

Rho kinase activation and ROS production contributes to the cooling enhanced contraction in cutaneous equine digital veins

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Zerpa H, Berhane Y, Woodcock H, Elliott J, Bailey SR. Rho kinase activation and ROS production contributes to the cooling enhanced contraction in cutaneous equine digital veins. *J Appl Physiol* 109: 11–18, 2010. First published April 1, 2010; doi:10.1152/jappphysiol.01301.2009.—A decrease in environmental temperature can directly affect the contractility of cutaneous vasculature, mediated in part by α_2 -adrenoceptors. Most of the cellular mechanisms underlying the cooling-enhanced contractility to α_2 -adrenoceptor agonists have been reported in cutaneous arteries but little information is available on cutaneous veins. To investigate the cellular mechanisms associated with the cooling-enhanced contraction to UK-14304 (α_2 -adrenoceptor agonist), isolated equine digital veins (EDVs) were studied at 30°C and 22°C. The effects of inhibitors were studied on the contractile response to UK-14304 (0.1 μ M). The cooling-enhanced responses were inhibited by Rho kinase inhibitors [maximum response to UK-14304 $95.2 \pm 8\%$ of response to depolarizing Krebs solution (DKS) in control vessels cooled to 22°C, compared with $31.4 \pm 6\%$ in the presence of fasudil 1 μ M and $75.8 \pm 6\%$ with Y-27632 0.1 μ M] and the effects of these inhibitors were considerably less at 30°C (control response $56.4 \pm 5\%$ of DKS; $34.9 \pm 6\%$ with fasudil 1 μ M and $50.6 \pm 9\%$ with Y-27632 0.1 μ M). Furthermore, Western blotting showed that one of the downstream targets for Rho kinase activity, ezrin/radixin/moesin, was phosphorylated after cooling and reduced by fasudil (1 μ M) only at 22°C. The activation of protein kinase C contributed to the contractile response, but predominantly at 30°C (maximum response $82.3 \pm 9\%$ of DKS for control; $57.7 \pm 10\%$ in the presence of chelerythrine 10 μ M) with no significant effect at 22°C. The reduction of the response at 22°C by antioxidants, rotenone (14% reduction), and tempol (21% reduction) suggested the contribution of reactive oxygen species (ROS). No evidence was obtained to support the participation of tyrosine kinase. These data demonstrate that Rho kinase activation and the production of ROS contributes to the cooling-enhanced contraction in these cutaneous digital veins.

cooling; α_2 -adrenoceptor; thermoregulation; reactive oxygen species

EQUINE LAMINITIS is a condition of the equine digit that compromises the integrity of the anatomical relationship between the third phalanx and the hoof and may be linked to peripheral vasoconstriction and ischaemia. It has been proposed that increased postcapillary resistance, due to venoconstriction, could be one of the pathophysiological mechanisms involved (6). This condition in the horse has been compared with Raynaud's phenomenon in humans, a similar condition that is characterized by digital ischemia-reperfusion injury, linked to a cooling induced enhancement of α_2 -adrenoceptor-mediated vasoconstriction (16, 23).

Redistribution of cutaneous blood from superficial to deeper blood vessels, including veins, contributes to heat conservation

on cooling, particularly in body appendages. These thermoregulatory mechanisms involve both an integrated autonomic response and the direct effect of temperature on the contractility of cutaneous vessels. Moderate cooling causes a selective potentiation of the contractile response evoked by agonists of 5-HT receptors and α_2 -adrenoceptors on the venous side of the equine digital circulation. Whereas cooling enhanced the responses to particular G protein receptor agonists, it did not modify the response mediated by α_1 -adrenoceptors or a depolarizing stimulus in the equine digital vein. These findings suggest that cooling might affect the response mediated by a specific group of receptors and their cellular downstream signal, but not the responses of other receptors or pathways (47).

Cooling-enhanced responses to UK-14304 (an α_2 -adrenoceptor agonist) in the mouse tail artery have been reported to be specifically mediated by the α_{2C} -adrenoceptor subtype, which may translocate from the Golgi compartment to the extracellular membrane. Such a mechanism involves the cooling-induced production of reactive oxygen species (ROS) and the activation of the Rho/Rho kinase pathway (4, 8, 12). The activation of Rho kinase promotes calcium sensitization of the vascular smooth muscle by inactivating the myosin light chain (MLC) phosphatase and maintaining the MLC phosphorylation state (40). Recently, it was shown that cold-induced cutaneous vasoconstriction *in vivo* was blocked by selective inhibitors of Rho kinase, confirming the participation of this pathway in the thermoregulatory response to cooling in mice and humans (22, 43).

Cooling activation of Rho kinase has also been found in the gastric smooth muscle; however, cooling-induced contraction was also inhibited by an inhibitor of tyrosine kinase, genistein (34). Similar results were reported in dermal arterioles from human patients with Raynaud's disease, where again genistein inhibited cooling-induced contraction and cooling-enhanced response to UK-14304 and 5-HT (17, 18). It is possible that a moderate reduction of temperature can activate both the Rho and/or tyrosine kinase pathways in the vascular smooth muscle and might be involved in the cooling-enhanced effect observed in the cutaneous equine digital vasculature.

While the cellular mechanisms mediating the regulatory effect of low temperature on the vasculature contractility by activation of α_2 -adrenoceptors has been extensively studied in arterial smooth muscle, the available information regarding the underlying mechanisms in cutaneous veins remains limited. Therefore, the aims of this study were to determine 1) whether cooling enhances the activity of Rho kinase in rings of equine digital veins, 2) the role of the Rho/Rho kinase pathway in the cooling-enhanced functional response to UK-14304 using selective Rho kinase inhibitors, and 3) the role of protein kinase C (PKC), tyrosine kinase, and the production of ROS in the

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cooling-enhanced response to UK-14304. Part of these results have been presented in abstract form elsewhere (48).

MATERIALS AND METHODS

Animals and tissues. Equine digital veins were collected in a local abattoir. The hindlimbs of healthy mixed breed adult horses of either sex were removed within 10 min of death. The digital artery was cannulated and 120 ml of ice-cold modified Krebs Henseleit (Krebs solution; composition in mM: 1.27 CaCl₂, 1.19 MgSO₄, 25.0 NaHCO₃, 118 NaCl, 1.19 KH₂PO₄, 4.57 KCl, 5.55 glucose) was infused through the catheter. The skin was then reflected from above the coronet band to reveal the digital coronet venous plexus. The digital coronet venous plexus was dissected and removed, placed in ice-cold Krebs solution, and transported to the laboratory. The veins were carefully cleared of connective tissue and cut into rings of 3–4 mm in length. Vessel rings that were not used immediately were stored in oxygenated Krebs solution at 4°C overnight.

Isometric tension recording in isolated equine digital vein. All the experiments were conducted in endothelium-denuded vessels, achieved by gently rubbing the luminal surface with a wooden cocktail stick (15). Rings of equine digital veins were suspended between two parallel stainless steel wires contained within a jacketed organ bath and bathed in modified Krebs solution that was aerated with 95% O₂ and 5% CO₂. One of the wires was fixed and the other connected to an isometric force transducer (HSE force transducer, Type K30; Linton Instrumentation, Diss, Norfolk, UK). The output of the force transducer was fed via an amplifier (HSE type 301, Linton Instrumentation) to a data acquisition system (Power Lab, ADI Instruments, Oxfordshire, UK). Twelve vessel rings could be examined simultaneously within the course of any one experiment. Equine digital vein rings were stretched to 2-g tension, and an equilibration period of 1 h was allowed prior to the administration of the selected agent. The Krebs solution was changed every 15–30 min during the equilibration period.

Pairs of equine digital veins rings from the same horse were studied simultaneously at two temperatures, 30°C and 22°C, established during the equilibration period (24). Thirty degrees centigrade represents a normal physiological temperature in this particular vascular bed (in a thermoneutral environment) and has been used in previous *in vitro* studies (rather than 37°C typically used for studying the function of blood vessels from the body core). The cutaneous temperature of the distal portion of the horse limb is considerably lower than the core temperature in a thermoneutral environment (7). The temperature of the organ bath was confirmed in preliminary experiments using a Thermistor Thermometer (Cole-Palmer Instrument, London, UK).

The viability of each vessel segment was tested initially by exchanging the Krebs solution for one in which the sodium chloride had been replaced with potassium chloride to produce a depolarizing Krebs solution (DKS; 118 mM KCl). Once the peak tension was obtained, the tissues were washed with Krebs solution and the active tension returned to baseline. Vessels that produced less than half of their resting tension were considered nonviable and discarded. The peak response to DKS in each vessel was also used to normalize the contractile response of the vessels to the agonist at both temperatures, thus eliminating any small direct effect of temperature on smooth muscle contractility as has been reported elsewhere (19). The DKS-induced contraction is predominantly mediated by a direct depolarization of the smooth muscle plasma membrane and is considered a valuable approach to normalize receptor-mediated responses in vascular preparations (31). At the end of each experiment, vessels were dried and weighed to normalize the DKS peak height response against the weight of the venous ring.

Ezrin/radixin/moesin immunoblotting. The phosphorylation of ezrin/radixin/moesin (ERM) family proteins has been extensively used as an indicator of the activity of Rho kinase in cardiovascular

research (20, 32, 42). Therefore, the phosphorylated and total levels of the Rho kinase substrate ERM were determined in equine digital vein homogenates treated at different temperatures. Equine digital vein rings were submitted to isometric tension recording as described above. After 1 h under a basal tension of 2 g at 30°C or 22°C, rings were removed from the chambers and snap-frozen in liquid nitrogen. Rings obtained from two to four different animals were pooled during homogenization using a pestle and mortar on liquid nitrogen. The homogenized material was recovered and submitted to further homogenization using a 2-ml glass tissue grinder and cold lysis buffer [63.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 1 mM Na₃VO₄, 1 mM 4-(2 aminoethyl) benzenesulfonyl fluoride, HCl (AEBSF), 50 µg/ml leupeptin]. The homogenate was recovered and centrifuged at 13,000 g for 25 s. An aliquot was taken for protein determination by a commercial kit (BCA, Pierce) and 5% β-mercaptoethanol and 0.02% bromphenol blue were added to the homogenates and heated at 100°C for 5 min. Equal amounts of homogenates (20–40 µg) from samples treated at 30°C or 22°C were separated on 10% SDS-PAGE gels. Samples from each temperature were run in pairs using different gels under the same experimental conditions and then transferred onto polyvinylidene fluoride microporous membranes (Millipore) by Western blotting. Membranes were blocked for 2 h in a blocking solution (5% powdered milk in Tris-buffered saline containing 0.1% Tween 20) followed by an overnight incubation at 4°C with polyclonal antibodies against total ERM (rabbit anti-human ERM; cell signaling technology; diluted 1:1,000) or phosphorylated-ERM (rabbit anti-human phospho-ezrin[Thr567]/radixin [Thr564]/moesin [hr558] cell signaling technology; diluted 1:1,000) in different blots, according to the manufacturer's instructions. After washing with Tris-buffered saline containing 0.1% Tween 20, bound antibodies were detected by a secondary antibody (affinity purified goat anti-rabbit IgG; cell signaling technology; diluted 1:1,000) conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence. Subsequently, densitometry was performed using computer imaging software ImageJ 1.34j (National Institutes of Health).

Effect of Rho kinase inhibitors in the augmented response to cooling mediated by UK-14304. The response to UK-14304 was evaluated in equine digital veins submitted to isometric contraction studies at 30°C and 22°C under control conditions or after incubation for 30 min with selective inhibitors of Rho kinase: fasudil (1–10 µM) and Y-27632 (0.1–1 µM) (4). Subsequently, the response to UK-14304 was estimated by using the peak height contraction evoked by a submaximal fixed concentration of UK-14034 (0.1 µM). This concentration was chosen based on those results obtained in concentration response curves to UK-14304 in this preparation (47) where 0.1 µM induces a submaximal contraction for the first phase of the concentration response curve, which is principally mediated by α₂-adrenoceptors. Therefore, at this concentration, the effect of the inhibitors was studied on the UK-14304 response mediated by α₂-adrenoceptors and the influence of the activation of α₁-adrenoceptors was minimized. Vessels treated with fasudil (1 µM), were snap-frozen in liquid nitrogen immediately after the peak height contraction was reached and were used to estimate the activity of Rho kinase by the phosphorylation of its target proteins, ERM (as above).

Functional role of protein kinase C, tyrosine kinase, and ROS in the augmented response to cooling. Experiments were conducted as described above. Vessels were incubated for 30 min with 1) a specific protein kinase C (PKC) inhibitor, chelerythrine (1–10 µM); 2) the nonspecific tyrosine kinase inhibitor, genistein (10 µM), or its inactive analog daidzein (10 µM) (38); a selective inhibitor of Src tyrosine kinases, PP2 (10 µM), or its inactive analog, PP3 (10 µM) (38). Additionally, the mitochondrial complex I inhibitor rotenone (10 µM), the antioxidant, *N*-acetylcysteine (NAC; 20 mM) and the cell-permeable superoxide dismutase mimetic, tempol (1 mM), were also evaluated.

Statistical analysis of data. The peak height contraction elicited by 0.1 µM of UK-14304 was compared in the absence or presence of the

inhibitors at each temperature. The ratios between phosphorylated ERM and total ERM were estimated at each temperature without the influence of any treatment or after stimulation with UK-14304 in control conditions or in the presence of fasudil 1 μM . Values were expressed as the arithmetic mean \pm SE. Statistical comparisons were performed using paired or unpaired *t*-test as appropriate and significance was accepted at $P \leq 0.05$. Multiple comparisons were performed by one-way ANOVA with Dunnett's post hoc test.

Drugs and solutions. Fasudil hydrochloride and UK-14304 [5-bromo-6-(2-imidazolin-2-ylamino) quinoxaline] were purchased from Tocris Cookson, Avonmouth, UK. AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride, HCl], chelerythrine chloride [1,2-dimethoxy-*N*-methyl(1,3)benzodioxolo(5,6-*c*)phenanthridinium chloride], daidzein [4',7-dihydroxyisoflavone, 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 7-hydroxy-3-(4-hydroxyphenyl) chromone], genistein [4',5,7-trihydroxyisoflavone, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], leupeptin hydrochloride, *N*-acetylcysteine, sodium orthovanadate (Na_3VO_4), rotenone, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl), Tris-HCl, Y-27632 dihydrochloride monohydrate [(R)-(+)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate] were obtained from Sigma Chemical, Poole, Dorset, UK. PP3 [4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine] and PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine] were purchased from Calbiochem. All drugs used in functional studies were freshly prepared on the day of the experiment. Unless otherwise stated, drugs were dissolved in distilled water and Krebs solution. UK-14304, chelerythrine, genistein, daidzein, PP2, PP3, and rotenone were diluted initially in DMSO. Appropriate vehicles were added to the control preparation and did not display a contractile effect on the preparation.

RESULTS

ERM immunoblotting. A representative example of the bands corresponding to phosphorylated and total forms of the Rho kinase substrate ERM is presented in Fig. 1. The phospho-to-total ERM ratio was estimated in tissues incubated under a basal tension of 2 g for 1 h at 30°C or 22°C. The phosphorylation of ERM was significantly ($P < 0.05$) greater at 22°C (ratio 1.6 ± 0.2 ; $n = 5$) vs. 30°C (1.3 ± 0.1 ; $n = 5$).

Effect of Rho kinase inhibitors in the augmented response to cooling mediated by UK-14304 in equine digital veins. The submaximal concentration (0.1 μM) of UK-14304 used in this group of experiments caused greater contractions at 22°C compared with 30°C in most of the venous preparations (71% of the 75 evaluated preparations). Incubation with 1 μM fasudil significantly ($P < 0.05$) inhibited the contraction elicited by 0.1 μM of UK-14304 at both temperatures (Fig. 2). However, the magnitude of the inhibitory effect was 1.8 times greater at 22°C. The inhibitory effect of fasudil was concentration dependent, since a higher concentration (10 μM) almost abolished the contraction at both temperatures. A similar pattern of inhibition was observed with the structurally unrelated selective Rho kinase inhibitor, Y-27632, which selectively inhibited the UK-14304 contraction at 22°C at the lowest concentration (0.1 μM) but abrogated the response at either temperature when the concentration was increased to 1 μM (Fig. 3). The effect of 1 μM fasudil at 22°C was consistent with an inhibition of Rho kinase activity, as suggested by the significant ($P = 0.01$) reduction in the phosphorylation of ERM at this temperature (control: 1.5 ± 0.1 vs. fasudil 1 μM : 1.1 ± 0.2 ; $n = 5$). A similar reduction was evident at 30°C (control: 1.8 ± 0.4 vs. fasudil 1 μM : 1.2 ± 0.3 ; $n = 5$) but this did not reach statistical significance ($P = 0.15$). It is worth

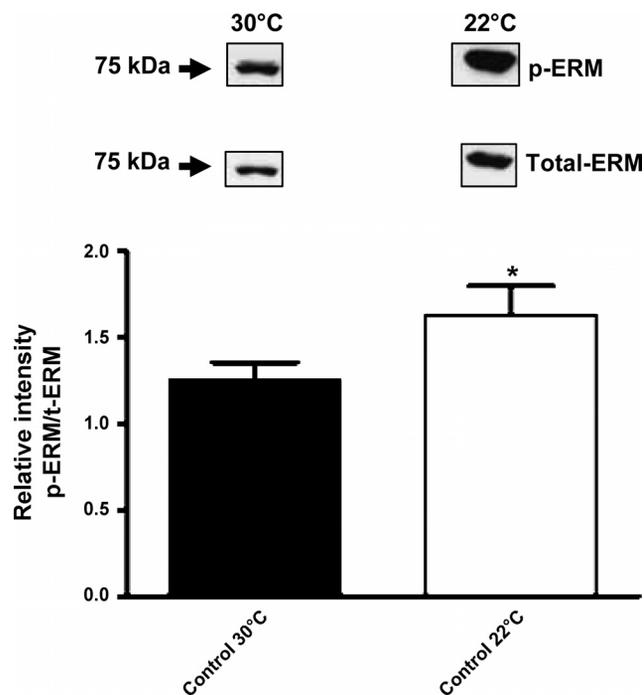


Fig. 1. Effect of temperature on the phosphorylation of the Rho kinase target family proteins ezrin/radixin/moesin (ERM) obtained from segments of equine digital veins (EDVs). Segments of EDVs were incubated for 1 h under 2 g of basal tension at 30°C or 22°C. Pooled tissue homogenates from 3 to 4 animals were separated by electrophoresis and the presence of phosphorylated and total ERM was determined by immunoblotting. The ratio between both forms was estimated by densitometry. Each point represents the arithmetic mean value \pm SE from densitometry analysis of 5 different pools of EDVs from 3–4 different animals in each. * $P \leq 0.05$ between temperatures by a paired Student's *t*-test.

noting that while the mean of the phospho-to-total ERM ratio in the presence of UK-14304 (α_2 -adrenoceptor agonist: 0.1 μM) was similar to the control without agonist at 22°C, it was ~ 1.3 times higher at 30°C (data not shown).

Functional role of protein kinase C, tyrosine kinase, and ROS in the augmented response to cooling mediated by UK-14304. The specific PKC inhibitor chelerythrine did not significantly modify UK-14304 (0.1 μM) responses at either temperature when used at a concentration of 1 μM . However, when the concentration was increased to 10 μM , there was a significant ($P < 0.05$) reduction ($\sim 30\%$) of the peak height contraction at only 30°C (Fig. 4).

The nonspecific tyrosine kinase inhibitor genistein (10 μM) and its inactive analog daidzein (10 μM) selectively inhibited ($P < 0.05$) by $\sim 46\%$ and 29%, respectively, the contraction caused by UK-14304 (0.1 μM) at 22°C with no significant effect at 30°C (Fig. 5). In contrast, PP2, a selective inhibitor of the Src family of tyrosine kinases and its inactive analog, PP3, did not significantly ($P < 0.05$) affect the response to UK-14304 at either temperature (Fig. 5).

Rotenone (10 μM) selectively ($P < 0.05$) reduced (by $\sim 14\%$) the contraction evoked by UK-14304 (0.1 μM) only at 22°C. Similarly, tempol (1 mM) also caused a significant reduction ($\sim 21\%$ inhibition; $P < 0.05$) of the UK-14304 response only after cooling. On the other hand, NAC did not affect the response at either temperature (Fig. 6).

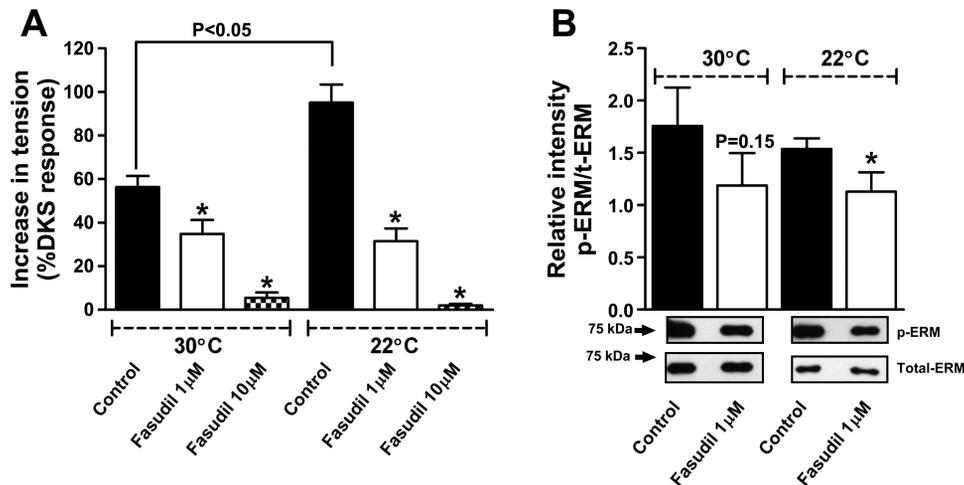


Fig. 2. Effect of the treatment with fasudil on the response to UK-14304 (0.1 μM) in EDVs at 30°C and 22°C. Peak contraction (A) to UK-14304 (0.1 μM) was obtained in EDV segments in the absence and presence of fasudil (Rho kinase inhibitor). The effect of fasudil (1 μM) on the phosphorylated/total ratio of the Rho kinase target family proteins ERM is also shown (B). Each column represents the arithmetic mean value \pm SE of either the peak contraction of 5–17 animals or the densitometry analysis of 5 different pools of 3–4 EDVs from different animals. The effect of 1 μM fasudil was estimated in 17 experiments using paired vessels from the same number of animals. The effect of 10 μM fasudil was estimated in 5 experiments and results were compared vs. control ($n = 17$) by using unpaired analysis. *Significant difference in response within treatments (in the presence of fasudil) by a one-way ANOVA with Dunnett's post hoc test ($P \leq 0.05$). Control values for the 2 temperatures were compared using a Student's t -test.

DISCUSSION

The cooling-enhanced contraction to UK-14304 in equine digital veins was reduced by specific inhibitors of Rho kinase or by inhibiting the production of ROS. Since the reduction of temperature resulted in the phosphorylation of the Rho kinase target family proteins, ERM, while the direct stimulation by UK-14304 only induced a minor increase in phosphorylation over the basal level, it is likely that cooling may increase the activity of the Rho kinase pathway in a cutaneous vein preparation.

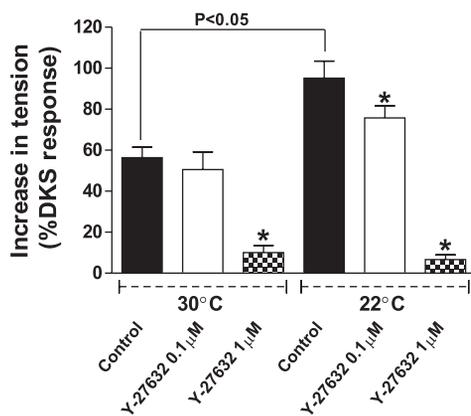


Fig. 3. The effect of treatment with Y-27632 on the response to UK-14304 (0.1 μM) in EDVs at 30°C and 22°C. Peak contraction to UK-14304 (0.1 μM) was obtained in EDV segments in the absence and presence of Y-27632 (Rho kinase inhibitor). Each column represents the arithmetic mean value \pm SE of the peak contraction obtained from blood vessels segments from 5–17 animals. The effect of Y-27632 (0.1 μM) was estimated in 17 experiments using paired vessels from the same number of animals. The effect of Y-27632 (1 μM) was estimated in 5 experiments and results were compared vs. control ($n = 17$) by unpaired analysis. *Significant difference in response within treatments (in the presence of Y27632) by a one-way ANOVA with Dunnett's post hoc test ($P \leq 0.05$). Control values for the 2 temperatures were compared using a Student's t -test.

The cellular mechanisms behind the cooling modulation of the α_2 -adrenoceptor-mediated response have been described in the mouse tail artery (4, 8). However, the information regarding these mechanisms in cutaneous venous smooth muscle is limited. The response to a fixed concentration of an agonist allows the efficacy of the receptor-coupled cellular signal to be evaluated. A fixed concentration (10 μM) of UK-14304 has previously been used by Roberts (38, 39) to investigate the cellular signals mediating the α_2 -adrenoceptor responses in the pig digital vein. In the present study, a concentration 100 times lower was chosen to reduce the likelihood of stimulating α_1 -adrenoceptors, which are functionally expressed in the equine digital vein and are activated by UK-14304 at higher concentrations (14, 46).

When the effect of fasudil (1 μM) was examined, the submaximal contraction to UK-14304 was reduced at both temperatures. However, the percentage of inhibition was almost twofold greater at 22°C vs. 30°C, with fasudil almost abolishing the cooling-induced enhancement of the response to UK-14304. This finding supports the role of the Rho kinase activity during cooling. Furthermore, the participation of Rho kinase in cooling-enhanced contraction by UK-14304 is also supported by the effect of the other structurally unrelated Rho kinase inhibitor, Y-27632, which selectively reduced the contraction caused by UK-14304 at 22°C at a low concentration (0.1 μM). This concentration of Y-27632 corresponds to the reported K_i value for Rho kinase (45).

While there are reports that support the activation of Rho kinase by α_2 -adrenoceptor agonists in the vasculature (11, 39), in some vascular beds there does not necessarily appear to be a direct link (4). This determination may be complicated by the selectivity of the Rho kinase inhibitors used. In the present study, fasudil and Y-27632 (10 μM) almost abrogated the contractile response to UK-14304. While these concentrations have been reported, to be relatively selective for Rho kinase (37), others have suggested that this may not be the case (9).

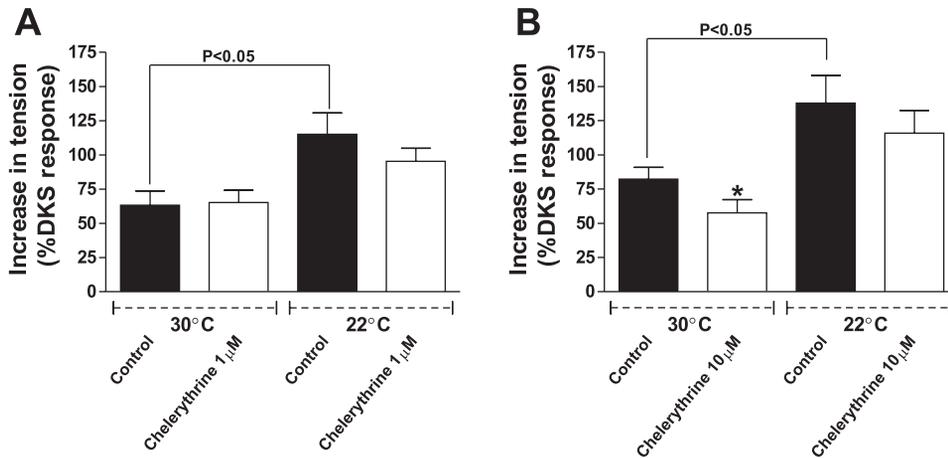


Fig. 4. The effect of treatment with chelerythrine on the response to UK-14304 (0.1 μM) in EDVs at 30°C and 22°C. Peak contraction to UK-14304 (0.1 μM) was obtained in EDV segments in the absence and presence of chelerythrine. Each column represents the arithmetic mean value \pm SE of the peak contraction obtained from blood vessels segments from 5–6 animals. The effect of chelerythrine (A, 1 μM ; B, 10 μM) was estimated in 5 and 6 separate experiments using paired vessels from the same number of animals. * $P \leq 0.05$ in response within treatments (in the presence of chelerythrine) by a paired Student's *t*-test.

For example, high concentrations of fasudil may inhibit other kinases, such as PKC (35) or other unspecific targets. PKC has also been implicated in the α_2 -adrenoceptor-mediated contraction in other venous preparations (1). If the contraction mediated by UK-14304 involves the activation of Rho kinase in EDV, then one would expect to observe a higher phospho-total ERM ratio on α_2 -adrenoceptor stimulation. This was not the case in the present study; at 30°C this increase was not significant.

Chelerythrine has been reported as a selective PKC inhibitor with a $K_i = 0.7 \mu\text{M}$ (2). The submaximal response to UK-14304 was resistant to the treatment with 1 μM chelerythrine at 30°C and 22°C. However, when the concentration was increased (10 μM), the response at 30°C was reduced by $\sim 30\%$. The higher magnitude of inhibition at 30°C suggests that the population of α_2 -adrenoceptors stimulated at this temperature are probably coupled more efficiently to the activation of PKC. On the other hand, considering the functional bioassays performed in the present study, the α_2 -adrenoceptor signal seems to depend less on the activation of PKC and more on the Rho kinase under cold conditions.

The activation of tyrosine kinases has been implicated in the cooling-induced vasoconstriction and the cooling-enhanced

contraction by various agonists in skin arterioles obtained from human patients with Raynaud's disease (17–18). Genistein, a phytoestrogen derivative, which is widely used as a nonspecific tyrosine kinase inhibitor, selectively reduced ($\sim 46\%$) the UK-14304 response at 22°C. However, daidzein, an analog of genistein that is inactive against tyrosine kinases also evoked a selective inhibition on cooling, although with a lower magnitude ($\sim 29\%$). This observation indicates that the effect of genistein could be partially associated with other pharmacological properties unrelated with the inhibition of tyrosine kinase activity.

While the effect of genistein might suggest a partial contribution by tyrosine kinase in the cooling-enhanced response to UK-14304 in these blood vessels, neither the more selective Src-tyrosine kinase inhibitor, PP2, nor the PP2 inactive analog (PP3) caused a significant reduction of the response to UK-14304 at either temperature. Therefore, the lack of significant effect of PP2 and its inactive analog, suggests a minor participation of the cytoplasmic Src family of tyrosine kinase in the enhanced response to α_2 -adrenoceptor in equine digital vein on cooling. These findings contrast with those published in the pig digital vein, where the Src family of tyrosine kinases and the epidermal growth factor (EGF) receptor tyrosine kinase seem to

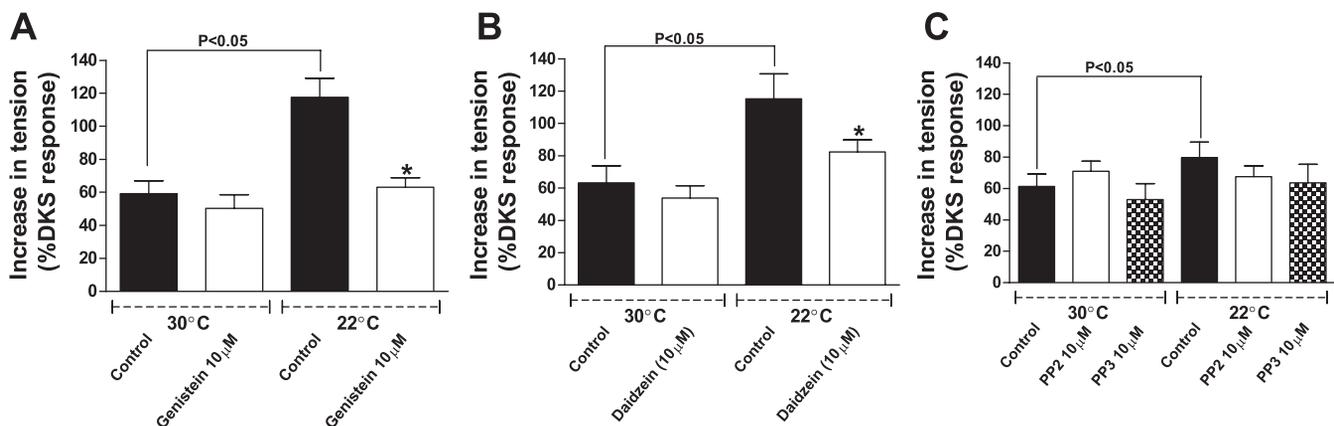


Fig. 5. Effect of the treatment with different tyrosine kinase inhibitors and their inactive analogs on the response to UK-14304 (0.1 μM) in EDVs at 30°C and 22°C. Peak contraction to UK-14304 (0.1 μM) was obtained in EDV segments in the absence and presence of genistein (A; 10 μM), daidzein (B; 10 μM), PP2 and PP3 (C; 10 μM). Each column represents the arithmetic mean value \pm SE of the peak contraction obtained from blood vessel segments from 5 (PP2-PP3), 6 (daidzein) or 12 (genistein) animals. Appropriate solvent controls were run in parallel experiments. Comparisons of the response within treatments (in the presence of the inhibitors) were made by a paired Student's *t*-test or one-way ANOVA with Dunnett's post hoc test. *Significant difference ($P \leq 0.05$). Control values for the 2 temperatures were compared using a Student's *t*-test.

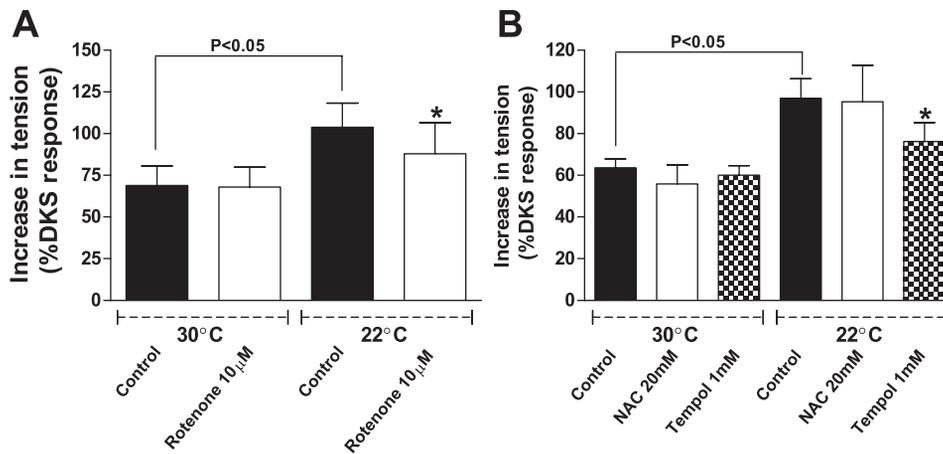


Fig. 6. Effect of the treatment with different inhibitors of reactive oxygen species production on the response to UK-14304 (0.1 μ M) in EDVs at 30°C and 22°C. Peak contraction to UK-14304 (0.1 μ M) was obtained in EDV segments in the absence and presence of rotenone (A; 10 μ M), *N*-acetylcysteine (B; NAC, 20 mM), and tempol (B; 1 mM). Each column represents the arithmetic mean value \pm SE of the peak contraction obtained from blood vessel segments from 5 (rotenone) or 6 (NAC and tempol) animals. Appropriate solvent controls were run in parallel experiments. *Significant difference in response within treatments (in the presence of the inhibitors) by a paired Student's *t*-test or a one-way ANOVA with Dunnett's post hoc test (for multiple comparisons in B; $P \leq 0.05$). Control values for the 2 temperatures were compared using a Student's *t*-test.

be involved in the UK-14304-mediated contraction (38). Differences between species could explain this discrepancy in venous samples from approximately the same digit region of two ungulates. Alternatively, the use of different UK-14304 concentrations in these two studies may have resulted in the activation of different subtypes of α -adrenoceptors and, consequently, activated different downstream kinases.

Beyond the inhibitory action on tyrosine kinase, genistein at the concentration used in the present study, also has antioxidant properties (27, 41). Therefore, it is also possible that by reducing the activity or production of ROS during cooling, genistein and daidzein caused a selective inhibition of the cooling-enhanced response mediated by UK-14304. Furthermore, since the production of mitochondrial ROS has been implicated in the cooling-enhanced response to UK-14304 (α_2 -adrenoceptor agonist) and α_1 -adrenoceptor-mediated contraction in arteries (8, 21) and to explore this further, the effect of antioxidants was assessed in this cutaneous venous preparation at different temperatures. Incubation of equine digital veins with rotenone, a selective mitochondrial complex I inhibitor, and the membrane permeable superoxide dismutase mimetic tempol (28, 30) reduced the contraction evoked by UK-14304 only at 22°C. However, NAC did not affect the response at either temperature. These results suggest that the contraction induced by α_2 -adrenoceptor activation at 22°C involves the production of ROS in the equine digital vein smooth muscle, probably from the mitochondrial respiratory electron transport chain. Since tempol inhibited the contraction, it is possible that superoxide is the main ROS involved in the observed cooling effect. The lack of effect of NAC was unexpected. NAC exerts its antioxidant action by directly scavenging ROS or indirectly by supplying cysteine for enhanced glutathione synthesis (33). However, NAC scavenging power for superoxide is limited and it reacts slowly with hydrogen peroxide (3).

The selective inhibitory effect of antioxidants on the cooling enhanced contraction to α_2 -adrenoceptor agonists in this cutaneous vein agrees with results published previously in the mouse tail artery (8). It was proposed that the production of

ROS from the mitochondrion acts as a temperature sensor in the vascular smooth muscle, with cooling causing uncoupling of the mitochondrial electron transport chain. Alternatively, a slower enzyme rate may reduce superoxide dismutase activity (10). Cooling can also activate uncoupling proteins (UCPs) to promote thermogenesis. However, most of the studies on hypothermic conditions have been conducted at lower temperatures than the ones used in the present study. Further analyses are required to establish whether these mechanisms operate under moderate cooling. The production of ROS has also been implicated in the activation of Rho kinase, not only in the cooling-enhanced contraction by α_2 -adrenoceptors but also in the phenylephrine-induced contraction in the rat aorta and the oxygen-induced contraction in the ductus arteriosus, under

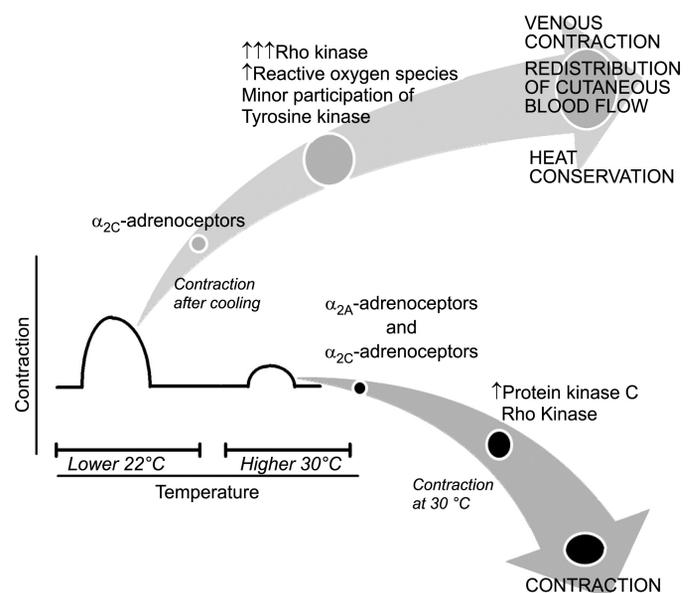


Fig. 7. Graphic representation of the cellular mechanisms involved in the enhanced contractility to UK-14304 in EDVs brought about by moderate cooling. The participation of the different subtypes of α_2 -adrenoceptors has been previously investigated (49).

physiological temperatures (8, 25, 26). Therefore, different sources of evidence indicate that the production of ROS by mitochondria might modulate the development of tension in the vasculature.

Taken together, the results presented above suggest that the production of ROS and increased Rho kinase activity in equine digital veins at 22°C could promote the enhanced contraction to UK-14304 (Fig. 7). This might occur either by promoting calcium sensitization or by the potential translocation of α_2C -adrenoceptors that might be located intracellularly. Most of the mechanistic studies that explain the effect of cooling in cutaneous vessels have been conducted in superficial arteries (16). The novel mechanisms described here for superficial cutaneous veins offer some insights in the physiological role of these veins during cooling and contribute to the thermoregulatory role of the equine digital vascular bed. The extent to which these mechanisms might contribute to the development of pathological conditions, such as laminitis, requires further investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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