Nitric Oxide–Induced Decrease in Calcium Sensitivity of Resistance Arteries Is Attributable to Activation of the Myosin Light Chain Phosphatase and Antagonized by the RhoA/Rho Kinase Pathway

Steffen-Sebastian Bolz, MD; Lukas Vogel; Daniel Sollinger; Roland Derwand, MD; Cor de Wit, MD; Gervaise Loirand, PhD; Ulrich Pohl, MD, PhD

Background—NO-induced dilations in resistance arteries (RAs) are not associated with decreases in vascular smooth muscle cell Ca^{2+}. We tested whether a cGMP-dependent activation of the smooth muscle myosin light chain phosphatase (MLCP) resulting in a Ca^{2+} desensitization of the contractile apparatus was the underlying mechanism and whether it could be antagonized by the RhoA pathway.

Methods and Results—The Ca^{2+} sensitivity of RA was assessed as the relation between changes in diameter and [Ca^{2+}]_{i} in depolarized RA (120 mol/L K^{+}) exposed to stepwise increases in Ca^{2+}_{ex} (0 to 3 mmol/L). Effects of 10 μmol/L sodium nitroprusside (SNP) on Ca^{2+} sensitivity were determined before and after application of the soluble guanylate cyclase inhibitor ODQ (1 μmol/L) and the MLCP inhibitor calyculin A (120 nmol/L) and in presence of the RhoA-activating phospholipid sphingosine-1-phosphate (S1P, 12 nmol/L). SNP-induced dilations were also studied in controls and in RAs pretreated with the Rho kinase inhibitor Y27632 or transfected with a dominant-negative RhoA mutant (N19RhoA). Constrictions elicited by increasing Ca^{2+}_{ex} were significantly attenuated by SNP, which, however, left associated increases in [Ca^{2+}]_{i} unaffected. This NO-induced attenuation was blocked by ODQ, calyculin A, and S1P. The S1P-induced translocation of RhoA indicating activation of the GTPase was not reversed by SNP. Inhibition of RhoA/Rho kinase by N19RhoA or Y27632 significantly augmented SNP-induced dilations.

Conclusions—NO dilates RA by activating the MLCP in a cGMP-dependent manner, thereby reducing the apparent Ca^{2+} sensitivity of the contractile apparatus. MLCP inactivation via the RhoA/Rho kinase pathway antagonizes this Ca^{2+}-desensitizing effect that, in turn, can be restored using RhoA/Rho kinase inhibitors. (Circulation. 2003;107:3081-3087.)

Key Words: microcirculation ■ muscle, smooth ■ nitric oxide ■ signal transduction ■ vasodilation

Smooth muscle relaxation results from a decrease in intracellular free Ca^{2+} ([Ca^{2+}]_{i}) or a decrease in myofilament Ca^{2+} sensitivity. Most studies in large arteries and cultured vascular smooth muscle cells (VSMCs) demonstrated that NO exerted its dilatory effect by a cGMP-mediated reduction of [Ca^{2+}]_{i}, leading to a decreased phosphorylation of the myosin light chain (MLC_{20}). However, this mechanism seems not to play a predominant role in resistance arteries (RAs). We have previously reported NO-induced dilations of hamster skeletal muscle RAs that occurred without changes in [Ca^{2+}]_{i}. In permeabilized rabbit ileum smooth muscle, Wu et al demonstrated dilations at constant [Ca^{2+}]_{i}, that resulted from activation of the myosin light chain phosphatase (MLCP) and subsequently reduced MLC_{20} phosphorylation. Because the state of MLC_{20} phosphorylation controls actin-myosin interaction, any increase in MLCP activity results in a rightward shift of the [Ca^{2+}]_{i} constriction curve reflecting a reduction of the apparent myofilament Ca^{2+} sensitivity. In the aforementioned study, MLCP was activated by cGMP, the intracellular second messenger of NO. More recent studies have shown that cGMP/cGMP-dependent kinase (cGKIα)-dependent activation of MLCP also occurs in cultured mesangial, VSMCs, and intact arterial smooth muscle, suggesting that the NO-induced reduction of Ca^{2+} sensitivity observed in RA could indeed result from an increase in MLCP activity. Additional evidence for a potential role of MLCP in controlling microvascular tone comes from an earlier study in which we showed that oxidized low-density lipoproteins (oxLDLs) induced contraction by increasing the myofilament Ca^{2+}...
sensitivity via a RhoA/Rho kinase–dependent mechanism. Rho kinase inactivates MLCP by phosphorylation of the myosin-binding subunit (MYPT1), a process that seems to involve a ZIP-like MLCP-associated kinase.

In this study, we investigated whether NO exerts its dilator effect in RA via an activation of MLCP and whether this effect was dependent on cGMP. We additionally studied whether a RhoA/Rho kinase–dependent inactivation of MLCP could modulate this effect of NO. Thus, in addition to the MLCP inhibitor calyculin A, the RhoA/Rho kinase–activating sphingolipid mediator sphingosine-1-phosphat (S1P) was used. To investigate the RhoA pathway, we not only used appropriate pharmacological inhibitors but also inhibited steps of the RhoA signaling cascade by transfecting the vessels with plasmids coding for the specific RhoA inhibitor C3 transferase or targeted mutations of RhoA.

We found that the Ca$$^{2+}$$-desensitizing effect of NO was dependent on cGMP and a functionally intact MLCP. It was antagonized by S1P-induced RhoA/Rho kinase–dependent inhibition of MLCP, suggesting a critical balance in the control of MLCP between NO and RhoA/Rho kinase. The NO-antagonizing effects of RhoA/Rho kinase may contribute to the pathogenesis of hypertension and the functional impairment of endothelium-dependent dilation.

Methods

Drugs

MOPS-buffered salt solution contained (in mmol/L) NaCl 145, KCl 4.7, CaCl$_2$ 1.5, MgSO$_4$ 1.17, NaH$_2$PO$_4$ 1.2, pyruvate 2.0, EDTA 0.02, MOPS 3.0, and glucose 5.0. In depolarizing solution with 120 mmol/L NaCl, CaCl$_2$ was compensatorily reduced to 29.7 mmol/L. Fura 2-AM was purchased from Molecular Probes, and norepinephrine (NE), acetylcholine (ACh), NS1619, and sodium nitroprusside (SNP) were from Sigma Chemicals.

Preparation of Small RA and [Ca$$^{2+}$$]i and Diameter Measurements

The care of the animals and the experimental procedures were in accordance with German animal protection laws. The preparation of the vessels and the technique of Ca$$^{2+}$$ (fura 2) and diameter measurements were previously described. Briefly, RAs (maximal outer diameter, 180 to 250 m) from gracilis muscle of female hamsters were cannulated with micropipettes and studied at the vessels and the technique of Ca$$^{2+}$$ physiological intracellular Ca$$^{2+}$$ store.

Transfection of Intact RA

To transfect plasmids containing C3 transferase or the respective mutated RhoA sequences (N19RhoA, RhoAAla-188) into VSMCs, arteries were incubated for 18 to 21 hours in an artery culture system with culture medium containing antibiotics, the transfectant Effectene (16 μL/mL), and 5 μg of the respective plasmid. Unspecific effects of the transfection procedure were assessed by comparing vascular responses of nontransfected RA and arteries transfected with green fluorescent protein (GFP). In arteries transfected with RhoA-GFP fusion protein, all VSMCs per microscopic field showed GFP-related fluorescence (confocal microscopy, excitation 488 nm, emission 523 to 565 nm; Figure 5b). The technique to transflect intact C3 transferase protein using trans LT was previously described.

Immunofluorescence and Digital Imaging

Arteries were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, blocked with 1% BSA, and incubated with the primary antibody (MLCP: rabbit anti-mouse, 1:200, Covance; RhoA: mouse monoclonal, 1:200, Santa Cruz Biotechnology). FITC-labeled goat anti-rabbit or donkey anti-mouse (1:200 each) were used as secondary antibodies. Images were obtained using a Zeiss LSM410 confocal microscope equipped with a Kr/Ar laser and a 40x/1.2W water immersion objective.

Immunoblotting

Tissue samples of hamster aorta were quick-frozen in liquid nitrogen and homogenized. Cytosolic and particulate fractions were separated by centrifugation of the homogenate at 100 000g (Beckman Coulter, Optima Max-E). Pellets were resuspended in lysis buffer plus 1% Triton-X 100. Protein–matched samples were electrophoresed by SDS-PAGE (7%), transferred to nitrocellulose membranes (Amer sham), and subjected to immunostaining using a polyclonal primary antibody (rabbit anti-mouse, 1:500). An HRP-labeled secondary antibody (goat anti-rabbit, 1:10 000, Santa Cruz) was used with ECLplus (Amersham) to visualize the signal.

Experimental Protocols

Changes in diameter and [Ca$$^{2+}$$], were continuously recorded in 70 vessels from 41 animals. All vessels studied developed spontaneous tone (9.6±1% of maximal diameter). The viability of each vessel was assessed by its constriction to NE (0.3 μmol/L) and a dilation >80% in response to 1 μmol/L ACh.

The apparent Ca$$^{2+}$$ sensitivity of the arteries was assessed by stepwise increasing the extracellular Ca$$^{2+}$$ concentration ([Ca$$^{2+}$$]$_{ex}$, 0 to 3 mmol/L) around the arteries kept in depolarizing solution (120 mmol/L K$^+$.). Depolarization-dependent opening of voltage-gated calcium channels allowed increases in Ca$$^{2+}$$ex to be reproducibly followed by increases in VSMC [Ca$$^{2+}$]. The Ca$$^{2+}$$ sensitivity was assessed under control conditions, in the presence of SNP and in the combined presence of SNP and the respective modulating substance or protein (ODQ, calyculin A, S1P, RhoAAla-188).

Additionally, dose-response curves for SNP were obtained in arteries preconstricted by 0.3 μmol/L NE under control conditions, in the presence of the Rho kinase inhibitor Y27632 (1 μmol/L), or in N19RhoA-transfected arteries.

Statistical Analysis

Dilations are expressed as the following: percent of maximum dilatation=[(dia$_{ex}$–dia$_{NE}$)/(dia$_{max}$–dia$_{NE}$)]×100, with dia$_{ex}$ and dia$_{NE}$ representing steady-state diameters 2 minutes after administration of NE or the respective vasodilator and dia$_{max}$ being the maximal diameter obtained in Ca$$^{2+}$$-free 1 mmol/L EGTA-containing MOPS buffer.

Because of methodological uncertainties in calculating exact values for [Ca$$^{2+}$$], in intact vessels, fluorescence ratios (F$_{510nm}$/F$_{380nm}$) are presented instead. Calibration curves obtained in a cell-free system indicated that the range of ratios observed here (0.4 to 6.3) fitted into the linear range of the curve that comprises physiological intracellular Ca$$^{2+}$$ concentrations (42.2 to 1520 mmol/L).

Steady-state values from different groups were compared with ANOVA followed by post hoc analysis of the means. Data are presented as mean±SEM. Differences were considered significant at $P<0.05$.

Curves were compared using a nonlinear regression analysis applied first to every individual curve and then to the pooled data. Curves were considered to be different if the F-test indicated a significantly smaller sum of squares for the deviations in each individual fit compared with the deviation in the fit to the pooled data.
Results

NO-Induced Desensitization of the Contractile Apparatus Is Dependent on cGMP

Stepwise constrictions of K⁺-depolarized arteries occurring in parallel to increases in Ca²⁺ᵪ were significantly attenuated in the presence of 10 μmol/L SNP (P<0.05, n=7, Figure 1). Increases in [Ca²⁺], were virtually identical in control and SNP-treated arteries for any given concentration of Ca²⁺ᵪ (Figure 2), suggesting that NO decreased the myofilament Ca²⁺ sensitivity. This NO effect was entirely mediated by cGMP because it was blocked after inhibition of the soluble guanylate cyclase with ODQ (1 μmol/L, P<0.05, n=7, Figure 1). [Ca²⁺], was not significantly different in control, SNP-treated RA, or SNP/ODQ-treated RA.

MLCP Mediates the Ca²⁺-Desensitizing Effect of NO

The potential involvement of the MLCP in NO-induced Ca²⁺ desensitization was assessed in RA pretreated with the MLCP inhibitor calyculin A at a concentration (120 nmol/L) considered to be specific for the MLCP. Calyculin A almost abolished the desensitizing effect of NO (P<0.05, n=7, Figure 2), suggesting that this effect requires a fully functional MLCP. None of the myofilament Ca²⁺ sensitivity–modulating treatments affected VSMC [Ca²⁺], (Figure 2).

Activation of RhoA/Rho Kinase Antagonizes NO-Induced Desensitization and Dilations

At concentrations <1 μmol/L, the sphingolipid mediator S1P induced constrictions of RA that were abolished after treatment with the RhoA inhibitor C3 transferase (n=7) or the Rho kinase inhibitor Y27632 (n=7, Table). S1P-induced activation of RhoA/Rho kinase induced a translocation of the MLCP subunit MYPT1 to the VSMC plasma membrane (Figure 3), an effect that has recently been linked to inhibition of MLCP. S1P-induced translocation was absent in arteries transfected with the dominant-negative RhoA mutant N19RhoA and those pretreated with Y27632 (1 μmol/L, Figure 3).

S1P (10 nmol/L, n=11), which per se increased the Ca²⁺ sensitivity only in a medium concentration range of Ca²⁺ᵪ (0.25 to 0.75 mmol/L), abolished the NO-induced Ca²⁺ desensitization over the whole range of Ca²⁺ᵪ (Figure 4a).

RhoA showing a cytosolic localization under resting conditions was translocated to the membrane after stimulation with 100 nmol/L S1P (Figures 4b and 4c). This translocation

Effects of RhoA (C3 Transferase, n=7) or Rho Kinase (Y27632, n=7) Inhibition on S1P-Induced Vasoconstrictions

<table>
<thead>
<tr>
<th>S1P, μmol/L</th>
<th>Control, % of dia_max</th>
<th>Y27632, % of dia_max</th>
<th>C3 Transferase, % of dia_max</th>
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</thead>
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<td>0.7±0.3*</td>
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<tr>
<td>0.01</td>
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<td>0.4±0.2*</td>
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<td>3.4±1.8*</td>
<td>2.8±0.7*</td>
</tr>
<tr>
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<td>45.0±2.0</td>
<td>20.0±7.2*</td>
<td>−18.1±2.9*</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*Significant differences (P<0.05) vs control.
was not affected by subsequent addition of SNP (10 μmol/L, 3 minutes, Figure 4d).

SNP(1 μmol/L)-induced dilations after preconstriction with 1 μmol/L S1P were significantly smaller (by 64% vs 4% each, P<0.001).

**Discussion**

This study demonstrates that NO decreases the myofilament Ca²⁺ sensitivity in VSMCs of hamster skeletal muscle RA. This effect was attributable to an activation of smooth muscle MLCP, presumably resulting in a subsequent decrease in MLCP activity, cGK I phosphorylation and hence a vasodilation that did not require a decrease in [Ca²⁺]. Activation of the RhoA/Rho kinase pathway that has recently been shown to mediate Ca²⁺-sensitizing effects of a variety of agonists via inhibition of MLCP, opposed the NO-induced desensitization. Both pathways, the Ca²⁺-sensitizing NO pathway as well as the Ca²⁺-sensitizing RhoA/Rho kinase pathway, terminate in modulation of MLCP activity, suggesting that its level of activity is a major determinant of microvascular tone. The finding that dilations in response to NO occur independently of changes in VSMC [Ca²⁺], is at variance with several studies demonstrating NO-induced decreases in [Ca²⁺]. The latter were cGMP-mediated and involved various mechanisms such as activation of Ca²⁺-ATPases located either at the plasma membrane or intracellular Ca²⁺ stores, inhibition of phospholipase C or IP₃ receptors, and activation of Ca²⁺-activated K⁺ channels. None of these mechanisms seems to be effective in the microvessels studied here, because NO-induced dilations were not associated with decreases in VSMC [Ca²⁺]. Recent studies in other preparations also challenged the classical model of action for NO. However, the cellular mechanism of NO-induced, Ca²⁺-independent dilations in microvessels remains unclear. Wu et al demonstrated Ca²⁺-independent relaxations by 8-Br-cGMP in skinned rabbit ileum smooth muscle that were based on activation of MLCP. Accordingly, cGMP-dependent relaxations of mesangial cells were also shown to depend on MLCP activation. Recently, Surks et al provided a possible molecular basis demonstrating that CGK1α, which finally mediates the effects of NO and cGMP, is targeted to the contractile apparatus by a leucine zipper interaction with the MLCP myosin-binding subunit. The results of the present study strongly suggest that the Ca²⁺-desensitizing effect of NO in RA was also mediated by a cGMP-dependent activation of MLCP, because inhibition of the cGMP-generating enzyme soluble guanylate cyclase by ODQ as well as MLCP inhibition by calyculin A used in a concentration shown to be specific for the SMPP-1 abolished the NO effect.
We additionally hypothesized that RhoA/Rho kinase that inhibit the MLCP via phosphorylation of MYPT1 antagonize the NO-induced Ca\textsuperscript{2+} desensitization. The phospholipid S1P known to stimulate RhoA/Rho kinase–dependent processes such as proliferation, migration, matrix reassembly, and angiogenesis\textsuperscript{21–23} was used to activate RhoA/Rho kinase. S1P induced strong constrictions in RA that were abolished by C3 transferase or the Rho kinase inhibitor Y27632, indicating that they were mediated by RhoA/Rho kinase. Furthermore, S1P induced a translocation of MYPT1 to the plasma membrane, an effect that was absent in arteries transfected with N19RhoA or pretreated with Y27632 and, therefore, RhoA/Rho kinase dependent. The translocation of MYPT1 has recently been suggested to underlie the inhibition of MLCP.\textsuperscript{14} Indeed, NO-induced Ca\textsuperscript{2+} desensitization that depends on a functional MLCP was completely prevented in the presence of S1P.

Accordingly, the amplitude of NO-induced dilations mediated by MLCP activation was modulated by the respective constraining stimulus with significantly smaller dilations in S1P-preconstricted (activation of RhoA) than NE-preconstricted (increase in VSMC Ca\textsuperscript{2+}) arteries. ACh-induced dilations (in the presence of L-NA/indomethacin EDHF-mediated and strictly Ca\textsuperscript{2+} dependent)\textsuperscript{2} that do not require an activated MLCP remained almost unaffected in S1P-preconstricted arteries. The fact that these Ca\textsuperscript{2+}-dependent dilations were affected at all, albeit to a lesser extent, presumably reflects the reduced activity of MLCP in S1P-constricted arteries.

Inhibition of basal RhoA/Rho kinase activity significantly augmented NO-based dilations. However, it did not affect Ca\textsuperscript{2+}-dependent and exclusively EDHF-mediated dilations after ACh\textsuperscript{2} or dilations induced by the K\textsubscript{r} channel opener NS1619. This suggests that even in resting VSMC, part of the desensitizing NO effect is physiologically and specifically antagonized by RhoA. In fact, there seems to be a high RhoA activity in unstimulated arteries because C3 transferase substantially decreased myofilament Ca\textsuperscript{2+} sensitivity. Virtually identical constrictions at maximal Ca\textsuperscript{2+}, however, suggest that the number of potentially recruitable actin-myosin interactions were similar in control and C3 transferase-transfected arteries, arguing against unspecific cytoskeletal effects of the RhoA inhibition. Unaffected responses to NE and ACh in N19RhoA-, C3 transferase-, or Y27632-treated arteries add to this interpretation.

A high basal activity of RhoA/Rho kinase resulting in a low MLCP activity in unstimulated arteries may explain the weak effect of calyculin A on microvascular resting tone and the confinement of S1P-induced Ca\textsuperscript{2+}-sensitizing effects to low [Ca\textsuperscript{2+}]i concentrations. A moderately active MLCP could only antagonize the MLCK at low Ca\textsuperscript{2+} but would become increasingly ineffective with increasing [Ca\textsuperscript{2+}], and hence high MLCK activity. At this point any additional inhibition of MLCP as induced by S1P becomes irrelevant for the regulation of microvascular tone.

A physiological antagonism between NO and RhoA/Rho kinase in the microvasculature may be pathophysiologically important, because several cardiovascular diseases are associated with activation of RhoA/Rho kinase.\textsuperscript{24} Interestingly,
both pathways terminate in phosphorylating MYPT1\(^{5,6,17}\) suggesting that this protein subunit is the molecular substrate for this antagonistic crosstalk.

In contrast to our results pointing to a direct activation of MLCP underlying the NO-induced Ca\(^{2+}\) desensitization in RA, Sauzeau et al\(^{16}\) recently demonstrated direct inactivation of RhoA through NO-induced cGK1-mediated phosphorylation in large arteries. However, we found no differences in NO-induced Ca\(^{2+}\)-desensitizing effect between control and arteries that overexpressed RhoA Ala-188, a mutant that cannot be phosphorylated by cGK1, suggesting that a direct inactivation of RhoA is not effective in RA.

Interestingly, the slope of the control curve of RhoA\(^{Ala-188}\) transfected arteries differed from that of nontransfected arteries. Unspecific effects of the transfection method are unlikely, because overexpression of GFP did not affect vascular responses to NO in the microvasculature, allowing new pharmacological approaches to enhance the efficacy of NO under pathophysiological conditions.

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References


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