Role of Smooth Muscle cGMP/cGKI Signaling in Murine Vascular Restenosis


Background—Nitric oxide (NO) is of crucial importance for smooth muscle cell (SMC) function and exerts numerous, and sometimes opposing, effects on vascular restenosis. Although cGMP-dependent protein kinase type I (cGKI) is a principal effector of NO, the molecular pathway of vascular NO signaling in restenosis is unclear. The purpose of this study was to examine the functional role of the smooth muscle cGMP/cGKI signaling cascade in restenosis of vessels.

Methods and Results—Tissue-specific mouse mutants were generated in which the cGKI protein was ablated in SMCs. We investigated whether the absence of cGKI in SMCs would affect vascular remodeling after carotid ligation or removal of the endothelium. No differences were detected between the tissue-specific cGKI mutants and control mice at different time points after vascular injury on a normolipidemic or apoe-deficient background. In line with these results, chronic drug treatment of injured control mice with the phosphodiesterase-5 inhibitor sildenafil elevated cGMP levels but had no influence on the ligation-induced remodeling.

Conclusions—The genetic and pharmacological manipulation of the cGMP/cGKI signaling indicates that this pathway is not involved in the protective effects of NO, suggesting that NO affects vascular remodeling during restenosis via alternative mechanisms. (Arterioscler Thromb Vasc Biol. 2008;28:1244-1250)

Key Words: nitric oxide ■ PKG ■ atherosclerosis ■ carotid ligation ■ wire-injury

Oclusion of coronary arteries results from advanced atherosclerotic lesions or plaque rupture. A common therapy to restore the blood flow in such narrowed arteries is angioplasty. However, endovascular interventions may lead to a long-term risk of restenosis, i.e., the remodeling and eventually reocclusion of treated arteries. Although the exact pathogenesis of restenosis is uncertain, it is known that after the initial injury smooth muscle cells (SMCs) contribute together with other cell types to the remodeling of the vessel wall.

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Nitric oxide (NO) is an important regulator of cardiovascular homeostasis and, in particular, of SMC function. In addition to potent vasorelaxant properties of NO, its bioavailability and signaling have also been associated with vascular proliferative diseases, such as atherosclerosis and restenosis. Studies with transgenic mice that overexpress or lack the NO synthases indicated that NO can mediate beneficial effects on the vasculature under particular pathophysiological conditions. On the other hand, several studies reported that NO promoted the progression of vasculoproliferative processes, and, thus, that it was deleterious for the vasculature. In restenosis, the endogenous effector(s) and underlying molecular mechanism(s) that mediate the opposing effects of NO on vascular remodeling are not clear. Under physiological conditions, NO exerts many of its effects via stimulation of the soluble guanylyl cyclase (sGC), which generates the second messenger cyclic guanosine-3’-5’-monophosphat (cGMP). The cGMP-dependent protein kinase type I (cGKI) is presumably the major target of NO/cGMP signaling in SMCs. For example, mice with a homozygous deletion of the cGKI gene showed an impaired NO/cGMP-dependent relaxation of SMCs. The role of cGKI has also been implicated in the phenotypic modulation of SMCs, which occurs in atherosclerosis and restenosis. Several studies suggested that activation of cGKI-dependent pathways has antimitogenic effects in SMCs in vitro, and might be vascular-protective in vivo. Recent evidence suggested that elevation of cGMP by the phosphodiesterase-5 (PDE-5) inhibitor sildenafil, hence, activation of cGMP-dependent pathways, was beneficial for the treatment of hypertrophic heart disease and for vascular remodeling associated with pulmonary hypertension. These findings indicated that particularly activation of cGKI mediated the protective effects of sildenafil in vivo.

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From the Institut für Pharmakologie und Toxikologie (R.L., P.W., D.B., F.H.), TU München, Germany; Interfakultäres Institut für Biochemie (S.F., R.F.), Universität Tübingen, Germany; Max-Delbrück-Centrum für Molekulare Medizin (M.G.), Berlin-Buch, Germany; Department of Molecular Genetics (J.H.), UT Southwestern, Dallas, Tex; Deutsches Herzzentrum (S.M.), TU München, Germany; and Institut für Kardiologische Molekularbiologie (A.Z., C.W.), RWTH Aachen, Germany.

Correspondence to Dr Robert Lukowski, Institut für Pharmakologie und Toxikologie der TUM, Biedersteiner Str. 29, D-80802 München, Germany. E-mail lukowski@ipt.med.tu-muenchen.de

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Surprisingly, the analysis of cGKI-deficient mice produced some evidence that activation of cGKI in SMCs increased the growth of primary SMCs and promoted the formation of atherosclerotic lesions in the apolipoprotein E (apoE)-deficient mouse model of hyperlipidemia-induced atherosclerosis.27 These results supported a unique proatherogenic potential of SMC cGKI in atherosclerosis,18 whereas the direct role of endogenous cGMP/cGKI signaling in restenosis, to our knowledge, was not tested before.

In the present study, we addressed the question whether the cGMP/cGKI pathway in SMCs is also involved in the vascular pathology of restenosis. The Cre/loxP system was used to generate mice with a tissue-specific deletion of the cGKI gene in SMCs. To test the pathophysiological role of endogenous cGKI 2 established mouse models, which resemble restenosis in humans, were used: the unilateral cessation of blood flow by carotid ligation28 and the removal of the endothelium by wire-injury of the carotid artery.29

Materials and Methods
Detailed methods are described in the supplemental data (available online at http://atvb.ahajournals.org).

Experimental Animals
The SM22α-Cre mouse line30 was crossed to mice that carried loxP-flanked cGKI alleles (L2)31 to generate cGKI mutants with recombined cGKI-null alleles (L-). The tissue-specific knockout of the cGKI gene was generated in cardiac and smooth muscle cells (cGKIcsmko, genotype: SM22α-Creα/α; cGKIαL-L). For experiments these cGKIcsmko animals were compared to littermate controls (ctr, genotype: SM22α-Creα/α; cGKIαL-L). All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München and had access to water and standard chow ad libitum. All experimental procedures were conducted according to the local government’s committee on animal care and welfare in München.

Carotid Artery Ligation
The injury procedure was performed by adapting an established model.28 Briefly, the left common carotid artery of deeply anesthetized animals was dissected and completely ligated proximal to its bifurcation.

Endothelial Denudation
Surgery was performed as described previously.29 Endothelial denudation of the left common carotid artery was performed by withdrawal injury passing a 0.014 inch flexible angioplasty guide wire 3 times trough the vessel. This method very efficiently removed the endothelium.

Results
Tissue-Specific Deletion of cGKI in SMCs
The recombination properties, in particular the tissue-specificity, of the SM22α-Cre mouse line (SM22α-Cre)30 were evaluated by crossing Cre transgenic animals to ROSA26 Cre reporter (R26R) mice.32 X-Gal staining of tissues from SM22α-Cre; R26R Cre reporter mice. Recombination was detectable in (c) medial SMCs of the CCA before injury and (d) at 28 days after ligation in cells in the media (m) and in the neointima (NI) of the CCA. Scale bars, 100 µm.

Figure 1. Recombination properties of the SM22α-Cre mouse line. X-Gal staining for β-galactosidase activity of (a) the heart and (b) the CCA from the SM22α-Cre; R26R Cre reporter mice. Recombination was detectable in (c) medial SMCs of the CCA before injury and (d) at 28 days after ligation in cells in the media (m) and in the neointima (NI) of the CCA. Scale bars, 100 µm.
Figure 2. Tissue-specific deletion of cGKI in SMCs. a, Western blot analysis of cGKI protein expression in different organs of control (ctr, genotype: SM22α-Cre\textsuperscript{ERT2}; cGKI\textsuperscript{L/L}) and tissue-specific cGKI knockout mice (csmko, genotype: SM22α-Cre\textsuperscript{ERT2}; cGKI\textsuperscript{L/L}). A cGKI common antibody\textsuperscript{34} was used to detect the respective protein. By detecting the p42/p44 mitogen-activated protein kinase (MAPK) equal loading of the gel was monitored. b, Immunohistochemical analysis of cGKI expression in uninjured (upper) and injured CCAs (lower) of control (left) and cGKI\textsuperscript{csmko} (right) mice. Scale bars, 100 \( \mu \)m.

Figure 3. cGKI-dependent substrate phosphorylation. Western blot analysis of protein extracts from control and cGKI\textsuperscript{csmko} vascular SMCs. Stimulation was carried out in the absence (H\(_2\)O) and presence of 1.0 mmol/L 8-Br-cAMP (cA) or 0.1 and 1.0 mmol/L 8-Br-cGMP (cG). The antibodies used to detect cGKI substrate phosphorylation were specific for phospho-Ser239 of the vasodilator-stimulated phosphoprotein (pVASP\textsuperscript{239}) and the phospho-Ser92 residue of phosphodiesterase-5 (pPDE-5). A VASP common antibody was used, which detects both total VASP (lower band) and the cAK and cGKI phosphorylation site of VASP at serine 157 (VASP\textsuperscript{157}). A PDE-5 antibody was used on the same protein homogenates to demonstrate equal loading of the gels.

Vascular Remodeling After Carotid Ligation

Ligation of the left CCA induced intensive remodeling of the injured vessel segment in both control (genotype: SM22α-Cre\textsuperscript{ERT2}; cGKI\textsuperscript{L/L}) and cGKI\textsuperscript{csmko} (genotype: SM22α-Cre\textsuperscript{ERT2}; cGKI\textsuperscript{L/L}) mice. The histological analysis of hematoxylin and eosin (H&E)-stained sections showed the formation of a neointima within the IEL, but no obvious differences were found between the genotypes 28 days after injury (supplemental Figure Va). The presence of an intact endothelial cell layer on the luminal surface of the ligated vessels was demonstrated by immunoreactivity for the von Willebrand factor (vWF) at all time-points analyzed (data not shown). The positive vWF stain confirmed the existence of endothelial cells after injury, and, therefore, it was anticipated that the endothelial sources of NO were preserved during the entire injury period. Furthermore, by studying knockout mice it was found that loss of NO derived from the NO synthase (NOS) isozymes had a major impact on the remodeling process in the ligation model.\textsuperscript{9,35}

Quantitative assessment of the remodeling response was performed by morphometric analysis on H&E stained sections. Based on the mean NI/media ratios, no differences
could be detected between control mice (NI/media ratio 0.38±0.06; n=22) and littermate cGKI^{csmko} mice (NI/media ratio 0.45±0.08; n=10) 28 days after injury (Figure 4a). As expected, the extent of remodeling was less intensive after 14 days, but again no differences could be detected between both groups (ctr NI/media ratio 0.12±0.03; n=18; cGKI^{csmko} NI/media ratio 0.14±0.04; n=13; Figure 4a). To test whether an activation of the cGMP/cGKI pathway after injury had consequences for the vascular response to ligation, a control group of animals (genotype: SM22α-Cre^{cre}+/c; cGKI^{+/-}) was continuously treated with sildenafil in their drinking water. As determined by the NI/media ratio 28 days after injury, no significant difference was detected between animals treated with sildenafil (NI/media ratio 0.29±0.10; n=10) and the untreated control mice (Figure 4a), although cGMP levels were effectively elevated in the drug-treated mice (supplemental Figure Vb).

To focus on possible local consequences of the cGKI inactivation, continuous NI/media profiles of the injured arteries were generated (Figure 4b). These profiles revealed a gradual decline of the NI/media ratio with increasing distance to the point of ligation and confirmed a similar response to injury of control and cGKI^{csmko} animals. Additional vessel parameters, such as the area inside the external elastic lamina (EEL) (ctr 58.1±4.8×10³ µm²; n=22; cGKI^{csmko} 72.8±8.1×10³ µm²; n=10), the medial area (ctr 25.8±1.6×10³ µm²; n=22; cGKI^{csmko} 31.3±2.9×10³ µm²; n=10), the NI area (ctr 10.8±1.8×10³ µm²; n=22; cGKI^{csmko} 14.5±2.7×10³ µm²; n=10), and the area of the vessel lumen (ctr 21.6±2.3×10³ µm²; n=22; cGKI^{csmko} 26.9±3.9×10³ µm²; n=10) again demonstrated that controls and cGKI^{csmko} responded equally to the injury (Figure 4c). All vascular parameters were also determined after 14 days of injury, but again they were similar for both genotypes (data not shown).

As shown previously, proliferation of cells from the vessel wall was involved in the remodeling after vascular injury. To assess this issue, the density of a proliferation marker was evaluated in the remodeled vessel (supplemental Figure Vc). The percentage of positive stained cells in the NI and the medial cell layer was comparable between genotypes. This result indicated that proliferation of cells from the vessel wall contributed to remodeling, but the deletion of cGKI in SMCs showed again no influence.

Earlier reports suggested that SMC cGKI modulated the progress of atherogenesis. It was possible that a contribution of cGKI to vascular remodeling after mechanical injury required factors that are present in atherosclerosis-prone mice. Therefore, we reinvestigated vascular remodeling in apoE-deficient (apoE-ko) mice using the above model of CCA ligation. The morphometric data based on measurements of the NI/media ratio (apoE-ko ctr NI/media ratio 0.59±0.09; n=13 and littermate apoE-ko cGKI^{csmko} NI/media ratio 0.79±0.15; n=12; Figure 5a) and all other vessel parameters such as the EEL (apoE-ko ctr 86.8±6.6×10³ µm²; n=13; apoE-ko cGKI^{csmko} 94.1±10.9×10³ µm²; n=12), the medial area (apoE-ko ctr 36.7±2.8×10³ µm²; n=13;
apoE-ko cGKI<sup>cmko</sup> 37.5±4.3×10<sup>3</sup> μm<sup>2</sup>; n=12), the NI (apoE-ko cGKI<sup>cmko</sup> 30.9±7.4×10<sup>3</sup> μm<sup>2</sup>; n=12), and the lumen (apoE-ko cGKI<sup>cmko</sup> 28.7±2.6×10<sup>3</sup> μm<sup>2</sup>; n=13; apoE-ko cGKI<sup>cmko</sup> 25.7±3.7×10<sup>3</sup> μm<sup>2</sup>; n=12) revealed that both genotypes responded equally to the ligation (Figure 5b). As shown before in the NI and media of normolipemic mice, the cGKI protein was expressed 28 days after injury in apoE-deficient control mice, and this expression pattern was abolished in apoE-deficient cGKI<sup>cmko</sup> mutants (supplemental Figure VI). Thus, the comparable remodeling responses of apoE-deficient control and cGKI<sup>cmko</sup> mice did not result from an inadequate recombination efficiency of the SM22α-Cre transgene because vascular cGKI was absent in the mutants. We cannot exclude that the lack of cGKI during ontogenesis in the present injury model perhaps led to an undetected functional compensation of the pathway, whereas in the atherosclerosis model, a tamoxifen-inducible Cre was used to delete cGKI selectively only in SMCs of adult mice that were fed an atherogenic diet.27

**Vascular Remodeling After Wire-Injury of the CCA**

All experiments shown so far used ligation of the left CCA. It was therefore hypothesized that the inability to detect an effect of SMC cGKI on vascular remodeling was perhaps caused by the presence of an “intact” endothelium. We therefore removed the endothelium by the wire-injury procedure.29 In line with the abolished cGKI expression pattern observed in ligated vessels of cGKI<sup>cmko</sup> mutants, no cGKI protein was expressed in the cGKI<sup>cmko</sup> 28 days after wire-injury (supplemental Figure VII). The data based on a detailed analysis of the NI/media ratio profile (Figure 6a) and all morphometric parameters of the remodeled vessel (Figure 6b) revealed no difference between both genotypes. In the vessel segment analyzed, the areas of the EEL (ctr 63.9±6.6×10<sup>3</sup> μm<sup>2</sup>; n=9; cGKI<sup>cmko</sup> 64.6±4.3×10<sup>3</sup> μm<sup>2</sup>; n=11), the media (ctr 23.4±1.3×10<sup>3</sup> μm<sup>2</sup>; n=9; cGKI<sup>cmko</sup> 25.3±1.8×10<sup>3</sup> μm<sup>2</sup>; n=11), the NI (ctr 33.1±6.0×10<sup>3</sup> μm<sup>2</sup>; n=9; cGKI<sup>cmko</sup> 28.6±3.2×10<sup>3</sup> μm<sup>2</sup>; n=11), and the lumen (ctr 6.6±1.0×10<sup>3</sup> μm<sup>2</sup>; n=9; cGKI<sup>cmko</sup> 10.7±1.7×10<sup>3</sup> μm<sup>2</sup>; n=11) were similar between genotypes after denudation. However, in a small region at approximately 1.4 to 1.8 mm distance from the bifurcation, the detailed analysis of the profiles revealed a restricted but statistically significant decrease in the NI/media ratios of the cGKI<sup>cmko</sup> mutant mice.

**Discussion**

Recent analysis of vascular proliferative disorders in cGKI mouse mutants indicated that smooth muscle cGKI accelerates plaque formation in a hyperlipidemia-induced model of atherosclerosis.27 These results suggested that cGKI in SMCs potentially mediates proatherogenic properties of the NO/cGMP pathway in vivo, being thus, in contrast to the common view, not vasculoprotective. To solve the controversy whether the cGMP/cGKI pathway in SMCs accelerates lesion formation or mediates vascular protection, we generated different models of vascular restenosis. In comparison to control mice, we could not detect any influences of the cGKI deletion in SMCs on the NI/media ratio, proliferation, and various other vessel parameters at different time-points after injury in normolipemic and in apoE-deficient mice. In line with these results, activation of the cGMP/cGKI pathway by chronic administration of sildenafil had no influence on the remodeling response after carotid ligation. Further, no potentially vasculoprotective effects of endogenous cGKI on the remodeling were observed in the absence of a functional endothelium. Interestingly, the tissue-specific deletion of cGKI in SMCs had a minor beneficial impact after wire-injury in a spatially restricted vessel segment. The inability of smooth muscle cGKI to affect remodeling was not caused by an altered distribution of the enzyme between media and NI. Previous analysis of the NI formation after balloon injury of rat carotid arteries showed that the media and the NI express cGKI after injury.28 These results implied a potential role of the cGMP/cGKI pathway for injury-induced remodeling, and identical data have been obtained in the present study.

Neither genetic nor pharmacological manipulation of cGMP/cGKI signaling indicate that smooth muscle cGKI is of major importance during mechanically-induced restenosis of murine vessels. It has been suggested that the effects of NO are strongly concentration-dependent. Presumably, the spatio-temporal profile, the amount of NO synthesized, and the source of its production after vascular injury result in the activation of alternative mechanisms that are not mediated by cGKI or cGMP, such as redox regulation of target proteins.37,38 It has been reported recently that cGMP-independent redox regulation of the sarcoplasmatic reticulum calcium ATPase (SERCA) might be required for NO inhibition of SMC migration after arterial injury.29

The cGMP/cGKI pathway has been previously evaluated in models that differ from the ligation model. In rat the
balloon injury model has been used to examine the contribution of cGMP and cGKI to the restenosis process.\textsuperscript{3,30,41} Adenoviral transfer of a constitutively active kinase domain of cGKI\textsuperscript{23} and sGC\textsuperscript{41} in conjunction with the application of the NO-donor molsidomine reduced significantly the proliferation of cells in the NI. In contrast, adenoviral overexpression of the full-length cGKI\textbeta isoform did not affect restenosis induced by balloon injury.\textsuperscript{21} In the same model, YC-1, an activator of sGC, prevented NI formation.\textsuperscript{40} In these studies endogenous cGMP levels after drug treatment were not determined, and it was not clear whether or not the observed effects were cGMP-dependent. Interestingly, it was shown recently that YC-1 activates matrix metalloproteinases thereby inhibiting neointima formation in a cGMP-independent manner.\textsuperscript{22} Together, the pharmacological and genetic approach used in the present work differs significantly from balloon injury in conjunction with adenoviral gene transfer.

In conclusion, the present study demonstrated that neither a deletion of cGKI in SMCs nor an activation of the cGMP/cGKI pathway through pharmacological PDE-5 inhibition impaired the overall vascular remodeling after carotid ligation in normolipidemic and apoE-deficient or wire-stenting impaired the overall vascular remodeling after carotid ligation in normolipidemic and apoE-deficient or wire-stented mice. All experimental approaches tested the influence of endogenous SMC cGKI on vascular remodeling by locally restricted injuries resembling restenosis, whereas the analysis of atherosclerosis identified a proatherogenic role of SMC cGKI for systemic vessel disease.\textsuperscript{27} Based on the presented results, we conclude that the well-accepted effects of NO in restenosis\textsuperscript{35} are independent of smooth muscle cGKI and that the contribution of cGKI-mediated mechanisms to vascular remodeling appears to be context-specific, being more important in atherosclerosis than in restenosis.

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Disclosures
None.

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Role of smooth muscle cGMP/cGKI signaling in murine vascular restenosis

Robert Lukowski¹*, Pascal Weinmeister¹, Dominik Bernhard¹, Susanne Feil², Michael Gotthardt³, Joachim Herz⁴, Steffen Massberg⁵, Alma Zernecke⁶, Christian Weber⁶, Franz Hofmann¹, Robert Feil²

¹ Institut für Pharmakologie und Toxikologie, TU München, Germany
² Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
³ Max-Delbrück-Centrum für Molekulare Medizin, Berlin-Buch, Germany
⁴ Department of Molecular Genetics, UT Southwestern, Dallas, USA
⁵ Deutsches Herzzentrum, TU München, Germany
⁶ Institut für Kardiovaskuläre Molekularbiologie, RWTH Aachen, Germany

*Author for correspondence
**Figure 1.** Genomic PCR-analysis of the SM22α-Cre mediated inactivation of the cGKI gene in different tissues. PCR products amplified from the loxP-flanked (L2), wild type (wt), and knockout (L-) alleles of a SM22α-Cre^{+/+}; cGKI^{+/L2} mouse are indicated. Black triangles and open boxes denote loxP sequences and exon 10 of the cGKI gene, respectively.
Figure II. (a) Kaplan-Meier survival curves of control (n=9) and cGKI\textsuperscript{csmko} (n=11) mice. Survival was documented for up to 62 weeks. (b) Intestinal passage time of BaSO\textsubscript{4}. Passage time was measured in 12-h fasted control (n=7) and cGKI\textsuperscript{csmko} (n=5) mice. Passage time was normalized to body weight (bw).
Figure III. (a) Western blot analysis of cGKI protein expression in the aorta of four control (ctr, genotype: SM22α-Cre<sup>β<sup>+</sup>;</sup> cGKI<sup>+/L2</sup>) and four tissue-specific cGKI knockout mice (csmko, genotype: SM22α-Cre<sup>β<sup>+</sup>;</sup> cGKI<sup>L<sup>−</sup>/L2</sup>). A cGKI common antibody was used to detect the respective protein. By detecting the p42/p44 mitogen-activated protein kinase (MAPK), β-actin, and the protein kinase Akt in the same protein homogenates, equal loading of the gel was monitored. (b) Western blot analysis showing the expression of smooth muscle marker proteins, cGKI, and cGKI target proteins in the common carotid arteries of control and cGKI<sup>csmko</sup> mice. Vessels of three animals per genotype were pooled to obtain the respective protein extracts. Antibodies used on loaded lysates were specific for phosphodiesterase-5 (PDE-5), the IP<sub>3</sub>-receptor associated cGMP-kinase substrate (IRAG), cGKI, Vimentin, smooth muscle α-actin (α-actin), SM22α, and myosin light chain (MLC). By detecting MAPK, β-actin, and Akt in the same protein homogenates, equal loading of the gel is demonstrated.
**Figure IV.** Endogenous (a) cAMP and (b) cGMP levels of control (black bars; n=4) and cGKI^cmko^ (open bars; n=4) primary vascular SMCs in response to cGMP-elevating compounds. Cells were maintained on 10-cm dishes in serum-free medium for 48 h and then stimulated for 10 min in the absence (H_2O) and presence of 0.1 mM sildenafil (S) or 0.1 mM of the NO-donor 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA-NO) (***, p<0.001).
Figure V. (a) Histological analysis of H&E stained 6 μm cross sections from control (ctr) and cGKIcsmko (csmsko) mice 28 days after carotid ligation. Representative photomicrographs from the central region of the vessel segment studied are shown. Following the injury, a prominent neointima (NI) inside the internal elastical lamina (white broken line) was detectable in both genotypes. Arrows indicate the external elastic lamina separating the tunica media (m) from the surrounding adventitial tissue. Scale bars, 100 μm. (b) cGMP measurements from untreated control mice (ctr 34.39 ± 9.71 fmol/mg protein; n=4) and mice that continuously received sildenafil (50 mg·kg⁻¹·d⁻¹) for 14 days in their drinking water (ctr+S 75.90 ± 3.81 fmol/mg protein; n=4). Values were determined in extracts from whole hearts (**, p<0.01). (c) Quantification of cellular proliferation in remodeled vessels from control (black bars) and
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cGKI^{csmko} (open bars) mice at 28 days after injury. The statistics are based on the analysis of immunohistochemical staining for the proliferating cell nuclear antigen (PCNA). No significant differences were found between both genotypes in the media (ctr 3.06% ± 1.04%; n=12 and cGKI^{csmko} 2.83% ± 0.80%; n=9) and NI (ctr 3.13% ± 0.63%; n=12 and cGKI^{csmko} 3.03% ± 0.92%; n=9).
Figure VI. Immunohistochemical detection of cGKI protein 28 days after carotid ligation in apoE-deficient control (left) (ctr apoE-ko, genotype: SM22α-Cre\textsuperscript{tg/+}; cGKI\textsuperscript{+/L2}; apoE\textsuperscript{-/-}) and cGKI\textsuperscript{csmko} mice (right) (csmko apoE-ko; SM22α-Cre\textsuperscript{tg/+}; cGKI\textsuperscript{L/-L2}; apoE\textsuperscript{-/-}) on an apoE-deficient background are shown. Scale bars, 100 µm.
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Figure VII. Immunohistochemical staining for cGKI protein after wire-injury of the CCA in control mice (ctr) and cGKI^{csmko} (csmko) mutants. Scale bars, 100 µm.
Role of smooth muscle cGMP/cGKI signaling in murine vascular restenosis

Robert Lukowski\textsuperscript{1*}, Pascal Weinmeister\textsuperscript{1}, Dominik Bernhard\textsuperscript{1}, Susanne Feil\textsuperscript{2}, Michael Gotthardt\textsuperscript{3}, Joachim Herz\textsuperscript{4}, Steffen Massberg\textsuperscript{5}, Alma Zernecke\textsuperscript{6}, Christian Weber\textsuperscript{6}, Franz Hofmann\textsuperscript{1}, Robert Feil\textsuperscript{2}

\textsuperscript{1} Institut für Pharmakologie und Toxikologie, TU München, Germany
\textsuperscript{2} Interfakultäres Institut für Biochemie, Universität Tübingen, Germany
\textsuperscript{3} Max-Delbrück-Centrum für Molekulare Medizin, Berlin-Buch, Germany
\textsuperscript{4} Department of Molecular Genetics, UT Southwestern, Dallas, USA
\textsuperscript{5} Deutsches Herzzentrum, TU München, Germany
\textsuperscript{6} Institut für Kardiovaskuläre Molekularbiologie, RWTH Aachen, Germany

*Author for correspondence
Supplemental Materials and Methods

Experimental animals. Mice carrying the loxP-flanked (L2) or recombined (L-) cGKI allele\(^1\) and the ROSA26 Cre reporter mice (R26R) allele\(^2\) were described previously. For the recombination analysis, transgenic SM22\(\alpha\)-Cre mice expressing Cre recombinase under the control of a SM22\(\alpha\) promoter fragment\(^3\) were crossed with the R26R Cre reporter mice. Since the reduced life expectancy of the conventional cGKI null mice\(^4\) makes them inappropriate for long-term \textit{in vivo} studies tissue-specific cGKI mutants were generated. In order to generate mice with a tissue-specific knockout of the cGKI gene in cardiac and smooth muscle cells (cGKI\(^{csmko}\), genotype: \textit{SM22}\(\alpha\)-Cre\(^{tg/+}\), cGKI\(^{L/-L2}\)) and littermate controls (ctr, genotype: \textit{SM22}\(\alpha\)-Cre\(^{tg/+}\), cGKI\(^{+/L2}\)) the mice with modified cGKI alleles were crossed with the SM22\(\alpha\)-Cre mouse line. Because the cGKI protein was ablated in both cardiac and smooth muscle, the tissue-specific cGKI knockouts generated were termed cGKI\(^{csmko}\) mice, where “csmko” stands for “cardiac and smooth muscle knockout”. The apolipoprotein E-deficient mice\(^5\) were obtained from The Jackson Laboratory. Genotyping of animals was carried out as described.\(^1-^3\) The experiments were performed with litter-matched male and female mice aged 6-18 weeks on a mixed C57BL6/129Sv background. All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München and had access to water and standard chow ad libitum. All experimental procedures were conducted according to the local government’s committee on animal care and welfare in München.

Recombination analysis of the floxed cGKI gene. In order to obtain DNA samples from control animals (genotype: \textit{SM22}\(\alpha\)-Cre\(^{tg/+}\), cGKI\(^{+/L2}\)) tissue was isolated and washed in ice-cold phosphate-buffered saline (PBS) incubated in DNA lysis buffer (50 mM Tris-HCl, pH 7.4; 5 mM EDTA; 1% SDS; 0.2 M NaCl; 0.5 mg/ml proteinase K) at 55°C over night, and then extracted with phenol-/chloroform. The DNA was precipitated by adding 2 volumes of
100% ethanol and resuspended in H$_2$O. PCR was performed on the murine cGKI gene locus with specific primers to detect the L2, L- and wild type alleles.

**Western blot analysis.** For Western blot analysis, tissues were isolated, washed in PBS and homogenized in protein lysis buffer (20 mM Tris-HCl, pH 8.3; 0.67% SDS; 238 mM β-mercaptoethanol; 0.2 mM PMSF). To obtain the protein extracts of carotid arteries and aortas, material from six animals was pooled. Proteins (30 µg) were separated on 8-12% SDS-PAGE gels. Immunodetection was performed by using the cGKI common antibody (dilution 1:200), and primary antibodies specific for phosphodiesterase-5 (PDE-5) (dilution 1:500), the IP3-receptor associated cGMP-kinase substrate (IRAG) (dilution 1:300), Vimentin (Santa Cruz Biotech.) (dilution 1:200), smooth muscle α-actin (α-actin) (Sigma) (dilution 1:100,000), SM22α (Santa Cruz Biotech.) (dilution 1:250), myosin light chain (MLC) (Cell Signaling Technology) (dilution 1:1000), the p42/p44 mitogen-activated protein kinase (MAPK) (Cell Signaling Technology) (dilution 1:1000), β-actin (Abcam) (dilution 1:50,000), and the protein kinase Akt (Cell Signaling Technology) (dilution 1:1000).

**5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining and immunohistochemistry.** The β-galactosidase expressed from the recombined R26R Cre reporter allele was detected by staining of tissues with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) as described. X-Gal staining was performed on organs and sections obtained from the SM22α-Cre; R26R reporter mice (genotype: SM22α-Cre$^{+/-}$; R26R$^{+/+}$). Immunohistochemical staining was performed on paraffin embedded serial 6 µm sections. Briefly, sections were rehydrated, blocked in 1.5% normal goat serum in PBS for 1 hour, and incubated over night at 4°C in primary antibody dilutions. Biotinylated secondary antibodies (Vector Laboratories) (dilution 1:200) were applied and the detection was performed by the avidin-biotin method (Vector Laboratories) with diaminobenzidine or Vector Blue substrate (Vector Laboratories) as chromogen to visualize the complexes. Staining of sections was carried out with the
primary antibodies against cGKI (dilution 1:50), proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology) (dilution 1:100), and von Willebrand Factor (vWF) (Dako) (dilution 1:400). Stained sections were mounted in Aquatex (VWR) or in 1 µg/ml Hoechst 33258 (Sigma) in 80% Glycerol for the nuclear counterstain of the PCNA immunohistochemistry. To determine the proliferation index, the PCNA-stained cells and total cells were counted in three sections of n=3-4 animals for each genotype.

Survival curves. The Kaplan-Meier survival curves was generated according to the survival data of control (n=9) and cGKI csmko mice (n=11) using GraphPad Prism 4.0.

Intestinal passage time. For intestinal passage mice were fasted for 12 hours and then 200 µl of a BaSO₄ (3.5 g/ml) suspension were orally administered via stainless feeding needles. The mice were returned to individual cages and the transit time was determined as the time until deposition of white feces. Male and female animals were pooled for analysis of gastrointestinal passage time since no significant gender-dependent effects on the tested parameter were observed.

VASP and PDE-5 phosphorylation in vascular SMCs. Primary vascular SMCs isolated from control and cGKI csmko mice were grown to 80-90% confluence in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin before serum withdrawal for 48 h. Stimulations were carried out with with Tyrode’s solution (140 mM NaCl; 5 mM HEPES; 10 mM glucose; 5 mM KCl; 1.2 mM MgSO₄; 2 mM CaCl₂, pH 7.4) containing water or with Tyrode’s solution containing the membrane-permeable cyclic nucleotide analogues 8-Br-cGMP or 8-Br-cAMP for 10 min. After stimulation, cells were immediately lysed in SDS-lysis buffer and protein extracts were prepared for western blot analysis. As marker for cGKI activity, specific antibodies for VASP (Alexis Biochemicals) (dilution 1:4000), phospho-VASP (Alexis Biochemicals) (0.5 µg/ml) and phospho-PDE-5 (dilution 1:80) were used.

Carotid artery ligation. The injury procedure was performed by adapting an established model as described previously. In brief, animals were deeply anaesthetized using a mixture
of Midazolam (Ratiopharm) (5 mg/kg), Medetomidin (Pfizer) (0.5 mg/kg), and Fentanyl (Janssen-Cilag) (0.05 mg/kg), which was injected intraperitoneally. The left common carotid artery was dissected by a midline incision in the neck and completely ligated proximal to its bifurcation with a 8-0 nylon suture (Ethicon). During the whole procedure, the animals received a continuous oxygen/isoflurane (1-2%) (Forene) inhalation. After surgery, the anaesthesia was antagonised with Atipamezol (Pfizer) (2.5 mg/kg), Flumazenil (Roche) (0.5 mg/kg), and Naloxon (Curamed Pharma) (1.2 mg/kg). The animals were allowed to recover after surgery and showed no symptoms of a stroke. At 14 and 28 days after injury groups of animals were sacrificed and vascular remodeling was examined by morphometry and immunohistochemistry.

**Chronic drug treatment.** For PDE-5 inhibition, sildenafil citrat tablets (Pfizer) were used. Tablets were grounded into powder and dissolved in tap water at a final concentration of 0.2 mg/ml. Before use, the solution was filtered with 0.22 µm StericapPlus (Millipore) and 0.2 µm Acrodisc Syringe (Pall). This freshly prepared sildenafil solution was substituted for the drinking water beginning from the day of surgery, whereas a control group received normal water. The water intake was constantly monitored by weighing the bottle to assure a final drug dose of 50 mg·kg⁻¹·d⁻¹. For the cGMP measurements, mice were treated for 14 days with sildenafil, whilst the morphometric analysis was done after 28 days.

**Endothelial denudation.** Surgery was performed as described previously using a dissection microscope (Zeiss, Germany). Litter-matched mice were anaesthetized with a continuous oxygen/isoflurane (Forene) inhalation. A ventral midline incision was performed and the left common carotid artery was carefully exposed. The bifurcation of the left common carotid artery was localized and two non absorbable ligatures using a 7-0 braided silk fibre (Resorba) were placed around the external carotid artery. A transverse arteriotomy was made by introducing a 0.014 inch flexible angioplasty guide-wire via the external carotid artery. Endothelial denudation of the left common carotid artery was performed by withdrawal injury.
passing the wire 3 times through the vessel towards the aortic arch. This method removed very efficiently the endothelium from the common carotid artery. After injury, the wire was removed and the external common carotid artery was ligated completely. In contrast to the ligation model, the blood flow through the left common and internal carotid arteries was restored after removal of the endothel. The skin was closed with a 7-0 surgical silk (Resorba) and the animals were allowed to recover. At 28 days after injury, vascular remodeling was analysed by morphometry and immunohistochemistry.

**Cyclic nucleotide measurements.** For cyclic nucleotide determination primary vascular SMCs isolated from control and cGKI csmko mice were grown to 80-90% confluence in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin before serum withdrawal. Cyclic nucleotides from serum-starved (48 h) vascular SMCs left untreated, stimulated with sildenafil (0.1 mM), or DEA-NO (0.1 mM) for 10 min were extracted using ice-cold 100% ethanol. Nucleotides were concentrated by evaporating the alcohol. The resulting pellet was dissolved in 200 µl of EIA-buffer provided with the cAMP and cGMP EIA kit (Cayman chemical). cAMP and cGMP levels were determined successively from the same samples. Cyclic nucleotide concentrations were determined according to the manufacture’s recommendations. cGMP-levels were determined in hearts of control mice (genotype: SM22α-Cre 0/+, cGKI 0/1,2) that were either treated with sildenafil (50 mg·kg⁻¹·d⁻¹) for 14 days or left untreated. The heart of each mouse was homogenized in 1 ml of ice-cold 100% ethanol, and then cGMP was concentrated by evaporating the alcohol. Further procedure was as described for the cyclic nucleotide determination in primary vascular SMCs.

**Morphometric analysis.** Morphometric data of the left common carotid artery were evaluated with the investigator being unaware of the genotype and/or drug treatment of the mice. All experiments were performed with litter-matched control and cGKI csmko animals at an age of 6 to 18 weeks. Since there were no significant gender-dependent effects on tested parameters (data not shown), male and female mice were pooled for analysis. Animals were
sacrificed and perfused in situ via the left ventricle with formaldehyde (2%) and glutaraldehyde (0.2%) in PBS. The injured arterial segment proximal to the bifurcation was excised, postfixed for 1 h, and then embedded in paraffin. Starting from the point of ligation (ligation model) or from the bifurcation of the common carotid artery (wire-injury model), which were used as reference points (0 mm), serial sections of 6 µm thickness were obtained and hematoxylin & eosin (H&E) stained. In total, 30 H&E stained sections in 120 µm intervals covering the segment from 0 to 3.6 mm of the injured artery were included. This procedure ensured the analysis of a representative vessel from each animal. Digital images of the H&E stained sections were analyzed by UTHSCSA ImageTool, version 3.0. (University of Texas Health Science Center). The area within the external elastic lamina (EEL), internal elastic lamina (IEL), and the lumen were determined by tracing their perimeter. From these primary data all additional vessel parameters were calculated. In detail, the neointimal area (NI) was calculated by subtracting the luminal area from the area defined by the IEL. By similar means the media was defined as the area between the IEL and the EEL. The degree of NI formation was expressed by the NI/media ratio. Taking all NI/media ratio measurements of the entire vessel into account an individual mean value for each animal was calculated.

**Statistical analysis.** The OriginPro-Software, version 6.1, was used for statistical analysis. Data are presented as mean ± SEM. In order to compare groups an unpaired Student’s t-test was used and significance was determined at p<0.05.
cGKI and vascular restenosis

Supplemental References


