The Effect of Botulinum Neurotoxin-A on Blood Flow in Rats: A Potential Mechanism for Treatment of Raynaud Phenomenon

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Purpose Botulinum neurotoxin-A (BoNTA) is used to treat several disorders, including Raynaud phenomenon. Recent investigations cite toxin-induced increases in blood flow, but no mechanism for BoNTA's actions is proposed. This study hypothesized that local application of BoNTA causes arteriolar vasodilation through sympathetic blockade and results in increased blood flow.

Methods Microvascular effects of BoNTA were assessed using a rat cremaster preparation. Cremaster microvascular diameters were measured in the muscle before and after treatment with the muscle paralytic agent gallamine triethiodide. Preparations were then treated with one of the following: BoNTA (4, 6, or 10 units), BoNTA dilution vehicle, or denatured BoNTA. Arteriolar diameters were measured repeatedly over the observation period. Additional preparations were treated with either tetrodotoxin or prazosin and rauwolscine before BoNTA to confirm that the observed vasodilatory responses were the result of sympathetic neural inhibition.

Results The BoNTA application resulted in a significant dose-dependent vasodilation (13% to 15%) of observed cremaster arterioles. Control treatments did not cause vasodilation. Both tetrodotoxin and prazosin/rauwolscine treatments elicited similar vasodilatory effects, with no additional vasodilation elicited by BoNTA. Addition of sodium nitroprusside following BoNTA elicited further vasodilation. In addition, systemic arterial pressure was unaffected by the local administration of BoNTA.

Conclusions Local application of BoNTA results in arteriolar dilation that yields an approximate 69% increase in blood flow, without changing systemic arterial pressure. A BoNTA-mediated vasodilation through sympathetic blockade is a likely mechanism to explain the increase in blood flow reported after treatment with the toxin.

Clinical relevance The ability of BoNTA to inhibit sympathetic nervous input reduces vasoconstriction, which is the most likely mechanism for improvement seen in Raynaud phenomenon patients following BoNTA injection. *(J Hand Surg 2012;37A:795–802.*

Key words Adrenergic nervous system, arteriole, blood flow, Botulinum toxin, vasodilation.

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Botulinum neurotoxin-A (BoNTA) is used to treat a variety of orthopedic, dermatologic, and surgical disorders.[1–6] In addition, botulinum toxin is emerging as a treatment modality for Raynaud phenomenon.[7–10] The mechanism of action of BoNTA at the neuromuscular junction and the functional recovery of the nerve and muscle are well established[11–18]; however, little information is available regarding toxin effects on the local vasculature.[7–9,19] Arteriolar diameter and blood flow is predominantly controlled by the sympathetic nervous system, which releases norepinephrine after stimulation and results in α-adrenergic receptor-mediated vasoconstriction.[20–22] Local metabolites and growth factors also influence blood flow to a lesser degree.[21] Raynaud phenomenon results from a disruption of vascular control with subsequent ischemia and pain.

Current treatments for Raynaud phenomenon include calcium channel blockers, endothelin antagonists, phosphodiesterase-5 inhibitors, antioxidants, and statins; however, these are all systemic treatments.[23] Local administration of BoNTA was proposed to treat Raynaud's phenomenon, and initial results are promising.[8–10] The underlying mechanism of action of BoNTA for the treatment of Raynaud phenomenon remains poorly understood. In general, BoNTA blocks synaptic neurotransmission by cleaving the pre-synaptic soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins required for neuronal vesicle fusion with the pre-synaptic neuron membrane, thus blocking neurotransmitter release. The ability of BoNTA to block synaptic transmission and its effects on the neuromuscular junction are well characterized.[1–4,6,11–15,17–18,24–25] Although 2 basic science studies examined components of BoNTA's effects on muscle perfusion and metabolism, these studies did not examine the real-time pharmacologic effects of BoNTA on blood flow.[19,26] Clinical studies report improvement in patients with Raynaud phenomenon after BoNTA injection, and increased blood flow is implicated[6–10]; an underlying mechanism has not been proposed. This study hypothesized that local, topical application of BoNTA inhibits sympathetic neural transmission and results in arteriole vasodilation.

METHODS

Our institutional animal care and use committee approved this study. All portions of the protocol were performed in accordance with the institutional guidelines and the National Institute of Health animal care and use guidelines.

Preparation of the rat cremaster muscle

The validated rat cremaster model was used to study the microvasculature.[27–28] Male Sprague-Dawley rats (90–105 g; n = 47) were anesthetized with urethane (intraperitoneal injection, 1.2 g/kg body weight (Sigma-Aldrich, St. Louis, MO). Rats were placed supine on a stage equipped with a reservoir to superfuse the cremaster muscle. The right cremaster muscle was exposed as previously described.[27] After removal of the testicle, the cremaster muscle was stretched over an adjustable, raised pedestal surrounded by a 2-piece reservoir. The muscle was secured over the pedestal using 5–0 silk suture (Ethicon, Cornelia, GA) anchored to the platform with Châ-seal compound (Chase Instruments, Rockwood, TN). A notch in the upper portion of the reservoir protected the cremaster pedicle. The notch and reservoir seam were sealed with petroleum jelly (Fisher Scientific, Pittsburgh, PA). The rat and platform were secured to the microscope stage.

Microvascular measurements were obtained using live video microscopy. A camera (Hitachi charge-coupled device, black and white camera, KP-M1U, Hitachi Densi Ltd., Japan), attached through a 2/3X Optem extender to a Leitz LaborLux 12 microscope (Leica Microsystems, Inc., Buffalo Grove, IL) with a Zeiss 40× long-working-distance objective, transmitted images to a video home system recorder (Panasonic Professional S-VHS tapes [Secaucus, NJ], Mitsubishi S-VHS videocassette recorder [HS-U69, Japan]). Vessel internal diameters were measured from wall to wall using a video dimension micrometer (CIM Solutions, Advance, NC). The video micrometer was calibrated on-screen with a stage micrometer scale (Bausch and Lomb, Rochester, NY) before the start of each experiment.

A recirculating tissue bath approximated physiologic conditions and provided a method for local delivery of drugs in reasonable concentrations. The bath and pump reservoir were filled with buffered modified Krebs solution maintained at room temperature (24°C) and recirculated with a peristaltic pump and pressure servo control (Living Systems Instrumentation, Burlington, VT). All drug solutions were added directly to the tissue bath.

Drug treatment and vessel measurement

Arteriolar diameters were measured following the addition of the Krebs solution. Gallamine triethiodide (gallamine, 0.170 mM; Sigma-Aldrich, St. Louis, MO), a curareform acetylcholine receptor antagonist, minimized the metabolic demands of the cremaster muscle before the application of BoNTA.[29] Gallamine paraly-

sis eliminated the confounding effect of vascular responses to altered metabolic rate due to toxin-mediated muscle paralysis.[30] Baseline measurements were repeated 10 minutes after the addition of gallamine. Because gallamine did not cause a significant change in vessel diameter over the pre-gallamine treatment diameters, all vessel measurements were normalized to resting diameter in the presence of gallamine. Similar-sized vessels were measured in the microvascular preparations.

Lyophilized BoNTA (Botox, 100 units; Allergan, Irvine, CA) was freshly reconstituted with 1.0 mL 0.9% saline for each experiment. One of 3 doses of BoNTA in units (U) (4 U [n = 8], 6 U [n = 6], or 10 U [n = 13]) was added directly to the tissue bath. The doses were selected based on clinical doses used in muscle injections and our laboratory's experience with injection of mouse and rat gastrocnemius muscle.[4,8–12,14–18,31] The 10 U dose was known to elicit a maximal inhibition in other tissues, and the doses selected are within the relevant range to better determine the microvascular response. These doses are believed to be physiologically relevant to injections in humans. Vessel diameters were measured immediately after toxin addition and every 5 minutes thereafter, for a total of 20 minutes.

Six preparations were used as experimental controls. Vehicle control preparations were first treated with gallamine, and then 100 μL 0.9% saline was added to the bath. After a 20-minute observation period, 10 U denatured BoNTA was added to the bath. The BoNTA was denatured by boiling the reconstituted toxin until a clear-to-cloudy change in the solution was evident.

Data were analyzed with a mixed models analysis of variance with statistical significance at $\alpha \leq 0.05$. Vessel diameters were compared over time and between treatment groups relative to the vessel diameter obtained after the addition of gallamine. Diameters recorded after the addition of gallamine served as a covariate. Data were reported $\pm$ standard error of the mean.

Measurement of systemic arterial pressure

Four rats were prepared as described earlier. A pressure-tipped catheter (1.0 French; Millar Instruments, Houston, TX) was inserted through the femoral artery to the bifurcation of the aorta to measure systemic arterial pressure. Preparations were treated first with gallamine followed by 10 U BoNTA and 20 minutes observation. Vessel diameters and arterial pressure were recorded (pressures were continuously recorded with IOX software [EMKA Technologies, Falls Church, VA]).

Measurement of maximal arteriole dilation

Sodium nitroprusside (SNP, 30 μM; Nitropress, Abbott Labs, N. Chicago, IL) was added to the bath 20 minutes after the addition of BoNTA to assess the maximal arteriolar vasodilatory potential.[20] Vessel diameters were recorded until maximal dilation was achieved.

Measurement of adrenergic contribution to vasodilation

Tetrodotoxin (10 μM; Sigma-Aldrich, St. Louis MO) treatments assessed the sympathetic contribution to vasodilation. Four additional cremaster preparations were used to obtain gallamine baseline measurements, after which tetrodotoxin was added and vessel diameters measured at 5-minute intervals for 30 minutes. The BoNTA (10 U) was added, and diameters were recorded at 5-minute intervals for 20 minutes. Sodium nitroprusside was added, and the maximal arteriole dilation was recorded. The SNP was allowed to be degraded and the vessel returned to its post-BoNTA-treated diameter. Phenylephrine (50 μM; Baxter, Irvine, CA) was then added to the microvascular bath to demonstrate that the vessel was still capable of constriction.

The contributions of α1-AR and α2-AR mediated vasodilation were assessed using prazosin (α1 antagonist, 0.1 μM; Sigma) and rauwolscine (α2 antagonist, 1 μM; Sigma). Three preparations received prazosin following gallamine baseline measurements, and vessel diameters were recorded at 5-minute intervals for 20 minutes. Rauwolscine was added, and diameters were measured for another 20 minutes, and finally, 10 U BoNTA was added, with an additional 20-minute observation period. Sodium nitroprusside was added to elicit maximal vasodilation, followed by phenylephrine to verify inhibition of the αa-ARs. This protocol was repeated with 3 additional rats with the addition of prazosin and rauwolscine reversed.

RESULTS

Microvessel diameter did not change following Krebs and gallamine addition to the tissue bath ($P = .479$). The average arteriole diameter was 59.7 μm $\pm$ 2.0 μm. Similar-sized vessels were used in all preparations (Table 1).

The BoNTA treatment resulted in significant arteriolar vasodilation of 13% to 15% ($P < .001$; Fig. 1) with significant dose and time effects. Arteriolar diameters in the BoNTA treatment groups were statistically significant compared to each control treatment (denatured BoNTA and saline groups; $P < .001$). The BoNTA elicited a dose-dependent and time-dependent increase in vessel diameters that was significant compared to baseline at all time points ($P < .001$; Fig. 1). Maximal

TABLE 1. Vessel Diameters (μm) Studied in BoNTA Microvascular Preparations

Drug (+ min)	Denatured BoNTA	4 U BoNTA	6 U BoNTA	10 U BoNTA
Gallamine triethiodide	57.3 ± 5.5	60.0 ± 5.0	57.8 ± 5.2	60.0 ± 3.3
BoNTA +5	57.1 ± 5.4	63.9 ± 5.1	64.2 ± 6.7	66.5 ± 3.5
BoNTA +10	57.2 ± 5.5	65.5 ± 5.1*	65.1 ± 6.7*	67.1 ± 3.7*
BoNTA +15	57.2 ± 5.4	65.3 ± 5.1*	65.3 ± 6.7*	67.9 ± 3.4*
BoNTA +20	57.4 ± 5.5	65.4 ± 5.1*	65.3 ± 6.7*	68.2 ± 3.2*

*P < .001 when compared to denatured BoNTA vessel diameters. Reported with standard error of the mean.

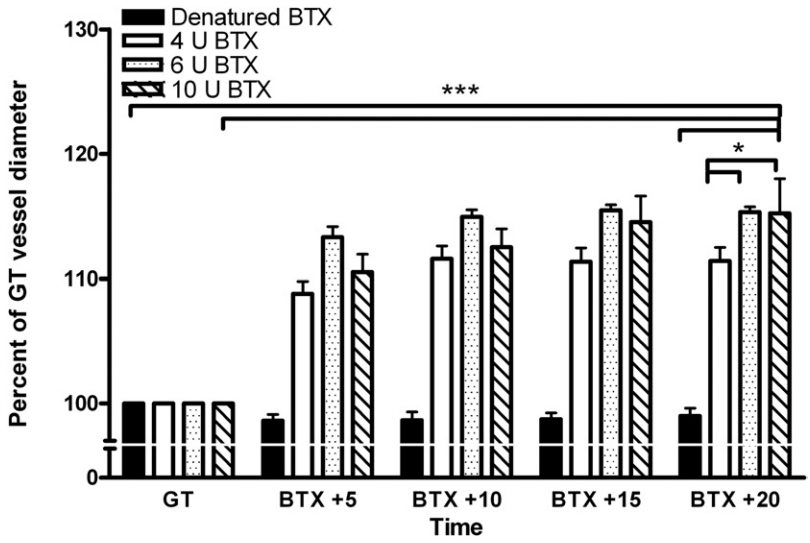

FIGURE 1: Addition of BoNTA results in dose-dependent vasodilation. The BoNTA induced a dose-dependent and time-dependent vasodilatory response. Maximal vasodilation is observed within 15 minutes after treatment and is sustained for the 20-minute observation period. The control denatured BoNTA (10 U) elicited no significant changes in vessel diameter. BTX, botulinum neurotoxin-A; *, $P < .05$; ***, $P < .001$. (Copyright Wake Forest University, Orthopedics.)

dilation occurred 10 minutes after BoNTA application and was maintained for the 20-minute observation period. Both 6 U and 10 U of the toxin produced a significantly greater dilatory response than 4 U ($P = .0335$ and $P = .0255$ for 6 U and 10 U versus 4 U, respectively).

Control animals were observed for 50 minutes to ensure that systematic time-dependent effects did not cause vasodilation. Neither the saline treatments (used to reconstitute BoNTA) nor the addition of denatured BoNTA elicited a significant change in vessel diameter over time (Fig. 1).

Local BoNTA (10 U) treatment did not alter systemic arterial pressure (n = 4). Mean arterial pressure was 75 ± 6 mm Hg before BoNTA and 74 ± 6 mm Hg after BoNTA. The average absolute difference between pre-BoNTA and post-BoNTA arterial pressure was 2 ± 1 mm Hg.

Tetrodotoxin treatment confirmed that vasodilation was the result of inhibition of neurotransmitter release. Arteriolar diameter increased 12% ± 1% ($P < .001$) after tetrodotoxin, which was similar to the vasodilation seen with larger BoNTA doses (Fig. 2). The BoNTA added to tetrodotoxin-treated muscle did not cause additional vasodilation. Vessel viability after toxin treatment was confirmed by treating with SNP to elicit maximal vasodilation (Fig. 3). After the SNP was metabolized, phenylephrine was used to cause vasoconstriction and confirm an intact vasoconstrictive mechanism.

To establish the adrenergic component of the observed response to BoNTA, α1-ARs and α2-ARs antagonists were used. Prazosin inhibition of α1-ARs resulted in vasodilation of 12% ± 3%, which increased to a total 13% ± 3% after treatment with α2-AR antagonist rauwolscine. When the order of drug addi-

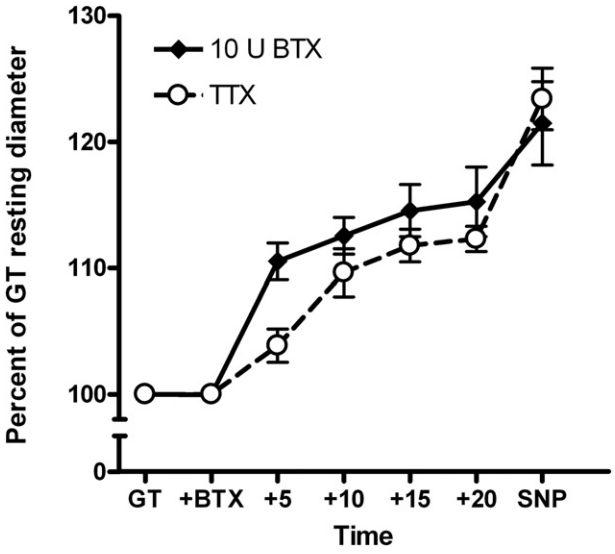

FIGURE 2: The BoNTA and tetrodotoxin induce similar vasodilatory responses. Vasodilation observed following the addition of tetrodotoxin parallels changes with BoNTA. Both tetrodotoxin and BoNTA attenuate neuronal release of neurotransmitters. Vasodilation induced by both agents might be due to the blockade of sympathetic control. Vasodilatory responses following sodium nitroprusside reflect maximal vasodilation. TTX, tetrodotoxin; BTX, botulinum neurotoxin-A; SNP, sodium nitroprusside. (Copyright Wake Forest University, Orthopedics.)

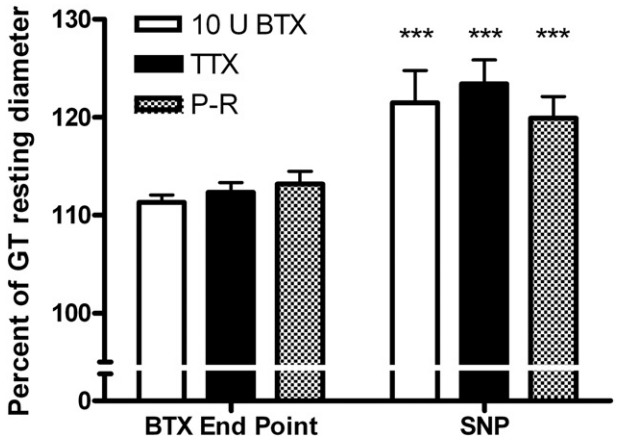

FIGURE 3: The SNP is able to further elicit vasodilation beyond the maximal dilation achieved with all drug treatments. To demonstrate that additional vessels' dilatory mechanisms were still intact following drug addition, SNP was added to achieve maximal vessel dilation. All SNP diameters were statistically significant from their respective BTX end point diameters. GT, gallamine triethiodide; BTX, botulinum neurotoxin-A; TTX, tetrodotoxin; P-R, prazosin, rauwolscine; SNP, sodium nitroprusside; ***, $P < .001$). (Copyright Wake Forest University, Orthopedics.)

tion was reversed, rauwolscine increased vessel diameter by 4% ± 1% and to a maximum of 12% ± 2% after prazosin treatment. Addition of 10 U BoNTA to the α-AR antagonist–treated microvascular preparations did not result in statistically significant increases in vessel diameter (Fig. 4). The addition of SNP caused further vasodilation (Fig. 3). The subsequent addition of phenylephrine to the α-AR–inhibited microvascular preparation did not result in vasoconstriction (data not shown).

The topical application of SNP elicited a maximal vasodilatory response for all drug treatments tested ($P < .001$; Fig. 3). The previously reported dose of SNP used in the current study (30 μM) was sufficient to maximally dilate the vessel.[20]

DISCUSSION

Treatment of the microvasculature with BoNTA causes an increase in arteriolar diameter and a consequent theoretical increase in blood flow. This study supported the hypothesis that the vasodiliatory response observed after BoNTA administration is a result of inhibition of the sympathetic nervous system on local blood vessels. Sympathetic nerves influence local peripheral vascular resistance through the vasoconstrictor, norepinephrine.[28] In addition to nervous system control, autoregulation is accomplished through myocyte paracrine signaling to meet local metabolic demand while buffering local transient changes in blood pressure. This autoregulatory component was eliminated by paralyzing the striated muscle with gallamine, a competitive muscarinic antagonist.[29] Gallamine treatment resulted in a slight vasoconstriction in arteriolar diameter that is likely attributable to the muscle's reduced metabolic demand. Pretreatment with gallamine minimized the confounding effects of BoNTA-mediated muscle paralysis and isolated BoNTA's blockade of sympathetic neural transmission.[30]

Inhibition of norepinephrine release from vascular smooth muscle nerve terminals resulted in relaxation of arterial smooth muscle and increased vessel diameters. The observed maximal response might have been due to a saturation of the nerve terminals or simply sufficient synaptosomal-associated protein 25 cleavage to block soluble N-ethylmaleimide-sensitive factor attachment protein receptor–mediated neurotransmission.[32] Maximal vasodilation as a result of BoNTA treatment was generally seen within 10 minutes of drug administration and was sustained for the duration of the protocol.

Several studies reported evidence of increased blood flow in both animals and humans following BoNTA injection.[6–10,19,26] A study of BoNTA for the treatment

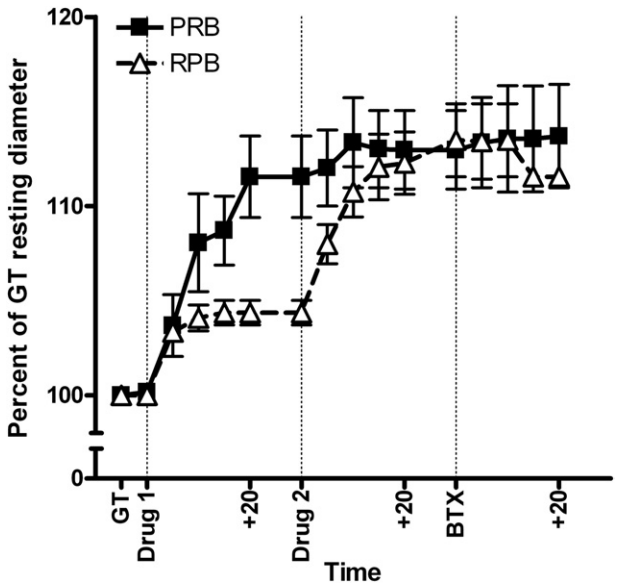

FIGURE 4: Assessment of adrenergic contribution to vasodilation through the addition of α-AR antagonists. The α1-AR antagonist prazosin and the α2-AR antagonist rauwolscine were added sequentially to the preparation. Then BoNTA was added. Vasoactive responses were predominately α1-AR mediated with a smaller α2-AR component. The order of the drug addition is indicated in the legend. One α-AR antagonist was added at point Drug 1, followed by a 20-minute observation period. The second α-AR antagonist was added at point Drug 2, followed by another 20-minute observation. Then BoNTA was added. (Order of addition: PRB, prazosin, rauwolscine, BoNTA; RPB, rauwolscine, prazosin, BoNTA; BTX, botulinum neurotoxin-A). (Copyright Wake Forest University, Orthopedics.)

of lateral epicondylitis reported increased blood flow at 3 and 12 months following injection.[6] The BoNTA increased blood flow in a rabbit model for 8 weeks, and BoNTA effects are evident for 3–6 months in rats and mice depending on the dose and volume injected.[11,17–18] Based on the proposed mechanism of action, BoNTA should have similar effects on vasodilation through sympathetic inhibition as it does on the nerve terminals at the neuromuscular junction. In humans, BoNTA could be expected to be effective for at least 3–6 months.[4–5]

Treatment with tetrodotoxin and α-AR antagonists prazosin and rauwolscine confirmed that the vasodilation observed with BoNTA resulted from vasomotor sympathetic inhibition. Tetrodotoxin selectively binds to sodium channels and prevents terminal membrane depolarization and subsequent neurotransmitter release. Tetrodotoxin also blocks vasoconstrictor nerves to cause vasodilation and increased blood flow.[33–35] Although there is a statistically significant increase in vessel diameter with tetrodotoxin over resting diameters ($P < .001$), no significant difference existed between the maximal dilation achieved with tetrodotoxin and BoNTA. Treatment with SNP elicited further vasodilation significantly greater than endpoint values obtained after administration of BoNTA and tetrodotoxin treatment ($P < .001$, Fig. 3). The observations following tetrodotoxin treatment suggest that the vasodilation resulting from treatment with BoNTA is the result of attenuating the release of the neurotransmitters participating in maintenance of vessel tone.

The α-adrenergic contribution to vasoconstrictor tone was assessed after establishing that the vasodilation was the result of neural inhibition. The α-AR antagonists, prazosin (α1 inhibitor) and rauwolscine (α2 inhibitor), were sequentially added to the preparations (Fig. 2). Selectively inhibiting the α-ARs demonstrated the characteristic dilatory pattern associated with dual mediation of vasoconstriction consisting predominately of an α1-AR regulatory component and showed that drug concentrations were appropriate. The SNP treatment confirmed the vessel's ability to vasodilate (Fig. 3), and treatment with phenyleprine demonstrated sufficient blockade of the α-ARs (data not shown). Because treatments with the adrenergic antagonists resulted in vasodilation similar to that elicited by BoNTA, and BoNTA treatment of the blocked vessels did not cause additional vasodilation, the observed vasodilation likely results from BoNTA's blockade of the sympathetic control of vascular tone.

The observed vasodilation with BoNTA treatment should substantially increase local blood flow. Increases of 12% to 14% in vessel diameter cause concurrent increases of 25% to 30% in cross-sectional area. The Poiseuille law established the reduction in resistance to flow as a function of the pressure difference between 2 ends of the vessel and the radius of the blood vessel to the fourth power. The BoNTA treatment did not alter systemic pressure (Fig. 3), so when the Poiseuille law is applied to a 14% increase in vessel radius with an unchanged systemic arterial pressure, the result would be a 69% reduction in resistance and a concomitant 69% increase in blood flow.

The increase in flow is even more impressive because metabolic demand of the muscle tissue was held constant by striated muscle paralysis. Normal metabolic autoregulation of blood flow to the muscle would be expected to reduce perfusion after paralysis,[21,36] but the observed BoNTA effects are consistent with vasodilation following tetrodotoxin administration.[34–35] Similar responses were seen in rats with sympathetic denerva-

tion of the cremaster muscle; these vessels dilated but remained active to topical administration of vasoactive substances.[37] Because BoNTA did not change systemic pressure and elicited vasodilation, the actions of BoNTA would be expected to produce increases in blood flow in a manner similar to those of tetrodotoxin or sympathectomy with denervation.

Several physiologic parameters were investigated in this study. The BoNTA is reported to result in an increase in the angiogenic vascular endothelial growth factor, which might increase long-term blood flow.[19] Further investigation into the molecular mechanisms is warranted. The interpretation of our results is somewhat limited by the bath application of the toxin; however, the cremaster preparation is a well-established model for studying the microvasculature that closely approximates physiologic conditions and allows *in vivo* analysis of the real-time pharmacologic treatments.[27]

These data demonstrate that BoNTA rapidly alters the skeletal muscle microcirculation by causing vasodilation that results in a calculated increase in blood flow. Abnormal participation of adrenergic receptors on the cutaneous vasculature in response to environmental and emotional stimuli is suggested to be responsible for inducing vasoconstriction in Raynaud patients.[38] Increased blood flow and improvement in patients' symptoms is seen after periarterial sympathectomy in both animal studies and patients with Raynaud phenomenon.[39–40] The ability of BoNTA to inhibit sympathetic nervous input reduces vasoconstriction, which is the most likely mechanism for improvement seen in Raynaud phenomenon patients following BoNTA injection.[7–10] The increased blood flow accompanying BoNTA administration might also be considered for other vaso-occlusive disorders and might serve as an adjunct therapy to promote healing.

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