

Effect of Temperature and Modulators of Protein Tyrosine Kinase Activity on the Reactivity of Isolated Venules in Secondary Raynaud's Phenomenon

PHILIP B. FURSPAN, MAUREEN D. MAYES, and ROBERT R. FREEDMAN

ABSTRACT. **Objective.** To investigate the response of skin venules from healthy controls and scleroderma patients with Raynaud's phenomenon (RP/SSc) to cooling and to modulators of protein tyrosine kinase (PTK) activity at normal and reduced temperature.

Methods. We used the microvessel perfusion technique to characterize the response of isolated dermal venules (200–400 μm outside diameter) from normal ($n = 10$) and RP/SSc ($n = 8$) subjects to cooling and to contractile agents at 37 and 31°C.

Results. The response to clonidine at 37°C was less in venules from patients with RP/SSc compared to controls; the contraction to serotonin was greater in venules from RP/SSc patients versus controls; at 31°C, venules from RP/SSc patients contracted to both clonidine and serotonin to a greater extent versus controls; and contraction to these agonists was reversed by cumulative addition of genistein (1–100 μM). Venules from controls and patients with RP/SSc exhibited slight vasodilation to cooling from 37 to 31°C. In the presence of the protein tyrosine phosphatase inhibitor sodium orthovanadate (10 μM), venules from controls now exhibited a small contraction ($-5.1 \pm 3.2\%$) and venules from RP/SSc subjects a significantly greater contraction ($-38.7 \pm 9.0\%$; $p < 0.05$).

Conclusion. Our study supports the view that RP/SSc is the result of defects in the peripheral vasculature. (J Rheumatol 2001;28:2263–8)

Key Indexing Terms:

HUMAN
VEIN

RAYNAUD'S DISEASE
COLD

PROTEIN TYROSINE KINASE
GENISTEIN

It is estimated that as many as 90–95% of individuals with scleroderma are subject to the cold induced vasospasm that is characteristic of Raynaud's phenomenon (RP). The etiology and pathophysiological mechanism underlying this hyperresponsiveness and the reason for its frequent association with scleroderma remain obscure. Much of the research in this area has focused on the dermal microcirculation. These studies have reported changes in the responsiveness of these blood vessels to a variety of vasoconstrictors (adrenergic, serotonergic, and endothelin). We postulate that in RP associated with scleroderma (RP/SSc) this altered responsiveness involves a protein tyrosine kinase (PTK) transduction pathway. Our reasoning: (1) PTK pathways are important mediators of G protein coupled agonists of vascular smooth muscle^{1,2}. (2) The contractile effects of the G protein coupled agonists listed above have been reported

to be mediated, at least in part, by PTK transduction pathways^{3–5}. (3) Recent work has suggested an etiologic role for PTK transduction pathways in vascular diseases such as atherosclerosis and cerebral and coronary vasospasm^{6–8}. (4) A PTK transduction pathway has been implicated in the modulation of cold induced vasoconstriction^{9–11}. (5) The enzymes of these pathways can be influenced by circulating cytokines and growth factors^{8,12–14}. (6) The concentrations of many circulating cytokines and growth factors are abnormal in scleroderma^{15,16}. We investigated the response of skin venules from healthy controls and from patients with scleroderma and RP (RP/SSc) to cooling and modulators of PTK activity at normal and reduced temperature.

MATERIALS AND METHODS

Subject characteristics. Ten patients with RP/SSc (8 women, 2 men) and 8 healthy volunteers (7 women, one man) participated in this study. Because of the number and length of the various protocols not every one could be performed for every subject. Each protocol was carried out on vessels from 6 subjects. The protocols were randomized among subjects. The average age was 42 ± 5.0 years for patients with RP/SSc. Scleroderma (systemic sclerosis) was defined according to the American College of Rheumatology criteria¹⁷. Average duration of the disease was 11.5 ± 4 years. Five patients had the limited form of the disease and 5 had the diffuse form. All patients had RP: defined as episodic, bilateral, digital color changes (2 out of 3 possible colors: blanching, cyanosis, rubor) provoked by cold and/or emotional stress. The patients were recruited from a registry of scleroderma patients in Michigan administered by one of us (MDM) and sponsored by

From the Departments of Psychiatry and Behavioral Neurosciences, and Internal Medicine (Rheumatology), Wayne State University, Detroit, Michigan, USA.

Supported by NIH grant HL-30604.

P.B. Furspan, PhD, Assistant Professor; M.D. Mayes, MD, MPH, Professor; R.R. Freedman, PhD, Professor.

Address reprints requests to Dr. P. Furspan, C.S. Mott Center, Wayne State University, 275 E. Hancock Ave., Detroit, MI 48201.
E-mail: aa1985@wayne.edu

Submitted September 26, 2000; revision accepted April 10, 2001.

the National Institutes of Health. No patient had active digital ulcers. Subjects were not hypertensive or hypercholesterolemic. Medications taken by the patients varied: antimetabolites, methotrexate in one; gastrointestinal medications, lansoprazole in one, cisapride in one, omeprazole in 2; thyroid hormones, levothyroxine in 2; calcium antagonists, nifedipine in one; blood flow enhancers, pentoxifylline in one; cholinergic agents, pilocarpine in one. All medications were withdrawn at least one week prior to study.

Healthy volunteer controls (average age 30.8 ± 3.7 yrs, not significantly different from mean patient age) were recruited from signs posted on our medical campus requesting subjects for research on blood vessels. They were screened by giving a medical history and completing an extensive symptom questionnaire. They were free of all medication. All patients and controls gave written informed consent according to procedures approved by our institutional review board and were paid for their participation.

Vessel preparation. Using lidocaine (without norepinephrine) as a local anesthetic, skin biopsies (6 mm in diameter) were taken from the medial forearm of controls and patients with RP/SSc. In the latter, biopsies were taken from clinically uninvolved skin as determined by the dermatologist performing the procedure. Venules of usable size (mean diameter 244 ± 21 μm controls; 278 ± 37 mm RP/SSc patients) were dissected from the dermal-subcutaneous boundary. Only one vessel was used from each biopsy. They were stored in Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO, USA) overnight at 4°C . Preliminary experiments found no difference in reactivity between vessels used the same day and vessels from the same biopsy used the next day. Similar results have been reported^{18,19}. The next morning the skin sample was placed in a dissecting dish and covered with physiological salt solution (PSS): in mM, NaCl, 118; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄–7H₂O, 1.17; CaCl₂–2H₂O, 1.6; NaHCO₃, 25.0; dextrose, 5.5; and CaNa₂ EDTA, 1.2; aerated with 95% O₂ and 5% CO₂. Isolated dermal venules were cannulated (perfusion pressure = 10 mm Hg, no flow) in a microvascular chamber (Living Systems Instrumentation, Burlington, VT, USA) placed on the stage of an inverted microscope. Changes in lumen diameter in response to vasoactive substances added to the superfusate were observed and quantified with a video dimension analyzer (Living Systems Instrumentation) connected to a chart recorder. After the bath temperature was raised to 37°C the vessel was allowed to equilibrate for 45 min. All vessels were then exposed to PSS containing 95 mM KCl (equimolar substitution for NaCl). Only vessels exhibiting a reduction in diameter > 50% were used.

Experimental protocol. Concentration-response curves were constructed for clonidine, a predominantly α_2 -adrenergic agonist, (10^{-9} to 3×10^{-6} M), and serotonin, a non-adrenergic agonist (10^{-9} to 10^{-5} M) at 37°C and then at 31°C . This temperature was chosen because preliminary results indicated that lower temperatures significantly inhibited contraction to these agonists. No contractile response was seen at 24°C in venules from either group of subjects. Both clonidine and serotonin have been reported to exert their contractile effects, at least in part, via the PTK pathway. Results were expressed as percentage change in lumen diameter. The effect of PTK inhibition on agonist induced contraction was determined by cumulatively adding genistein (1–100 μM) to the bath after contraction to the highest concentration of the agonist had reached a plateau.

The response of vessels to cooling from 37°C to 31°C was determined in the absence and presence of sodium orthovanadate (10 μM), an inhibitor of tyrosine phosphatase. Superfusion fluid temperature was reduced quickly (< 2 min) by adding a predetermined quantity of ice to the circulating water bath. Venules exhibiting significant contraction (> 10%) to cooling were then exposed to increasing concentrations of genistein (1–100 μM).

Data analysis. Results were analyzed by t test or one-way analysis of variance (ANOVA) supported by the Bonferroni test when pairwise between-group comparisons were performed. In all cases, $p < 0.05$ denoted statistical significance between groups. All results were expressed as means \pm standard error. Sensitivity to a particular agonist was expressed as the

concentration causing a change in diameter that is equal to 50% of the plateau minimum diameter, i.e., maximum contraction (EC₅₀).

Drugs. All drugs and chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. Genistein was dissolved in dimethyl sulfoxide.

RESULTS

In experiments performed at 37°C , the difference in sensitivity and maximum contraction to clonidine of venules from patients with RP/SSc was significantly less compared to those from control subjects ($p < 0.05$; Table 1). The sensitivity and maximum contraction to serotonin, however, were significantly greater in venules from RP/SSc subjects than in those from control subjects ($p < 0.05$; Table 1). When the PTK inhibitor genistein was applied to venules maximally contracted to either clonidine or serotonin it caused a concentration dependent relaxation (Figure 1). Venules contracted with PSS containing 95 mM KCl did not relax in response to genistein (data not shown). In venules contracted using serotonin, those from patients with RP/SSc exhibited greater relaxation at most concentrations of genistein than those from controls (Figure 1).

At 31°C , the response of venules from control subjects to clonidine and serotonin was attenuated compared to that observed at 37°C ($p < 0.001$ and $p < 0.05$, respectively) whereas the response of venules from RP/SSc patients was not (Table 1). As at 37°C , the contraction of venules from RP/SSc patients to clonidine and serotonin at 31°C was reversed by cumulative addition of genistein (Figure 1). The curves at 31°C , however, were shifted to the left compared to those at 37°C . Because of the minimal contraction of venules from control subjects to clonidine and serotonin at 31°C responsiveness to genistein was not tested.

When the bath temperature was reduced from 37 to 31°C , untreated venules from controls and RP/SSc patients dilated slightly, $2.3 \pm 0.73\%$ and $0.96 \pm 0.76\%$, respectively (Figure 2). Pretreating the venules with the protein tyrosine phosphatase inhibitor sodium orthovanadate (SOV), however, caused venules from controls and RP/SSc to now contract to cooling, $-5.1 \pm 3.2\%$ and $-38.7 \pm 9.0\%$, respectively ($p < 0.001$; Figure 2). The contraction of the venules from RP/SSc subjects to cooling in the presence of SOV was reversed by cumulative addition of genistein (1 μM : $17 \pm 7\%$ to 100 μM : $88 \pm 4\%$ reversal of contraction). Because of the minimal contraction of venules from control subjects to cooling, responsiveness to genistein was not tested.

DISCUSSION

Our results support our hypothesis that abnormalities in agonist and cooling mediated contraction associated with RP/SSc are secondary to an abnormality of a PTK transduction pathway.

The diminished responsiveness to clonidine at 37°C of venules from RP/SSc patients (Table 1) appears to be consistent with the results of an *in vivo* study by Freedman, *et al*²⁰. They measured finger blood flow (FBF) using

Table 1. EC₅₀ and maximum contraction values for clonidine and serotonin at normal and reduced temperature.

	37°C Clonidine	37°C Serotonin	31°C Clonidine	31°C Serotonin
Control				
EC ₅₀ , μM	0.018 \pm 0.006	0.087 \pm 0.012	0.28 ^{†††} \pm 0.013	0.14 [†] \pm 0.026
Maximum change in diameter, %	-48 \pm 6.7	-30.1 \pm 4.9	-16.3 ^{††} \pm 3.3	-9.3 [†] \pm 7.6
RP/SSc				
EC ₅₀ , μM	0.038* \pm 0.01	0.042* \pm 0.011	0.031*** \pm 0.007	0.011** [†] \pm 0.008
Maximum change in diameter, %	-22.1* \pm 9.1	-56.2* \pm 11.4	-36.5* \pm 4.7	-43.4** \pm 9.4

* p < 0.05, ** p < 0.01, *** p < 0.001; RP/SSc value vs control value.

† p < 0.05, †† p < 0.01, ††† p < 0.001; 31°C value vs 37°C value.

Values are expressed as mean \pm SEM. Sample size is 6 for all groups.

venous occlusion plethysmography (VOP) in RP/SSc patients and controls. In response to intraarterial infusion of clonidine FBF increased in RP/SSc and decreased in controls. In a separate study, isolated arterioles from RP/SSc and control subjects contracted equally to clonidine (unpublished results). Therefore, the increased FBF *in vivo* in RP/SSc patients may reflect decreased venule compliance, i.e., diminished venule contraction. The lesser responsiveness of venules from RP/SSc patients contrasts with the greater responsiveness of arterioles reported by Flavahan, *et al*²¹. They reported that isolated skin arterioles from scleroderma subjects exhibited greater maximum contraction to the specific α_2 -adrenergic agonist UK 14,404 than those from healthy subjects. The difference in results between the 2 studies may be attributable to differences in agonist specificity and/or differences in the responsiveness between venules and arterioles based on anatomy, function, receptor types, and numbers.

The greater sensitivity and maximum contraction to serotonin at 37°C observed in venules from patients with RP/SSc compared to controls (Table 1) is consistent with the results reported by Winkelmann, *et al*²². They observed that isolated wrist cutaneous blood vessels from subjects with scleroderma exhibited hypersensitivity to serotonin compared to those from control subjects.

The relaxation of both clonidine and serotonin induced contractions by genistein (Figure 2) is consistent with the results of studies implicating a PTK transduction pathway as a mediator of their contractile effects. The greater responsiveness of venules from RP/SSc contracted with serotonin may indicate a greater dependence on the PTK pathway in these vessels. Although the venules contracted with clonidine responded similarly to genistein, the magnitude of contraction in the venules from patients with RP/SSc was less versus controls. These results are also consistent with the possibility that venules from the RP/SSc patients are more dependent on the PTK pathway for the mediation of contraction.

Reducing bath temperature elicited changes in the response of venules to the 2 agonists. The diminution of the

response in venules from controls is opposite to the response reported in 2 studies by Harker and colleagues for human saphenous vein^{23,24}. They reported significant potentiation of the contractile response to serotonin and the α_2 -adrenergic agonist B-HT 920 at 24°C. Similar findings have been reported for canine saphenous vein^{25,26} and human hand veins^{27,28}. Although the authors of some of these studies link the effects of cooling on α_2 -adrenergic induced contraction to changes in the receptors for this agonist, no explanation is provided for the effect of cooling on serotonin induced contraction. In fact, the similar effect of cooling on 2 such dissimilar agonists suggests that cooling is exerting its effects "downstream" of the receptors. We have postulated that this occurs in the PTK transduction pathway.

The involvement of PTK transduction in the contractions elicited by clonidine and serotonin at 31°C is evidenced by the relaxation of these contractions caused by genistein (Figure 1). In fact, the sensitivity to genistein appears to be greater at 31°C than at 37°C (Figure 1). The greater sensitivity may indicate that a PTK pathway mediates a greater proportion of the contraction at the lower temperature. A recent report suggests that cooling significantly increases tyrosine phosphorylation in lung tissue²⁹. Lung tissue maintained at 4°C exhibited much greater tyrosine phosphorylation, as determined by Western blotting, than the same tissue after rewarming.

Enhanced PTK activity is consistent with the results of the experiment in which venules from controls and RP/SSc patients were cooled from 37 to 31°C in the absence and presence of the protein tyrosine phosphatase (PTP) inhibitor SOV. In the absence of SOV, venules from control and RP/SSc subjects dilated slightly when cooled (Figure 2). Eliminating the dephosphorylating action of PTP with SOV, however, caused venules from controls to contract slightly in response to cooling and those from RP/SSc patients to contract more forcefully (Figure 2). The PTK dependence of the forceful contraction of the venules from RP/SSc patients is indicated by its reversal by genistein.

Our results support the view that Raynaud's phenomenon associated with scleroderma is the result of defects in the

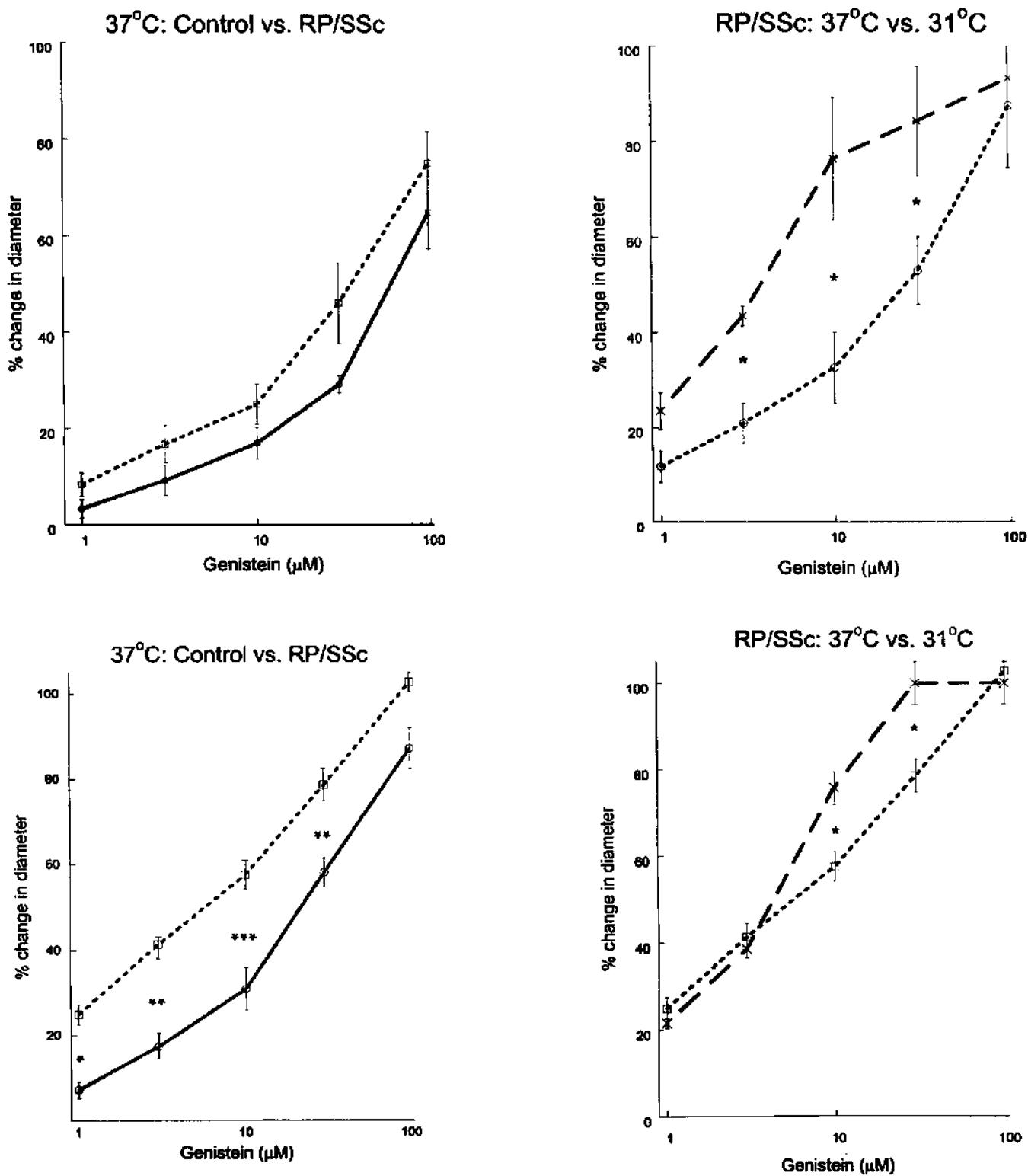


Figure 1. Left panels. The effect of increasing concentrations of the PTK inhibitor genistein on the contraction induced at 37°C by 3 μM clonidine (top) or 10 μM serotonin (bottom) in venules from controls (solid lines) and patients with RP/SSc (broken lines). Right panels. The effect of increasing concentrations of the PTK inhibitor genistein on the contraction induced by 3 μM clonidine (top) or 3 μM serotonin (bottom) in venules from RP/SSc subjects at 37°C (small broken lines) and 31°C (large broken lines). *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA. Sample size = 6 for all.

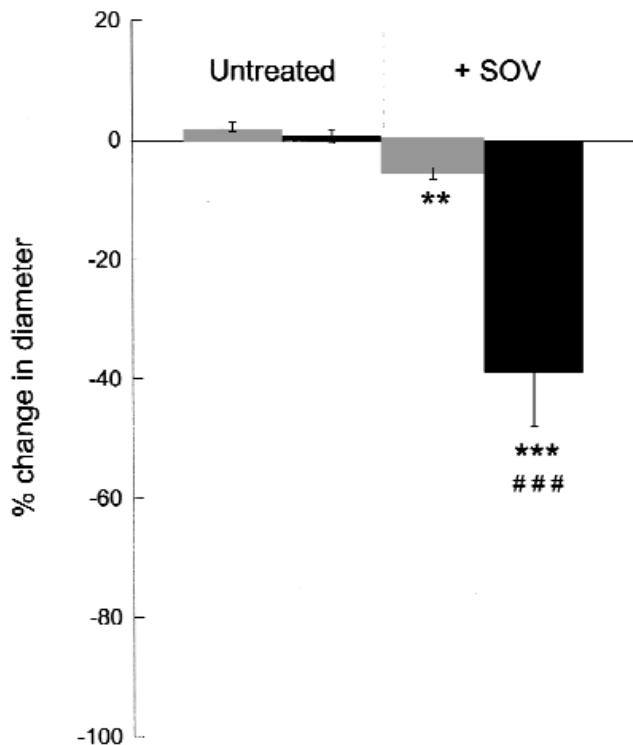


Figure 2. The effect of cooling from 37 to 31°C on the contractile response of venules from healthy controls (shaded bars) and patients with RP/SSc (black bars) in the absence and presence of 10 μM sodium orthovanadate (SOV), an inhibitor of protein tyrosine phosphatase. The response of the venules from controls and RP/SSc patients in the presence of SOV was significantly greater than their responses in its absence, **p < 0.01 and ***p < 0.001, respectively. The contraction of the venules from RP/SSc patients in the presence of SOV was significantly greater versus control subjects, ###p < 0.001. Sample size = 6.

peripheral vasculature. The major defect is consistent with the hypothesis of an alteration in a protein tyrosine kinase transduction pathway that mediates G protein coupled agonist induced contraction. This defect may underlie many of the secondary vascular abnormalities reportedly associated with RP/SSc. The precise nature of the PTK pathway and specific defect(s) are impossible to determine from the current results. Studies using more specific PTK and PTP inhibitors and molecular biology techniques may pinpoint the defect.

REFERENCES

- Laniyoun AA, Saifeddine M, Yang SG, Hollenberg MD. Tyrosine kinase inhibitors and the contractile action of G-protein-linked vascular agonists. *Can J Physiol Pharmacol* 1994;72:1075-85.
- Hollenberg MD. Tyrosine kinase-mediated signal transduction pathways and the actions of polypeptide growth factors and G-protein-coupled agonists in smooth muscle. *Mol Cell Biochem* 1995;149-50:77-85.
- Jinsi A, Deth RC. Alpha 2-adrenoceptor-mediated vasoconstriction requires a tyrosine kinase. *Eur J Pharmacol* 1995;277:29-34.
- Liu CY, Sturek M. Attenuation of endothelin-1-induced calcium response by tyrosine kinase inhibitors in vascular smooth muscle cells. *Am J Physiol* 1996;270:C1825-33.
- Watts SW, Yeum CH, Campbell G, Webb RC. Serotonin stimulates protein tyrosyl phosphorylation and vascular contraction via tyrosine kinase. *J Vasc Res* 1996;33:288-98.
- Zou Y, Hu Y, Metzler B, Xu Q. Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells [review]. *Int J Mol Med* 1998;1:827-34.
- Fujikawa H, Tani E, Yamaura I, et al. Activation of protein kinases in canine basilar artery in vasospasm. *J Cereb Blood Flow Metab* 1999;19:44-52.
- Ito A, Shimokawa H, Kadokami T, et al. Tyrosine kinase inhibitor suppresses coronary arteriosclerotic changes and vasospastic responses induced by chronic treatment with interleukin-1 beta in pigs *in vivo*. *J Clin Invest* 1995;96:1288-94.
- Wagerle LC, Kim SJ, Russo P. Protein tyrosine kinase signaling in cold-stimulated contraction of newborn lamb cerebral arteries. *Am J Physiol* 1996;270:H645-50.
- Wagerle LC, Kim SJ, Levchuk SG, Russo P. Biochemical mechanisms of cold-induced vascular contraction. *FASEB J* 1996;10:A7.
- Furspan PB, Freedman RR. Effect of modulators of protein tyrosine kinase activity on gender-related differences in vascular reactivity at reduced temperature. *J Cardiovasc Pharmacol* 1998;32:728-35.
- Neumann C, Zehentmaier G, Danhauser-Riedl S, Emmerich B, Hallek M. Interleukin-6 induces tyrosine phosphorylation of the Ras activating protein Shc, and its complex formation with Grb2 in the human multiple myeloma cell line LP-1. *Eur J Immunol* 1996;26:379-84.
- Sauro MD, Thomas B. Tyrphostin attenuates platelet-derived growth factor-induced contraction in aortic smooth muscle through inhibition of protein tyrosine kinase(s). *J Pharmacol Exp Ther* 1993;267:1119-25.
- Touyz RM, Schiffrin EL. Growth factors mediate intracellular signaling in vascular smooth muscle cells through protein kinase C-linked pathways. *Hypertension* 1997;30:1440-7.
- Fagundes DM, Leroy EC. Cytokines and systemic sclerosis. *Clin Dermatol* 1994;12:407-17.
- Kantor TV, Friberg D, Medsger TA Jr, Buckingham RB, Whiteside TL. Cytokine production and serum levels in systemic sclerosis. *Clin Immunol Immunopathol* 1992;65:278-85.
- Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. *Arthritis Rheum* 1980;23:581-90.
- Ingemansson R, Budrikis A, Bolys R, Sjoberg T, Steen S. Effect of temperature in long-term preservation of vascular endothelial and smooth muscle function. *Ann Thorac Surg* 1996;61:1413-7.
- Iwaki H, Sakamoto A, Tanaka S. Effects of temperature and preservation time on the pharmacological response of isolated vascular endothelial and smooth muscle function. *Nippon Ika Daigaku Zasshi* 1999;66:15-20.
- Freedman RR, Girgis R, Mayes MD. Endothelial and adrenergic dysfunction in Raynaud's phenomenon and scleroderma. *J Rheumatol* 1999;26:2386-8.
- Flavahan NA, Flavahan S, Liu Q, et al. Increased alpha2-adrenergic constriction of isolated arterioles in diffuse scleroderma. *Arthritis Rheum* 2000;43:1886-90.
- Winkelmann RK, Goldyne ME, Linscheid RL. Hypersensitivity of scleroderma cutaneous vascular smooth muscle to 5-hydroxytryptamine. *Br J Dermatol* 1976;95:51-6.
- Harker CT, Ousley PJ, Harris EJ, Edwards JM, Taylor LM, Porter JM. The effects of cooling on human saphenous vein reactivity to adrenergic agonists. *J Vasc Surg* 1990;12:45-9.
- Harker CT, Taylor LM Jr, Porter JM. Vascular contractions to

- serotonin are augmented by cooling. *J Cardiovasc Pharmacol* 1991;18:791-6.
25. Vanhoutte PM, Flavahan NA. Effects of temperature on alpha adrenoceptors in limb veins: role of receptor reserve. *Fed Proc* 1986;45:2347-54.
 26. Flavahan NA, Lindblad LE, Verbeuren TJ, Shepherd JT, Vanhoutte PM. Cooling and alpha 1- and alpha 2-adrenergic responses in cutaneous veins: role of receptor reserve. *Am J Physiol* 1985;249:H950-5.
 27. Bodelsson M, Arneklo-Nobin B, Tornebrandt K. Cooling augments contractile response to 5-hydroxytryptamine via an endothelium-dependent mechanism. *Blood Vessels* 1989;26:347-59.
 28. Bodelsson M, Arneklo-Nobin B, Nobin A, Owman C, Sollerman C, Tornebrandt K. Cooling enhances alpha 2-adrenoceptor-mediated vasoconstriction in human hand veins. *Acta Physiol Scand* 1990;138:283-91.
 29. Keshavjee S, Zhang XM, Fischer S, Liu M. Ischemia reperfusion-induced dynamic changes of protein tyrosine phosphorylation during human lung transplantation. *Transplantation* 2000; 70:525-31.