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Supplementary Material

Title: Hexokinase II knockdown in the mouse heart results in exaggerated hypertrophy via increased ROS production

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Supplemental Materials and Methods

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Transverse aortic constriction

Mice were placed supine under anesthesia with 2% isoflurane. A 7-0 silk suture was placed around the transverse aorta between the origin of the right innominate and left common carotid arteries against an externally positioned 27 gauge needle to yield a narrowing 0.4 mm in diameter when the needle was removed after ligation. The sham procedure was identical except that the aorta was not ligated. No mortality was observed in this group. Mice from all the groups were sacrificed at 2, 4 and 8 weeks after surgery to perform molecular or histological analyses. The efficacy of the pressure overload was tested by measuring the arterial pressure in the right and the left carotid artery.

Hemodynamic studies

Hemodynamic studies were performed 4 weeks after TAC. A high-fidelity transducer-tipped pressure-volume catheter (Scisense Inc., London, Ontario, Canada) was calibrated in 37°C saline and was introduced into left and right carotid artery. After stabilization, 30 seconds of data (arterial pressure) was collected. Signals were digitized by use of a data translation series analog-digital converter and then stored and analyzed. Values derived from pressure tracings were averaged over a minimum of 20 beats. TAC Animals with systolic pressure gradients less than 40 mmHg were excluded in our study.

Screening of $HKII^{+/-}$ knockout mice

Genomic DNA was prepared form tail clips using the PureGene DNA isolation kit according to the manufacturer's protocol (Gentra Systems, USA). Approximately 10 ng of the genomic DNA was used for PCR using primers against the HKII genomic DNA. The offspring carrying disrupted HKII alleles was identified by PCR amplification with forward primer HK2KO-F2 (5'-ACTCTCCTGCCGCCCTGC-3') and reverse primers Neo-R1(5'-GTGCCCAGTCATAGCCGAATAGC-3') and HK2KO-R1 (5'-CCC-CTC-ATC-GCC-ACC-GC-3').

Echocardiography

Cardiac function was non-invasively monitored by transthoracic echocardiography using Vevo 770 high-resolution imaging system with a 30 MHz scan head at 2, 4 and 8 weeks after TAC as described (Bunck et al, 2009). At least 10 independent cardiac cycles per each experiment were obtained.

Histological analysis

Hearts were fixed in 10% formalin (PBS buffered), dehydrated, and embedded in paraffin. Heart architecture was determined from transverse 5μm deparaffinized sections stained with H&E. For cell size measurement, cell surface areas were quantified across centered myocytes using a computer-assisted image analysis system (Image J, NIH). A minimum of 400 myocytes from 3 different animals for each group was quantified. Fibrosis was detected with Masson's Trichrome staining. The fibrotic area (blue) was measured using Image J and was determined as a percentage of total area of ventricle (for at least 3 in each group). TUNEL positivity after TAC was determined using an In Situ Detection Kit according to the manufacturer's instructions (Roche Diagnostics).

RNA isolation and quantitative real-time PCR

Total RNA samples from cultured cardiomyocytes were isolated using the RNA stat-60 reagent (Tel-Test, Inc), and RNA samples from mouse heart were isolated using RNEASY Fibrous Tissue Mini Kit from Qiagen according to manufacturer's instructions. Samples of total RNA (500 ng) were reverse-transcribed using the Taqman reverse transcription reagents PCR Kit (Applied Biosystems), and the resulting cDNA was used as a PCR template. The mRNA levels of HKII, ANP, and BNP were determined by quantitative real-time PCR of SYBR green with the 7500 Fast Real-Time PCR system (Applied Biosystems), according to the manufacturer's instructions. 18S and β2M RNA was amplified as an internal control. The relative gene expression level was calculated using the comparative Ct method formula: $2^{-\Delta\Delta Ct}$. Primers used in our studies are as follows: For mouse HKII, forward 5'-AGTGGAACCCAGTGA-3', reverse 5'-ATGAAGTTGGCCAGGCATT-3'; ANF, forward 5'-GGAGGAGAAGATGCCGGTAGA-3', reverse 5'-GCTTCCTCAGTCTGCTCACTCA-3'; BNP, forward 5'-GCATGGATCTCCTGAAGG-3', reverse 5'- GCGCTGCCTTGAGACCGA AGG-3'; for rat HKII, forward 5'-CAACATTCTCATCGATTTCACGAA-3', reverse 5'-GTTAGTTTCAAAGATTCCCCTTGTCT-3'; ANF, forward 5'-TGGGCTCCTTCTCCATCACC-3', reverse 5'- GCCAAAAGGCCAGGAAGAGG-3'; BNP, forward 5'-CAGAACAATCCACGATGCAG-3', reverse 5'- GCTGTCTCTGAGCCATTTCC-3'.

Isolation of mitochondria

For tissue samples, we used the Pierce Mitochondria Isolation Kit for Tissue according to the manufacturer's reagent-based protocol for hard tissue (Pierce, USA). For mitochondria isolation from NRCM, we used the Pierce isolation kit for cultured cells, and the protocol described previously (Weiss et al, 2003). Briefly, cells were resuspended in lysis buffer (68mM sucrose, 200mM mannitol, 50mM KCl, 1mM EGTA, 1mM EDTA, 5mM Hepes, pH 7.4), supplemented with protease inhibitors. The cells were removed with a scraper, sonicated gently for 30 seconds, incubated on ice for 30 minutes, and then centrifuged at 500 *xg* for 5 minutes at 4 °C to remove cellular debris. Following differential

centrifugation at 4 °C, the supernatant (cytosolic fraction) is removed, and the remaining pellet containing the mitochondrial enriched fraction is dissolved in the above buffer with the addition of Triton X-100 (Invitrogen). The purity of the fractionated lysates was assessed via Western blot using appropriate markers for each compartment; Actin: cytosolic; SDH 70kDa subunit: mitochondria (Santa Cruz, Molecular Probes respectively).

Western blotting

For western blotting of fractionated lysates, the mitochondria were isolated as described. For western blotting of total protein, hearts were homogenized in a modified RIPA buffer containing protease and phosphatase inhibitors (Roche). Total protein lysates were isolated from NRCM in RIPA buffer containing protease and phosphatase inhibitors. Samples were run on and SDS-PAGE gel and transferred to nitrocellulose membrane (Invitrogen). Antibodies against HKII and Actin were purchased from Santa Cruz Biotechnology. The protein bands were developed with an enhanced chemiluminescence substrate kit. Quantification of blots was performed with Image J Software (NIH).

Cell viability

Viability was determined by flow cytometry using AnnexinV labeling and DAPI exclusion (Molecular Probes). After treatment, cells were trypsinized, and resuspended in Annexin buffer (10mM HEPES, 140mM NaCl, and 2.5mM $CaCl_2$, pH 7.4) followed by incubation with AnnexinV-APC conjugate and 1μM DAPI. Total cell death, defined as cells positive for Annexin, DAPI, or both, was determined by flow cytometry using the LSRIII (Becton Dickinson).

Antioxidant Expression and Activity

Antioxidant expression levels were evaluated via Western Blot of ventricular and cellular lysates. Catalase, SOD1, and SOD2 antibodies were obtain from Chemicon. Antioxidant activity was assessed using a commercially available kit from Cell Biolabs Inc. according to manufacturers' instructions. For total activity, protein levels were determined by BCA assay (Pierce) and equilibrated for each condition.

NADPH and Glutathione Levels

Levels of NADPH in NRCM were reported relative to total NADPH/NADP+ as previously described (Nisselbaum & Green, 1969; Zerez et al, 1987). Briefly, cardiomyocytes were trypsinized, washed with ice cold PBS, resuspended in ice cold isolation buffer (20mM nicotinamide, 20mM $NaHCO_3$, 100mM Na_2CO_3), and immediately placed into a dryice/acetone bath. After 2 freeze/thaw cycles, lysates were centrifuged at 14,000 x*g* for 10min at 4 ^oC and the supernatant was separated into 2 aliquots. Total NADPH/NADP levels were immediately quantified as changes in absorbance at 570 nm with the SpectraMax Pro (Molecular Devices) using an enzymatic cycling assay coupling NADPH production with MTT reduction. NADPH alone was quantified using the same assay, after heating the sample for

30min at 60 °C in the dark to destroy NADP+. Total glutathione (GSH:GSSG) and GSSG were analyzed in separate aliquots from the same sample using a commercially available kit according to the manufacturer's instructions (Cayman Chemical).

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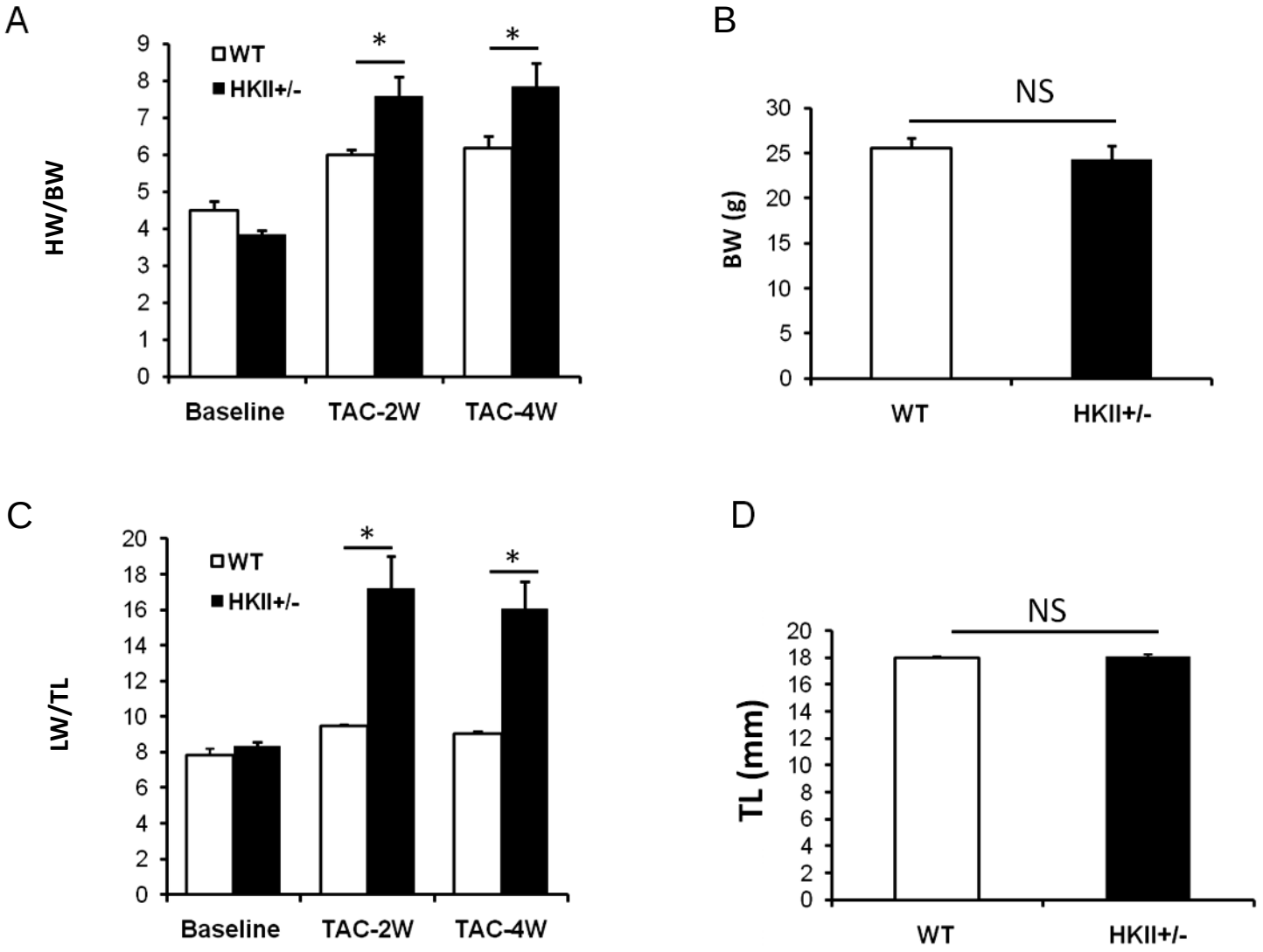

Figure S1. Exacerbated hypertrophy in HKII$^{+/-}$ hearts in response to pressure overload hypertrophy. **(A)** Quantification of heart weight to body weight ratio (HW/BW) in 10 week old WT and HKII$^{+/-}$ mice subjected to TAC for 2 and 4 weeks. *P< 0.01 vs WT, n=8-12. **(B)** Body weight of WT and HKII$^{+/-}$ mice (n=12). **(C)** Quantification of lung weight to tibia length ratio (LW/TL) in WT and HKII$^{+/-}$ mice. mice subjected to TAC for 2 and 4 weeks. *P< 0.01 vs WT, n=8-12. **(D)** Tibia length in WT and HKII$^{+/-}$ mice (n=12). Data are presented as mean ± SEM.

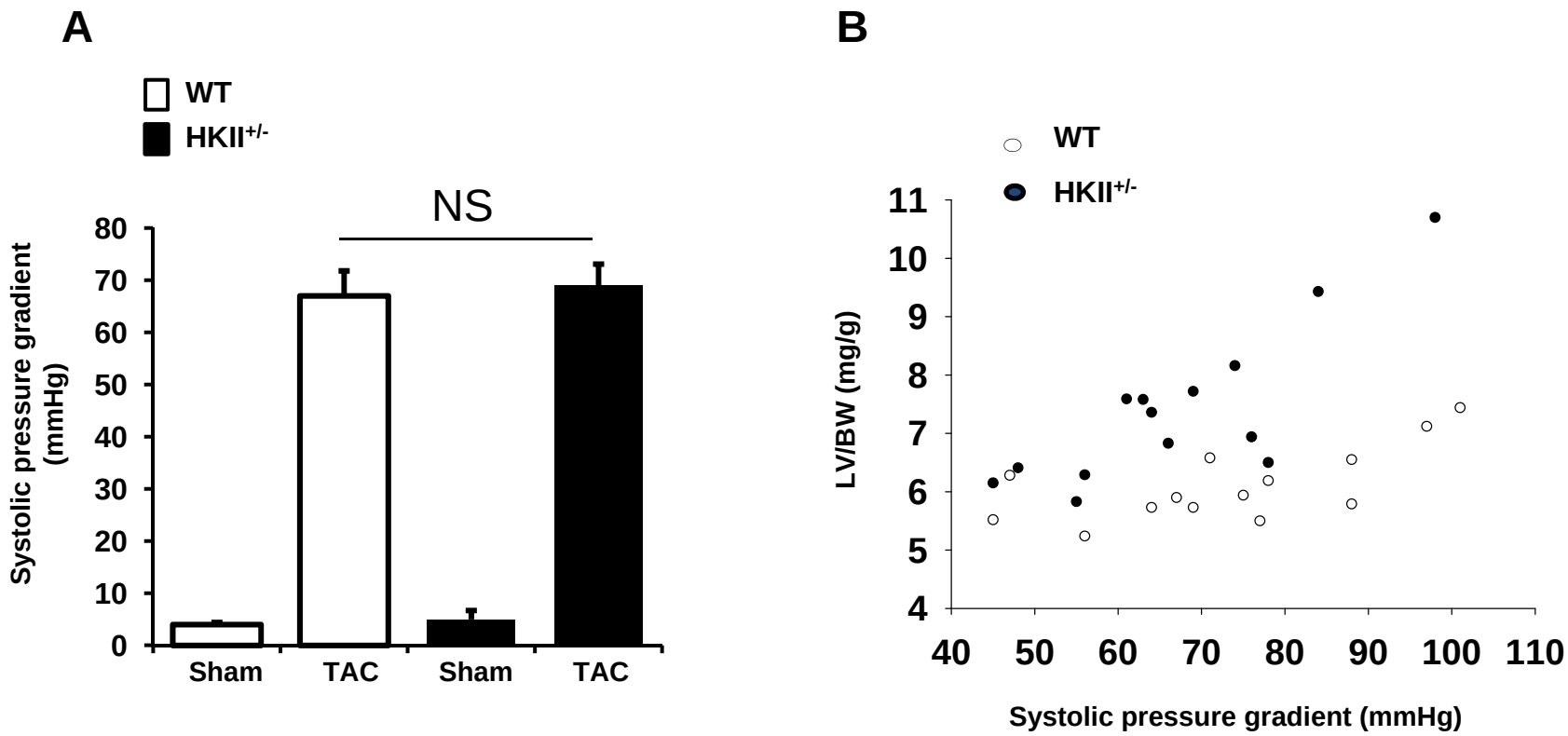

Figure S2. Hypertrophic response to pressure overload in WT and HKII$^{+/-}$ mice. **(A)** Systolic pressure gradient between HKII$^{+/-}$ and WT mice after TAC (n=14). Data are presented as mean ± SEM. **(B)** The index of left ventricular mass over body weight (LV/BW) is plotted against the systolic pressure gradient produced by TAC for WT (n = 14) and HKII$^{+/-}$ (n = 14) mice (open and black circles, respectively). The slopes of the linear regressions for WT and HKII$^{+/-}$ animals were significantly different ($P < 0.001$, ANOVA).

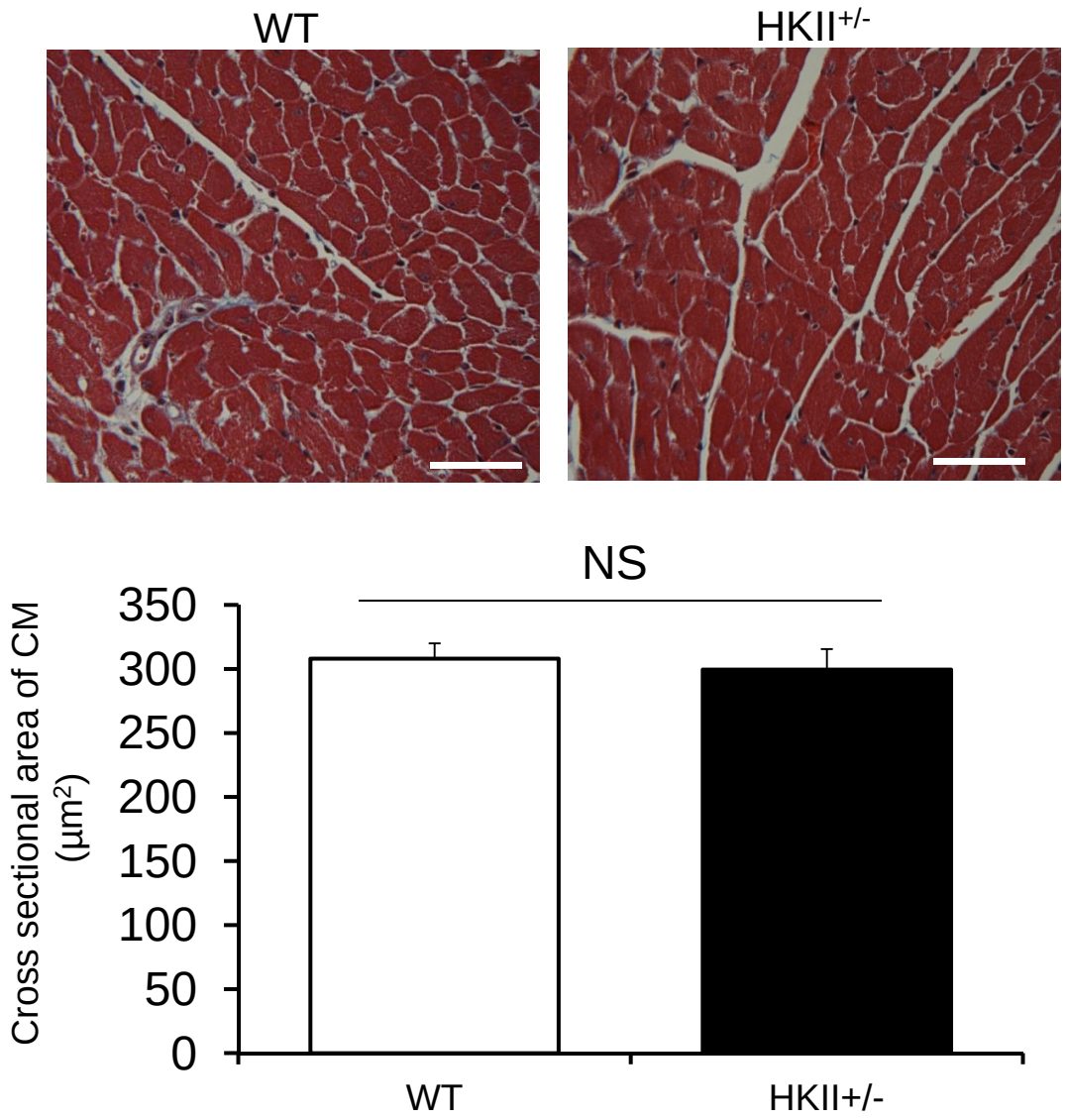

Figure S3. Hematoxylin and eosin (H&E) staining of WT and HKII$^{+/-}$ mice at 12 weeks old**.** Quantification of myocyte size from cardiac histological sections of WT and HKII$^{+/-}$ hearts is shown on the bottom, n>400 cells from three animals in each group. Scale bar=50μm.

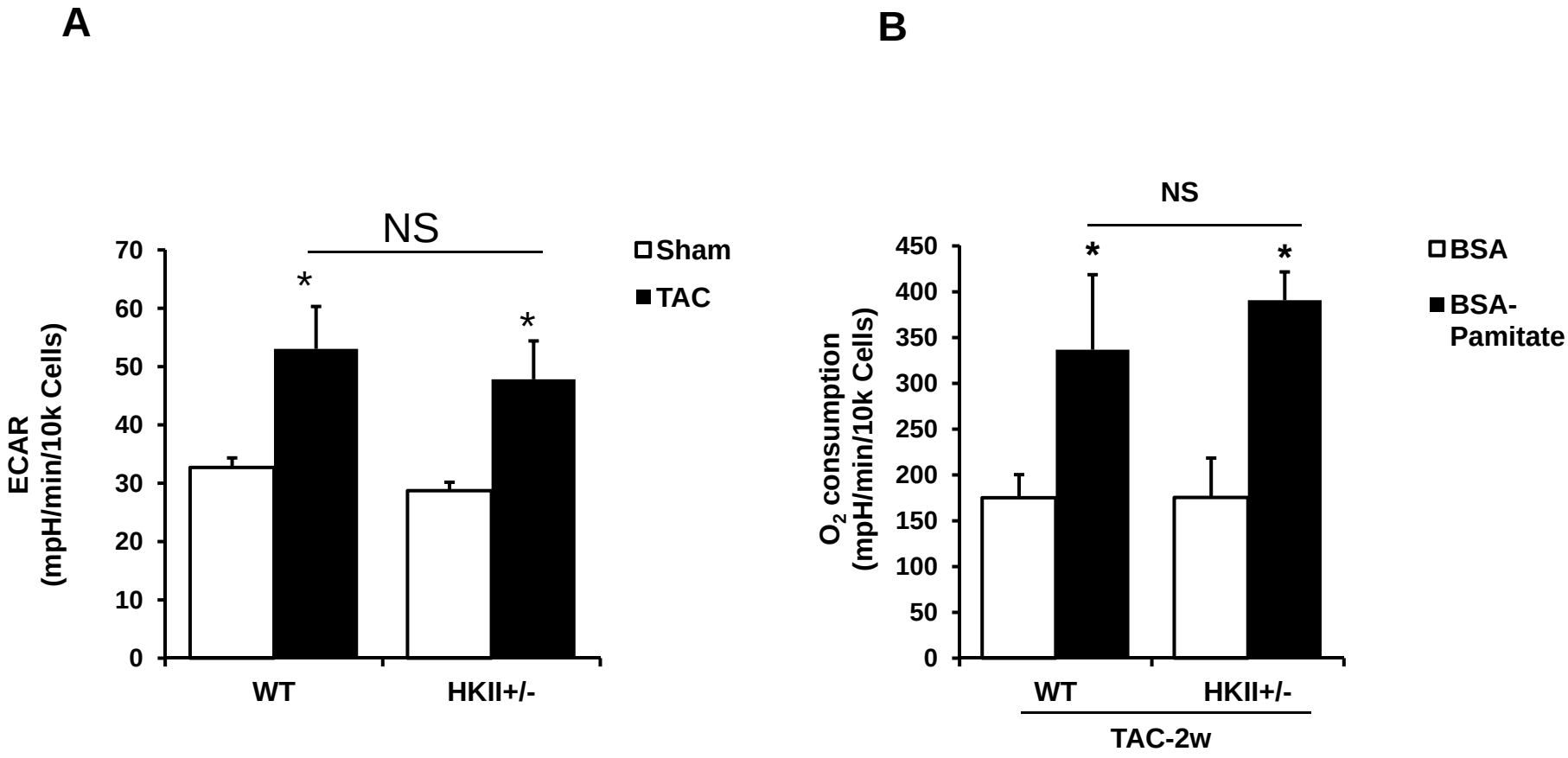

Figure S4. Metabolic changes in response to TAC in isolated adult cardiomyocytes from WT and HKII$^{+/-}$ hearts. **(A)** Measurement of extracellular acidification (ECAR), a proxy of the rate of glycolysis, increased in cells from WT and HKII$^{+/-}$ after TAC. There was no significant difference between the groups after TAC treatment, although cells from HKII$^{+/-}$ mice displayed a trend towards a decrease. **(B)** Fatty acid oxidation, as assessed by a rise in oxidative respiration in the presence of exogenously added palmitat in cells from both WT and HKII+/- mice after TAC, there was no difference between the two groups. The studies were performed by using the XF assay from Seahorse Biosciences. n = 3-5. Data are presented as mean ± SEM.

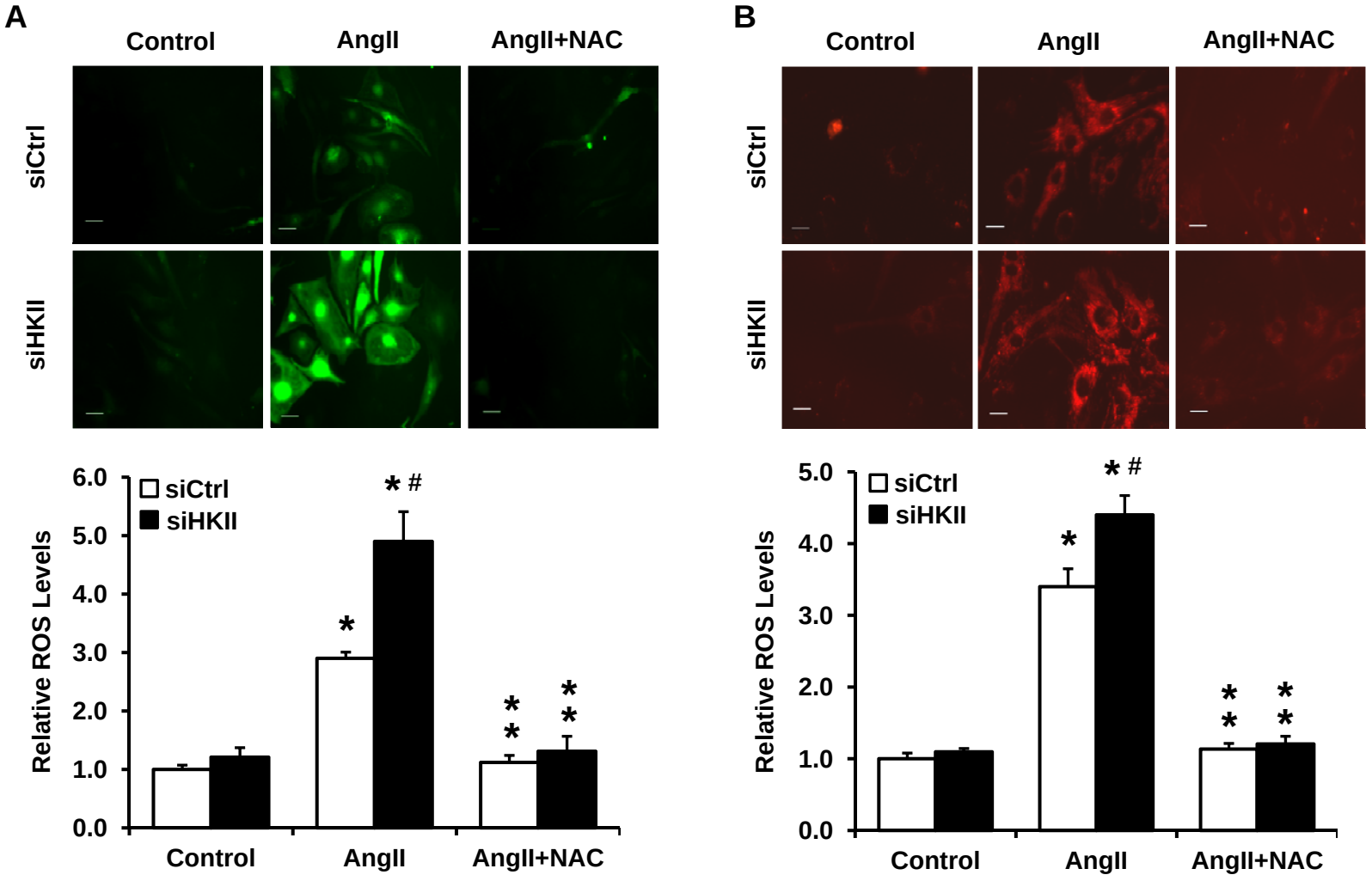

Figure S5. ROS levels after HKII knockdown and treatment with the antioxidant NAC. NRCM were treated with scrambled or HKII siRNA and exposed to AngII (250nM, 12h). NAC (1mM) was added to the media 2h after the addition of AngII. **(A)** ROS levels were assessed using DCF (green), and **(B)** mitochondrial superoxide levels were assessed using MitoSox (red). Data represent the average fluorescent intensity relative to untreated control (3 different samples, minimum of 3 fields analyzed per sample). Scale bars: 20μm. **p* < 0.05 vs untreated controls, #*p* < 0.05 vs siCtrl +AngII, ***p* < 0.05 vs AngII. Data are presented as mean SEM.

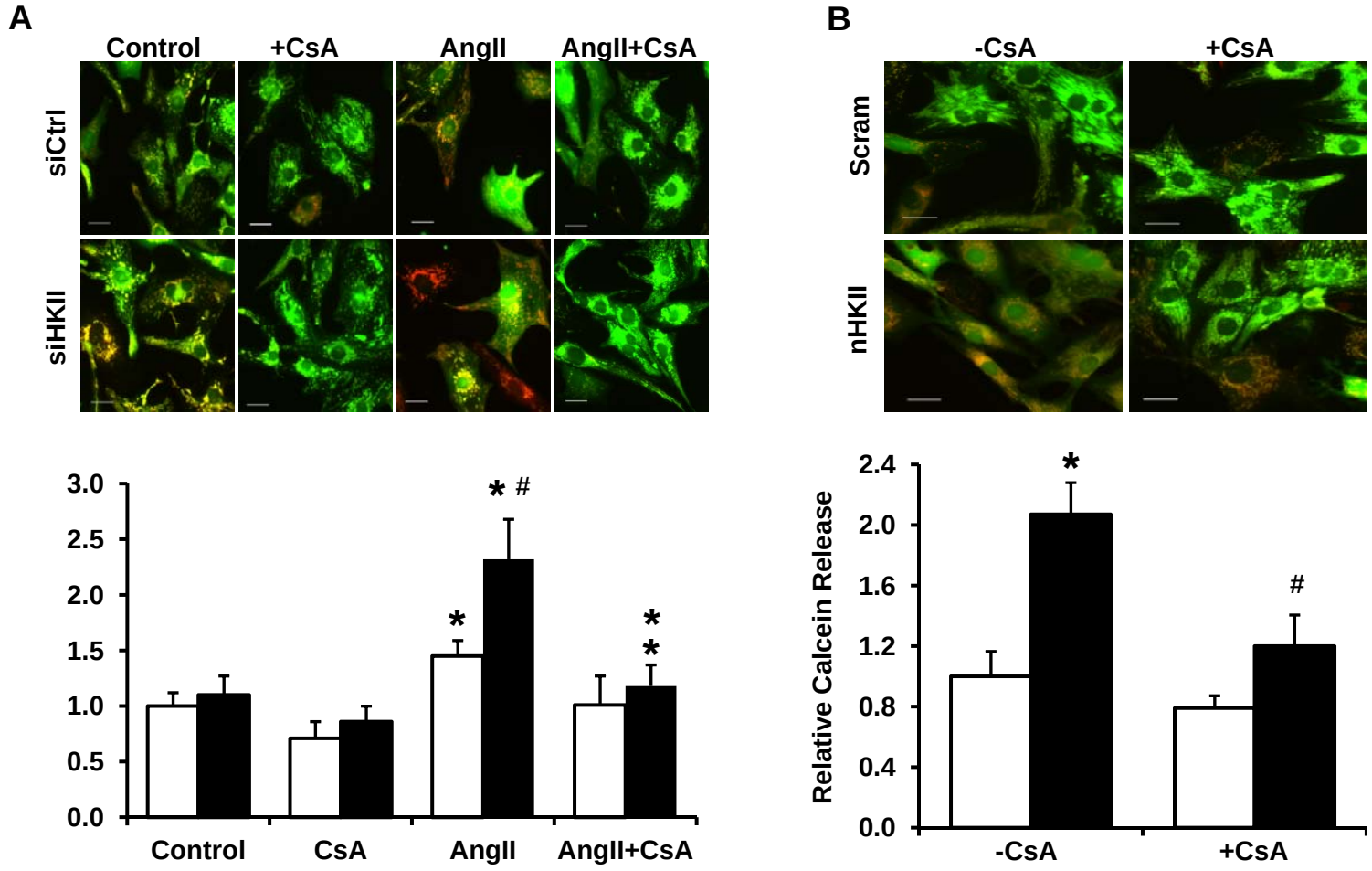

Figure S6. MPT inhibition with CsA. **(A)** Representative images and quantification of cells demonstrating mitochondrial calcein release after AngII treatment +/- CsA. NRCM were loaded with calcein, quenched, and treated with AngII (250nM, 2h). CsA (.2μM) was added 10min prior to treatment as indicated. *p<0.05 vs control, #p<0.05 vs siCtrl+AngII, **p<0.05 vs AngII. **(B)** Representative images and quantification of cells demonstrating mitochondrial calcein release after peptide treatment +/- CsA. CsA (.2μM) was added 10min prior to treatment as indicated. *p<0.05 vs scram, #p<0.05 vs n-HKII. A minimum of 50 cells from at least 3 independent samples were analyzed for each condition and are presented relative to untreated scrambled siRNA or peptide controls. Mitotracker Red was used as a mitochondrial marker. Scale bar: 20μM. Data are presented as mean ± SEM.

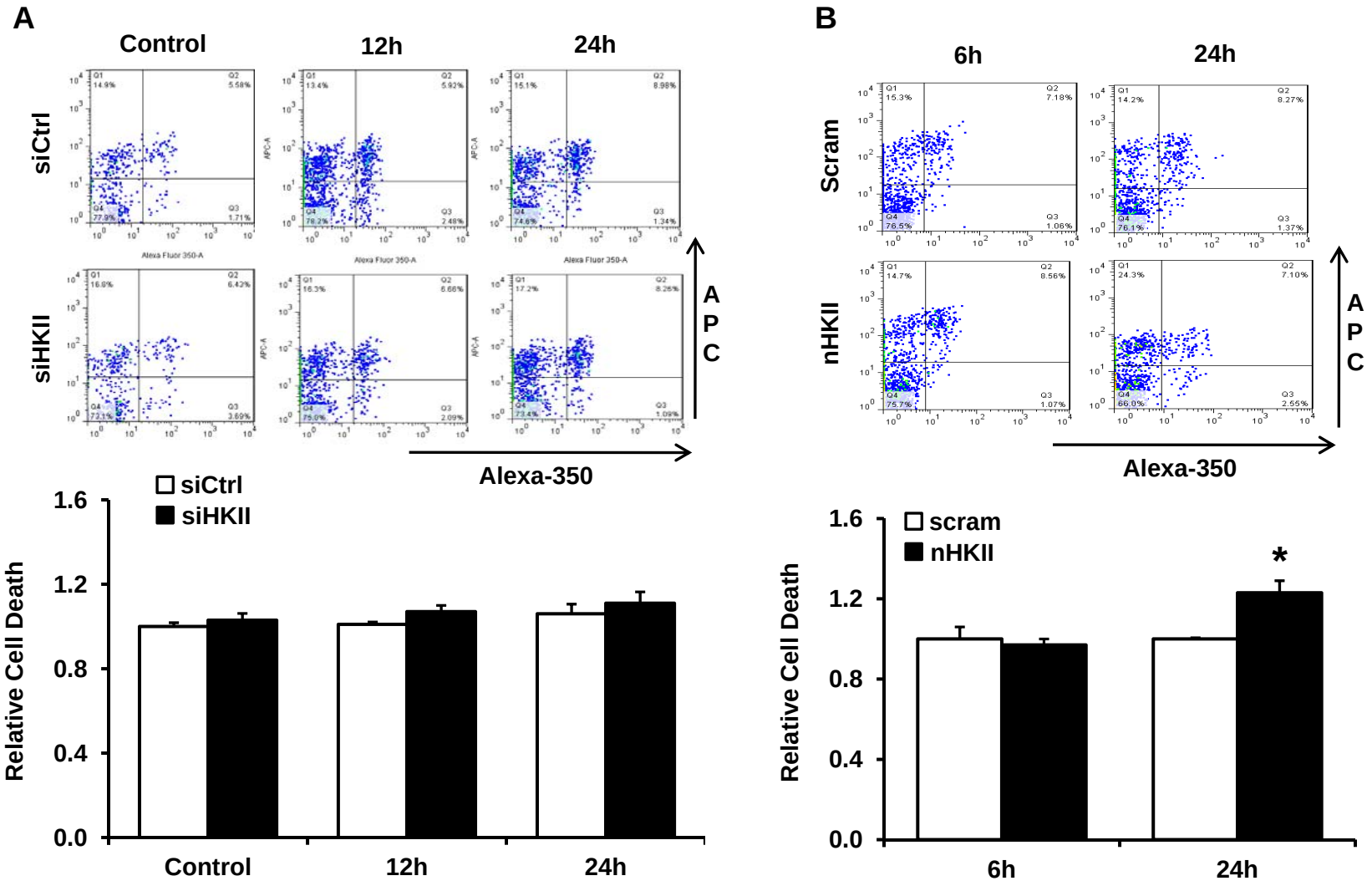

Figure S7. Analysis of cell death. **(A)** Representative flow output and cell death analysis after knockdown in NRCM +/- AngII (250nM), n=3. **(B)** Representative flow output and cell death analysis after peptide treatment (5μM), n=3. Cell death was measured via flow cytometry using Annexin V (APC) and DAPI exclusion (Alexa 350) to determine apoptosis and necrosis, respectively. Data represent total cell death (cells positive for AnnexinV, DAPI, or both) relative to untreated or scrambled control. $^{*}p < 0.05$ vs scram (24h). Data are presented as mean ± SEM.

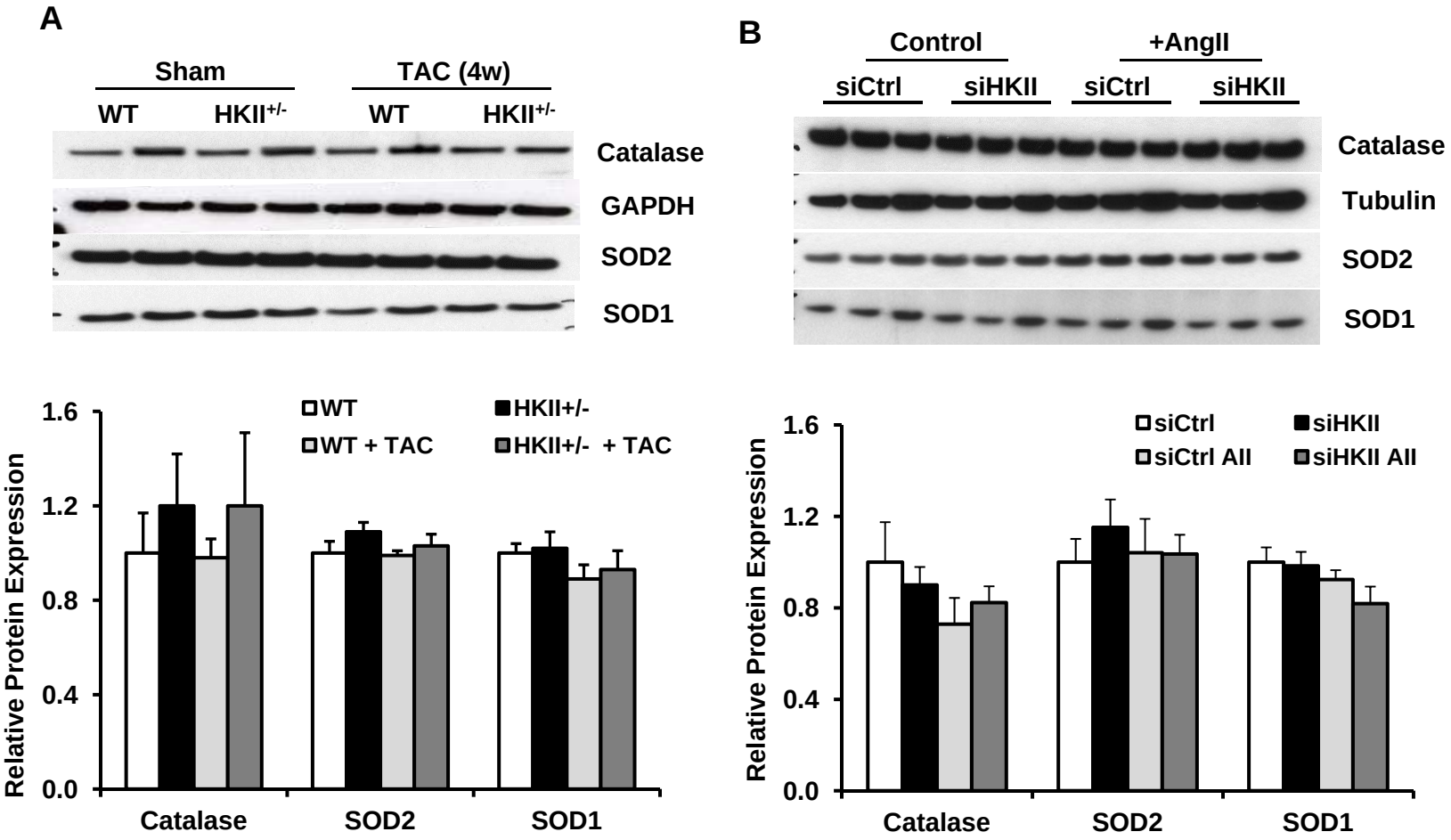

Figure S8. Antioxidant expression levels. **(A)** Western blot demonstrating antioxidant expression levels in ventricular lysates from WT and HKII$^{+/-}$ hearts after sham operation or TAC (4W). Antioxidant expression levels were normalized to GAPDH and normalized sham control, n=3. **(B)** Western blot demonstrating antioxidant expression levels in NRCM after knockdown and treatment with AngII (250nM, 24h). Antioxidant expression levels were normalized to Tubulin and are presented relative to untreated siRNA control, n=3.

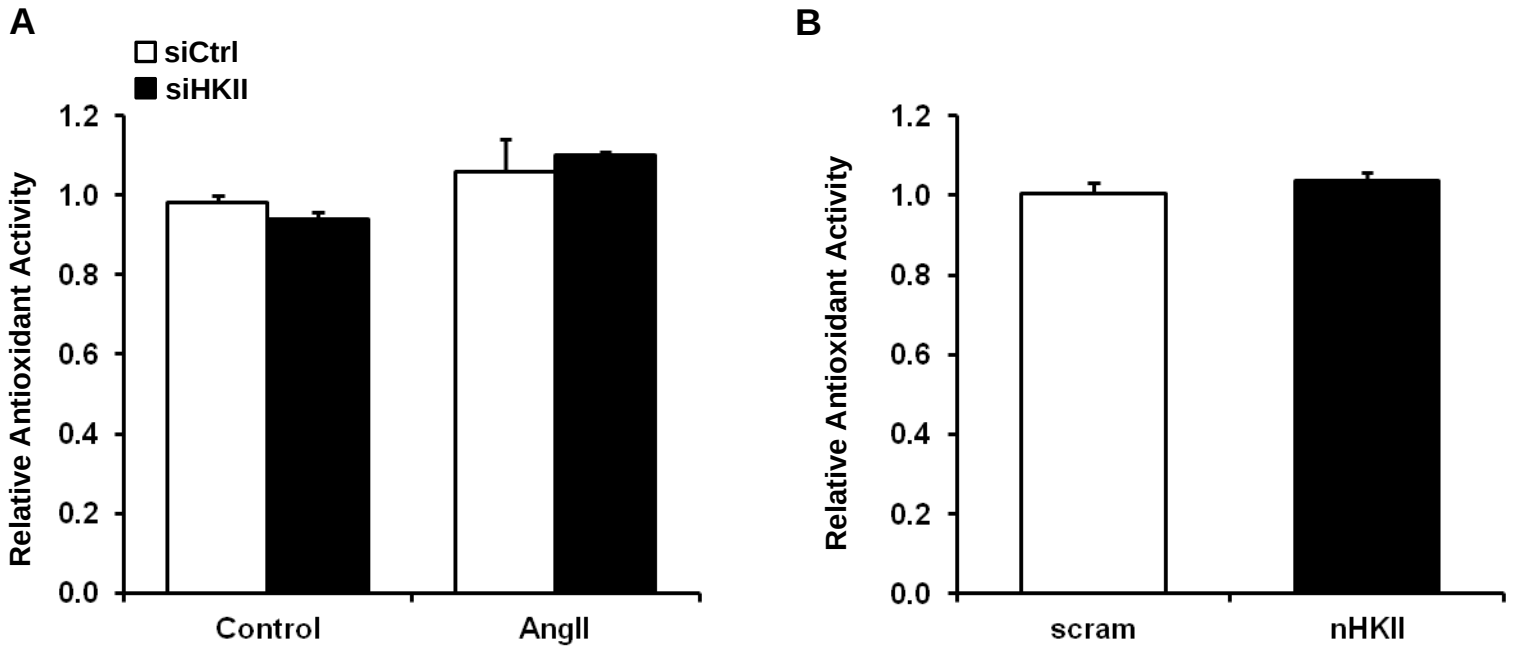

Figure S9. Total antioxidant activity. **(A)** Antioxidant activity in NRCM after knockdown and treatment with AngII (250nM, 24h). **(B)** Antioxidant activity in NRCM after treatment with the scrambled or HKII dissociation peptide (5μM, 24h). Total activity was determined using a commercially available kit and is presented relative to untreated siRNA or scrambled peptide control, n=3.

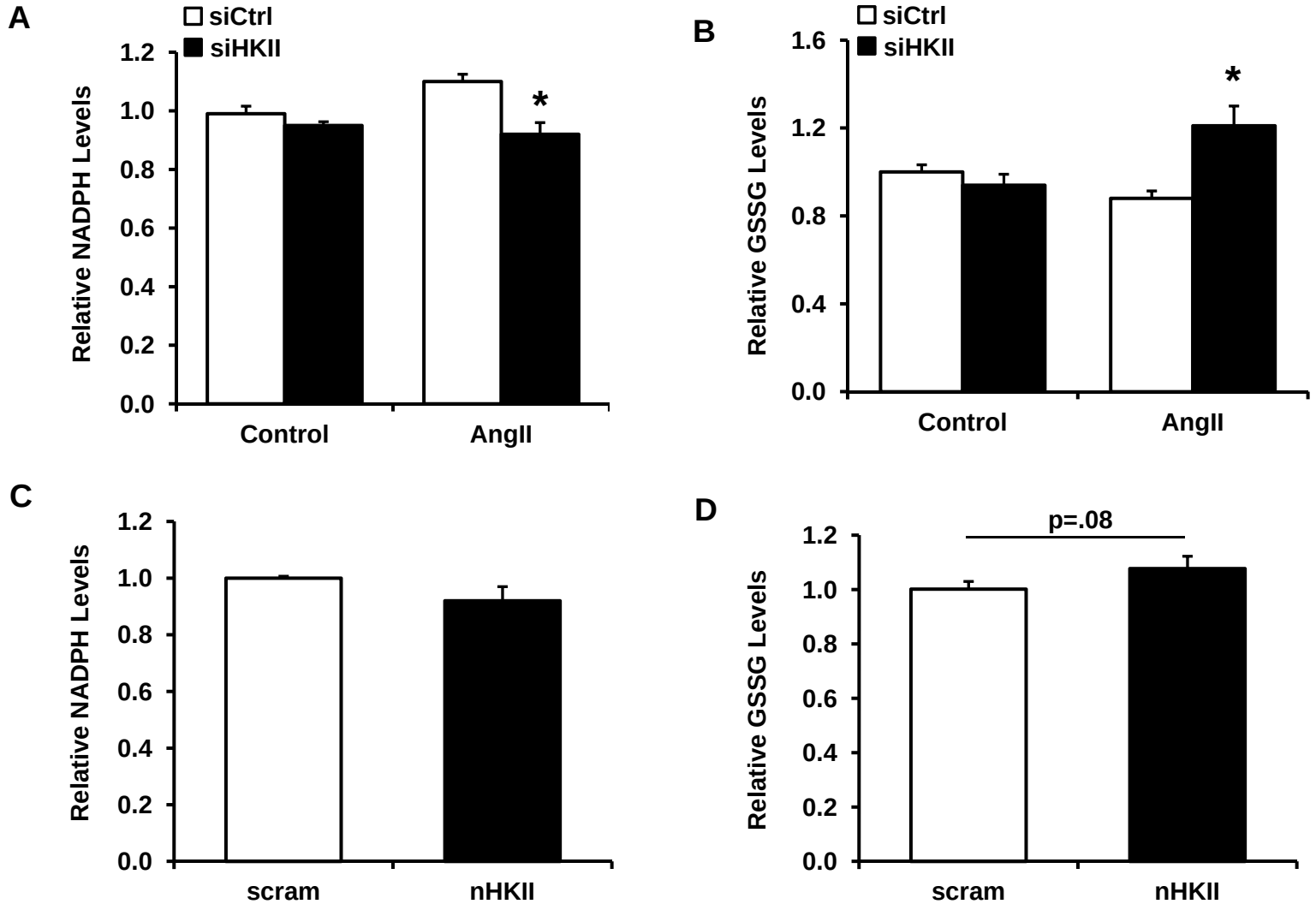

Figure S10. Assessment of NADPH and oxidized glutathione (GSSG) levels in NRCM. **(A)** Relative NADPH and **(B)** GSSG levels after treatment with AngII (250nM, 24h), n=4-5, **p*<0.05 vs control siRNA +AngII. **(C)** Relative NADPH and **(D)** GSSG levels after treatment with either the scrambled control or HKII dissociation peptide (5μM, 24h), n=3-4, Data represent the ratios for NADPH and GSSG relative to siRNA or scrambled control. Data are presented as mean ± SEM.