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-M_3 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 % O_2 and 5 % CO_2, 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

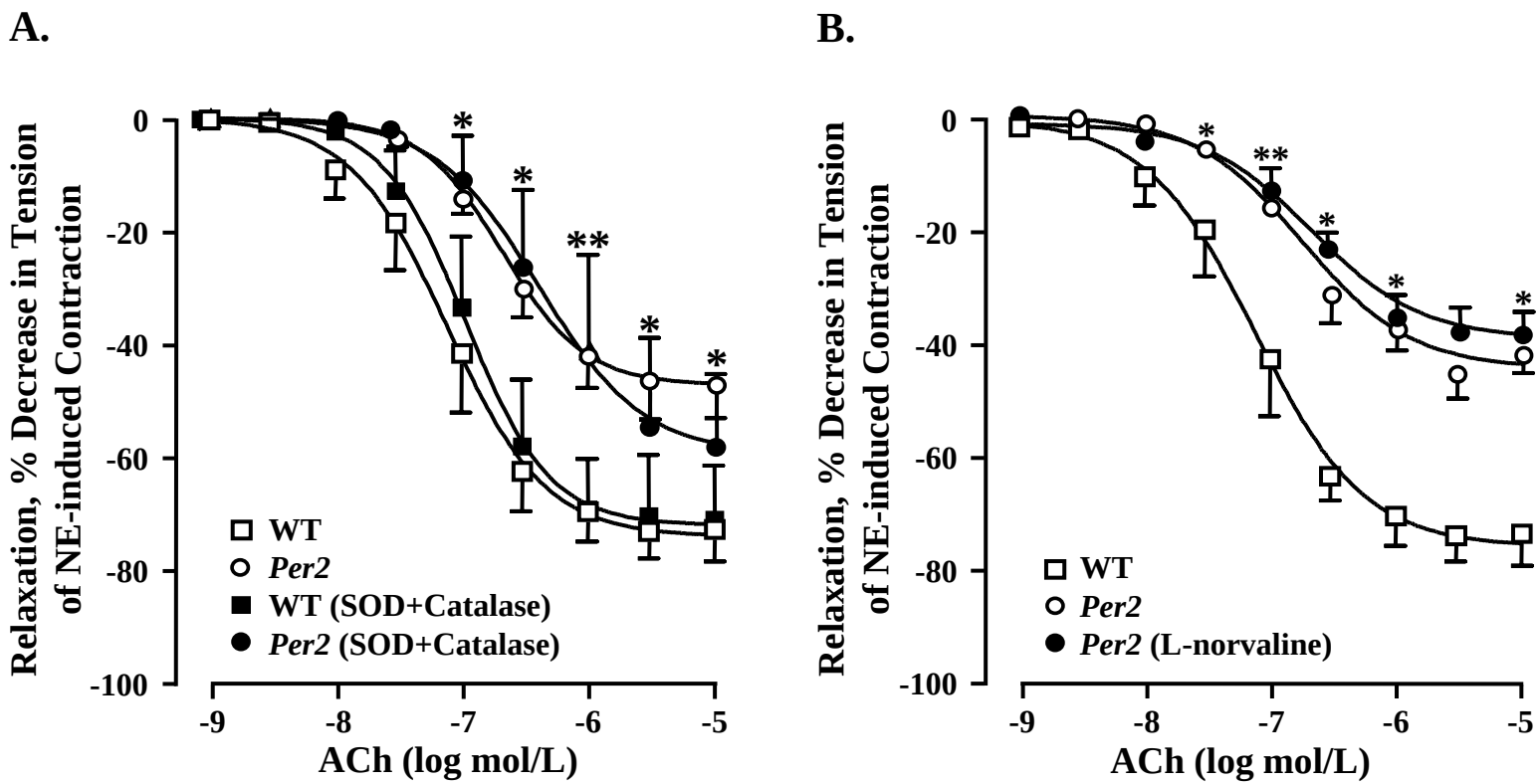
Suppl. Fig. 1: **Neither ROS, nor arginase, are involved in endothelial dysfunction in response to ACh in *Per2* mutant mice:** (A) Neither treating aortic rings with superoxide dismutase (SOD, 150U/ml) plus catalase (1000 U/ml) (B) nor with the arginase inhibitor L-norvaline (0.2 mmol/L), improve the response to ACh (1 nmol/L to 10 μmol/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 μmol/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.

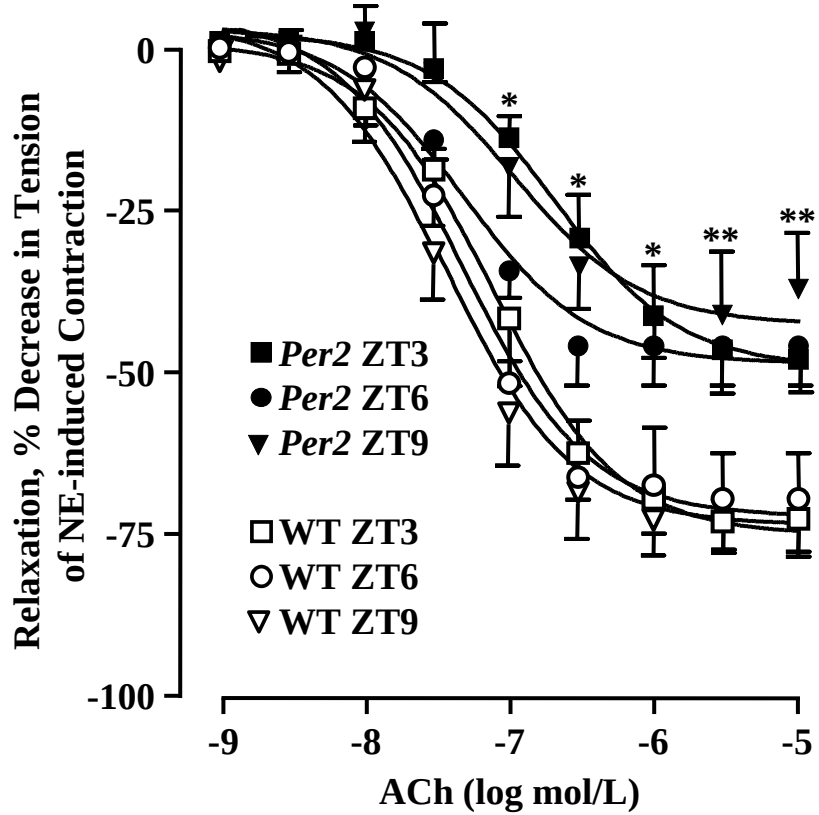
Suppl. Fig. 3: Impairment of endothelium-dependent relaxations to ionomycin in the *Per2* mutant mice at ZT15. The *Per2* mutant mice demonstrate decreased endothelium-dependent relaxations to ionomycin at ZT15 in the aortas pretreated with indomethacin (1 mmol/L, 30 minutes). n=7, p<0.05 for the AUC 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).

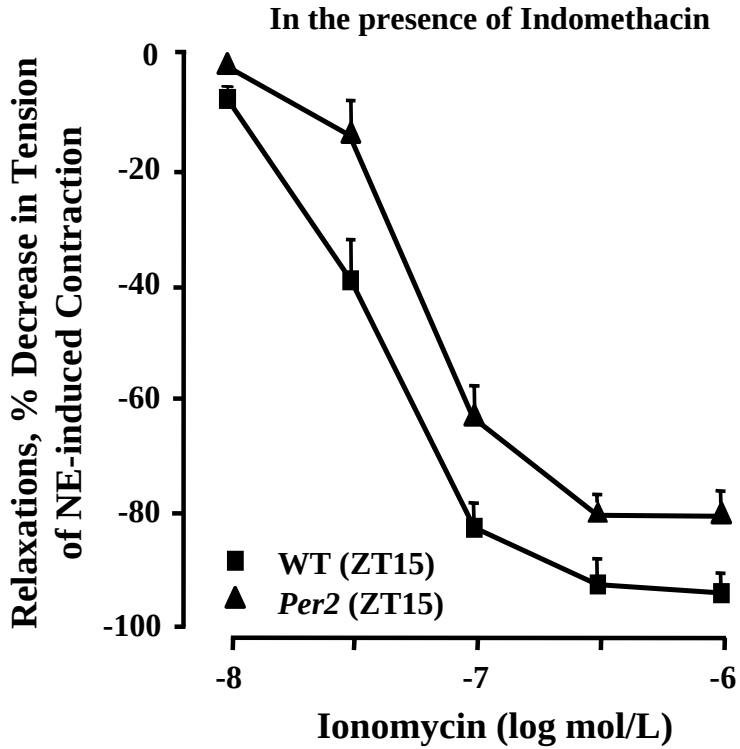
Suppl. Fig. 5: Plasma concentrations of total cholesterol and triglyceride in the WT and *Per2* mutant mice (n=4).


A.
B.
Relaxation, % Decrease in Tension of NE-induced Contraction
0
-20
-40
-60
-80
-100
-9
-8
-7
-6
-5
ACh (log mol/L)
WT
Per2
WT (SOD+Catalase)
Per2 (SOD+Catalase)
Per2 (L-norvaline)
*
**

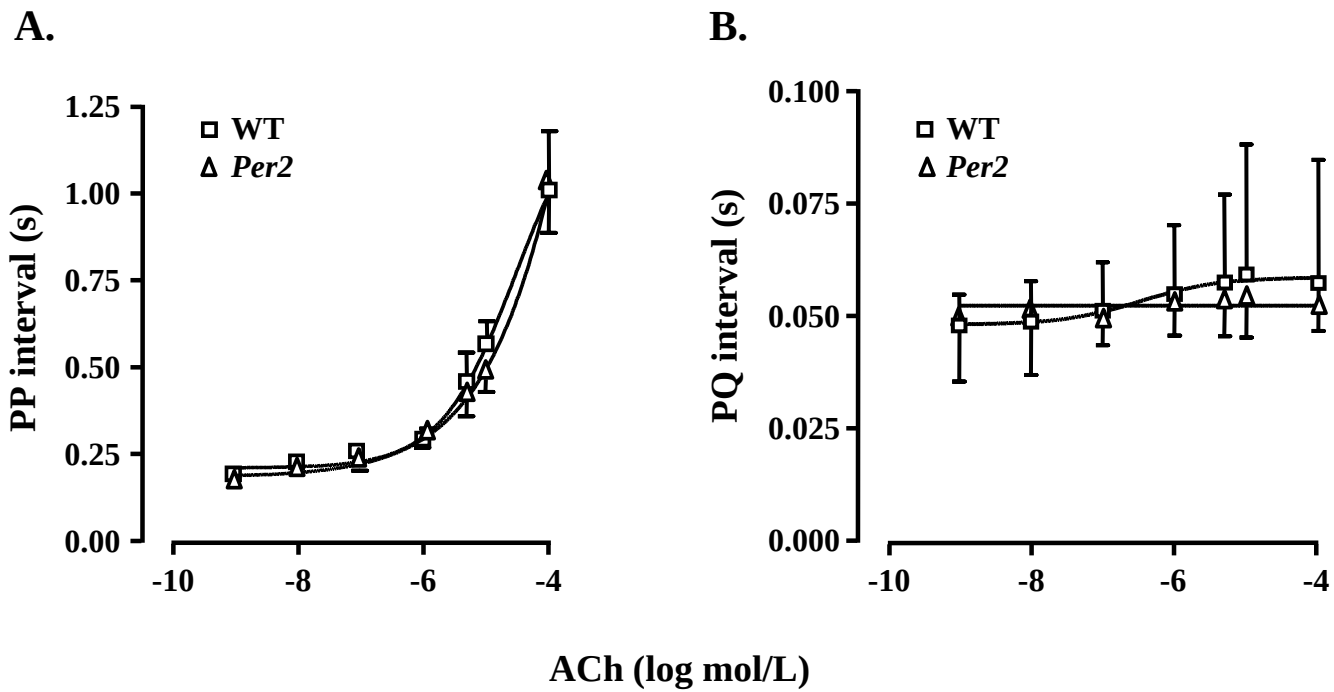
Suppl. Fig. I


Relaxation, % Decrease in Tension
of NE-induced Contraction
0
-25
-50
-75
-100
-9
-8
-7
-6
-5
ACh (log mol/L)
*
*
*
**
**
Per2 ZT3
Per2 ZT6
Per2 ZT9
WT ZT3
WT ZT6
WT ZT9

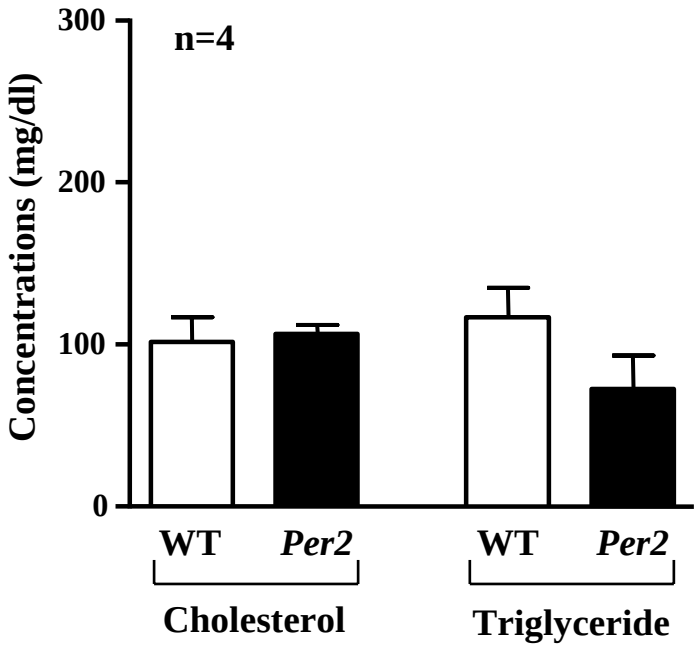
Suppl. Fig. II



Suppl. Fig. III


A.
B.
PP interval (s)
1.25
1.00
0.75
0.50
0.25
0.00
WT
Per2
-10
-8
-6
-4
PQ interval (s)
0.100
0.075
0.050
0.025
0.000
ACh (log mol/L)

Suppl. Fig. IV


n=4
300
200
100
0
Concentrations (mg/dl)
WT
Per2
WT
Per2
Cholesterol
Triglyceride

Suppl. Fig. V