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

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

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
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 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: SM22\(\alpha\)-Cre\(^{tg/+}\); cGKI\(^{L-/L2}\)) and littermate controls (ctr, genotype: 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: 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
cGKI and vascular restenosis

100% ethanol and resuspended in H₂O. 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 antibody⁶ (dilution 1:200), and primary antibodies specific for phosphodiesterase-5 (PDE-5)⁷ (dilution 1:500), the IP₃-receptor associated cGMP-kinase substrate (IRAG)⁶ (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.⁵ X-Gal staining was performed on organs and sections obtained from the SM22α-Cre; R26R reporter mice (genotype: SM22α-Cre<sup>39+</sup>; R26R<sup>+/−</sup>). 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\(^6\) (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 \(\mu\)g/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 \(\mu\)l of a BaSO\(_4\) (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 MgSO\(_4\); 2 mM CaCl\(_2\), 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\(^{157}\) (Alexis Biochemicals) (dilution 1:4000), phospho-VASP\(^{239}\) (Alexis Biochemicals) (0.5 \(\mu\)g/ml) and phospho-PDE-5\(^7\) (dilution 1:80) were used.

**Carotid artery ligation.** The injury procedure was performed by adapting an established model as described previously.\(^9\) 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 previously¹⁰,¹¹ 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<sup>csmko</sup> 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 sildeanfil (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<sup>β<sup>+</sup></sup>, cGKI<sup>+/L2</sup>) that were either treated with sildenafil (50 mg·kg<sup>−1</sup>·d<sup>−1</sup>) 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<sup>csmko</sup> 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.
Supplemental References