cGMP-Dependent Protein Kinase Mediates NO- but not Acetylcholine-Induced Dilations in Resistance Vessels In Vivo

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Abstract—cGMP and cGMP-dependent protein kinase type I (cGKI) mediate the dilation of large vessels in response to NO and acetylcholine (ACh). However, the physiological significance of the NO/cGMP/cGKI pathway in resistance vessels is controversial. Here, we analyzed NO- and ACh-induced dilations of arterioles in cGKI-deficient (cGKI<sup>−/−</sup>) or endothelial NO synthase–deficient (eNOS<sup>−/−</sup>) mice. Mean arterial pressure was similar in cGKI<sup>−/−</sup> and wild-type mice (∼105 mm Hg). Pressure drops in response to intracarotid bolus application of the NO donor sodium nitroprusside (SNP) were almost abolished in cGKI<sup>−/−</sup> mice, whereas ACh-induced pressure decreases remained intact in cGKI<sup>−/−</sup> and eNOS<sup>−/−</sup> mice. The direct observation of arterioles in the cremaster muscle by intravital microscopy showed impaired SNP-induced dilations in cGKI<sup>−/−</sup> mice (by ∼80%) and normal ACh-induced dilations in cGKI<sup>−/−</sup> and eNOS<sup>−/−</sup> mice. ACh-induced dilations in eNOS<sup>−/−</sup> mice were attenuated by iberiotoxin (by ∼50%), indicating that they were mediated in part by Ca<sup>2+</sup>-activated K<sup>+</sup> channels, but not by inhibitors of cyclooxygenase or p450-monoxygenases. We conclude that cGMP and cGKI are the major effectors of NO to induce acute dilations of murine resistance vessels. However, the NO/cGMP/cGKI pathway is not essential for ACh-induced dilation of arterioles and for basal blood pressure regulation in mice. (Hypertension. 2004;44:952-955.)

Key Words: nitric oxide ■ endothelium ■ microcirculation

Nitric oxide plays an integral role in the control of vascular tone. Relaxation of smooth muscle by NO is mediated, at least in part, by a rise in cyclic GMP, which has several targets, such as cGMP-regulated cyclic nucleotide phosphodiesterases, cGMP-dependent protein kinase type I (cGKI), and, at high concentrations, cAMP-dependent protein kinase. In vitro studies with isolated vessels of cGKI-deficient (cGKI<sup>−/−</sup>) mice showed that cGKI contributes to relaxation of the aorta and arteria tibialis in response to NO. However, the role of the NO/cGMP/cGKI pathway in resistance vessels in vivo is controversially discussed. Endothelium-dependent dilation of arterioles triggered by acetylcholine (ACh) might involve NO, prostanoids, and, as endothelium-derived hyperpolarizing factor (EDHF). Although ACh dilations are mediated in large vessels in mice nearly exclusively by NO/cGMP/cGKI, EDHF seems to play an important role in smaller arteries and arterioles, possibly via activation of Ca<sup>2+</sup>-dependent K<sup>+</sup> channels, which suggests that dilator pathways might be very distinct with respect to vessel size. In this study, we investigated the physiological relevance of the NO/cGMP/cGKI pathway to the regulation of tone in resistance vessels by recording arterial pressure and by intravital microscopy of the cremaster microcirculation in cGKI<sup>−/−</sup> or endothelial NO synthase–deficient (eNOS<sup>−/−</sup>) mice.

Materials and Methods

Animals and Arterial Pressure Recording

Experiments were performed in 6- to 8-week-old cGKI<sup>−/−</sup> or eNOS<sup>−/−</sup> mice and litter- or age-matched wild-type (wt) controls on a 129/Sv (cGKI<sup>−/−</sup>) or C57BL/6 (eNOS<sup>−/−</sup>) genetic background, in accordance with the German animal protection law. Mean arterial pressure (MAP) was measured in awake female animals via a catheter that was inserted in the right carotid artery during short anesthesia as described. After obtaining baseline pressure values during a 1-hour period, sodium nitroprusside (SNP) or ACh was injected as a single bolus into the carotid artery at increasing dosages (from 0.05 to 15 nmol) in a volume of 50 μL. Pressure was allowed to return to control values before the next bolus was applied, which normally occurred within 5 to 10 minutes depending on the dosage used. All mice were killed by an overdose of anesthesia at the end of the protocol.

Microcirculatory Studies

Intravital microscopy was performed in the cremaster muscle of anesthetized mice (droperidol [20 mg/kg], fentanyl [0.1 mg/kg], midazolam [2 mg/kg] IV) as described. In each animal, 7 to 12 arterioles were observed using a microscope (Metallux; Leitz) equipped with a video camera. Diameters were measured shortly before and during the local superfusion of ACh (0.1 to 10 μmol/L),...
SNP (0.1 to 10 μmol/L), or S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 1 to 10 μmol/L). Increasing concentrations of vasoactive drugs were applied consecutively, with a recovery period of 5 minutes after washout. In some experiments, dilator responses were restudied in the presence of the cyclooxygenase inhibitor indomethacin (3 μmol/L), the reversible inhibitor of p450-monoxygenases sulfaphenazole (20 μmol/L), the suicide inhibitor of the p450-monoxygenase 17-octadecynoic acid (ODYA; 100 μmol/L), or the specific blocker of large-conductance Ca2+-dependent K+-channels (BKCa) iberiotoxin (0.1 μmol/L). The applied concentrations of these inhibitors have been shown previously to be effective in hamster microcirculation.11 The inhibitors were added continuously to the superfusion 30 minutes before the protocol was repeated, except for iberiotoxin, which was applied for only 15 minutes before restudying the dilator responses. Experiments lasted typically between 3 and 5 hours. The maximal diameter of the arterioles was measured during superfusion of a combination of different vasodilators (adenosine [100 μmol/L], SNP [100 μmol/L], and ACh [100 μmol/L]) at the end of the experimental protocol, and the animal was euthanized by an overdose of anesthesia.

Statistics and Calculations
Vascular tone is given as the quotient of the resting diameter of the vessel divided by its maximum. Changes of the inner diameter of the vessels were normalized to the maximal possible constriction or dilation according to the following relationship. Percentage of maximal response = \((D_{m}-D_{o})/(D_{m}-D_{o})\times100\), where \(D_{m}\) is the diameter observed after treatment and \(D_{o}\) is the control diameter before treatment. \(D_{o}\) is (for dilator responses) the diameter at maximal dilation or (for constrictions) the minimal luminal diameter (zero). Comparisons within groups were performed using paired t tests, and for multiple comparisons, \(P\) values were corrected according to Bonferroni. Data between groups were compared by ANOVA followed by post hoc analysis of the means, with \(P<0.05\) considered significant. Data are presented as mean±SEM.

Results
Responses on Systemic Application of SNP and ACh
MAP in conscious cGKI−/− mice (106±6 mm Hg; n=6 animals; age 51±7 days) was not different from wt littermates (104±6 mm Hg; n=8 animals; age 48±5 days). Heart rate was also similar in both genotypes (wt 393±34 bpm; cGKI−/− 369±50 bpm). Bolus injections of the NO donor SNP into the carotid artery induced dose-dependent transient decreases of MAP in wt mice (Figure 1A). With increasing concentrations, magnitude as well as duration of the response increased. Both factors contributed to the increment of the calculated area under the curve (AUC) with higher SNP concentrations (Figure 1C). In contrast, SNP-induced pressure drops were nearly absent in cGKI−/− animals (Figure 1B and 1C). MAP was elevated in eNOS−/− mice (127±8 mm Hg). How-
ever, SNP-induced decreases of MAP in eNOS−/− mice were comparable to wt controls (Figure 1C). Surprisingly, systemic application of ACh induced similar maximal pressure drops in wt, cGKI−/−, and eNOS−/− mice (Figure 1D).

**Dilations in the Cremaster Muscle Microcirculation In Vivo**

Arterioles with a diameter between 14 and 94 μm were studied (wt 31±3, n=42 in 4 animals; cGKI−/− 27±2 μm, n=67 in 6 animals; P=0.22). Arteriolar resting tone was not different between genotypes (wt 0.34±0.03; cGKI−/− 0.35±0.02) reflecting the normotension in cGKI−/− mice. SNP induced a concentration-dependent arteriolar dilation in wt mice (Figure 2A). Significant dilations were already observed at the lowest SNP concentration used (0.1 μmol/L) and strongly reduced at higher (≥1.0 μmol/L) SNP concentrations compared with wt controls (Figure 2A). Similar results were obtained using the NO donor SNAP. Dilations in response to 1 μmol/L and 10 μmol/L SNP in wt mice were 30±5% and 63±5%, and in cGKI−/− mice, 11±3 and 15±3% (P<0.05 versus wt). Endothelium-dependent dilations on ACh application were preserved in cGKI−/− and eNOS−/− arterioles (Figure 2B and 2C).

**Effect of Different Inhibitors on ACh-Induced Dilations**

Indomethacin (3 μmol/L) did not induce significant diameter changes in cGKI−/− mice or attenuate ACh-induced dilations (data not shown). Likewise, indomethacin did not attenuate ACh-induced dilations in eNOS−/− mice (Figure 2C). Additional application of inhibitors of p450-monoxygenases (20 μmol/L sulfaphenazole or 100 μmol/L ODYA) did not affect arteriolar resting diameter (data not shown) or ACh-induced dilations in eNOS−/− mice (Figure 2D). However, Iberiotoxin (0.1 μmol/L), a specific blocker of large-conductance Ca2+-activated K+ (BKCa) channels, significantly attenuated ACh dilations in eNOS−/− mice (Figure 2C), whereas vascular tone (before 0.24±0.02; after 0.23±0.03) or dilations in response to SNP (10 μmol/L) were not altered (71±4 versus 62±4%).

**Discussion**

Results of the MAP measurements and intravital microscopy of arterioles correlate well with each other and strongly support the following 2 conclusions. First, the acute dilation of resistance vessels by NO is mediated predominantly by cGKI. Dilation is in part cGKI independent only at high, presumably pathological, concentrations of NO. The dilation of cremaster arterioles by NO does not require activation of BKCa channels. These findings are in line with previous results showing that NO can relax the arteria tibialis (which may behave similar to resistance vessels) via cGKI-dependent and cGKI-independent mechanisms that do not absolutely require the activation of BKCa channels. Arterial dilation by the NO/cGMP/cGKI pathway might involve translocation of the small GTPase RhoA, activation of the myosin phosphatase, a decrease in the Ca2+ sensitivity of the contractile machinery, and/or an inhibition of intracellular Ca2+ release. Despite the critical role of cGKI,
conscious cGKI−/− animals were normotensive. This is in agreement with a previous study that reported that cGKI−/− animals were hypertensive only at very young age (≈32 days) but normotensive at older age (≈43 days). Thus, cGKI is apparently not essential for basal blood pressure regulation in 6–8-week-old mice.

Second, endothelium-dependent dilations of resistance vessels triggered by ACh do not require eNOS-derived NO or cGKI because dilations initiated by ACh were preserved in eNOS−/− and cGKI−/− mice, together with previous reports showing normal ACh responses in various vascular beds of eNOS−/− mice. Our findings that ACh-induced decreases in MAP as well as ACh-induced dilations of cremaster arterioles were almost intact in eNOS−/− and cGKI−/− mice, together with previous reports showing normal ACh responses in various vascular beds of eNOS−/− mice, strongly suggest that the NO/cGMP/cGKI pathway is not essential for ACh-triggered dilation of resistance vessels. The ACh-induced dilation of cremaster arterioles in eNOS−/− mice was not affected by indomethacin but attenuated by iberiotoxin. This indicates that the ACh response was not mediated by prostaglandins but at least in part by an EDHF-like activity resulting in the opening of BKCa channels, hyperpolarization, and smooth muscle relaxation. In contrast to coronary and skeletal muscle arteries, this EDHF was apparently not related to metabolites of the cytochrome p450 pathway because 2 chemically distinct blockers of this pathway were without effect. The identity of the EDHF-like activity because 2 chemically distinct blockers of this pathway in arterioles is not essential for ACh-induced dilation of resistance vessels. However, the NO/cGMP/cGKI pathway is not essential for basal blood pressure regulation in 6–8-week-old mice.

Perspectives

This study shows that hypotheses about vasodilator pathways cannot be generalized and need critical evaluation in different vascular beds, especially in vessels of different sizes. As in larger arteries, cGKI is an important effector of the acute effects of NO in resistance vessels. However, the NO/cGMP/cGKI pathway in arterioles is not essential for ACh-induced dilations and basal blood pressure control in mice. These results support the notion that cGMP-dependent pathways in smooth muscle may be more important in acute versus chronic blood pressure regulation and that the systemic hypertension of eNOS−/− mice might be caused not only by a defect in endothelium-dependent vasorelaxation but also by other changes in the knockout animals, such as a disturbed baroreceptor response or an increased release of renin from the kidney into the bloodstream.

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