

Supplement data:

Methods and Materials

Materials

All chemicals including those for Western blotting were obtained from Sigma, unless otherwise indicated: norepinephrine bitartrate, acetylcholine chloride (ACh), sodium nitroprusside (SNP), adenosine 5'-triphosphate (ATP), ionomycin, superoxide dismutase (SOD), catalase, L-norvaline, indomethacin, and ponceau S. Anti-eNOS monoclonal antibody was purchased from Transduction Laboratories; anti-mouse IgG (H+L) alkaline phosphatase (AP) conjugate and the BCIP/NBT stabilized substrate for AP were from Promega. Anti-ACh-M₃ receptor, anti-COX1, and anti-COX2 antibodies were purchased from Santa Cruz Biotechnology, Inc. Secondary antibodies IRDye 800 conjugated affinity purified goat anti-rabbit IgG F(c) was purchased from BioConcept (Allschwil, Switzerland), Alexa fluor 680 conjugated goat anti-mouse IgG (H + L) was from Invitrogene (Lucerne, Switzerland). Concentrations of the drugs are expressed as final concentrations in organ bath solution.

***In vitro* ECG**

Mice were heparinized (100 IU) and 10 minutes later anesthetized using sodium pentobarbital (50 mg kg⁻¹ intraperitoneally). After midline sternotomy, hearts were excised rapidly and the aorta was cannulated using a 20 gauge metallic cannula. Retrograde perfusion in the Langendorff mode was performed at 37°C at a perfusion pressure of 70 mm Hg on a commercial Langendorff system (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). All hearts were perfused with a filtered (pore size 0.65 µm) non-recirculating modified Krebs-Henseleit solution as described above saturated with a mixture of 95 % O₂ and 5 % CO₂, at pH 7.4. During all experiments, the hearts were immersed in perfusate that was maintained at 37.0°C.

After a 20-min stabilization period, hearts underwent dose-response curves of ACh (0.01 to 100 µmol/L, 10 minutes perfusion time each). Coronary flow was measured continuously within the aortic cannula using an inline flowprobe (Transonic 2N) connected to a transit time flowmeter (Transonic TTFM-SA type 700, Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Simultaneously, a bipolar electrocardiogram (ECG) was recorded from electrodes implanted superficially in the right atrium and the apex. A digitized readout of the ECG was recorded at 1 kHz sampling rate throughout the experiment using PowerLab 4/20 (AD Instruments, Castle Hill, Australia) connected to a Macintosh computer (Apple, Cupertino, CA, USA) running Chart software (version 5, AD Instruments, Castle Hill, Australia). On the ECG, the rate of sinus node depolarization (P-P interval) and the atrioventricular nodal conduction time (P-Q interval) was analyzed in response to increasing concentrations of ACh.

Supplement data:

Supplemental Figure legends

Suppl. Fig. 1: Neither ROS, nor arginase, are involved in endothelial dysfunction in response to ACh in *Per2* mutant mice: (A) Neither treating aortic rings with superoxide dismutase (SOD, 150U/ml) plus catalase (1000 U/ml) (B) nor with the arginase inhibitor L-norvaline (0.2 mmol/L), improve the response to ACh (1 nmol/L to 10 μ mol/L) in the *Per2* mutant mice at ZT3 (n=6, n.s.) *p<0.05, **p<0.01 for *Per2* vs. WT. ANOVA with Bonferroni adjustment for comparison of ACh responses at the indicated corresponding concentrations among 3 or 4 groups.

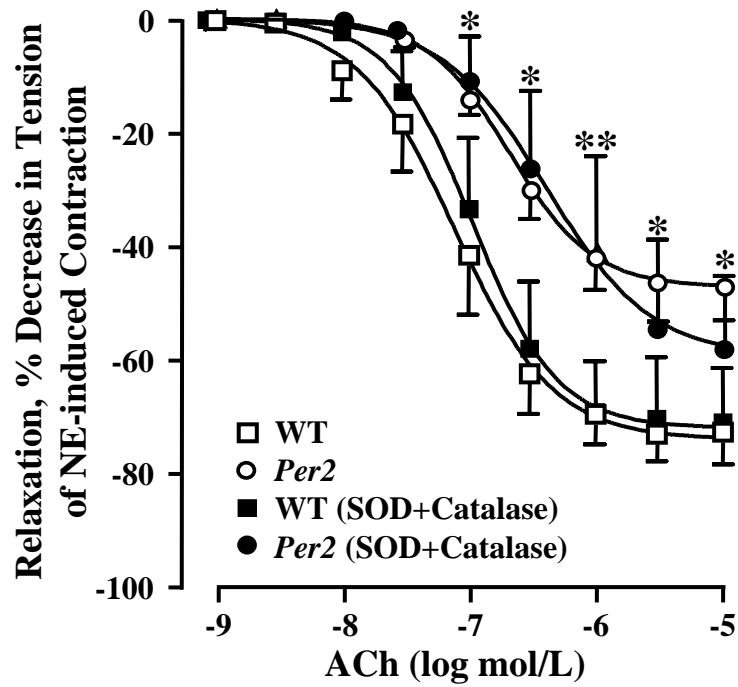
Suppl. Fig. 2: Impairment of endothelium-dependent relaxations to acetylcholine in the *Per2* mutant mice at different ZT times. *Per2* mutant mice demonstrate decreased endothelium-dependent relaxations to acetylcholine (ACh, 1 nmol/L to 10 μ mol/L) at ZT3, ZT6 and ZT9 compared to wild type (WT) mice, n=9; *p<0.05, **p<0.01 for *Per2* vs. WT at the corresponding ZT. Student's unpaired t test for comparison of ACh responses at the indicated corresponding concentrations between *Per2* mutants and WT mice at the corresponding ZT.

Suppl. Fig. 3: Impairment of endothelium-dependent relaxations to ionomycin in the *Per2* mutant mice at ZT15. The *Per2* mutant mice demonstrate decreased endothelium-dependent relaxations to ionomycin at ZT15 in the aortas pretreated with indomethacin (1 mmol/L, 30 minutes). n=7, p<0.05 for the AUC between the two groups. Student's unpaired t test for comparison of AUC between WT and *Per2* mutant mice at ZT15.

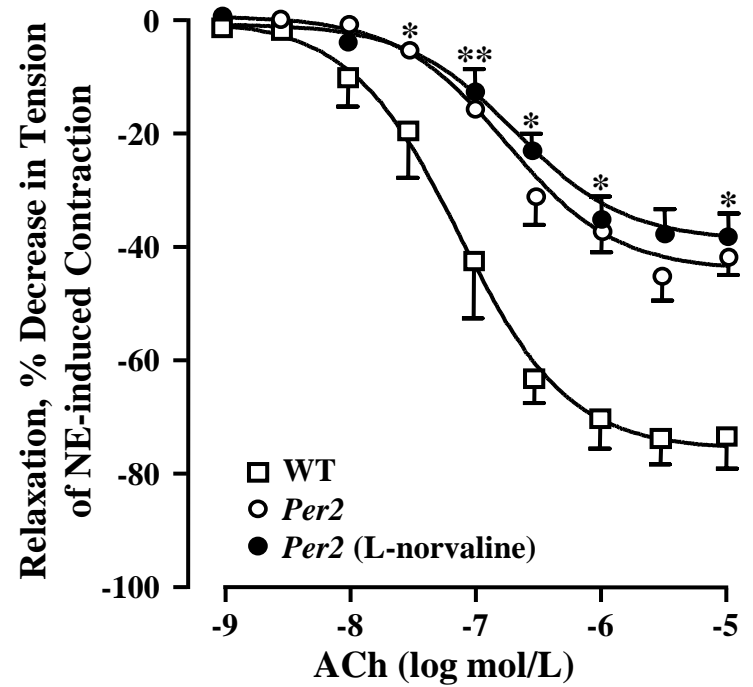
Suppl. Fig. 4: Effects of ACh on isolated heart. In vitro ECG showed no difference in changes of PP interval or PQ interval in response to increasing concentrations of ACh (1 nmol/L to 0.1 mmol/L) between the two groups (n=8).

Suppl. Fig. 5: Plasma concentrations of total cholesterol and triglyceride in the WT and *Per2* mutant mice (n=4).

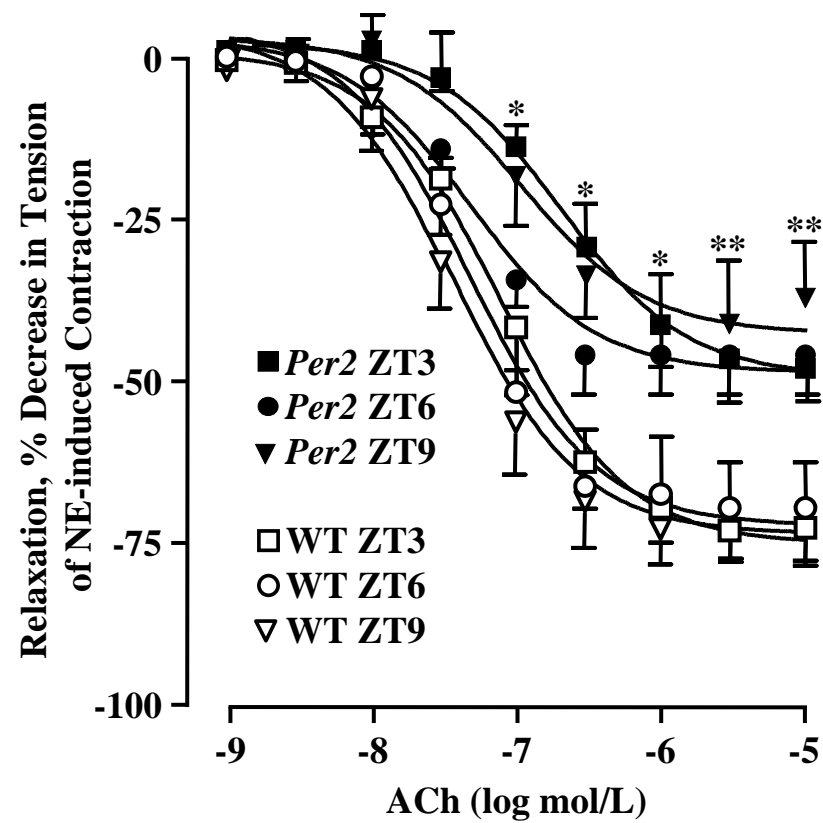
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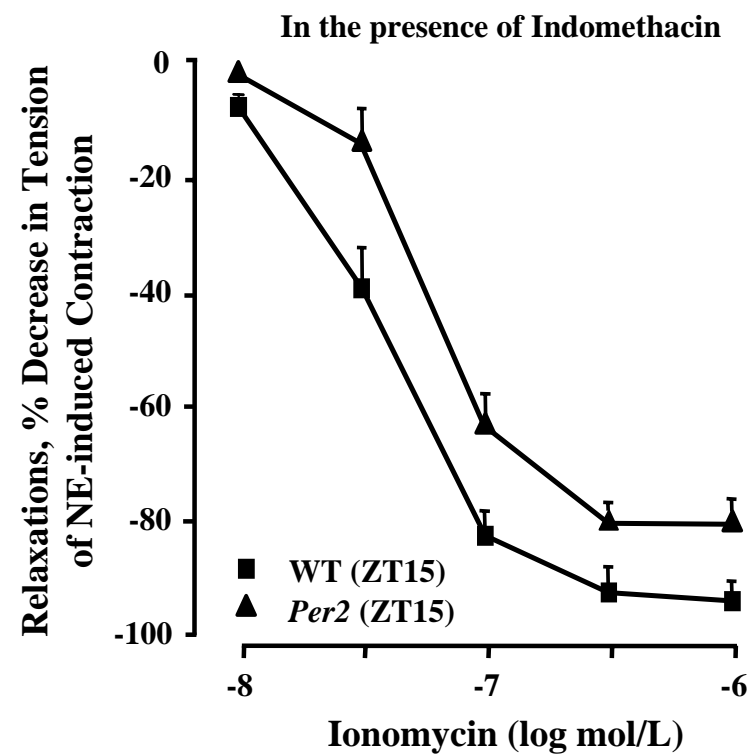
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Suppl. Fig. I

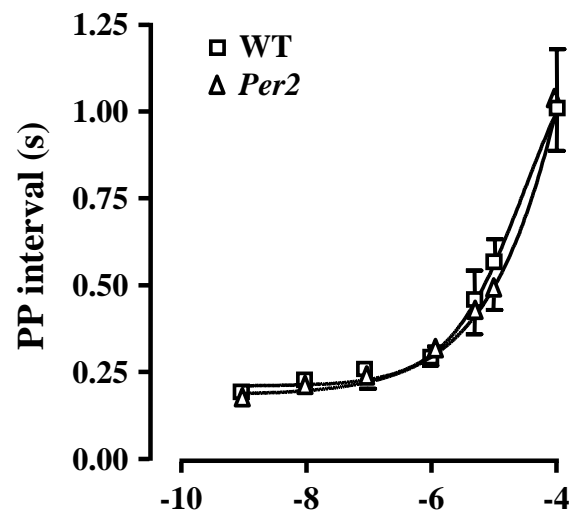


Suppl. Fig. II

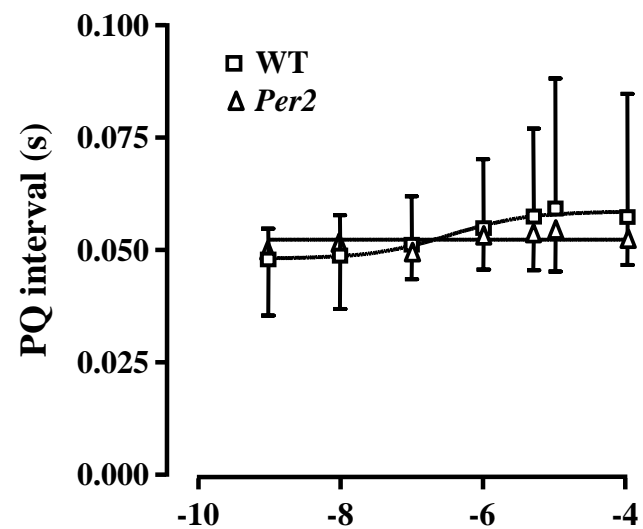


Suppl. Fig. III

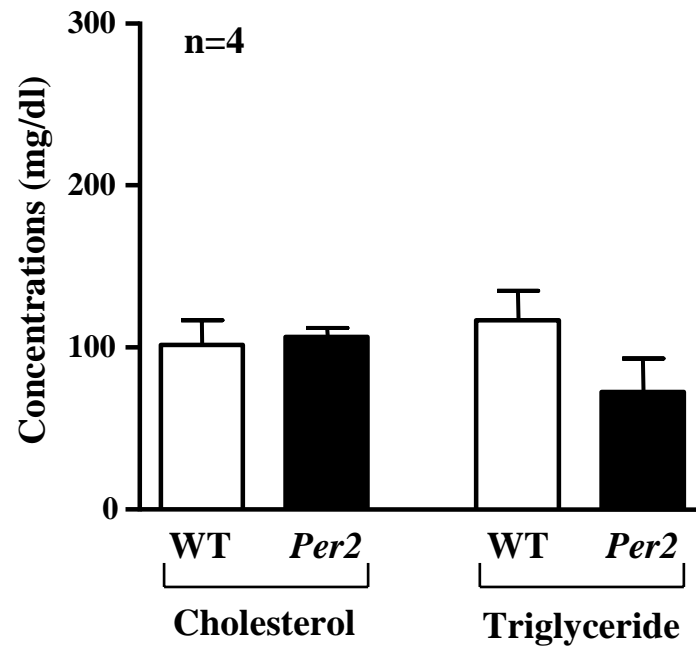
A.



B.



Suppl. Fig. IV



Suppl. Fig. V