $O_2^{\cdot-}$ production (Eto et al., 2001; Ming et al., 2002; Takemoto et al., 2002). Further increases in $O_2^{\cdot-}$ production from uncoupled eNOS and other hypertension-associated elevations in the activity of enzymes NAD(P)H oxidase and xanthine oxidase (Feletou et al., 2009; Tang and Vanhoutte, 2008) lead to a relative vasoconstriction.

Our laboratory has previously demonstrated that locally-mediated VC becomes less adrenergic and more ROCK dependent with advancing age (Thompson-Torgerson et al., 2007b), reflecting a compensatory pathway to preserve the VC response and highlighting the shift towards a 'pre-clinical' pro-constrictor vascular state. More recently, we reported an attenuation of cutaneous eNOS-derived NO-dependent vasodilation in essential hypertensive human skin (Smith et al., 2011), consistent with the ROCK-dependent downregulation of the NO pathway. The purpose of the present study was to examine the role of Rho-Kinase in pre- to stage 1 hypertension-induced cutaneous microvascular dysfunction, using the ROCK-specific stimulus of local skin cooling (Bailey et al., 2004; Thompson-Torgerson et al., 2007a). We hypothesized that *in vivo* augmented cutaneous VC in pre- to early stage essential hypertensive humans results from upregulation of Rho-Kinase during local cooling compared to normotensive age-matched controls. We further hypothesized that *in vitro* Rho-

Kinase expression and activity would be increased in skin samples obtained from pre- to stage 1 essential hypertensive humans.

Materials and Methods

Subjects

Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the *Declaration of Helsinki*. Verbal and written consent were voluntarily obtained from all subjects prior to participation. Nine unmedicated pre- to stage 1 essential hypertensive (50 ± 1 years, 5 men and 4 women) and eleven normotensive age-matched control subjects (51 ± 1 years, 3 men and 8 women) participated in the study. Each subject reported to the laboratory on three separate occasions to determine their blood pressure status in accordance with the guidelines set forth by the American Heart Association (AHA, 2006). Hypertensive status was further assessed using a 24 hour ambulatory blood pressure monitor to obtain values every hour over a 24 hour period (Ambulo 2400, Tiba Medical, Portland OR, USA). Subject classification in the pre- to stage 1 hypertension group was assessed based on American Heart Association guidelines for abnormal ambulatory pressure defined as a sustained pressure greater than 130 mmHg systolic and 80 mmHg diastolic values (Izzo et al., 2008; Pickering et al., 2005). Subjects underwent a complete medical screening, including blood chemistry, complete lipid, renal, and liver enzyme profile evaluation (Quest Diagnostics Nichol Institute, Chantilly, VA), resting electrocardiogram, and physical examination. All subjects were screened for the presence of cardiovascular, dermatological, and neurological disease. Subjects were nonobese, nonsmokers, nondiabetic, normally active (neither sedentary nor highly exercise trained), and were not taking medications including antihypertensives or other drugs that may affect the cardiovascular system, including antioxidants, hormone replacement therapy, or oral contraceptives. Eight out of the nine pre- to stage 1 essential hypertensive subjects had never taken antihypertensive pharmacotherapy and one subject had previously been on antihypertensive drugs but had not taken them for over a year. All women were postmenopausal except three normotensive females.

In Vivo Vasoreactive Studies

Protocols were performed in a thermoneutral laboratory with the subject semi-supine and the experimental arm at heart level (Figure 1). Four intradermal microdialysis fibers (MD 2000, Bioanalytical Systems) (10 mm, 20-kDa cutoff membrane) were inserted into the left ventral forearm skin as previously described (Holowatz and Kenney, 2007b), and separated by at least 4.0 cm to ensure no cross-reactivity of pharmacological agents being delivered. Initial insertion trauma was allowed to subside for 60–120 minutes, during which time lactated Ringer solution was perfused through all fibers at a rate of 2 μ L/min (Bioanalytical Systems Bee hive and Baby Bee microinfusion pumps, West Lafayette, IN).

Skin blood flow was measured using laser-Doppler flowmetry probes (MoorLAB, Moor Instruments, UK) placed over each microdialysis site to provide an index of cutaneous red blood cell flux, which was measured continuously during the experiment. Arterial blood pressure was measured via brachial auscultation every 5 minutes following hyperemia.

Mean arterial pressure (MAP) was calculated as $[1/3 \text{ systolic blood pressure} + (2/3 \text{ diastolic blood pressure})]$. Skin blood flow was expressed as cutaneous vascular conductance (CVC; red blood cell flux/MAP) and expressed as absolute CVC and percent change from baseline CVC values ($\% \Delta \text{CVC}_{\text{base}}$). Local skin temperature (T_{skl}) was controlled at each site by using Peltier elements (Newport Temperature Controller 350B) with a laser Doppler probe secured through the center of each device.

Local cooling protocol—Following hyperemia (90–120 minutes), local skin temperature was clamped at 34°C and laser Doppler probes were attached over each site. ‘Drug-free’ baseline values were recorded for 15 minutes before microdialysis sites were perfused with Ringers to serve as control, 5mM yohimbine + 1 mM propranolol (Y + P) to antagonize α - and β -adrenoceptors (USP, Rockville, MD), 3 mM fasudil to inhibit Rho-Kinase (Tocris, Bioscience, Ellisville, MO), or Y + P + fasudil to simultaneously antagonize adrenoceptors and inhibit Rho-Kinase activity at a rate of 2 $\mu\text{L}/\text{min}$ for 60 minutes. All pharmacological solutions were mixed immediately prior to usage, dissolved in lactated Ringer solution, and sterilized using syringe microfilters (Acrodis4c, Pall, Ann Arbor, MI, USA). The efficacy of the Rho-Kinase specific antagonism and concentrations of the pharmacological agents used in this study have been demonstrated in other studies and using the same intradermal microdialysis technique (Fasudil $K_i = 0.33 \mu\text{M}$) (Lang et al., 2009; Thompson-Torgerson et al., 2007b; Thompson and Kenney, 2004; Uehata et al., 1997).

Following 60 minutes of drug perfusion, and 15 minutes of ‘drug perfusion’ baseline data, 1 μM norepinephrine + 1 mg/ml L-ascorbate (preservative; Sigma-Aldrich, St. Louis, MO) (Hughes and Smith, 1978) were perfused through all sites for 4 minutes to test vascular responsiveness and the integrity of the of the Y + P adrenoceptor antagonism. Following a 30 minute washout period to allow CVC to recover to pre-norepinephrine values, all sites were cooled at a rate of 3°C/min, and T_{skl} was clamped at 24°C for 40 minutes. A representative tracing illustrating the phases of the local cooling response from a normotensive and a hypertensive subjects’ control sites are illustrated in Figure 3. Sites were rewarmed to 34°C before local temperature was increased to 39°C and 28 mM sodium nitroprusside (SNP) was perfused to induce maximal cutaneous vasodilation (CVC_{max}) (Holowatz and Kenney, 2007c; Minson et al., 2002). This temperature was selected due to the large surface area of the peltier devices causing discomfort at higher temperatures, and no further increase in SkBF was elicited above 39°C in combination with 28 mM SNP during pilot testing.

In Vitro Skin Sample Analysis

Ventral forearm skin samples were obtained on a separate day from the *in vivo* functional assessment of vasoreactivity and on the opposite arm. Using sterile technique two 3mm diameter skin samples were obtained. The skin was anesthetized using 2% lidocaine without epinephrine. Samples were rinsed in lactated Ringers and immediately frozen in liquid nitrogen and stored at –80°C until analysis.

ROCK activity and expression—ROCK activity was measured in skin biopsy lysates using the ROCK Activity Immunoblot Kit (Cat# STA-415, Cell Biolabs). Skin samples were

homogenized in RIPA buffer containing protease and phosphatase inhibitors (Kim et al., 2009; Smith et al., 2011). Samples were centrifuged at 12,000 rpm for 10 min. Supernatants were collected and protein concentrations were determined using the BioRad Protein Assay Reagent (BioRad). Equal amounts of protein from each sample (=18 ug) were withdrawn from each sample and the volume was made up to 25 ul with RIPA buffer. Kinase reaction was initiated by adding 50 ul of kinase buffer containing ATP and recombinant MYPT-1 substrate, prepared per vendor's instructions. For some of the samples, a second reaction was initiated with the ROCK inhibitor fasudil added at a final concentration of 50 uM. The reaction was allowed to proceed at 37 C for 60 min with gentle agitation and terminated by the addition of 16 ul of 5× SDS PAGE sample buffer. Samples were then boiled for 5 min and centrifuged at 12,000 rpm for 1 min. 35 ul of each sample were resolved by SDS PAGE and electro-transferred to nitrocellulose. The membranes were blocked for 1 h at room temperature (5% nonfat dry milk, in Tris-buffered saline containing 0.1% Tween-20; TBST). After rinsing, the membrane was cut at MW 75kDa and incubated with a primary antibody to phospho-MYPT1 Thr696 (bottom half, 1:1000, provided in the kit) or ROCK (top half, 1:1000; SantaCruz Biotech). Bound antibody was detected with horseradish peroxidase conjugated secondary antibody (1:1000; from kit for pMYPT; BioRad for ROCK) and visualized using enhanced chemiluminescence. The pMYPT half was then stripped with Restore Plus Western blot Stripping buffer (Thermo), and used to examine GAPDH (1:1000, Novus) abundance to normalize protein loading. Densitometry analysis was performed using ImageJ software (NIH).

Data and Statistical Analysis

Data were digitalized at 40 Hz, recorded and stored for offline analysis using Windaq software and Dataq data acquisition system (Windaq; Dataq Instruments, Akron, OH). Baseline values were determined as the last 5 minutes of each baseline phase. Time course local cooling data are presented as mean values over 5 minute intervals throughout the duration of the cooling protocol. Early and Late phase data are expressed as mean values for 1–5 and 35–40 minutes of cooling, respectively.

Student's unpaired *t*-tests were used to compare physical characteristics between subject groups. CVC data were analyzed using three-way, mixed model, repeated measures ANOVA protocol (group * pharmacological site * local cooling VC phase; proc mix SAS 9.2). Specific planned comparisons were performed when appropriate to determine where differences between groups and pharmacological sites occurred with appropriate Bonferroni correction. The level of significance was set at $\alpha = 0.05$. Values are presented as means \pm SE.

Results

The physical characteristics of the subjects are presented in Table 1. Groups were matched for anthropometric parameters, lipid profile, and total cholesterol. Pre- to stage 1 hypertensive subjects showed a significantly higher resting systolic, diastolic ($p < 0.01$) and mean arterial blood pressure ($p < 0.001$) compared to normotensive age-matched controls. A significant positive correlation for LDL cholesterol and MAP was present within the HT

group ($r = 0.85$, $p < 0.05$), but ceased to be significant when groups were combined ($r = 0.40$, $p = 0.08$).

Figure 2 shows densitometric analysis and representative Western blots of ROCK expression and activity (pMYPT-1) from the skin biopsy samples for both groups. There was no difference in ROCK expression between groups. ROCK activity was significantly increased in skin samples from the HT group, as evidenced by increased phosphorylation of recombinant MYPT-1. Fasudil (50 μ M) inhibited ROCK dependent MYPT-1 phosphorylation in this assay (pMYPT-1 not detected in the presence of Fasudil; data not shown).

Figure 3 illustrates representative skin blood flow tracings during local cooling for a normotensive (A) and essential hypertensive subject (B) with ROCK and adrenergically-mediated vasoconstriction labeled.

Table 2 shows the absolute CVC values prior to pharmacological drug infusion and after localized drug infusion when local skin temperature was clamped at 34°C. There were no differences at the control site or at the adrenoceptor antagonized site between groups. In both pre- to stage 1 hypertensive and normotensive subjects fasudil caused a significant increase in CVC ($p < 0.0001$) but there were no differences between the groups. The combination of fasudil and adrenoceptor antagonism resulted in vasodilation in both groups ($p < 0.0001$), and the vasodilation was greater in the hypertensive group ($p = 0.002$).

Figure 4 illustrates the local cooling response ($\Delta\text{CVC}_{\% \text{baseline}}$) normalized to baseline 3 (drug baseline) at different pharmacological sites in normotensive (A) and pre- to stage 1 hypertensive (B) groups. A greater vasoconstriction was observed in the HT group during minutes 6–25 of local cooling with ROCK and adrenoceptor antagonism. When data are normalized as a percentage of change from baseline after perfusion of site specific pharmacological agents, with exception of a greater vasoconstriction in the HT group at 5–9 minutes of local cooling at the control site (HT: -57 ± 5 versus NT: -45 ± 12 $\Delta\text{CVC}_{\% \text{baseline}}$; $p = 0.003$), no further differences were observed between groups at control, ROCK-inhibited, or adrenoceptor antagonized sites.

Figure 5 shows absolute cutaneous vascular conductance (flux/MAP) calculated ROCK-dependent (control – fasudil site) and adrenergically-mediated (control – Y + P site) vasoconstriction in both groups during early (1–5 min) and late (36–40 min) phases of local cooling ($\Delta\text{CVC}_{\text{baseline}}$). In absolute terms during early phase local cooling pre- to stage 1 hypertensive subjects showed greater ROCK-dependent (HT: -0.8 ± 0.2 versus NT: -0.3 ± 0.2 $\Delta\text{CVC}_{\text{baseline}}$; $P < 0.0001$) and adrenergic vasoconstriction (HT: -0.2 ± 0.1 versus NT: 0.04 ± 0.10 $\Delta\text{CVC}_{\text{baseline}}$; $P < 0.0001$). During late phase local cooling the HT group continued to display greater ROCK-dependent (HT: -0.9 ± 0.1 versus NT: -0.5 ± 0.2 $\Delta\text{CVC}_{\text{baseline}}$; $P < 0.0001$) and adrenergic vasoconstriction (HT: -0.2 ± 0.1 versus NT: 0.1 ± 0.1 $\Delta\text{CVC}_{\text{baseline}}$; $P < 0.0001$) compared to normotensives.

Finally, there was no effect of localized microdialysis treatment within each group, however, absolute maximal CVC was attenuated in the pre- to stage 1 hypertensive group (HT: 1.46 ± 0.21 versus NT: 1.78 ± 0.19 CVC, $p = 0.025$).

Discussion

The major new findings of the present study were that 1) ROCK activity but not ROCK expression was increased in pre- to stage 1 essential hypertensive compared to age-matched normotensive skin, 2) at thermoneutral baseline the combination of ROCK and adrenoreceptor antagonism results in greater vasodilation in the pre- to stage 1 hypertensive compared to the normotensive group, and 3) absolute ROCK and adrenergic-dependent vasoconstriction were greater during early and late phases of local cooling in the pre- to stage 1 hypertensive compared to normotensive group. Together these *in vitro* molecular and *in vivo* functional findings are the first to suggest that upregulation of ROCK-dependent mechanisms contributes to cutaneous microvascular dysfunction in pre- to stage I essential hypertensive humans. Further these data suggest that there is an interaction between the ROCK and adrenergic pathways contributing to basal vasomotor tone in the cutaneous vasculature of humans with pre- and early stage essential hypertension.

In vitro analysis of the skin biopsies showed that pMYPT/ROCK, a surrogate for ROCK activity, was increased in samples obtained from the hypertensive compared to normotensive subjects; however, ROCK expression did not differ between groups. This finding supports the functional *in vivo* data of augmented ROCK-dependent vasoconstriction during early and late phases of local cooling in the pre- to stage 1 hypertensive compared to normotensive subjects. Taken together, these data suggest that locally-mediated cutaneous vasoconstriction becomes increasingly ROCK-dependent in essential hypertensive skin, likely resulting from a post-translational modification upregulating ROCK activity. The early stage of disease progression in these subjects may explain the increase in ROCK activity but not expression, with animal and *in vitro* data supporting upregulation of ROCK activity by inflammatory stimuli in pathophysiological states, including superoxide anion, endothelin-1, (Anggrahini et al., 2009), thromboxane and angiotension II (Seko et al., 2003). Furthermore, the mutual inhibition of NO-dependent vasodilation and RhoA/ROCK-dependent vasoconstriction pathways, contributes to the upregulation of ROCK activity in the absence of ROCK expression via post-translation modification (Eto et al., 2001; Ming et al., 2002; Takemoto et al., 2002). *In vitro* animal models have similarly shown an increase in RhoA and ROCK activity with no difference in ROCK expression between normotensive versus hypertensive (Ang II, salt-sensitive, stroke-prone and renal hypertensive models) rats (Seko et al., 2003), suggesting changes in ROCK expression may potentially occur later in disease progression.

During thermoneutral baseline measurements when local skin temperature was clamped at 34°C there were no differences between the groups in absolute CVC (Table 2) at the control site or when ROCK or adrenoreceptors were independently antagonized. This is in contrast to data from both animal and human conduit vessel studies which show increased basal ROCK and adrenergic-dependent VSMC hypercontractility with hypertension (Masumoto et al., 2001). Because factors known to stimulate ROCK include reactive oxygen species (ROS) and a number of inflammatory stimuli, the early stage of disease progression in our subjects may explain the similarity in cutaneous basal tone observed between groups, and the lack of a difference in baseline vasomotor tone after independent fasudil or adrenoreceptor antagonism. However, with dual ROCK and adrenoreceptor antagonism the

hypertensive group vasodilated significantly more than the normotensive group. These data suggest that at this pre- to early stage of hypertension there is an interaction between adrenoceptors and ROCK contributing to basal vascular tone.

During our physiological stimulus to induce adrenergic and ROCK-dependent vasoconstriction both early and late phase ROCK and adrenergically-mediated VC were augmented in the pre- to stage I hypertensive compared to normotensive group. These data support the present *in vitro* evidence of increased ROCK activity in the hypertensive subjects and are in agreement with animal models which identify the involvement of augmented ROCK activity in hypertension (Mukai et al., 2001). Work from our laboratory has previously demonstrated no difference in the pattern or magnitude of local cold-induced VC in aged compared to young skin, however, there was a shift in the underlying mechanisms towards a greater reliance on ROCK-dependent pathways (Thompson-Torgerson et al., 2007b). This compensatory mechanism acts to maintain the magnitude of VC with aging due to the well characterized attenuation of adrenergically-mediated mechanisms resulting from receptor desensitization (Thompson et al., 2005). In contrast to what is observed in primary aged skin, both ROCK and adrenergic VC are upregulated in pre- to stage I hypertensives compared to age-matched normotensive subjects in the present study. The current cooling and baseline data also suggest an interaction between the two pathways via ROCK-mediated α_2c -adrenoceptor translocation increasing the receptor availability on the VSMC surface for norepinephrine binding.

In the present study we examined adrenergic and ROCK-dependent mechanisms in response to local cooling. ROCK and endothelium-dependent mechanisms, specifically eNOS, are mutually inhibitory (Takemoto et al., 2002). In a similar cohort of human subjects we have recently demonstrated that essential hypertensives display an attenuated eNOS-dependent vasodilation to local heating. In combination with *in vitro* analysis of skin biopsies, we showed that iNOS-dependent mechanisms were upregulated and limited eNOS-dependent vasodilation. One potential mechanism underlying these observations is through an iNOS-mediated upregulation of arginase activity thereby limiting NO synthesis through eNOS (Smith et al., 2011). In animal models and cell culture, ROCK increases arginase activity, inhibiting eNOS gene expression, eNOS activation, and induces NOS uncoupling resulting in further superoxide production (Takemoto et al., 2002). Taken together the human and animal data strongly suggest that ROCK-dependent mechanisms downregulate the eNOS pathway and contribute to endothelial/microvascular dysfunction in essential hypertension.

In the present study we examined a potential mechanism underlying microvascular function using the cutaneous circulation as our model. Using physiologically relevant stimuli our results are consistent with findings from other circulations (Masumoto et al., 2001) and demonstrate a globalized vascular dysfunction. In essential hypertensive vascular pathology changes indicative of end organ damage in the cutaneous circulation occur in parallel with those in the renal vascular bed (Coulon et al., 2011). In the present study, while the magnitude of absolute basal and local cold-induced vasoconstriction were similar between groups, *in vitro* molecular data clearly showed increased ROCK activity. With more advanced stages of vascular pathology ROCK expression is increased via inflammatory stimuli including angiotensin II, IL-1 β , and remnant lipoproteins (Hiroki et al., 2004; Oi et

al., 2004). These inflammatory pathways and their involvement with augmented ROCK activity have yet to be explored in essential hypertensive humans.

Finally, in this cohort of pre-to stage I hypertensives we did find a significant difference in absolute maximal cutaneous vascular conductance. We and others have demonstrated this using similar techniques in unmedicated essential hypertensive humans (Holowatz and Kenney, 2007a; Holowatz and Kenney, 2007c). A reduction of maximal cutaneous vascular conductance has been suggested to be the most appropriate way to assess the degree of inward vessel remodeling suggestive of more significant and long term microvascular dysfunction. In the present study, maximal cutaneous vascular conductance was reduced, indicating that vascular smooth muscle cell remodeling has occurred despite the relatively young age and pre- to stage I hypertensive status of our cohort. Moreover, these data support the notion of the disease progression with a relative endothelial dysfunction occurring in parallel with vascular smooth muscle hypertrophy and inward vessel remodeling.

Limitations

Fasudil is the only ROCK inhibitor currently available for use in humans. Fasudil causes considerable vasodilation upon administration raising the need for data normalization and careful interpretation of the data. Because of the significant vasodilation in response to fasudil when data are presented as a relative change from baseline there were no differences between the groups. However, as a change in absolute CVC there were differences between the groups for both adrenergic and ROCK-dependent vasoconstriction. In the present study a 'drug free' baseline was obtained prior to establishing a secondary drug perfusion baseline. Cutaneous vasodilation to fasudil in both groups was considered and data are presented in accordance with the results of the *in vitro* molecular data.

Conclusions

Hypertension-associated vascular dysfunction is a complex, multifaceted condition which occurs simultaneously in multiple vascular beds. Augmented ROCK activity and ROCK-mediated vasoconstriction in the cutaneous circulation may precede alterations in conduit vessels, making the skin an easily accessible, generalizable vascular bed for assessing *in vivo* vascular function and dysfunction in pre-clinical and cardiovascular disease groups (Abularrage et al., 2005; Holowatz et al., 2008; Rossi et al., 2006; Stewart et al., 2004). The present data are the first to indicate a role for ROCK in cutaneous microvascular dysfunction in pre- to stage 1 essential hypertension. Because the magnitude of VC to local cooling did not differ between groups, coupled with a greater ROCK activity, and attenuated NO-dependent vasodilation reported from our previous studies, detectable endothelial dysfunction may either precede or occur simultaneously with VSMC dysfunction in the disease progression of essential hypertension. Alterations in ROCK expression and an increase in the magnitude of locally-mediated VC may occur later in the disease progression. Considering the pre- to stage 1 hypertensive status of the present subjects, these data are important in understanding early disease progression (Jablonski et al., 2012), in which early changes in the ROCK signaling pathway occurs in advance of decrements in vascular function. The higher cardiovascular disease risk associated with 'pre-hypertension'

(120–129 mmHg), makes this particularly important for early detection and identifying preventative strategies (Kokubo et al., 2008; Mancia, 2007; Vasani et al., 2001).

In summary, ROCK activity was increased in skin samples from pre- to stage I essential hypertensive humans. Moreover both ROCK and adrenergically-mediated vasoconstriction were upregulated in the hypertensive group. Taken together these findings suggest that ROCK contributes to cutaneous microvascular dysfunction in essential hypertensive humans and may be a potential molecular target in the treatment of hypertension.

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Highlights

- ROCK and adrenergically-mediated VC were examined in hypertensive human cutaneous circulation.
- ROCK and adrenergic VC were upregulated in HT vs. NT despite a similar magnitude of VC.
- ROCK activity but not expression was increased in skin samples from hypertensives.
- ROCK contributes to cutaneous microvascular dysfunction in early stage essential hypertension.

Fiber placement	Hyperemia	BL 1 (34°C)	Drug perfusion	BL 2 (34°C)	NE	Wash out	BL 3 (34°C)	Local cooling 24°C	Re-warm (34°C)	SNP + local heat (39°C)
20 min	60 min	10 min	60 min	10 min	4 min	30 min	10 min	40 min	10 min	20 min

Figure 1. Schematic of the local cooling protocol. Drug-free baseline (BL1), drug baseline (BL2), post norepinephrine (NE) baseline (BL3).

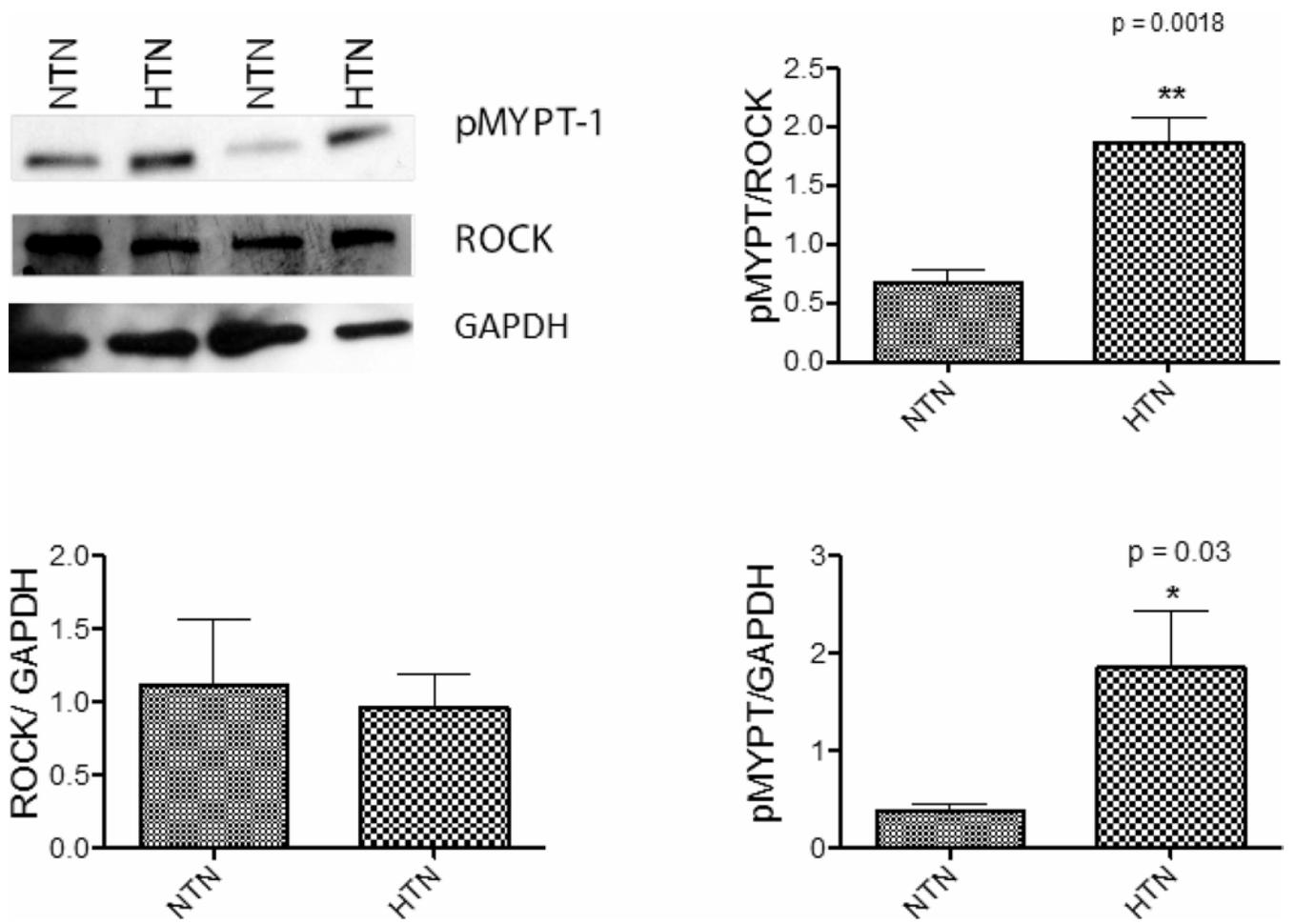


Figure 2. Rho-Kinase (ROCK) and pMYPT-1. ROCK expression and activity (MYPT-1 phosphorylation) were determined by Western blotting. Sample blots are shown in top left panel. Densitometry analysis was performed using ImageJ software (NIH). * $p < 0.05$, ** $P < 0.01$ difference from the normotensive group.

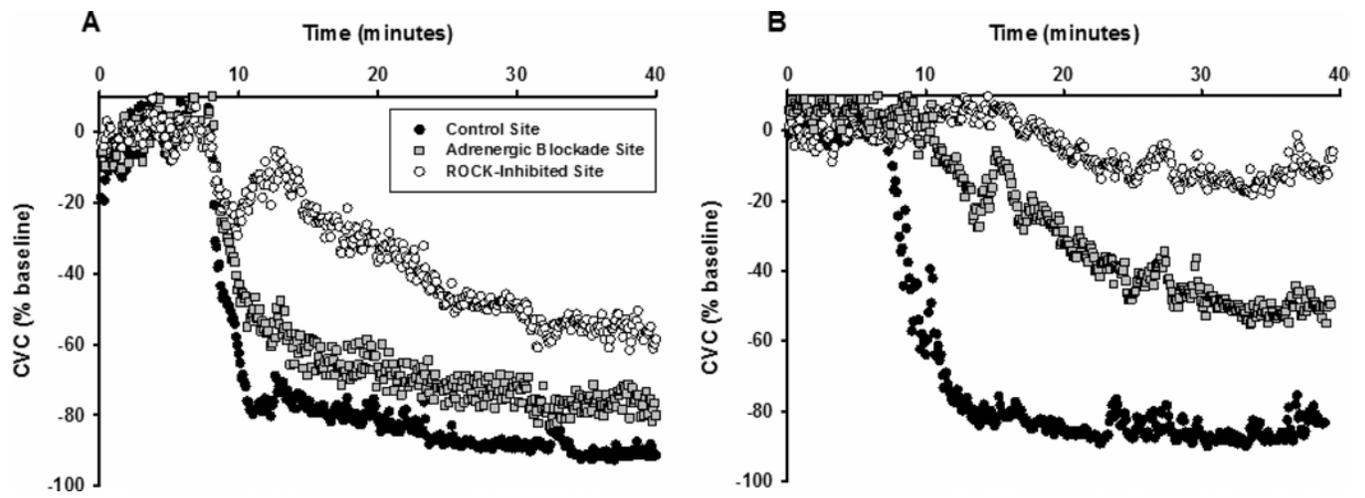


Figure 3. Representative skin blood flow tracings during local cooling for a normotensive (A) and essential hypertensive (B) subject. Adrenergic and ROCK-mediated vasoconstriction are labeled.

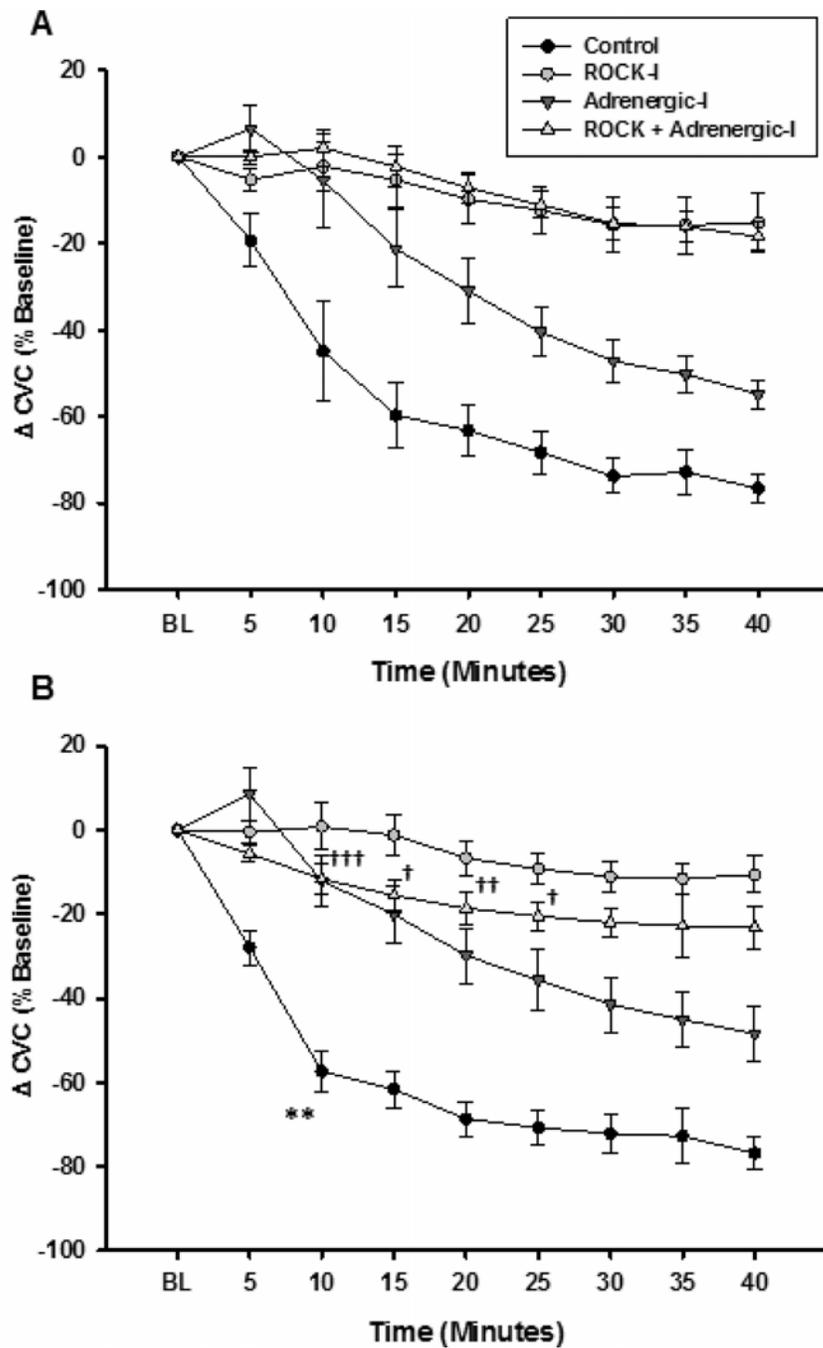


Figure 4. Cutaneous vascular conductance (Δ CVC %baseline 3)- in (A) normotensive controls and (B) essential hypertensive subjects at baseline (BL; during drug perfusion), and during a local cooling protocol to 24°C at the control site, ROCK inhibited, adrenoceptor inhibited, and ROCK + adrenoceptor inhibited. Values significantly different from normotensive group: Control site ** $p < 0.001$; ROCK + Adrenergic Inhibited site † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$.

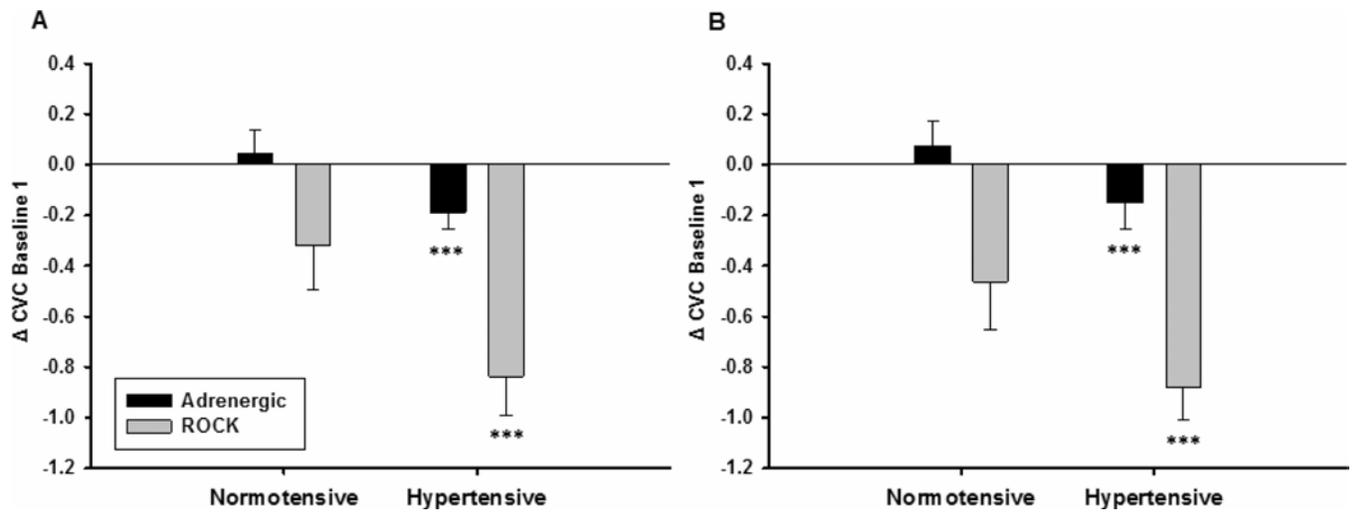


Figure 5. Cutaneous vascular conductance (Δ_{BL1}) in normotensive controls and essential hypertensive subjects for ROCK-mediated (control – fasudil site) and adrenergically-mediated (control – Y + P site) vasoconstriction during (A) early phase local cooling (1–5 minutes), and (B) late phase local cooling (35–40 minutes) Values significantly different from normotensive group: *** $p < 0.001$.

Table 1

Participant characteristics (mean \pm SE). Blood pressure values represent a mean of the initial 5 readings during the experiment.

	Normotensive	Pre/ stage 1 Hypertensive
Subjects (male, female)	(3, 8)	(5, 4)
Age (years)	51 \pm 1	50 \pm 1
BMI	25 \pm 0	27 \pm 0
Systolic BP (mmHg)	114 \pm 1	136 \pm 1 **
Diastolic BP (mmHg)	72 \pm 1	88 \pm 1 **
MAP (mmHg)	86 \pm 1	106 \pm 3 ***
Glucose (mg·dL ⁻¹)	91 \pm 1	96 \pm 1
HDL (mg·dL ⁻¹)	52 \pm 1	57 \pm 2
LDL (mg·dL ⁻¹)	108 \pm 2	112 \pm 2

**

P<0.01,

P<0.001 difference from the normotensive age match control group.

Table 2

Cutaneous vascular conductance (CVC) at all microdialysis sites after 40 minutes of perfusion with site specific antagonists when local temperature was clamped at 34°C in normotensive and hypertensive subjects.

Site	Baseline (CVC)	
	NT	HT
Control	0.43 ± 0.07	0.36 ± 0.07
ROCK-inhibited	1.11 ± 0.17 [†]	1.09 ± 0.21 [†]
Adrenoceptor-inhibited	0.47 ± 0.07	0.40 ± 0.05
ROCK + Adrenoceptor-inhibited	0.99 ± 0.12 [†]	1.31 ± 0.23 ^{†*}

*
p=0.002 versus the normotensive ROCK + Adrenoceptor-inhibited site

[†]
p<0.0001 versus the control site