Mechanisms of NO/cGMP-Dependent Vasorelaxation
Matthias Sausbier, Rudolf Schubert, Viktor Voigt, Christoph Hirneiss, Alexander Pfeifer, Michael Korth, Thomas Kleppisch, Peter Ruth, Franz Hofmann

Abstract—Both cGMP-dependent and -independent mechanisms have been implicated in the regulation of vascular tone by NO. We analyzed acetylcholine (ACh)- and NO-induced relaxation in pressurized small arteries and aortic rings from wild-type (wt) and cGMP kinase I–deficient (cGKI–/–) mice. Low concentrations of NO and ACh decreased the spontaneous myogenic tone in wt but not in cGKI–/– arteries. However, contractions of cGKI–/– arteries and aortic rings were reduced by high concentrations (10 μmol/L) of 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA-NO). Iberiotoxin, a specific blocker of Ca2+-activated K+ (BKCa) channels, only partially prevented the relaxation induced by DEA-NO or ACh in pressurized vessels and aortic rings. DEA-NO increased the activity of BKCa channels only in vascular smooth muscle cells isolated from wt cGKI+/+ mice. These results suggest that low physiological concentrations of NO decrease vascular tone through activation of cGKI, whereas high concentrations of DEA-NO relax vascular smooth muscle independent of cGKI and BKCa. NO-stimulated, cGKI-independent relaxation was antagonized by the inhibition of soluble guanylyl cyclase or cAMP kinase (cAK). DEA-NO increased cGMP to levels that are sufficient to activate cAK. cAMP-dependent relaxation was unperturbed in cGKI–/– vessels. In conclusion, low concentrations of NO relax vessels by activation of cGKI, whereas in the absence of cGKI, NO can relax small and large vessels by cGMP-dependent activation of cAK. (Circ Res. 2000;87:825–830.)

Key Words: cGMP-dependent protein kinase I ■ arteries ■ K+ channels

The NO/cGMP signaling cascade plays an essential role in vascular smooth muscle relaxation, and clinical studies indicate that endothelium-derived NO is involved in normal and pathological blood pressure regulation in humans.1 Targeted inactivation of endothelial NO synthase,2,3 atrial natriuretic peptide (ANP),4 and the ANP receptor guanylyl cyclase-A,6,6 causes hypertension. Animal and in vitro studies also show that NO and ANP decreases blood pressure by relaxation of small arteries and arterioles.7 In most studies, this decrease in vascular tone was associated with increases in cGMP. Some studies suggested that NO reduces vascular tone independent of cGKI.8–11 The mechanisms that are activated by NO or cGMP in smooth muscle are controversial.12–16

The important effector of cGMP, cGKI, is highly expressed in smooth muscle. Deletion of the gene for cGKI can lead to multiple phenotypes, including high blood pressure in young mice.17 Interestingly, and in contrast to previous suggestions,13 adenosine A1 receptor- and cAMP-dependent relaxation of aortic rings was not affected by the lack of cGKI.17 Older cGKI-deficient (cGKI–/–) mice, many of which have multiple infections, have normal or only slightly elevated blood pressure,17 suggesting that cGKI is not absolutely required to lower vascular tone and can be bypassed. A potential alternative pathway could be an increased production of endothelium-derived hyperpolarizing factors (EDHF).18 Activation of large and small KCa channels, calcium-activated chloride channels, or voltage-dependent K+ channels by EDHF or NO would hyperpolarize the membrane, close calcium channels, and reduce cytosolic calcium concentrations ([Ca2+]i).14,18

Thus far, the in vitro analysis of hypertensive knockout mice has been performed on isolated aortic segments. In our studies, we noticed that aortic segments can be relaxed by high concentrations of NO in the absence of cGKI. To investigate whether this is also valid for other vessels, we used a small artery (arteria tibialis) that may behave similar to resistance vessels and develop a spontaneous myogenic tone in wild-type (wt) and cGKI–/– mice. In this study, we show that high concentrations of NO relax vascular smooth muscle independent of the activation of Ca2+-activated K+ (BKCa) channels by cGMP-dependent cross-activation of cAMP kinase (cAK). This pathway may be important in situations where high concentrations of NO prevail, such as in endotoxin shock.19,20

Materials and Methods

Materials and Animals
1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 2-(N,N-diethy lamino)-diazenolate-2-oxide (DEA-NO) and N ω-nitro-L-arginine
(L-NOARG) were from Alexis, Rp-8-bromo-adenosine-3',5'-cyclic monophosphothioate (Rp-8-Br-cAMPS) was from Biolog, and 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethyl-carboxamido-adenosine hydrochloride (CGS21680) and ibotriptin (IBTX) were from RBI.

Four-week-old inbred (129sv) male wt and cGKI –/– mice, 17 either litter-mate or age-matched, were used. All experiments were approved by German legislation. Statistical differences were analyzed by Student’s paired or unpaired t tests.

Measurement of Contractility

Arteria Tibialis

The arteria tibialis, a second order branching from arteria femoralis, was dissected and transferred to the experimental chamber containing ice-cold physiological buffer. The chamber was perfused at 37°C and a rate of 2 mL/min. A permanent intravascular pressure of 80 mm Hg was applied at nonflow conditions, resulting in an inner solution. The diameters of fully relaxed wt and cGKI –/– vessels were 78±6 μm (n=6) and 68±5 μm (n=7), respectively. These values are not significantly different. All compounds were applied to the adventitial side. Final concentrations are always reported. Diclofenac (1 μmol/L) was present in all buffers. To block endothelial NOS, 30 μmol/L L-NOARG was added 60 minutes before DEA-NO.

Aorta

Aortic rings from male mice were prepared as described elsewhere. For other experimental details, see our previous study.

Analysis of BK<sub>Ca</sub> Channel Activity

Aortic smooth muscle cells were isolated as described elsewhere. Membrane currents were recorded in whole-cell configurations. The bath solution contained (in mmol/L) NaCl 140, KCl 5.6, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, glucose 10, and HEPES 10 (pH 7.4). The pipette solution contained KCl 40, potassium aspartate 100, NaCl 10, MgATP 3, glucose 10, HEPES 10, and 300 mmol/L free calcium (pH 7.4). The holding potential was −20 mV. Test pulses lasted 500 ms to potentials from −30 to +80 mV. Amplitudes of the final 50 ms of depolarizing test pulses were averaged from 3 to 5 consecutive trials.

Determination of Cyclic Nucleotide Levels

Aortic rings were incubated for 10 minutes with norepinephrine (NE) followed by vehicle (100 μmol/L NaOH) or 100 μmol/L or 100 μmol/L DEA-NO for 3 minutes. Aortic rings were transferred into liquid nitrogen and pulverized together with 400 μL 10% trichloracetic acid (TCA) under liquid nitrogen. After thawing and centrifugation, the supernatant was extracted 5 times with 5 volumes of ether. The ether was evaporated at 70°C for 10 minutes. cGMP and cAMP were determined by specific enzyme immunoassays (Cayman Chemical).

Results

Lack of cGKI Impairs Acetylcholine- and NO-Induced Relaxation in Small Arteries

Small arteries and arterioles determine peripheral resistance and blood pressure. Isolated small arteries develop a spontaneous myogenic tone under isobaric conditions. We analyzed pressurized segments of the arteria tibialis from wt and cGKI–/– mice, which developed a spontaneous myogenic tone, suggesting that tonic regulation of such vessels is similar or identical to that of other small vessels. Arteria tibialis from both phenotypes developed a similar myogenic tone and had a similar internal diameter. The width of the vessel wall was 6.0±0.3 μm and 6.0±0.2 μm in the arteria tibialis from wt and cGKI–/– mice, respectively. A change in the ratio of the width of the wall to the width of the lumen has been considered an indicator of vascular remodeling. Our measurements indicate that the higher blood pressure of cGKI–/– mice had not altered grossly the structure of the arteria tibialis up to the age of 4 weeks.

Acetylcholine (1 nmol/L to 1 μmol/L) that stimulates the endothelial NO synthesis induced a concentration-dependent relaxation only in small arteries of wt but not cGKI–/– mice (Figures 1A and 1B), suggesting that cGKI is essential for endothelial NO synthesis induced (D) relaxation of small arteries derived from 6 to 7 wt (•) and cGKI–/– (□) mice are presented. Relaxation has been calculated as vessel diameter change in percentage of diameter change to the fully relaxed state (see Materials and Methods). *Significantly different at P<0.05.
cGKI –/– mice (Figure 1D). Adenosine acting via cAMP/cAK elicited a similar relaxation in wt and cGKI –/– vessels, confirming that cAMP relaxes arteries independent of cGKI. 17

BK Ca Channels Are Involved in Acetylcholine-Induced Relaxation

The above results suggest that high concentrations of NO relaxed the vessels by mechanisms independent of cGKI. Previously, it was shown that NO can affect smooth muscle contractility by hyperpolarizing the membrane potential through direct or indirect activation of BKCa channels, which are widely distributed in vascular smooth muscle. 14 We tested the contribution of BKCa channels to the cGMP/cGKI-mediated relaxation in small vessels using the specific BKCa channel blocker IBTX. Relaxation of wt small arteries induced by 100 nmol/L ACh was significantly reduced by 200 nmol/L IBTX (n=6, P<0.05; Figure 2A), indicating that ACh-induced relaxation was at least in part mediated by BKCa channels in murine small vessels. However, the inhibitory effect of IBTX was no longer observed when the concentration of ACh was raised to 1 mmol/L (Figure 2A). Similar results were obtained in the presence of DEA-NO. IBTX partly inhibited vasorelaxation induced by 1 mmol/L DEA-NO but had no effect on the relaxation induced by 10 μmol/L DEA-NO (Figure 2B). These results confirm that high concentrations of NO can relax murine arteries independent of BKCa channels and cGKI.

Figure 2. Effect of the BKCa channel blocker IBTX (■, IBTX 200 nmol/L; ■, control) on ACh-dependent (A and C) and DEA-NO-dependent (B) relaxation in wt arteria tibialis (A and B) and aorta (C) (n=6). The concentration of ACh was 10 μmol/L in C. Relaxation has been calculated as described in Material and Methods. Please note that in this series of experiments, the arteria tibialis was slightly more sensitive to 1 μmol/L DEA-NO than in Figure 1C. *Significantly different at P<0.05.

In cGKI–/– mice (Figure 1D), Adenosine acting via cAMP/cAK elicited a similar relaxation in wt and cGKI–/– vessels, confirming that cAMP relaxes arteries independent of cGKI. 17

CgKI Is Required to Activate BKCa Channels

A direct activation of BKCa channels by NO has been reported. 11 An electrophysiological analysis of the BKCa channels in isolated murine smooth muscle cells from small vessels was not feasible because of the extremely low amount of cells obtained. Others have used isolated aortic smooth muscle cells to investigate the regulation of BKCa channels. 11 Therefore, we tested whether the relaxation of murine aortic rings was also affected by inhibition of BKCa channels (Figure 2C). ACh-induced (10 μmol/L) relaxation of aortic rings was attenuated by 50% in the presence of 200 nmol/L IBTX.

This finding encouraged us to analyze the regulation of BKCa channels in isolated aortic wt and cGKI–/– smooth muscle cells (Figures 3A and 3B). In wt cells, BKCa currents were increased by 250 nmol/L and 5 μmol/L DEA-NO (Figure 3A), and the DEA-NO–increased current was inhibited by 100 nmol/L IBTX. The current increase was significant at a membrane potential of +20 mV (Figure 3A, inset). In contrast, DEA-NO up to a concentration of 50 μmol/L had no effect on the activity of BKCa channels in cGKI–/– smooth muscle cells (Figure 3B and inset). In agreement with other reports, 25–28 this finding indicates that NO regulates murine BKCa channels only in the presence of cGKI.

Figure 3. Current-voltage relations of BKCa currents in aortic smooth muscle cells from wt (WT, open symbols) (A) and cGKI–/– mice (filled symbols) (B). Whole-cell current was measured at test potentials from −30 to +80 mV under control conditions (squares) and in the presence of the indicated DEA-NO concentrations. All values are mean±SEM of 3 to 10 cells. The insets show current at the membrane potential of +20 mV. *Significantly different at P<0.01.

NO-Dependent Relaxation of Aortic Rings

To gain more insight into the molecular mechanism of NO-dependent relaxation in cGKI–/– vessels, rings from wt and cGKI–/– thoracic aorta were precontracted with NE and then incubated with increasing concentrations of DEA-NO.
DEA-NO relaxed the wt and cGKI−/− aorta with EC50 values of 35 and 850 nmol/L, respectively (Figures 4A and 4B). These results are in agreement with those obtained from small arteries and show that NO at higher concentrations induced vascular relaxation by an unidentified mechanism in small and large vessels.

In the next series of experiments, we investigated whether the relaxing effect of NO depended on the synthesis of cGMP. WT and cGKI−/− aortic rings were preincubated with the competitive inhibitor at the cAMP binding sites of cAK, or 3 μmol/L of the guanylyl cyclase inhibitor ODQ (upward triangles), C, CGS21680-induced relaxation of wt aortas (n=5) precontracted with 100 nmol/L NE in the absence (inverted triangles) and presence (diamonds) of 100 μmol/L Rp-8-Br-cAMPS. Relaxation was calculated as the percentage of the initial NE-induced increase in tension and expressed as percent of remaining contraction. All experiments were done in the presence of 1 μmol/L diclofenac. Experiments in panels A and B were done in the presence of 30 μmol/L L-NOARG.

Figure 4. Relaxation of aortic rings. Aortic rings from 4 to 5 wt (A) and cGKI−/− (B) mice were precontracted with 100 nmol/L NE and then incubated in the presence of increasing concentrations of DEA-NO in the absence (squares) and presence (circles) of 100 μmol/L Rp-8-Br-cAMPS, a competitive inhibitor at the cAMP binding sites of cAK, or 3 μmol/L of the guanylyl cyclase inhibitor ODQ (upward triangles). C, CGS21680-induced relaxation of wt aortas (n=5) precontracted with 100 nmol/L NE in the absence (inverted triangles) and presence (diamonds) of 100 μmol/L Rp-8-Br-cAMPS. Relaxation was calculated as the percentage of the initial NE-induced increase in tension and expressed as percent of remaining contraction. All experiments were done in the presence of 1 μmol/L diclofenac. Experiments in panels A and B were done in the presence of 30 μmol/L L-NOARG.

cGMP relaxed the aortic rings by cross-activation of the cAMP-signaling pathway. Addition of Rp-8-Br-cAMPS, a competitive antagonist of cAMP at the cAMP-binding sites of cAK, did not affect the dose-response curve for DEA-NO in wt aortas. Rp-8-Br-cAMPS inhibited effectively relaxation of aortic rings by the adenosine-A2 receptor agonist CGS21680 (Figure 4C). The A2 receptor has been shown to relax murine aortic rings through activation of cAK and independent of cGKI.17,29 Therefore, these results support the notion that low concentrations of NO relaxed vascular smooth muscle exclusively via cGKI and not via cAK. In contrast, preincubation of the cGKI−/− aortic rings with Rp-8-Br-cAMPS increased the EC50 of DEA-NO 10-fold, from 0.85 μmol/L to 9.4 μmol/L (Figure 4B). This 10-fold shift supported the hypothesis that cGMP relaxed cGKI−/− aorta by cross-activation of cAK.

Cyclic Nucleotide Levels in WT and cGKI−/− Aorta
The experiments carried out so far are compatible with the hypothesis that NO-increased cGMP levels can affect vascular tone by activation of the cAMP/cAK signaling pathway. However, the above finding did not determine whether cGMP inhibited the activity of a cAMP-hydrolyzing phosphodiesterase and thereby increased the cellular cAMP concentration or activated directly the cAK. Direct activation of cAK by cGMP requires cGMP concentrations around 10 μmol/L, whereas phosphodiesterase III is inhibited by cGMP with an IC50 value of 0.13 μmol/L.31 Using the difference in required cGMP concentration as criterion should allow differentiation between a direct and indirect activation mechanism by measuring NO-stimulated cGMP and cAMP levels. The cyclic nucleotide levels were measured in aortic rings of wt and cGKI−/− mice before and after the addition of a low (100 nmol/L) and high (100 μmol/L) concentration of DEA-NO under the same conditions used for the relaxation experiments (Figure 5). DEA-NO at a concentration of 100 nmol/L increased cGMP levels 2-fold in both genotypes but had no significant effect on the cAMP levels. These cGMP levels

Figure 5. Cyclic nucleotide levels. cGMP and cAMP concentrations were measured in 7 wt (□) and 3 cGKI−/− (■) aortic rings in the absence and presence of 0.1 μmol/L and 100 μmol/L DEA-NO. cGMP values in the presence of 0.1 and 100 μmol/L DEA-NO were significantly different (P<0.02 and P<0.001) from control values of both phenotypes. Values for the two phenotypes were not different at 0, 0.1, and 100 μmol/L DEA-NO.
activate cGKI in wt vessels.\textsuperscript{32} The high concentration of DEA-NO stimulated cGMP level over 80-fold in both tissues. cAMP levels were not increased significantly. Assuming a 60% water content of the aortic rings, the final cGMP concentration of 30 pmol/mg wet weight yields a cytosolic cGMP concentration of 50 μmol/L. This cGMP concentration exceeds 5-fold the \( K_e \) values for activation of cAK.\textsuperscript{30} These values strengthen additionally the conclusion that high concentrations of DEA-NO relaxed small and large vessels in the absence of cGKI by cross-activation of cAK.

**Discussion**

NO coordinates the blood-flow distribution between arterioles and the microvasculature by regulating the diameter of small arteries.\textsuperscript{7} The importance of NO and cGMP for the regulation of vascular tone and blood pressure has been recently strengthened by the observation that mice deficient in eNOS, ANP, the ANP receptor guanylyl cyclase A, or cGKI develop hypertension.\textsuperscript{2–6,17} However, the mechanism by which NO or cGMP regulates arterial tone has been discussed controversially.\textsuperscript{12–16} We used pressurized small arteries that develop spontaneous myogenic tone to analyze the mechanism of NO/cGMP-induced smooth muscle relaxation. In a previous publication\textsuperscript{17} and in this study, we show that in the murine system, NO and, most likely, ACh relax arterial smooth muscle through cGMP/cGKI. It was suggested previously that the vascular tone is regulated by the activity of the BK\textsubscript{Ca} channel.\textsuperscript{14} In agreement with this hypothesis, we found that smooth muscle tone is regulated at least partially by the activity of BK\textsubscript{Ca} channels.

The molecular mechanisms involved in NO-mediated regulation of BK\textsubscript{Ca} channels presently are not clear. It was reported that the activity of these channels is regulated directly by NO\textsuperscript{11} by cGKI-dependent phosphorylation of the channel protein\textsuperscript{25–27} or of a protein phosphatase.\textsuperscript{28,33,34} NO-dependent activation of BK\textsubscript{Ca} channels was only observed in the presence of cGKI. Even a concentration of 50 μmol/L DEA-NO did not activate BK\textsubscript{Ca} channels in mice lacking cGKI (Figure 3). In agreement with these results, IBTX did not prevent relaxation induced by high NO concentrations. These findings establish firmly that NO activates BK\textsubscript{Ca} channels in murine vascular smooth muscle via cGKI. This implies that BK\textsubscript{Ca} channels or closely associated proteins are in vivo targets for cGKI.

ACh induced relaxation in the presence of IBTX. Similar experiments led to the detection of EDHF, a family of endothelial-derived hyperpolarizing factors.\textsuperscript{18} It is possible that ACh induced relaxation by stimulating the production of EDHF and not by that of NO. However, IBTX-independent relaxation was also observed when NO was applied directly to the vessels. NO has not been reported to stimulate the production of EDHF, suggesting that relaxation in the presence of IBTX was caused through a different pathway involving cGMP. Possible targets of cGKI that regulate vascular tone could be IRAG, a protein modulating calcium release from IP\textsubscript{3}-sensitive stores,\textsuperscript{35} and myosin phosphatase.\textsuperscript{36} Phosphorylation of these proteins could be involved in the IBTX-insensitive relaxation of wt vessels.

Numerous cGMP-independent effects of NO have been described.\textsuperscript{37} Experiments designed to test alternative pathways suggested that NO effects were mediated by cGMP even in the cGKI\textsuperscript{−/−} mice. The inhibition of the soluble guanylyl cyclase by the competitive inhibitor ODQ shifted the dose relaxation curves to higher concentrations of NO. Activation of cAK by cGMP was identified as the most likely pathway leading to relaxation. cGMP-dependent activation of cAK has been observed previously in isolated smooth muscle cells after downregulation of cGKI.\textsuperscript{10,11} The mechanism by which cAK relaxes vascular tone is not known. It is unlikely that cAK activated BK\textsubscript{Ca} channels, because relaxation of cGKI\textsuperscript{−/−} vessels occurred in the presence of IBTX.

The cGKI\textsuperscript{−/−} mice develop hypertension that could be caused by endothelial dysfunction.\textsuperscript{24} A change in endothelial function associated with a reduced but not abolished vascular responsiveness to ACh as well as vascular remodeling has been proposed as a common phenomenon in hypertension. The wall thickness as potential index of vascular remodeling\textsuperscript{23} was not altered in cGKI\textsuperscript{−/−} small arteries, demonstrating no compensatory effects in the young mutant mice. The data presented here do not exclude an impaired endothelium-dependent relaxation in older cGKI\textsuperscript{−/−} mice. The evidence presented in this study and a previous one\textsuperscript{17} supports the hypothesis that the lack of smooth muscle cGKI caused hypertension. The relaxation induced by DEA-NO or 8-Br-cGMP did not depend on a functional endothelium.\textsuperscript{17} Hence, it is very likely that the cGKI deficiency and not endothelial dysfunction led to the impaired ACh/NO-dependent vasorelaxation.

There is clear evidence from this and a previous study\textsuperscript{17} that the NO/cGMP and adenosine/cAMP cascades regulate vascular tone in mice independent of each other under physiological conditions. cGMP-dependent activation of cAK was responsible for the relaxation obtained at high concentrations of NO. This pathway is not operative under physiological conditions, as shown in Figure 4A. However, under pathophysiological circumstances, for example, during endothelin shock when NO production is high, activation of cAK by cGMP may be an additional regulatory mechanism that produces generalized hypotension.\textsuperscript{10,20}

**Acknowledgments**

This work was supported by grants from Deutsche Forschungsgemeinschaft, Thyssen-Stiftung (grant 9.26/91), and Fond der Chemie. We thank M. Wöckner, S. Kamm, and A. Salusky for excellent technical assistance.

**References**


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Circ Res. 2000;87:825-830
doi: 10.1161/01.RES.87.9.825

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