SUPPLEMENTAL MATERIAL

I. SUPPLEMENTAL METHODS

Oligomer generation and Western blot analysis

The following peptides were synthesized by RS Synthesis, LLC (Louisville, KY), with HPLC and mass spectrometry performed to confirm identity and >95% purity: ANP (1-28; SLRRSSCFGGRMDRIGAQSGLGCNSFRY-disulfide bond [C7-C23]); mutANP (SLRRSSCFGGRMDRIGAQSGLGCNSFRYRITAREDKQGWA-disulfide bond [C7-C23]); and BNP (SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH-disulfide bond [C10-C26]). To assay for time-dependent oligomerization, peptides (10μM) were prepared in 1xPBS buffer (pH 7.4) and incubated at room temperature (RT) for 24hr, 6d, or 10d. Separate samples were incubated for 24hr with either 2 molar equivalent of synthetic 15-E_2-isolevuglandins (IsoLG)[31] or DMSO (vehicle) as described. [32] After incubation, peptides were subjected to Western analysis using an anti-α-ANP antibody (1:500, Phoenix Pharmaceuticals, Inc, Burlingame, CA) and an HRP-conjugated secondary (goat anti-rabbit) antibody (1:10,000, Jackson ImmunoResearch, West Grove, PA), with protein bands detected using an enhanced chemiluminescent kit (Pierce ECL Substrate, Thermo Fisher, Waltham, MA). Concentration-dependent oligomerization was assessed by Western blot analysis following incubation of peptides in 1xPBS at concentrations of 10, 30 and 100μM for 24h.

Animal use and mouse atrial cardiomyocyte isolation

All animal procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee, with animal care in accordance with the Guide for the Care and Use of

Laboratory Animals, US Department of Health and Human Services. C57Bl/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and studied at 3-4 months of age. Hypertension was induced by continuous infusion of angiotensin (ang) II (490ng/kg per minute) for 2wks as described previously.[14] Blood pressure was monitored using tail cuff measurements preceded by acclimation.

To isolate mouse cardiomyocytes, the animal was injected with heparin (100U per mouse) intraperitoneally 10min prior to the procedure. Terminal anesthesia was induced in an induction chamber with inhaled isoflurane (3-4%) under a fume hood, using a physically-isolated isoflurane-soaked gauze pad within the chamber. The mouse was placed in the chamber and monitored closely until deep anesthesia was achieved (determined by the loss of pedal withdrawal reflex) and subsequently euthanized by cervical dislocation. A midline thoracotomy was rapidly performed, the heart was excised, and the aorta was quickly cannulated and perfused on a Langendorff apparatus with a calcium-free perfusion buffer containing (in mM): NaCl 113, KCl 4.7, KH_2PO_4 0.6, $MgSO_4$ 1.2, glucose 5.5, Na_2HPO_4 0.6, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, 2,3-butanedione monoxime 10, and taurine 30, at a rate of 3ml/min using a constant peristaltic pump for 4 min at 37°C, followed by perfusion at the same flow rate with a digestion buffer containing collagenase II (Worthington; 300U/ml) and 12.5 μM $CaCl_2$, for 8-12 min at 37ºC, until the heart became pale and flaccid. The heart was removed from the Langendorff apparatus, and the atria were dissected from the ventricles and gently teased into small pieces with fine-tip forceps, with further dissociation using a wide-bore (2 mm) Pasteur pipette for 5 min. Subsequently, the enzymatic digestion was terminated with a buffer containing 10% FBS and 12.5 μM $CaCl_2$ and the atrial cardiomyocyte suspension was filtered through 200 μm mesh and allowed to settle by gravity sedimentation for 20 minutes at room temperature. The

final atrial cardiomyocyte pellet was resuspended in perfusion buffer with 5% FBS and 12.5 µM $CaCl_2$ and final $CaCl_2$ concentration was gradually increased to 1mM. Immediately following each isolation, cell viability was determined with the Trypan blue exclusion test. Only rod-shaped atrial cardiomyocytes with clear striations were used to study electrophysiologic properties.

Cell Preparations

Atrial HL-1 cardiomyocytes were cultured under a 5% CO_2 atmosphere in supplemented Claycomb medium (Sigma-Aldrich, St. Louis, MO) as previously described.[15, 33-35] *KCNA5* was stably expressed in mouse fibroblasts (*Ltk*$^-$ cells).[36] *Ltk*$^-$ cells were split in 100mm culture dishes and maintained at 37°C (5% CO_2) in DMEM medium (Thermo Fisher Scientific, Waltham, WA) containing 10% fetal horse serum, 1% penicillin-streptomycin, 1% glutamine and 400µg/ml geneticin (Thermo Fisher Scientific).

Cytotoxicity

Oligomers derived from mutANP were generated by incubating the peptide at RT for 24hr at a concentration of 30µM in 1xPBS. Atrial HL-1 cardiomyocytes were plated at density of 25,000 cell per 100µl Claycomb Medium/well in a 96-well microplate (Perkin Elmer, Waltham, MA) precoated with gelatin and fibronectin and incubated overnight (37°C, 5% CO_2). Cells were then treated with either freshly-prepared mutANP peptide or oligomers (diluted to a concentration equivalent to 0.45µM monomers, or 0.45µM$_{(m)}$) for 24hr. At the end of the treatment, cytotoxicity was determined by measuring cellular ATP levels with an ATPlite assay

(Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. Luminescence was measured using a Lumicount microplate reader (Global Medical Instrumentation, Ramsey, MN).

Bioenergetic profiling

The Seahorse XF Cell Mito Stress Test (Agilent Technologies, Santa Clara, CA) was used to measure oxygen consumption rate (OCR) to assay bioenergetic parameters in atrial HL-1 cells in response to natriuretic peptide monomers/oligomers. Briefly, oligomers were generated by incubating freshly-prepared peptides (30μM) in 1xPBS at RT for 1d. Cells were cultured in Seahorse XF96 tissue culture microplates at a density of 1.5×10^4 cells per well in 80μL of Claycomb media overnight. On the next day, cells were treated with different amounts of oligomers (diluted to a concentration equivalent to 0.45, 1.5 or 4.5$\mu M_{(m)}$) for 24hr. At the end of the treatment, the Claycomb medium was changed to Seahorse base medium and subsequently OCR was measured with a Seahorse XFe96 Analyzer following sequential injection of oligomycin (1μM), FCCP (1μM), and antimycin A/rotenone (1μM). Following the analysis, cells were stained with Hoechst 33342 and imaged with ImageXpress Micro XLS System (Molecular Devices; **Supplemental Figure IV**). Subsequently, Hoechst 33342 positive nuclei in each well were counted with MetaXpress High Content Image Acquisition & Analysis Software (Molecular Devices, San Jose, CA) employing the Multi Wavelength Cell Scoring function. Cell number in each well was then used to normalize OCR readings from the Seahorse XF Analyzer. The Multi-File XF Cell Mito Stress Test Report Generator (Agilent Technologies) was used to combine and quantify final OCR results from multiple independent experiments with at least 4 Seahorse 96-well XF Cell Culture Microplate wells per condition. Specific statistical methods are defined in the figure legends. For natriuretic peptide monomers, the same protocol was

employed except that, unlike the oligomers, the indicated concentrations of monomers were freshly prepared and used immediately to treat the cells without the RT incubation step.

Immunostaining

Atrial HL-1 cardiomyocytes were grown on sterilized coverslips at 37°C in 5% CO_2. After 24hr treatment with monomers or oligomers (equivalent to 0.45μM monomer concentration), cells were fixed in 1% formalin for 15min at RT, permeabilized with 0.2% Triton X-100 in 1xPBS, and blocked with 5% goat serum prior to incubation with anti-TOMM20 antibody (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. Cells were washed three times with 1xPBS and incubated with secondary antibody (Alexa Fluor 594) for 1hr at RT in the dark. Cells were washed three times and Hoechst was used to visualize the nuclei. Samples were analyzed using identical settings with a Zeiss LSM880 Laser Scanning Confocal Microscope (Carl Zeiss Microimaging, Inc., Oberkochen, Germany). Experiments were repeated at least 3 times.

Detection of reactive oxygen species (ROS) generation

To measure intracellular ROS levels, cells were grown in chamber slides (μ-slide 8-well glass bottom; Ibidi, Grafelfing, Germany) and exposed to natriuretic peptide monomers or oligomers for 24hr at 37°C in 5% CO_2. Thereafter, cells were washed with 1xPBS twice and incubated in 5 μM dihydroethidium (DHE; Thermo Fisher Scientific) for 30min in the dark at 37°C in 5% CO_2. Cells were then washed twice with 1xPBS and live-cell images were immediately acquired using identical settings with a Zeiss LSM880 Laser Scanning Confocal Microscope (Carl Zeiss Microimaging, Inc.). Experiments were repeated twice.

Real-time quantitative PCR (RT-qPCR)

Following treatment with monomers or oligomers for 24hr, total RNA was isolated from cells with TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Next, 1μg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). cDNAs were stored at -20°C until analysis. Real-time PCR was performed in a CFX96 PCR system using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Three technical replicates were measured for each treatment in 4-6 independent experiments. Reactions were carried out using the following conditions: an initial step of 2min at 50°C and 2min at 95°C, followed by 40 cycles of 10s at 95°C and 30s at 60°C. Fold changes were calculated using ΔΔCT method with GAPDH for normalization. The average of control samples was then set to 1 for each gene. Primer sequences are as follows: PGC-1α (forward: 5' TTCGGGAGCTGGATGGCTTG 3'; reverse: 5' CAGGAAGATCTGGGCAAAGAGG 3'), NRF1 (forward: 5' GGTGGGGGACAGATAGTCCT3'; reverse:5' ATCTGGACCAGGCCATTAGC 3'), and GAPDH (forward: 5' TGCCAAGTATGATGACATCAACAAG 3'; reverse: 5' AGCCCAGGATGCCCTTTAGT 3').

Intracellular calcium measurements

Following culture (Delta TPG, Fisher) for 1-2 days, atrial HL-1 cells were exposed to mutANP oligomers (10μM) in the culture medium. After 24hr, cells were loaded with Fura-2 acetoxymethyl ester (Fura-2, AM 2μM; Invitrogen) for 8min in normal Tyrode's solution (NT) containing (in mM): NaCl 134, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 1, Glucose 10, and HEPES 10 (pH

adjusted to 7.4 with NaOH). Cells were then washed twice for 10min in NT with 250 µmol/L probenecid to retain the indicator in the cytosol. After Fura-2, AM loading, intracellular Ca^{++} was measured at baseline for 20 seconds from single cells in NT containing 2 mM Ca^{++} using a dual-beam excitation fluorescence photometry setup (IonOptix Corp.). Cells were then exposed to caffeine 10mM in NT for 5 seconds. The amplitude and decay rate of the caffeine-induced Ca^{++} transient was used to estimate total sarcoplasmic reticulum (SR) Ca^{++} content and Na^{+}-Ca^{++} exchanger function, respectively. Ca^{++} measurement was analyzed using commercially available data analysis software (IonWizard, IonOptix, Milton, MA). All experiments were conducted at room temperature.

Electrophysiology

Action potentials. Action potentials were recorded from single mouse atrial or HL-1 cells at 37°C using the current clamp technique as described previously.[15] The extracellular Tyrode's solution was bubbled with 100% O_2 and contained (in mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, and HEPES 10 (pH 7.4). Data were acquired using the current clamp mode of an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) connected to a Digidata 1320A interface (Molecular Devices). Pipettes were pulled (Sutter Instrument, Novato, CA) using capillary tubes (Fisherbrand Micro-Hematocrit Capillary Tubes Cat #:AF-22-362-574) having tip resistances of 0.8 to 1 MΩ. The pipette-filling solution contained (in mM): K-DL-aspartate 120, KCl 25, $MgCl_2$ 1, EGTA 10, Na_2phosphocreatine 2, Na_2ATP 4, NaGTP 2, and HEPES 5 (pH 7.2). Cells were selected for experimentation if the resting membrane potential was below -55mV and the overshoot exceeded 20mV.

Ionic currents. The whole cell configuration was used to record ionic currents from atrial HL-1 cells (I_{Na}, I_{K1}, and I_{To}), *Ltk*$^{-}$ cells (I_{sus}, represented by KCNA5 current), and mouse atrial myocytes ($I_{Ca,L}$) at room temperature (22±1°C). Mouse atrial cells were used to study $I_{Ca,L}$ given that the current is often not present in HL-1 cells,[15] as well as the multiplicity of subunits that constitute $I_{Ca,L}$ in native myocytes. Currents were low-pass filtered at 5 kHz using an Axopatch 200B amplifier and digitized at 10 kHz with a Digidata 1320A A/D converter. Capacitance and 80–95% series resistance were routinely compensated. Capacitance values of the HL-1 cells averaged 54.5±2.2 pF (n=47). To calculate ionic current conductance:

$G_i = \frac{I_{ion}}{(Vm - E_{rev})}$ where, Gi is conductance, I_{ion} is ionic current, and (V_m-E_{rev}) is the driving force, where V_m is the conditioning voltage and E_{rev} is the reversal potential of the ion.

Recording I_{Na}. To record Na^+ currents, the holding potential was -120mV. The voltage dependence of channel activation was measured using 100ms test pulses from -90mV to +60mV in 10 mV increments. The pipette solution contained the following (in mM): NaF 10, CsF 110, CsCl 20, EGTA 10, and HEPES 10, pH 7.2. The extracellular solution was composed of (in mM): NaCl 10, CsCl 110, TEA-Cl 5, $CaCl_2$ 0.1, $MgCl_2$ 1, HEPES 10, and glucose 10, pH 7.4. To eliminate L- and T-type calcium currents, nimodipine 2μM and mibefradil 10μM were added to the bath solution.

Recording I_{K1}. For I_{K1}, a holding potential of -30mV was used, and the cycle time for pulse protocols was 3sec. Voltage was sequentially stepped for 150ms from -130mV to 0mV in 10mV increments. The current recorded was confirmed to be I_{K1} based on its elimination following application of 500 μM $BaCl_2$. The pipette solution consisted of: KCl 140, K_2ATP 5, EGTA 1, and HEPES 10, pH 7.2. The bath solution was Tyrode's with: NaCl 137, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 0.3, NaH_2PO4 0.16, $NaHCO_3$ 3, HEPES 5, glucose 5, ivabradine 10μM, pH

7.4. Correction for minor linear leak was performed off-line by bringing the reversal potential of corrected currents to -80mV.

Recording $I_{Ca,L}$. To record $I_{Ca,L}$, a holding potential of -40mV was used, and the cycle time for pulse protocols was 5sec. Voltage was sequentially stepped for 300ms from -40mV to +70mV in 10mV increments. The pipette solution consisted of (in mM): Cs-aspartate 120, EGTA-CsOH 10, tetraethylammonium chloride (TEA-Cl) 10, $CaCl_2$ 1, Mg_2ATP 1, phosphocreatine 5, NaGTP 1, and 10 HEPES, pH 7.2. The bath solution contained (in mM): TEA-Cl 140, $MgCl_2$ 2, $CaCl_2$ 5, glucose 10, and HEPES 10, pH 7.4.

Recording I_{To}. For I_{To}, a holding potential of -70mV was used and the cycle time for pulse protocols was 2sec. Voltage was sequentially stepped for 500ms from -70mV to +60mV in 10mV increments. The pipette solution consisted of (in mM): K-DL-aspartate 120, KCl 25, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 10, HEPES 5, Na_2phosphocreatine 2, NaATP 4, and NaGTP 2, pH 7.2. The bath solution contained (in mM): NMDG 160, KCl 4, $MgCl_2$ 1, Glucose 10, and HEPES 10, pH 7.4.

Recording I_{sus}. To record $K_v1.5$ currents from Ltk^- cells, a holding potential of -80 mV was used and the cycle time for pulse protocols was 2sec. Test potentials (100ms) were stepped from -80mV to +60mV, with tail currents recorded upon repolarization to -30mV for 100ms. The pipette solution consisted of (in mM): K-DL-aspartate 120, KCl 25, $MgCl_2$ 1, Na_2 phosphocreatine 2, EGTA 10, Na_2ATP 4, NaGTP 2, and HEPES 5, pH 7.2. The bath solution was Tyrode's solution with (in mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.8, and HEPES 10, pH 7.4.

Statistics

All electrophysiologic data were analyzed using Clampfit 10.0 software (Molecular Devices), compiled in Excel Office 365 (Microsoft, Redmond, WA), and plotted and fitted in OriginPro 2020 (OriginLab Corporation, Northampton, MA, USA). For statistical analysis, results are presented as mean ± SEM. Unless specified, nonparametric tests (Paired Samples Wilcoxon signed rank test or Mann-Whitney U test for 2 independent samples) were used when appropriate, and $P < 0.05$ level was considered significant. The effect of peptides/oligomers on membrane currents is presented as the percentage change in current (Δ) relative to the control value:

$\Delta\ (\%) = \frac{I_{after} - I_{before}}{I_{before}} \times 100\%$, (+) and (-) values indicate increase and decrease in current, respectively.

For other results, statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc test for pairwise comparisons using Graph Pad Prism software (Version 8.03). For qPCR analysis, statistical analysis was performed with Mann-Whitney test using GraphPad Prism. Details of the statistical analysis are defined in the figure legends. Differences with a P value < 0.05 were considered significant.

II. SUPPLEMENTAL TABLES

Supplemental Table I: Effects of extracellular exposure on atrial HL-1 cells

	RMP	APD_{90}	APD_{50}	V_{max}
	mV	ms	ms	mV/ms
Control	-71.1±0.6	64.3±1.3	22.9±1.9	21.0±0.2
ANP	-69.7±0.7	63.1±1.3	21.9±1.5	20.8±0.2
Control	-69.9±1.2	64.8±1.1	22.9±0.7	21.1±0.5
ANP oligo	-65.5±1.4*	50.8±1.1*	16.6±0.7*	19.9±0.2*
Control	-72.6±0.3	64.3±1.3	22.9±0.7	20.8±0.3
mutANP	-70.7±0.2*	55.3±1.3*	18.4±0.8*	19.4±0.3*
Control	-69.1±0.4	64.8±1.1	22.5±0.8	21.3±0.3
mutANP oligo	-65.6±1.4*	52.8±1.1*	16.4±0.8*	20.3±0.3*
Control	-71.1±0.7	64.6±1.0	22.9±1.7	21.2±0.3
BNP	-70.6±0.7	63.6±1.0	21.9±1.7	20.8±0.3
Control	-71.8±0.5	64.5±1.3	22.9±0.6	22.5±0.3
BNP oligo	-70.4±0.4*	58.6±1.4†	19.8±0.7†	21.6±0.2*

oligo represents oligomer

* $P<0.05$; † $P<0.01$; n=9-20

Initial peptide concentration=500nM

Supplemental Table II: Effects of intracellular exposure on atrial HL-1 cells

	RMP	APD_{90}	APD_{50}	V_{max}
	mV	ms	ms	mV/ms
Control	-72.8±0.8	64.2±1.8	22.7±0.8	21.2±0.7
ANP	-70.5±0.8	64.8±1.0	22.1±0.8	19.9±0.2
ANP oligo	-70.9±0.5*	61.8±1.6*	20.0±1.3	20.0±0.3
mutANP	-70.9±0.5	64.3±1.9	22.8±1.2	19.3±0.3
mutANP oligo	-69.9±0.5*	61.4±1.6*	22.0±1.3	20.5±0.3
BNP	-72.1±1.2	63.5±1.2	22.3±1.5	20.9±0.2
BNP oligo	-70.2±0.5*	61.9±4.9*	22.4±0.8	18.7±0.8*

oligo represents oligomer

* *P*<0.05; n=9-31

Initial peptide concentration=500nM

Supplemental Table III: Effects on the voltage dependence of ion channel activation

	$I_{Kv1.5}$	I_{To}	I_{K1}	I_{Na}	$I_{Ca,L}$
Control	10.7±0.6	35.2±0.4	-93.6±0.4	-40.2±0.8	4.8±0.1
ANP oligo	9.2±1.2*	34.0±0.3†	-95.0±0.3†	-38.6±0.7	4.5±0.1*
Control	10.2±0.9	32.4±0.3	-93.7±0.6	-39±0.7	5.4±0.3
BNP oligo	8.5±0.9*	30.5±0.5†	-95.7±0.5†	-37.2±0.6†	5.9±0.4
Control	12.6±3.3	32.3±8.9	-93.4±0.5	-39.4±0.5	5.5±0.4
mutANP	9.9±2.3†	29.1±8.4	-96.1±0.1*	-37.4±0.5†	6.6±0.6*
Control	11.7±1.6	32.4±0.4	-94.1±0.8	-38.5±0.7	4.1±1.1
mutANP oligo	9.9±1.6†	29.2±0.6†	-96.3±0.8†	-35.7±0.4†	5.9±0.9†

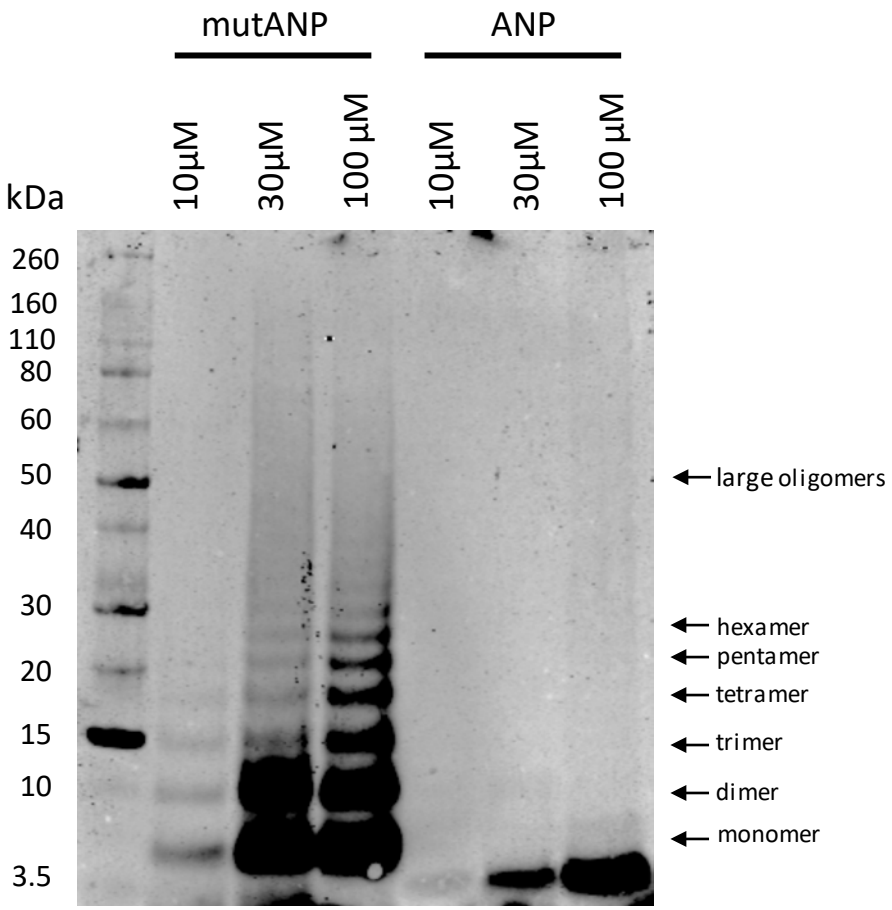
All data (in mV) are expressed as the midpoint ($V_{1/2}$ or half-maximal) of the conductance (or tail current for K_v1.5)-voltage activation curve (n).

oligo represents oligomer

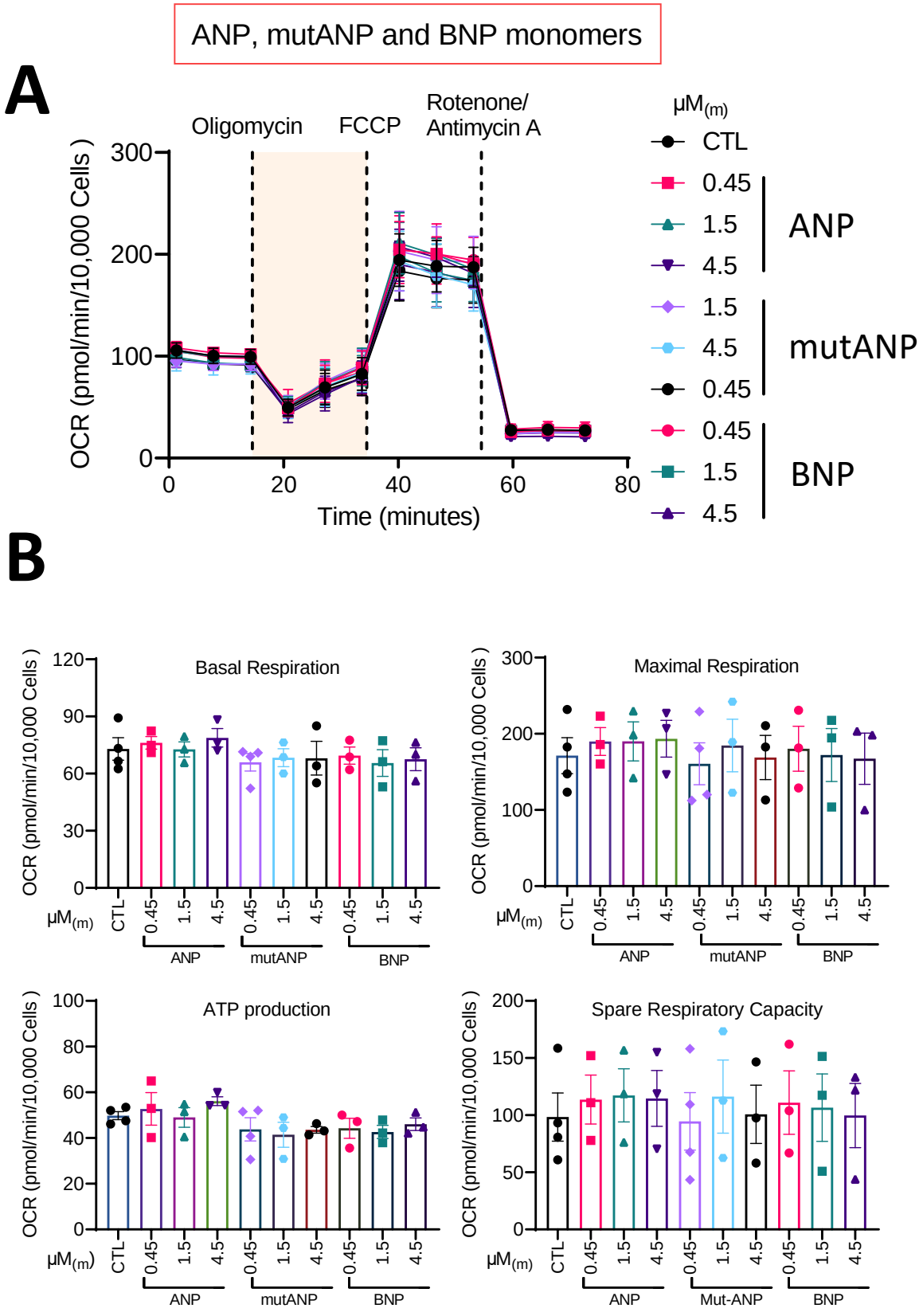
* *P*<0.05; † *P*<0.01; n=7-16

Initial peptide concentration=500nM

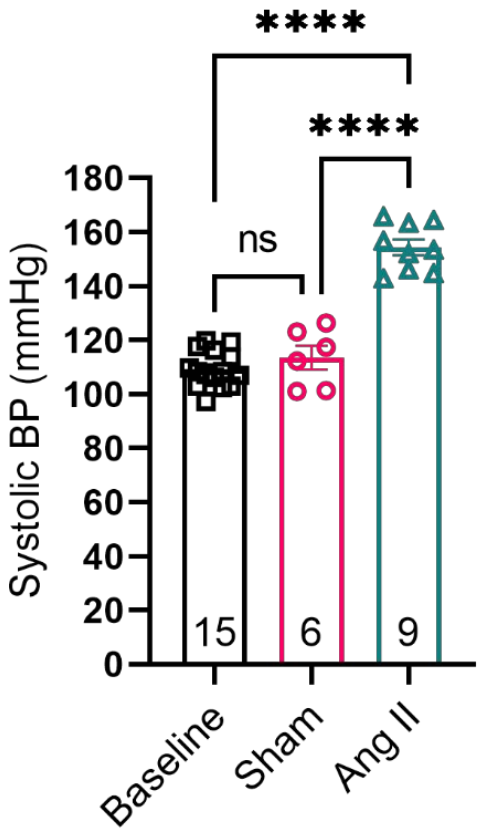
III. SUPPLEMENTAL FIGURES



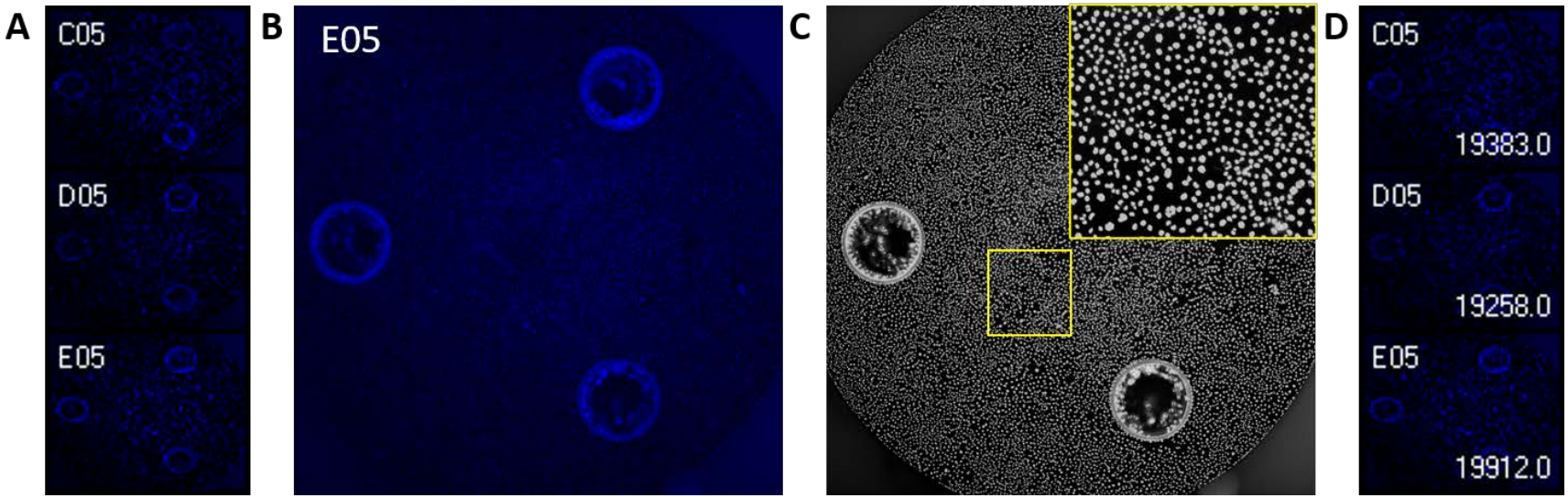
Supplemental Figure I: Concentration-dependent oligomerization is accelerated for mutANP compared to ANP. WT ANP and mutANP peptides were oligomerized at RT for 24hr at the indicated initial monomer concentrations, incubated with 2.3% glutarylaldehyde for cross-linking, and subjected to Western blot analysis that included incubation with a rabbit anti-α-ANP antibody (1:500) and an IRDye 680RD anti-rabbit (1:5000) secondary antibody followed by detection on an Odyssey CLx Near-Infrared Fluorescence Imaging Systems (LI-COR).



Supplemental Figure II: Monomers derived from ANP, mutANP, and BNP have no effect on mitochondrial function. Data are shown for bioenergetic profiling using the mitochondrial stress test for atrial HL-1 cardiomyocytes incubated in the absence (control or CTL) or presence of freshly-prepared monomers for 24h, using the same format as in Figures 1 and 2. All OCR values were corrected for Hoechst positive nuclei. Data are expressed as mean ± SEM. The figure is the result of n=3-4 independent experiments.



Supplemental Figure III: Systolic BP in normotensive and hypertensive mice. The systolic BP for the 15 mice studied is shown at baseline. Mice then received osmotic pumps filled with either buffer (sham) or angiotensin II (ang II, 490 ng/kg/min) for 2 weeks. Systolic BPs are illustrated at this time point just prior to sacrifice for atrial myocyte isolation. ****P<0.0001.



Supplemental Figure IV: Data normalization of Mito Stress Test results using in situ Hoechst 33342 staining and nuclear segmentation. **A.** Typical in situ nuclear staining of HL-1 cells is shown for 3 Seahorse XF 96-well cell culture plate wells. Immediately after the metabolic assay, cells were washed with PBS and incubated with Hoescht 33342 (5µg/ml) in the dark for 15 min at room temperature. Cells were then washed again with PBS and imaged with ImageXpress Micro equipped with a DAPI filter set. **B.** Enlarged view of the well E05 in panel A. **C.** Multiwavelength Cell Scoring function of MetaExpress high content analysis software was used for nuclear segmentation of Hoescht 33342-stained nuclei in E05 (panel B). Inset: enlarged view of bordered area. **D.** Actual cell counts of the wells following nuclear segmentation shown in panel A.