Data Supplement

Methods

Echocardiography. The mice were anesthetized with 1.5% isoflurane by inhalation. The left ventricular (LV) ejection fraction (EF) was calculated from the LV end diastolic diameter (EDD) and LV end systolic diameter (ESD) as follows: $EF= (EDD^3-ESD^3)/EDD^3 *100\%$.

Western blots. NOS Protein content was analyzed using Western blots as previously described [14]. Primary antibodies against iNOS, eNOS, nNOS, ANP, protein arginine methyltransferase 1 (PRMT1), 4-HNE, nitrotyrosine, total mTOR, phospho-mTOR, Akt, phospho-Akt, phosphor-S6 and total p70S6K were purchased from Transduction Laboratories, Santa Cruze Inc, Sigma, Upstate, or Cell Signaling Technology, respectively. Dimethylarginine dimethylaminohydrolase 1 (DDAH1) antibody was a gift from Dr. Kimmoto [10].

Measurement of reactive oxygen species (ROS): Relative ROS production was determined by chemiluminescence of coelenterazine (4μM, Molecular Probe- 1) [11] and the red fluorescent dye dihydroethidium (DHE, 2 μM; Invitrogen). To assess ROS production with chemiluminescence of coelenterazine, flash-frozen myocardium (~40mg) was cut into fine pieces (about ~1mm^3) on ice, and added to 500μl ice cold Krebs bicarbonate buffer containing the following reagents, in mmol/l: NaCl, 118; KCl, 4.7; $CaCl_2$, 1.5; $MgSO_4$, 1.1; KH_2PO_4, 1.2; glucose, 5.6; and $NaHCO_3$, 25. Coelenterazine (4μM) was added to the tissue suspension and chemiluminescence was assessed for 60 seconds at 5 min intervals over 60 min at room temperature in a Lumat LB 9507 tube luminometer. Data were normalized by sample weight. The superoxide anion production was also determined after nonselective NOS inhibition with LNME (1 mmol added 20 minutes before the assay) as previously described [16], or selective iNOS inhibition with 1400W (100 μmol added 20 minutes before the assay) [39]. 1400W at a concentration of 100 μmol had no effect on NO production in cultured endothelial cells but attenuated iNOS activity, indicating that this concentration of 1400W does not inhibit eNOS activity[39]. In addition, fresh frozen LV myocardium (8 μm slices) was incubated for 1 hour at 37°C with the red fluorescent dye DHE (2 μM; Invitrogen) to assess O_2^- formation (typically nuclear localization). Imaging was performed on a Zeiss microscope (Carl Zeiss Inc.).

MMP activity. In vitro gelatin lysis by MMP-2 and MMP-9 was assessed by zymography [12]. Briefly, LV tissue was homogenized in modified buffer containing 20mM Tris-HCl pH7.4, 0.3% Triton-X100, and spun down at 1000 X g for 10 min at 4℃. Modified Laemmli buffer without mercaptoethanol was added to 25 μg of the protein samples and incubated for 10 min before loading on 10% SDS-PAGE with 1mg/ml gelatin. 1ng of collagenase type 2 was loaded as a positive control. After electrophoresis, gels were washed twice with renaturing buffer, followed by developing buffer and stained with SimplyBlue (Invitrogen).

Histological staining and measurement of myocardial fibrosis. Tissue sections (8μm) from the central portion of the LV were stained with H&E (Sigma) for overall morphology, Masson's Trichrome (Sigma) or Sirius Red (Sigma) for detection of fibrosis, and FITC-conjugated wheat germ agglutinin (AF488,

Invitrogen) to evaluate myocyte size. For mean myocyte size, the short diameter and cross sectional area of at least 120 cells/sample (from 4 areas) and at least 4 samples of each group were averaged. Relative myocardial fibrosis was expressed as the percent volume fibrosis determined with a method described in Unbiased Stereology. For eNOS or iNOS staining, the sections were first fixed with 3.7% paraformaldehyde for 20 minutes, rinsed with PBS, followed by permeablization with 0.2% Triton X100 for 10 minutes. The sections were incubated with 0.3% H_2O_2 in PBS for 1 hour followed by 1% BSA solution for 1 hour. Sections were then incubated with primary antibodies (1:100) against iNOS or eNOS (Transduction Labs) (or nonselective IgG as a negative control) for 1 hour. iNOS staining was done using Tyramide Signal Amplification Kits (Molecular Probes). AF555 - labeled secondary anti mouse-IgG (1:1000) was used for eNOS staining. All sections were incubated with DAPI for nucleus staining and wheat germ-AF555 (during iNOS stain) or wheat germ-AF488 (during eNOS stain) for cell membrane and connective tissue staining, before mounting with Anti-Fade Gold (Molecular Probes). The slides were examined using single photon confocal microscopes (FluoView 1000 Olympus).

Supplementary Table 1. Effect of iNOS deletion on left ventricular function and dimensions by 2-D guided M-mode echocardiography.

	Wt-sham	iNOS$^{-/-}$-sham	Wt-TAC	iNOS$^{-/-}$-TAC
Body weight (g)	25.6 ±0.5	26.2 ±0.8	25.7 ±0.5	25.0±0.4
AWTD (μm)	69.8±0.9	74.7±0.2	110±3.3*	96.0±3.2*#
PWTD (μm)	70.6±0.7	74.7±0.1	109±2.7*	95.3±2.9*#
AWTS (μm)	102±4.5	110±3.0	134±4.3*	120±2.8*#
PWTS (μm)	102±4.5	109±2.9	132±4.2*	115±2.2*#

Data are Mean ± SE from 9 to 14 mice per group. AWTD: anterior wall thickness at end diastole. PWTD: posterior wall thickness at end diastole. AWTS: anterior wall thickness at end systole. PWTS: posterior wall thickness at end systole. *P<0.05 as compared to the corresponding sham group. #P<0.05 as compared to Wt-TAC group.

Supplementary Figure-1. Systolic aortic pressure and pressure gradient across TAC site were not different between iNOS KO and wild type mice immediately after moderated TAC.

Supplementary Figure-2. DHE staining shows that TAC resulted in an increase of ROS production in wild type mice, while iNOS deficiency attenuated the TAC-induced ROS production.

Supplementary Figure-3. Myocardial iNOS distribution in wild type mice after TAC. Membrane was stained for wheat germ agglutinin with FITC-conjugated AF555.