Supplementary Data

Supplementary Materials and Methods

Mutation of bovine endothelial NO synthase

The cDNA from bovine endothelial NO synthase (eNOS) was used for primer overlap extension polymerase chain reaction (PCR) to replace the bases TG in position 301/302 (starting with ATG) into GC, resulting in a change of the coding triplet for the amino acid cysteine 101 to alanine. The primers used to change the bases were 5′-CTGCACTCCC AGGTGCGCCCTGGGCTCCCTG-3′ and 5′-CAGGGAGC CCAGGGCGCACC TGGGAGTGCAG-3′. The primers, 5′-GTAATACGACTCACTATAGGGC-3′ and 5′-CGATCCA GGCCCAGTCGGCG-3′, were used to amplify the overlapping PCR products in a second PCR reaction. The mutation of C101A-eNOS was confirmed by sequencing. The cDNAs for the WT bovine eNOS and the mutant C101A-eNOS were cloned into the pcDNA3 vector (Invitrogen Corporation, Carlsbad, CA) for stable transfection.

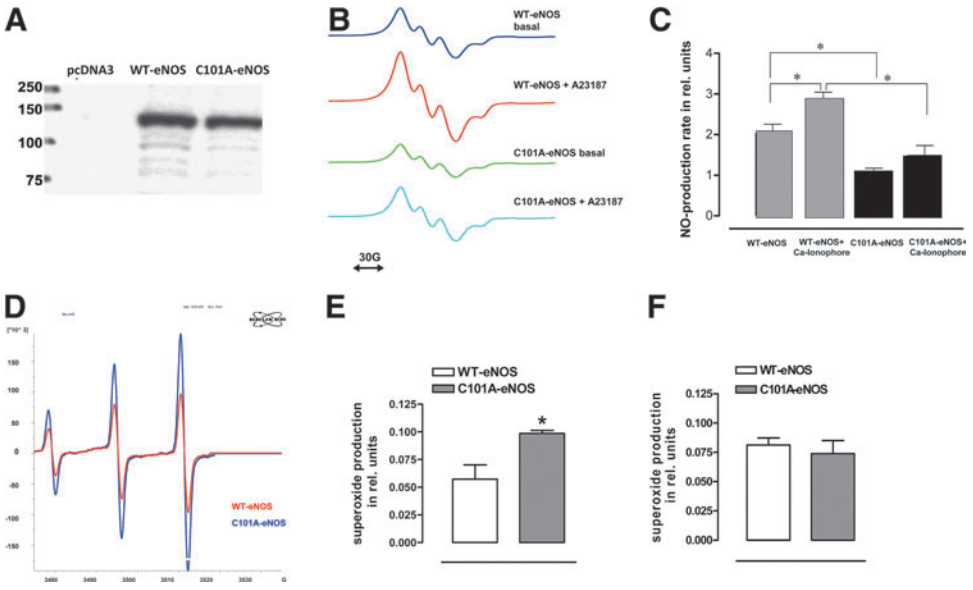
Stable transfection of human embryonic kidney 293 cells

Human embryonic kidney 293 cells (HEK 293 cells; Clontech, BD Bioscience, San Diego, CA) were cultured in T75 flasks in Dulbecco's modified Eagle's medium (DMEM; Fisher, Suwanee, GA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. One hundred-millimeter dishes were transfected with WT and MT bovine eNOS and the pcDNA3 vector using Superfect (Qiagen, Valencia, CA). Successfully transfected cells were selected by addition of 800 μg/ml of Geneticin (G418-sulfate; Fisher) to the culture medium for the first 6 weeks of culture and passage. After 10–15 days, when cells had become confluent, the dose of G418 was reduced to 400 μg/ml DMEM.

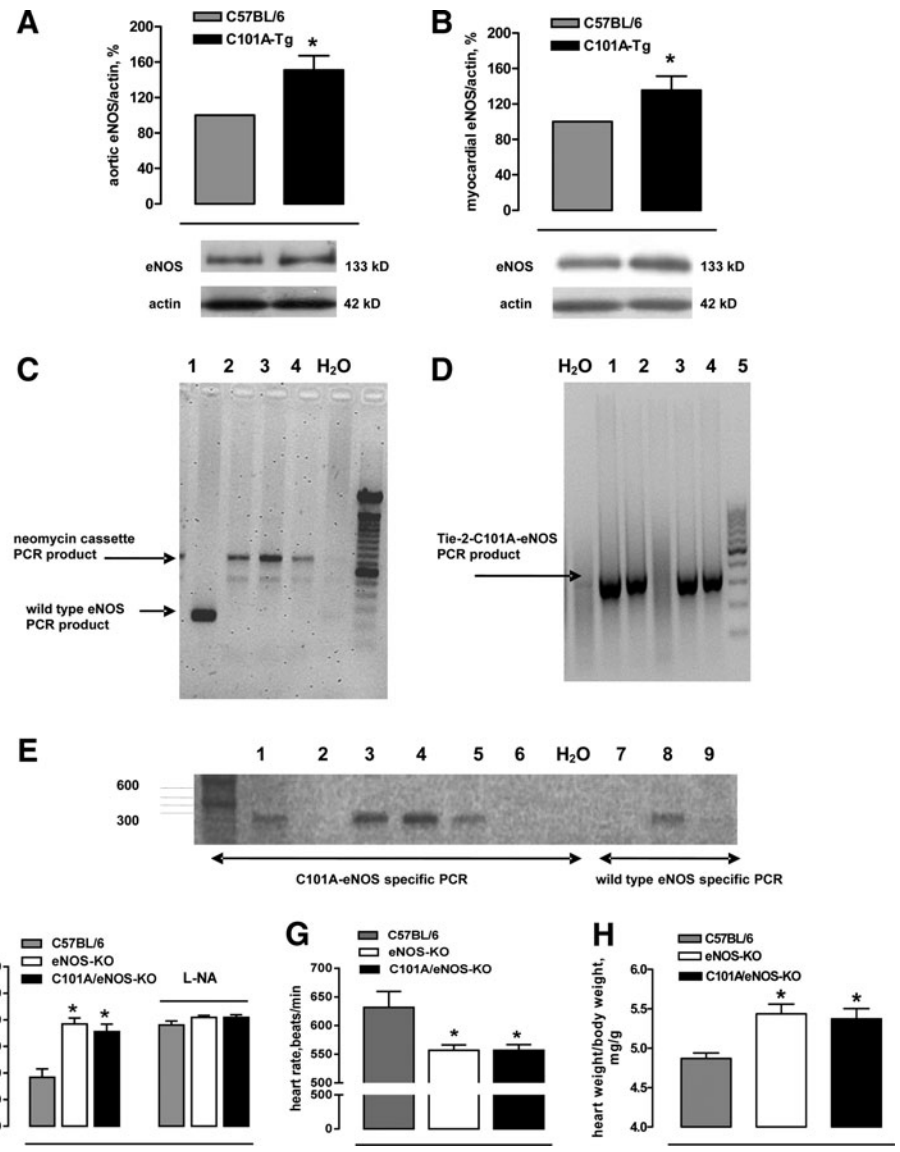
Preparation of cell and tissue homogenates

Stably transfected WT- and C101A-HEK 293 cells were washed twice with 0.1 *M* phosphate-buffered saline (PBS; pH 7.4) containing 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, and 8.3 m*M* Na_2HPO_4. The cells were then harvested in lysis buffer containing 50 m*M* Tris-HCl, pH 7.5, 1% Triton-X 100, 1 tablet Complete® (protease inhibitor cocktail tablets; Roche Molecular Biochemicals, Indianapolis, IN) in 50 ml buffer, and 100 μ*M* protease inhibitor cocktail (BD Pharmingen, San Diego, CA). The cell suspension was sonicated 4 × for 10 s with 150 W, and after 30 min of lysis on ice, the protein concentration was measured as described previously (9).

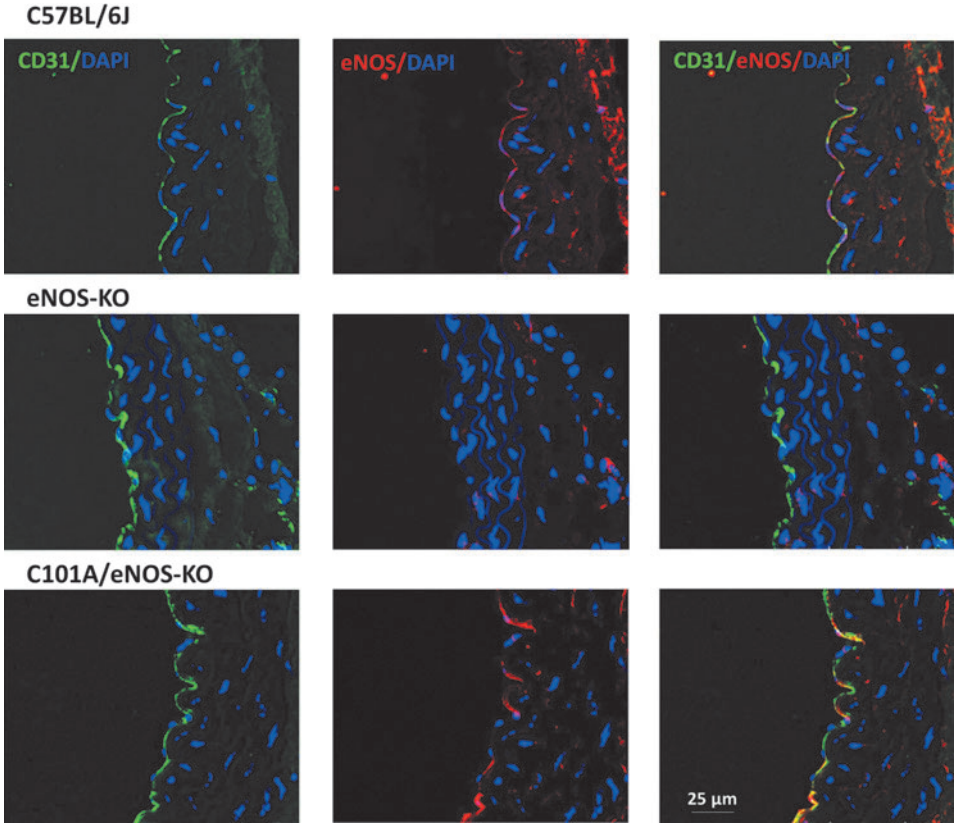
Mice organs were flash frozen in liquid nitrogen, homogenized, solubilized in lysis buffer (3), and centrifuged for



SUPPLEMENTARY FIG. S1. Characterization of C101A-eNOS in HEK cells. (A) Western blot analysis of eNOS protein (140 kDa) in stably transfected HEK 293 cells. Approximately, the same amount of eNOS protein is expressed in the wild-type (WT) and C101-eNOS (C101A-MT) cells. No eNOS protein was detectable in control cells transfected with the pcDNA3 vector alone. **(B)** Example of ESR spectra from NO production under basal conditions or stimulated with 10 μ*M* Ca^{2+} ionophore A23187 in WT and C101A-MT and **(C)** mean data showing NO production in rel. units. Basal and stimulated NO production was significantly higher in WT-eNOS-transfected HEK cells than in C101A-MT cells ($*p < 0.05$, $n = 3$). **(D)** Typically recorded superoxide triplet signals in WT and C101A-MT cells. **(E)** Increased superoxide production (in rel. units) by C101A-MT eNOS ($*p < 0.02$, $n = 4$) and **(F)** its inhibition after incubation of WT and C10A-MT cells with 1 m*M* L-NAME. There was no significant difference in the superoxide production between the two eNOS enzymes anymore. While superoxide production of WT was unchanged, the amount of superoxide produced by the C101A-MT cells was decreased. eNOS, endothelial NO synthase; ESR, electron spin resonance; HEK, human embryonic kidney; L-NAME, NG-nitro-L-arginine methyl ester.



SUPPLEMENTARY FIG. S2. Generation, genotyping and characterization of C101A-Tg and C101A/eNOS-KO. Aortic (**A**) and myocardial (**B**) eNOS protein expression standardized to actin in C101A-Tg (mean ± SEM $*p < 0.05$ *vs.* controls, $n = 5$ for aorta, $n = 7$ for myocardium). (**C–E**) Genotyping and characterization of C101A/eNOS-KO. **(C)** Two percent agarose gel with genotyping PCR products of the eNOS-KO background of C101A/eNOS-KO. Lane 1: wild-type DNA; lanes 2–4: PCR products of C101A/eNOS-KO DNA; lane 5: negative water control; last lane: DNA ladder. **(D)** Two percent agarose gel with genotyping PCR product for Tie-2 identifying the insertion of C101A/eNOS in C101A/eNOS-KO. *Left* lane: water control. Lanes 1–5: the Tie-2-C101A-eNOS PCR products. C101A/eNOS-KO numbers 1, 2, 4, 5 showed the correct size fragment of 351 kb, while lane 3 shows the result for a transgene-negative mouse. **(E)** One percent agarose gel with the products of 2 different PCRs. Lanes 1–6: products of PCR using a primer pair identifying the 310 bp fragment specific for the mutated C101A eNOS. Lanes 7–9: products of PCR using a primer pair complementary to wild-type bovine eNOS (326 bp fragment). Only C101A/eNOS-KO showed the correct size PCR product using primers specific for the C101A-eNOS transgene (lanes 1, 3–5), while no signal was detectable in the mice overexpressing wild-type eNOS (lanes 2, 6). Vice versa, primers complementary to wild-type eNOS did not give any signal in C101A/eNOS-KO (lanes 7, 9) while detecting the 326 bp fragment for the wild-type eNOS (lane 8). **(F)** Systolic blood pressure in C57BL/6, eNOS-KO, and C101A/eNOS-KO measured by the tail-cuff method before and during treatment with the NO synthase inhibitor, L-NA ($*p < 0.05$ *vs.* C57BL/6, $n = 6$). **(G)** Decreased heart rate in awake C101A/eNOS-KO and eNOS-KO compared with C57BL/6 ($*p < 0.05$ *vs.* C57BL/6, $n = 6$). **(H)** Myocardial hypotrophy in C101A/eNOS-KO and eNOS-KO as evident by an increased heart weight/body weight ratio ($*p < 0.01$ *vs.* C57BL/6, $n = 19$–24). L-NA, N^{ω}-nitro-L-arginine; PCR, polymerase chain reaction.



SUPPLEMENTARY FIG. S3. Endothelial-specific localization of C101A-eNOS. Images showing immunofluorescent staining for eNOS (*red*) and CD31 (pseudocolored in *green*) in the aorta of C57BL/6J, eNOS-KO, and C101A/eNOS-KO. Nuclei are stained with DAPI (*blue*). There was a lack of signal for eNOS in the aorta of the eNOS-deficient mouse, while C101A/eNOS-Tg showed an endothelial-specific expression of eNOS as evident by its overlay with CD31.

10 min at 100 *g*. Supernatants were stored at −80°C until they were used for Western blots. Total protein levels were determined by the Bradford method (1).

Immunoprecipitation and Western blotting

Protein expression of eNOS in cells and organ lysates was detected using Western blot analysis as described previously (4, 7, 10). Briefly, stably transfected WT- and MT-HEK 293 cell homogenates or crude mouse tissue extracts were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% acrylamide), transferred to a nitrocellulose membrane, and probed with the monoclonal eNOS antibody (BD Transduction Laboratories, Lexington, KY), diluted 1:1000. Western blot for nitrotyrosine residues in tissue lysates was done using a monoclonal antibody detecting nitrotyrosine residues (Abcam, Cambridge, United Kingdom), and three bands at around 50 kDa were evaluated densitometrically as previously described (11). For soluble guanylyl cyclase (sGC), Western blot 100,000 *g* supernatants of mouse tissues were prepared as described (8) and 10 μg of total protein per lane was fractionated on denaturing 7.5% polyacrylamide gels, blotted on polyvinylidene difluoride (PVDF) membranes (Millipore, Berlin, Germany), and stained with polyclonal antibody directed against sGC-β1 (Cayman/Biozol, Eching, Germany). Antibodies specific for actin (1:5000; Sigma, Munich, Germany) or GAPDH (1:5000; Sigma) were used as loading controls, the antibody against von Willebrand factor (1:2500; DakoCytomation, Glostrup, Denmark) was used as the endothelial cell control. Antibodies were detected by horseradish peroxidase-conjugated anti-rabbit IgG (Calbiochem, for polyclonal antibodies) or anti-mouse IgG (Bio-Rad, Munich, Germany, for monoclonal antibodies) and visualized by chemiluminescence and exposure to x-ray film. The autoradiographs were analyzed by densitometry (Geldoc; Bio-Rad). Comparative quantitative evaluation was performed with signals appearing on the same blot only.

Immunoprecipitation of eNOS was done using magnetic Dynabeads® (Invitrogen, Darmstadt, Germany). Rabbit IgG-coated beads were prepared following the manufacturer's instruction. Probes (500 μg total protein) were incubated overnight at 4°C with $\sim 10^6$ beads precoupled with anti-eNOS polyclonal antibody. The beads with any attached proteins were separated magnetically from unbound material and subjected to detection of either eNOS with monoclonal antibody specific for eNOS (BD Transduction Laboratories) or with antibody specific for eNOS phosphorylated at Ser 1176 (Ser 1179 in bovine eNOS) (Cell Signaling, Beverly, FL). Quantification of phosphorylated eNOS was performed using anti-rabbit and anti-mouse fluorescent secondary antibodies and the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE). Control experiments using a purified nonspecific rabbit IgG and the application of secondary antibodies in the absence of primary antibodies were carried out to

assess specificity of protein binding. nNOS protein level was determined in aortic and skeletal muscle homogenates using anti-nNOS antibody (1:1000; BD Transduction Laboratories).

Purification of WT- and C101A-eNOS protein

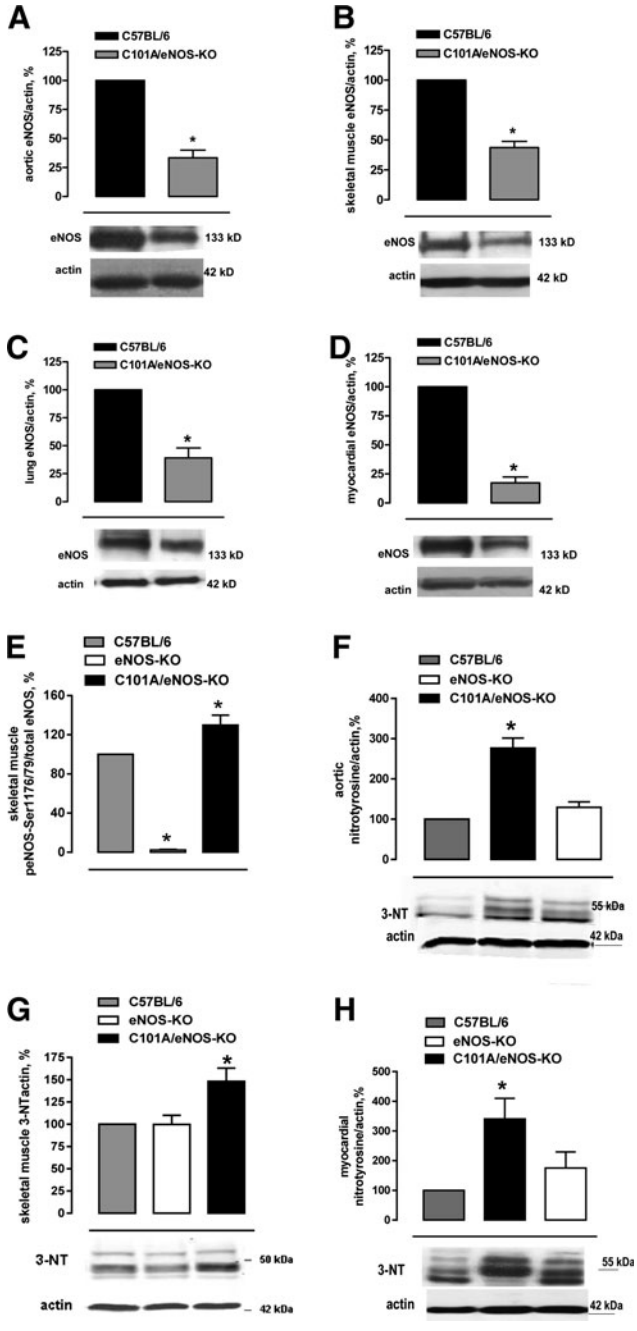
Stably transfected WT and C101A-HEK 293 cells were harvested in lysis buffer containing 50 m*M* Tris-HCl, pH 7.5, 1%Triton-X 100, 1 tablet Complete in 50 ml buffer, and 100 μl of protein inhibitor cocktail (BD Pharmingen). After 30 min on ice, the lysate was separated from insoluble materials by centrifugation with 3000 *g* for 15 min. The supernatant was added to 2′5′-ADP-Sepharose 4B (Amersham, Piscataway, NJ) and equilibrated in wash buffer A (50 m*M* Tris-HCl, pH 7.5, 20% glycerol, 0.5 *M* NaCl, 2 mM EGTA, 1 tablet Complete in 50 ml buffer, 100 μl of protein inhibitor cocktail, 50 μl β-mercaptoethanol). The suspension was incubated in Poly-Prep® chromatography columns (BioRad, Hercules, CA) at 4°C for 2 h continuously inverting the columns. The columns were washed 2× with wash buffer A and 2× with wash buffer B (like wash buffer A, without NaCl and EGTA). eNOS protein was eluted thrice by adding NADPH buffer (wash buffer B with 10 m*M* NADPH). The eluate was concentrated 15-fold by centrifugation in an Ultrafree-15 column with polyethersulfone membranes (Millipore, Bedford, MA). The purity of the samples was determined by Coomassie staining after separation on SDS-PAGE.

Measurement of NO release using electron spin resonance

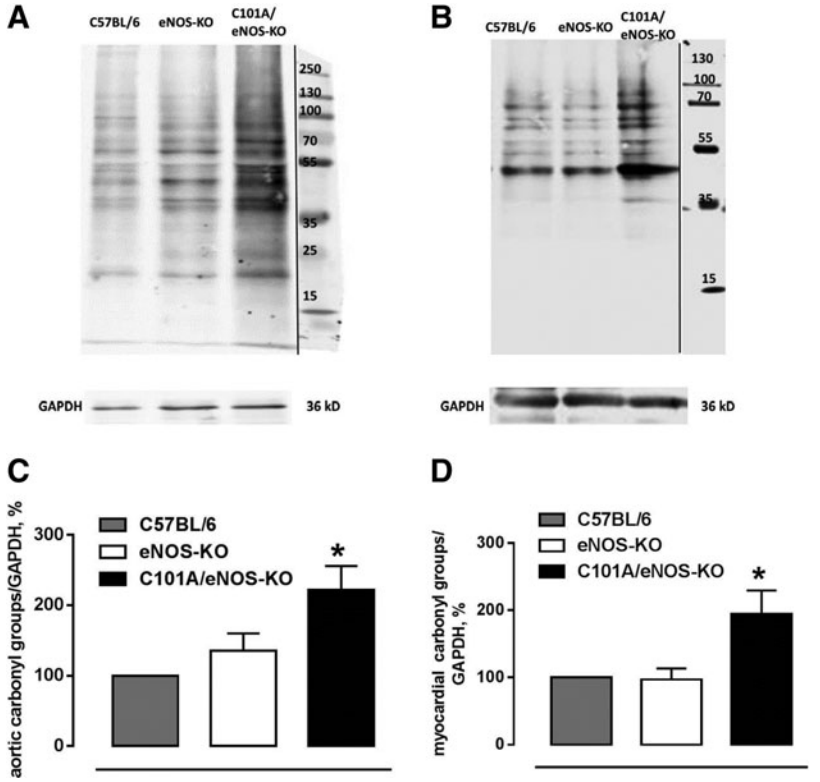
NO produced by stably transfected WT and C101A-HEK 293 cells was measured by electron spin resonance (ESR) using a NO-specific spin trap iron diethyldithiocarbamate ($Fe^{2+}(DETC)_2$) as described previously (5). Briefly, post-confluent stably transfected WT and MT HEK 293 cells were fed with only 5% DMEM 24 h before the experiment. The cells were rinsed with PBS and modified Kreb's/HEPES buffer (in m*M*: NaCI 99.01, KCI 4.69, $CaCI_2$ 1.87, $MgSO_4$ 1.20, $NaHCO_3$ 25.00, K_2HPO_4 1.03, Na-HEPES 20.00, D-glucose 11.1) before incubation with 0.5 *M* $Fe^{2+}(DETC)_2$ in Kreb's/HEPES buffer containing the agonist (10 μM Ca^{2+} ionophore A23187). The $Fe^{2+}(DETC)_2$ colloid was prepared freshly every time as described previously (5). After incubation at 37°C for 30 min, the cells were gently scraped and collected as a suspension in 1-ml syringes. These were then snap-frozen with liquid nitrogen. The frozen sample column was loaded into a finger Dewar and analyzed at low temperature with a Bruker EMX ESR spectrometer (Bruker Instruments, Billerica, MA) at microwave power of 10 mW and modulation amplitude of 3 G.

Measurement of superoxide production in intact cells

ESR was used to measure superoxide production by intact WT and C101A-HEK cells. The ESR spectrometer settings were as follows: field sweep: 60 G; microwave frequency:



SUPPLEMENTARY FIG. S4. Expression of eNOS, eNOS phosphorylation at Ser 1176/79 and nitrotyrosine residues formation in C101A/eNOS-KO. Protein expression of eNOS in the aorta (**A**, $n=12$), skeletal muscle (**B**, $n=11$), lung (**C**, $n=6$), and myocardium (**D**, $n=7$) of C101A/eNOS-KO (*$p<0.05$ *vs.* C57BL/6) given as mean ± SEM (*upper panel*), representative Western blot signals for eNOS (*middle panel*) and for actin (*lower panel*) (*$p<0.05$ *vs.* C57BL/6). eNOS protein expression level in C57BL/6 was set to 100%. **(E)** C101A/eNOS-KO showed a higher ratio of phosphorylated eNOS at Serine 1176 (Ser 1179) to total eNOS after immunoprecipitation of eNOS from the skeletal muscle homogenates compared with wild-type controls. No phosphorylated eNOS Ser 1176 (Ser 1179) signal was detectable in eNOS-KO ($n=6$ per group, *$p<0.05$ by Student's *t*-test). Significant increase in nitrotyrosine residues (3-NT) (**F**) in aortic, (**G**) in skeletal muscle, and (**H**) in myocardial homogenates of eNOS-KO and C101A/eNOS-KO given as mean ± SEM (*upper panel*, $p<0.05$ by one-way ANOVA, $n=7$). The *middle panel* shows representative nitrotyrosine Western blot, the *bottom panel* shows β-actin expression as loading control. ANOVA, analysis of variance.



SUPPLEMENTARY FIG. S5. Increased protein carbonylaton in C101A/eNOS-KO. Representative Western blot in aortic **(A)** and myocardial **(B)** tissue of C57BL/6, eNOS-KO, and C101A/eNOS-KO showing carbonyl groups as a marker for protein oxidation. All the bands were used for densitometric analysis. The *bottom panel* shows GAPDH expression as loading control. Relative optical density (%) from Oxyblots in (**C**) the aorta ($p < 0.05$ by one-way ANOVA, $n = 7$) and (**D**) left myocardium ($p < 0.05$ by one-way ANOVA, $n = 4$). The amount of carbonyl groups in the C101A/eNOS-KO was significantly higher than in C57BL/6 and eNOS-KO (*$p < 0.05$ $n = 6$).

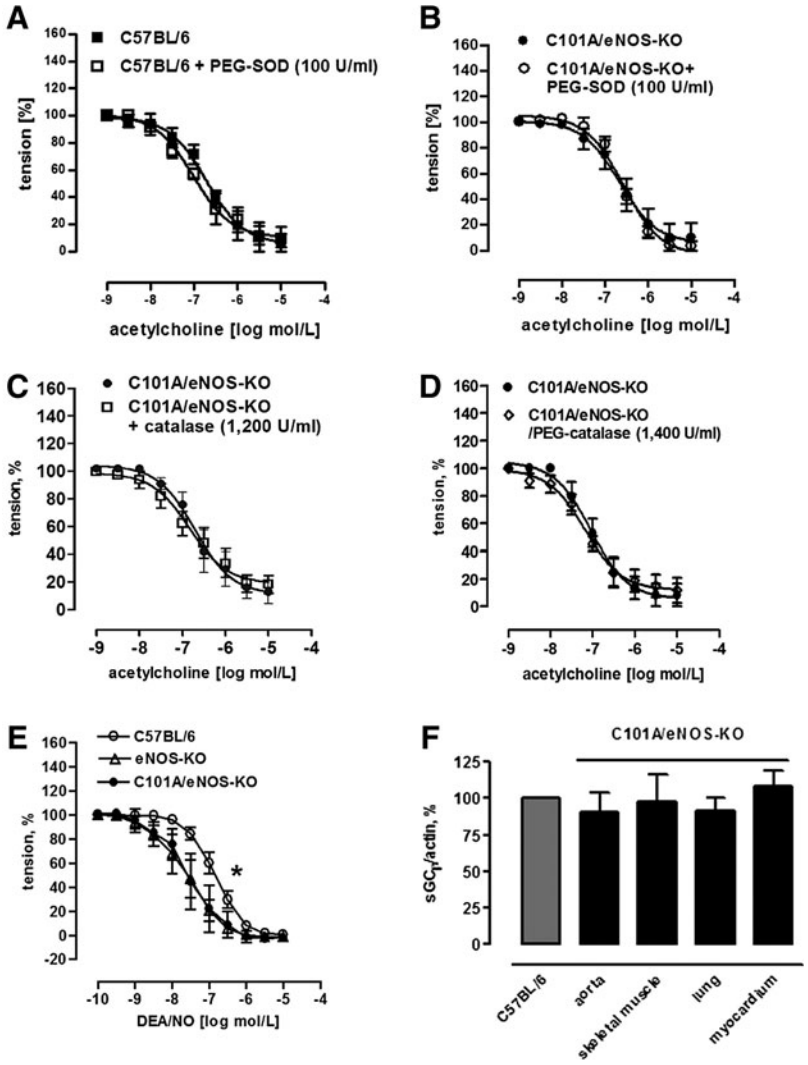
9.82 GHz; microwave power: 20 mW; magnetic field modulation amplitude: 2 G; conversion time: 655 ms; and constant time: 328 ms. Stably transfected WT and C101A HEK cells were cultured on 100-mm dishes. Twenty-four hours before the experiments, the DMEM was changed to 5% fetal bovine serum. At the time of the study, the cells were washed and harvested in incubation buffer containing (in m*M*) 5.5 glucose, 1.4 $CaCl_2$, 0.015 diethylenetriaminepentaacetic acid, 2.6 NaCl, and 5.0 KCl in sodium phosphate buffer (19.5 m*M* NaH_2PO_4 and 53.5 m*M* Na_2HO_4, pH 7.4). After low-speed (100 *g*) centrifugation, the pellet was resuspended in 100 μl incubation buffer, and 5 μl of the cell suspension was mixed with 95 μl incubation buffer containing 1 m*M* 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH; Alexis Corporation, San Diego, CA) as the spin trap. ESR spectra were recorded in 50-μl glass capillaries. Superoxide formation was determined by following the oxidation of CM-H to paramagnetic $CM^{\bullet}$. The intensity of ESR spectra was quantified and adjusted to cell numbers.

Determination of eNOS activity in cell homogenates

Cells were washed twice with 0.1 *M* PBS (pH 7.4) containing 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, and 8.3 m*M* Na_2HPO_4. They were harvested with cell scrapers in homogenization buffer containing 50 m*M* Tris HCl buffer (pH 7.4), 0.1 m*M* EDTA, 0.1 m*M* EGTA, 0.5 m*M* DTT, 1 μM pepstatin A, 2 μM leupeptin, 2 μM bestatin, and 1 m*M* PMSF in DMSO. The cell suspension was sonicated 4 × for 10 s with 150 W, and after 30 min of lysis on ice, the amount of protein was measured using Bradford reagents (1). The specific eNOS activity in stably transfected WT and C101A cells was determined by conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline as described previously (2). The cell homogenate (100 μl) was added to a reaction buffer containing 50 m*M* triethanolamine-HCl, pH 7.4, 0.1 U/μl calmodulin, 100 μM L-arginine, 2.5 m*M* $CaCl_2$, 1 m*M* citrulline, 4 μM flavin adenine dinucleotide, 4 μM flavin adenine mononucleotide, 1 m*M* NADPH, and 0–50 μM BH_4 to a total volume of 185 μl. After addition of 15 μl ^{14}C-L-arginine (50 μCi, 1,85 MBq; NEN, Boston, MA), the mixture was incubated for 15 min at 37°C, and the reaction stopped with a buffer containing 20 m*M* Na-HEPES, 2 m*M* EDTA, and 2 m*M* EGTA. The formed ^{14}C-citrulline was isolated using AG 50W resin columns and quantified in a scintillation counter.

Detection of oxidative protein modifications

Protein modifications caused by additional carbonyl groups were detected using the Oxyblot™ system (Intergen, Burlington, MA). During the incubation of cell homogenates with 2,4-dinitrophenylhydrazine (DNPH) for 15 min, carbonyl groups reacted with DNPH and formed 2,4-dinitrohydrazone residues. The proteins were then denatured in 6% SDS and loaded on 7.5% SDS-PAGE. After transfer to the nitrocellulose membrane, the 2,4-dinitrohydrazone residues were detected by the



SUPPLEMENTARY FIG. S6. Aortic reactivity and sGC-β1 protein expression in C101A/eNOS-KO. (A) Relaxation response of aortic ring segments of C101A/eNOS-KO and **(B)** C57BL/6 induced by cumulative addition of acetylcholine in the absence and presence of 100 U/ml of pegylated SOD (*$p < 0.05$, $n = 4$), **(C)** conventional and **(D)** pegylated catalase ($p > 0.05$, two-way ANOVA, $n = 4$–6). **(E)** Aortic relaxation of C101A/eNOS-KO, eNOS-KO, and C57BL/6 to cumulative addition of the NO donor, DEA/NO (*$p < 0.05$ for pD_2 and two-way ANOVA, $n = 4$–7). **(F)** Unchanged sGC-β1 protein expression standardized to actin in cytosols of the aorta, skeletal muscle, lung, and myocardium of C101A/eNOS-KO and C57BL/6 ($p > 0.05$ *vs.* C57BL/6, Student's *t*-test, $n = 4$–5). DEA/NO, diethylamine/nitric oxide; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase.

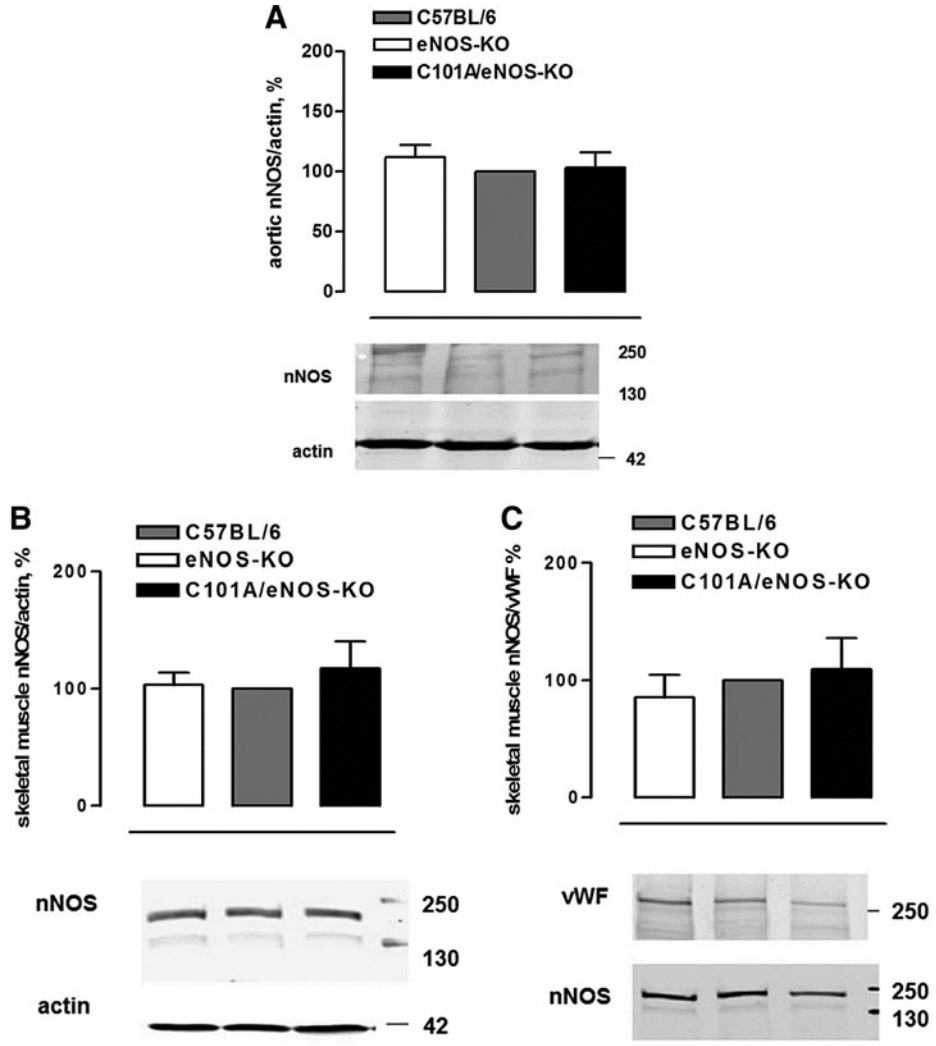
specific Oxyblot antibody system and visualized on Hyperfilm (see Immunoprecipitation and Western blotting).

Detections of eNOS monomers and dimers

WT- and C101A-eNOS cell lysates or purified enzyme preparations were separated using low-temperature SDS-PAGE. Laemmli sample buffer without β-mercaptoethanol was added and the nondenatured proteins were loaded on 6% SDS gel. Gel separation of proteins was performed on ice in a refrigerated room for ~ 3 h at 30 mA. The protein transfer and antibody incubations were done as described in the Western blot analysis.

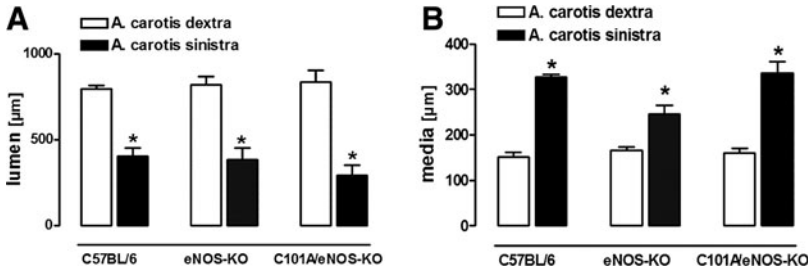
Generation of transgenic mice

An MluI restriction site was added to the 3′ site of mutated bovine eNOS cDNA. An EcoRV-C101A-eNOS-MluI fragment was isolated and inserted into the pBluescript II SK(+) backbone plasmid downstream of the Tie-2 promoter and upstream of the Tie-2 enhancer. The plasmid was confirmed by restriction analysis and excised from the vector with SpeI. The resulting Tie-2 promoter-C101A-eNOS-enhancer construct was purified and microinjected in fertilized eggs of C57BL/6 × C3H/He mice. The eggs were transferred into the oviducts of pseudopregnant mice after incubation for one night at 37°C and allowed to develop to term. Founder mice were crossed for 15 generations to the C57BL/6J genetic background. Male mice were used at 12 to 20 weeks of age. Transgene-negative littermates served as controls. In some experiments, C57BL/6 served as nontransgenic controls. Mice were sacrificed by inhalation of carbon dioxide and tissues were used for organ bath studies (aorta) or lucigenin measurements or immediately frozen in liquid nitrogen. The frozen tissues were taken to prepare total protein for Western blotting.



SUPPLEMENTARY FIG. S7. Lack of upregulation of nNOS in C101a/eNOS-KO. nNOS protein expression in C101A/eNOS-KO, eNOS-KO, and C57BL/6. There was no upregulation of nNOS protein in aortic ($n=4$) (**A**) and skeletal muscle homogenates standardized to actin (**B**) ($n=5$–9) or to an endothelial marker von Willebrand factor (vWF), $n=5$–6 (**C**). One hundred micrograms of total protein was loaded per lane.

Transgene-positive mice were identified by standard PCR in DNA isolated from tail biopsies at 4–5 weeks of age. The sense primer was designed to bind inside the Tie-2 promoter (5′-GGGAAGTCGCAAAGTTGTGAGTT-3′) and the antisense primer inside the eNOS gene (5′-GCTCCCAGTTCTTCA CGCGAGG-3′), identifying a 351 bp transgene-specific fragment. eNOS-KO were identified by the presence of the neomycin cassette and the absence of endogenous murine eNOS. Primers complementary to exon 12 of the eNOS gene were used to identify wild-type mice (sense, 5′-GCATCACCAGG



SUPPLEMENTARY FIG. S8. Luminal narowing and media thickening in C101A/eNOS-KO. Largely decreased lumen area **(A)** and enlarged media **(B)** in ligated left carotid artery of C57BL/6, eNOS-KO, and C101A/eNOS-KO (*$p<0.05$ *vs.* nonligated right carotid arteries, $n=4$–5).

AAGAAGACC-3′, and antisense, 5′-GAGCCATACAGAT GGTTGCC-3′), and primers complementary to the neomycin-resistance cassette were used to identify the presence of the disrupting genetic insert (sense, 5′-CTCGACGTTGTCACTG AAGC-3′, and antisense, 5′-TCAAGAAGGCGATAGAA GGC-3′). In addition, transgene-specific PCR was done to distinguish between mutated and wild-type bovine eNOS using a primer recognizing the mutated coding triplet of bovine eNOS (TG in position 301/302 into GC): the sense primer, 5′-TTGAAGAGTGTGGGCCAGGA-3′, the antisense primer, 5′-ACACCAGGGAGCCCAGGGC-3′, and identifying the 310 bp mutation-specific fragment. As a control, primers complementary to wild-type eNOS were used (the sense primer, 5′-CAGCGACATGGGCAAGA-3′, and the antisense primer, 5′-ACACCAGGGAGCCCAGGCA-3′), identifying the 326 bp wild-type eNOS fragment. Finally, the presence of mutated coding triplet was confirmed in selected breeding pairs by sequencing.

Lucigenin superoxide detection

Lucigenin-enhanced chemiluminescence detection of superoxide in intact aorta and left ventricular myocardium of C101A/eNOS-KO, eNOS-KO, and C57BL/6 was measured as described previously (6). Briefly, freshly cleaned and harvested thoracic aortas were cut into two parts and equilibrated in Krebs-HEPES buffer for 30 min at 37°C, with one half of each vessel being incubated in the presence of NG-nitro-L-arginine methyl ester (L-NAME; 1 m*M*). Aortic and left ventricle myocardial segments were placed in albumin buffer enriched with lucigenin 5 μM, and lucigenin chemiluminescence was recorded every 2 min for 20 min. Background readings were subtracted from sample reading and results expressed as counts per min per mg dry weight of tissue.

Immunohistochemistry

Mice were sacrificed with CO_2. Aortas were excised and embedded in Tissue Tek® medium at −40°C according to routine procedure. Endothelium-specific overexpression was analyzed by eNOS/CD31 double staining. For this purpose, frozen aortic sections (5 μm) were incubated with a primary polyclonal antibody against eNOS (1:100; Abcam) and rat monoclonal antibody against CD31 (1:50; Abcam) and subsequently detected by goat anti-rabbit Alexa Fluor® 594 (1:500; Life Technologies GmbH, Darmstadt, Germany) and goat anti-rat Alexa Fluor 647 (1:500; Life Technologies GmbH) conjugates. Nuclear staining was performed by using Roti®-Mount FluorCare DAPI reagent (Carl Roth GmbH, Karlsruhe, Germany). Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an AxioImager M2 microscope (Carl Zeiss, Göttingen, Germany).

Supplementary References

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