SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL MATERIALS AND METHODS

Enhance histidyl dipeptide levels within the cardiac tissue

***β-Alanine and carnosine feeding*.** To enhance histidyl dipeptide pool within cardiac tissue, we treated WT C57BL/6 mice (12-14 week) with β-alanine (20 g/L) or carnosine (10 g/L) in drinking water or water alone for seven days. Heart and skeletal muscle from the treated and untreated mice were collected and analyzed for different histidyl dipeptides levels using LC/MS.

Generation of cardio specific carnosine synthase (ATPGD1) transgenic mice*.* To enhance endogenous production of histidyl dipeptides within cardiac tissue, we generated cardio specific ATPGD1 transgenic (Tg) mice. Full protein-coding sequence of the mouse (m) ATPGD1 cDNA (2.84 kb a kind gift from Dr. Emile Van Schaftingen, Ludwig Institute for Cancer Research, Universite Catholique de Louvain, Belgium) was cloned under the control of mouse alpha-myosin heavy chain promoter (α-MHC; 5.5 kb), 0.6 Kb HGH (human growth hormone) poly A was ligated at the 3' end of the minigene construct. Nucleotide sequencing and comparisons with published sequences verified the construct as m*ATPGD*1 cDNA. The resultant transgene (9.04 Kb) composed of α-MHC promoter, the entire protein-coding region of ATPGD1 and HGH polyadenylation signal sequence. The transgene construct was linearized, purified, and microinjected into the pronuclei of fertilized ova to generate α-*MHC*-ATPGD1-Tg mice at the University of Cincinnati, Cincinnati, OH Transgenic Core. Four transgenic founders with the varying copy number of the transgene were generated on the C57Bl/6 background. Founder mice were identified by genomic PCR using the following primers: Forward-5'- *AGT CCT GGT GGG AGA GCC ATA* -3' and Reverse 5' – *GCC AAG CAG GGG ACA GGC AAA* -3' corresponding to *α-MHC* 5'UTR and the *ATPGD1* coding region, respectively. Two transgenic (TG) lines were established and distinguished from their non-transgenic wild type littermates (WT) cohorts by PCR.

Myocardial ischemia and reperfusion (I/R) injury in-vivo and assessment of area at risk and infarct size.

The I/R injury was produced by subjecting mice to a 30 min of coronary occlusion followed by 24 h of reperfusion as described previously[69-71]. Briefly, the mice (10-14 weeks of age) were anesthetized with pentobarbital sodium (50mg/kg, i.p) intubated and ventilated at room air supplemented with oxygen. The body temperature was monitored with a rectal probe and maintained at 37±0.2°C with a heating pad. To replace blood loss, blood from a donor mouse was given intravenously. The chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was applied on the left anterior descending coronary artery 2-3 mm from the tip of left auricle. Successful performance of the surgery was verified by noting the development of a pale color in the distal myocardium on inflation of the balloon and return of a bright red color due to hyperemia after deflation and by observing the ST-segment elevation and widening of the QRS complex on the ECG during ischemia and their resolution after reperfusion. Following the coronary occlusion/reperfusion, the chest was closed in layers, and the mice were allowed to recover under close monitoring. Heart was excised and perfused with Krebs-Henseleit (KH) solution through an aortic cannula. To determine the area of infarcted myocardium from the viable myocardium, the heart was perfused with 1% triphenyl tetrazolium chloride in phosphate buffer. To delineate the area of occlusion, the coronary artery was tied at the site of previous occlusion, and the aortic root was perfused with 10% Pthalo blue dye. The region of risk was identified by the absence of blue dye, whereas the rest of left ventricle was stained dark blue. The left ventricle was cut into transverse slices, which were fixed in 10% neutral buffered formaldehyde, weighed, and photographed under a microscope. The corresponding areas were measured by computerized planimetry and from these measurements; the infarct size was calculated as the percentage of region at risk.

In the second set of experiments, coronary ligation was performed, to analyze the formation of aldehyde protein adduct during ischemic injury. Hearts from the WT and ATPGD1-Tg mice were collected following 40 min of ligation. The ischemic zone of ligated mice and the anterior zone of sham-operated mice were clamp frozen and analyzed by Western blotting.

Langendorff perfusion

***Global ischemia-reperfusion (I/R) ex vivo*.** To exclude the neurohormonal effects associated with *in-vivo* model of I/R, we performed I/R in a Langendorff mode as described previously[27]. Mice were anesthetized (sodium pentobarbital, 60mg/kg body weight, heparin, 10 units/g body weight), the thorax of mice was opened and the hearts were removed, placed immediately in the ice-cold KH buffer; mM 118 NaCl, 4.7 KCl, $MgCl_2$, $CaCl_2$, KH_2PO_4, $NaHCO_3$ and 11 glucose. The buffer was heated at 37°C and continuously gassed (95% O_2, 5%CO_2). Hearts from the WT and ATPGD1-Tg, mice were continuously perfused at a constant pressure of 80 mmHg in the Langendorff mode and after 20-30 min of perfusion, the isolated hearts were subjected to 30 min of ischemia followed by 45 min of reperfusion. Functional recovery of the hearts was measured by placing a food grade plastic wrap, fluid-filled balloon to a pressure of 5-8 mm Hg. Left ventricular pressure was recorded using an APT300 pressure transducer (Harvard Apparatus, Holliston, MA) connected to a ML221 bridge amplifier, a Power lab 16/30 A/D board, and a PC running Lab Chart Pro v7 (AD Instruments, Colorado Springs, CO). Perfusion flow rate was monitored using a 1PXN inline probe and TS410 flowmeter (transonic Systems Inc., Ithaca, NY) and was typically between 1.5 and 2 mL/min. Left ventricular developed pressure was derived from the pressure trace as the difference between systolic and diastolic pressures. Perfusates were collected on ice and the levels of creatinine kinase (CK) and lactate dehydrogenase (LDH) were measured using kits purchased from Thermo-Electron.

In a second line of experiments, we tested which dipeptide analogue could lead to recovery of function following ischemia. For these experiments, wild type (WT) C57/Bl6 mice (12-14 weeks old) were perfused with either balenine and anserine (1mM), followed by 30 min of ischemia and 45 min of reperfusion and the recovery of cardiac function was monitored in a Langendorff mode as described above.

Low flow ischemia and ^{13}C D-glucose perfusion: To determine whether ATPGD1 *o*verexpression influences the glycolytic fluxes during ischemic injury, isolated WT and ATPGD1-Tg mice hearts were perfused in the Langendorff mode for 10 min in a KH buffer; mM 118 NaCl, 4.7 KCl, $MgCl_2$, $CaCl_2$, KH_2PO_4, $NaHCO_3$ and 11 ^{13}C-D-glucose followed by 15 min of low flow ischemia (LFI, 10% of baseline). The effluent was collected at regular intervals and analyzed for ^{13}C D-glucose and ^{13}C lactate.

^{31}P Nuclear Magnetic Resonance Spectroscopy*:* Hearts isolated from the WT and ATPGD1-Tg mice were perfused in a Langendorff mode with KH buffer at constant coronary pressure of 75 mmHg at 37°C for 10 min followed by 20 min LFI (10% of baseline) and 50 min reperfusion. ^{31}P-NMR spectra were collected continuously and simultaneously during measurement of isovolumic contractile performance using ^{31}P-NMR spectroscopy. [72] Isolated perfused hearts and an external standard containing 4.51 μmol of phenylphosphonic acid (PPA) in a small sealed plastic tube were placed in a 10-mm glass NMR sample tube and inserted into a 10 mm Varian broad band probe situated in an 89-mm bore 9.4-T superconducting magnet. To improve homogeneity of the NMR-sensitive volume, the perfusate level was adjusted so that the heart was submerged in buffer. ^{31}P-NMR spectra were obtained using 60° pulses and a recycle time of 2.4 S, 6,000-Hz sweep width and 2 K data points at 161.94 MHz using a Varian Inova spectrometer (Varian Inc., Palo Alto, CA). Single spectra were collected during 10 min periods and consisted of data averaged from 256 free induction decays. Spectra were processed using 20-Hz exponential multiplication and zero and first-order phase corrections. The resonance areas of phosphocreatine (PCr), ATP, inorganic phosphate (Pi) and the chemical shifts of Pi were quantified using Bayesian Analysis Software (G.L. Bretthorst Washington University, St. Louis, MO) [73]. By comparing the amplitude under the peaks from fully relaxed (recycle time 15 sec) and those of partially saturated (recycle time 2.4 sec) spectra, the correction factors for saturation were calculated for ATP (1.041), PCr (1.285), Pi (1.157) and PPA (1.283).

Cell culture studies

Isolation of adult cardiomyocytes*.* Cardiac myocytes from the WT and ATPGD1-Tg mice hearts (age 12-16 weeks) were isolated using Langendorff perfusion as described previously.[74] [21] Isolated hearts from these mice were rinsed in physiological saline and perfused with oxygenated (95% O_2- 5%CO_2), Ca^{2+}- free modified Tyrode bicarbonate buffer (buffer A mM: NaCl 126, KCL 4.4, $MgCl_2$ 1.0, $NaHCO_3$ 18, glucose 11, HEPES 4, 2,3 butanedione monoxime 10, taurine 30, pH 7.35) at 37°C for 5 min. The extracellular matrix was digested by buffer containing buffer A with Liberase Blendzyme type I 0.25 mg/mL, 1mg/mL albumin, 0.015 mg/mL DNase, 0.015 mg/mL proteinase in the 50 mL of recirculating digestion buffer (buffer A with 25 μM $CaCl_2$) for 12-15

min. Hearts were separated in mincing buffer (10 mL digestion buffer with 9 mg/mL albumin and Liberase) and cells were allowed to settle. $CaCl_2$ was reintroduced in a graded fashion at 5- min intervals (five total steps) to sequentially increase the Ca^{2+} concentration to 500 μM.

Hypoxia reoxygenation of adult cardiomyocytes*.* Cardiomyocytes isolated from WT and ATPGD1-TG mice hearts were subjected to 2-3 h of hypoxia using a controlled hypoxic chamber (Billups-Rothenberg Modulao, Incubator Chamber) maintained at 37°C. Freshly isolated cells were maintained in serum free/ glucose free HEPES-buffered medium (mM: NaCl 113, KCL 4.7, HEPES 10, $MgSO_4$ 1.2, taurine 30, $CaCl_2$ 1, pH 6.2) at 37°C. Cells were allowed to settle down, supernatant was removed and replaced with hypoxia buffer (glucose free/serum free HEPES buffered medium that was bubbled with nitrogen (N_2). The dishes were filled with 1 mL of hypoxia buffer and incubated in a humidified hypoxic chamber containing: 1% O_2, 5% CO_2 and 94% N_2 at 37°C. Following hypoxia, the cells were re oxygenated with 1 mL of HEPES buffered medium supplemented with serum (10%) and glucose (11 mM) bubbled with O_2 and placed under normoxic conditions. For control conditions, freshly isolated cells were washed twice with a HEPES-buffered medium supplemented with serum (5%) and glucose (5.5 mM) and placed under normoxic conditions. Reoxygenation was mimicked by adding 1 mL of the medium. The supernatant was collected and analyzed for LDH release.

Superfusion of isolated cardiomyocytes with HNE and acrolein. To examine whether the increase in endogenous production of histidyl dipeptides protects against aldehyde induced hypercontracture, adult cardiomyocytes from the WT and ATPGD1-Tg mice hearts were superfused with 50-60 μM HNE or 5 μM acrolein for 60 min as described previously.[21] Digital images of approx. 100 cardiomyocyte/field were acquired at 5 min interval to quantify the number of hypercontracted and non-hypercontracted cells following aldehyde treatment.

Western blot analysis

The anterior zone of sham operated and ischemic zone of the ligated mice hearts were homogenized and separated by SDS-PAGE. Tissues were homogenized in RIPA buffer (20mM Tris-HCl pH7.5, 150 mM NaCl, 1mM EDTA, 1mM EDTA, 1mMEGTA, 1% NP-40) and separated by SDS-PAGE. Immunoblots were developed

using anti-acrolein (0.25 μg/ mL, Novus Biological), anti-HNE (0.5 μg/mL, Abcam) and anti- ATPGD1 (0.25 μg/ mL COSMO Bio Company) antibodies. Western blots were developed using ECL Plus reagent and detected with a Typhoon 9400 Variable mode imager. Band intensity was quantified by using an Image Quant TL software and bands were normalized to amido black or tubulin.

LC/MS studies

Measurement of histidyl dipeptides by LC/MS. Histidyl dipeptides in the heart and skeletal muscle were measured by LC/MS as described previously[36]. Briefly the isolated tissues from mice were homogenized in extraction buffer containing 10 mM HCl and 200 μM tyrosine-histidine as an internal standard (IS). Homogenates were sonicated on ice for 10 s, centrifuged at 16,000 × g for 10 min at 4°C. Supernatants were injected into TQ-S micro mass spectrometer in positive mode, the samples were diluted in 75% acetonitrile/25% water. The dipeptides were separated and identified by using Water ACQUITY UPLV H-Class system coupled with a Xevo TQ-S micro triple quadrupole mass spectrometer (MS). The analytes were separated by a Waters Acquity BEH HILIC column (1.7μm, 2.1× 50) equipped with an in-line frit filter unit. The analytes were eluted by using a binary solvent system consisting of 10 mM ammonium formate, 0.125% formic acid in 50% acetonitrile/50% water for mobile phase A and 10 mM ammonium formate 0.125% formic acid in 95% acetonitrile/5% water for mobile phase B at a flow rated of 0.55 ml/min. Initial conditions were 0.1:99.9 A/B ramping to 99.9:0.1 A/B over 5 min then quickly ramping to 0.1:99.9 A/B over 0.5 min. This initial composition was held from 5.5 to 8 min to equilibrate the column for the next injection. Dipeptides were quantified using the LC/MS calibration curve of relative area of carnosine and anserine to internal standard tyrosine-histidine and expressed as mole/mg wet wt. or mole/mg protein. For carnosine *m/z* 227→110, anserine *m/z* 241→110, and tyrosine-histidine m/*z* 319→110, Da MRM transitions were followed.

^{13}C glucose and ^{13}C lactate analysis.

Perfusates collected from the WT and ATPGD1-Tg hearts perfused with ^{13}C glucose and subjected to low flow ischemia as described above were dried and dissolved in 200 μL 20% acetonitrile and vigorously vortex-

mixed for 3 min. After centrifugation at 14,000 rpm, 4 ^{o}C for 20 min, the supernatant was collected for 2DLC-MS/MS analysis.

All samples were analyzed on a Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with a Thermo DIONEX UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The UltiMate 3000 HPLC system was equipped with a reverse phase chromatography (RPC) column and a hydrophilic interaction chromatography (HILIC) column. The RPC column was an ACQUITY UPLC HSS T3 column (150 × 2.1 mm i.d., 1.8 µm) purchased from Waters (Milford, MA, USA). The HILIC column was a SeQuant® ZIC®-cHILIC HPLC column (150 × 2.1 mm i.d., 3 µm) purchased from Phenomenex (Torrance, CA, USA). The two columns were configured in parallel 2DLC mode [75]. For 2DLC separation, H_2O with 0.1% formic acid was used as the mobile phase A for RPC and 10 mM ammonium acetate (pH 3.25) was used as the mobile phase A for HILIC. Acetonitrile with 0.1% formic acid was used as mobile phase B for both RPC and HILIC. The RPC gradient was as follows: 0 min, 5% B, hold for 5.0 min; 5.0 min to 6.1 min, 5% B to 15% B; 6.1 min to 10.0 min, 15% B to 60% B, hold for 2.0 min; 12.0 min to 14.0 min, 60% B to 100% B, hold for 13.0 min; 27.0 min to 27.1 min, 100% B to 5% B, hold for 12.9 min. The gradient for HILIC separation was: 0 to 5.0 min, 95% B to 35% B, hold for 1.0 min; 6.0 min to 6.1 min, 35% B to 5% B, hold for 16.9 min; 23.0 min to 23.1 min, 5% B to 95% B, hold for 16.9 min. The flow rate was 0.4 mL/min for RPC and 0.3 mL/min for HILIC. The column temperature was 40 °C for both columns. The injection volume was 2 µL for each column.

To avoid systemic bias, the samples were analyzed by 2DLC-MS in a random order. All samples were first analyzed by 2DLC-MS positive mode followed by 2DLC-MS negative mode, to obtain the full MS data of each metabolite. For quality control purpose, a group-based pooled sample was prepared by mixing a small portion of the supernatant from all unlabeled samples in one group. One pooled sample was analyzed by 2DLC-MS after injection of every 5 biological samples. All pooled samples were analyzed by 2DLC-MS/MS in positive and negative mode respectively to acquire MS/MS spectra of each metabolite at three collision energies (20 eV, 40 eV and 60 eV).

MetSign software was used for spectrum deconvolution, metabolite identification, cross-sample peak list alignment, normalization, and statistical analysis [76-78]. To identify metabolites, the 2DLC-MS/MS data of the

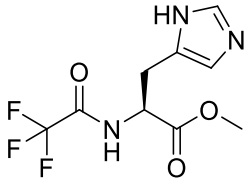
pooled samples were first matched to an in-house MS/MS database that contains the parent ion m/z, MS/MS spectra, and retention time of 205 metabolite standards. The thresholds used for metabolite identification were MS/MS spectral similarity ≥ 0.4, retention time difference ≤ 0.15 min, and m/z difference ≤ 4 ppm. The 2DLC-MS/MS data without a match in the in-house database were then analyzed using Compound Discoverer software (v2.0, Thermo Fisher Scientific, Inc., Germany), where the threshold of MS/MS spectrum similarity score was set as ≥ 40 with a maximum score of 100. The remaining peaks that did not have a match were then matched to the metabolites in -house MS/MS database using the parent ion m/z and retention time to identify metabolites that do not have MS/MS spectra. The thresholds for assignment were parent ion m/z ≤ 4 ppm and retention time difference ≤ 0.15 min.

Synthesis of histidyl dipeptide analogues anserine and balenine

(a) Balenine was synthesized as mentioned previously.[21]

(b) Anserine synthesis

(S)-Methyl 3-(1H-imidazol-5-yl)-2-(2,2,2-trifluoroacetamido) propanoate (3):

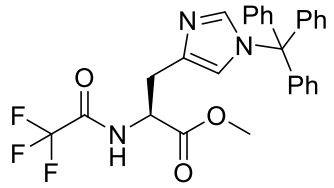


3

To L-histidine dihydrochloride (5.00 g, 20.65 mmol) in methanol (100 mL), we added ethyl 2,2,2-trifluoroacetate (2.70 mL, 22.72 mmol) and triethyl amine (1.52 mL, 82.6 mmol) and the reaction mixture was stirred for 5 h at room temperature (RT). The solvent was evaporated to dryness and the obtained solid was purified by silica gel chromatography to get the desired product, (*S*)-methyl 3-(1*H*-imidazol-5-yl)-2-(2,2,2-trifluoroacetamido) propanoate, (**3**, 4.10 g, 75% yield) as an off-white solid compound.

^{1}H NMR (D_2O, 400 MHz): δ 7.60 (s, 1H), 6.87 (s, 1H), 4.76 – 4.67 (m, 1H), 3.66 (s, 3H), 3.17 – 2.96 (m, 2H) ppm.

(S)-Methyl 2-(2,2,2-trifluoroacetamido)-3-(1-trityl-1H-imidazol-4-yl) propanoate (4):

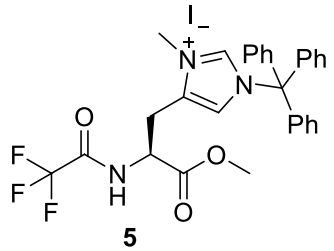


4

Trityl chloride (5.60 g, 20.36 mmol) and triethyl amine (8.53 mL, 61.53 mmol) were dissolved in anhydrous benzene (100mL) and (*S*)-methyl3-(1*H*-imidazol-5-yl)-2-(2,2,2-trifluoroacetamido) propanoate (**3**, 5.40 g, 20.36 mmol) was added. The resulting reaction mixture was refluxed for 1 h and the solvent was evaporated. Obtained semi pure compound was purified with silica gel column using dichloromethane and methanol was used as eluents to get (*S*)-methyl 2-(2,2,2-trifluoroacetamido)-3-(1-trityl-1*H*-imidazol-4-yl) propanoate (**4**, 5.80 g, 55% yield) as an off-white solid compound.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.89 – 8.87 (d, 1H), 7.40 (s, 1H), 7.37 – 7.30 (m, 9H), 7.13 – 7.07 (m, 6H), 6.57 (s, 1H), 4.85 – 4.79 (m, 1H), 3.63 (s, 3H), 3.16 – 3.11 (m, 1H), 3.06 – 3.00 (m, 1H) ppm.

(S)-4-(3-Methoxy-3-oxo-2-(2,2,2-trifluoroacetamido) propyl)-3-methyl-1-trityl-1H-imidazol-3-ium iodide (5):

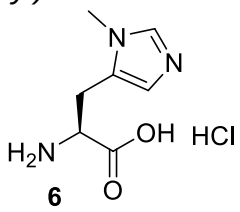


5

(*S*)-Methyl 2-(2,2,2-trifluoroacetamido)-3-(1-trityl-1*H*-imidazol-4-yl) propanoate (3.50 g, 6.90 mmol) was dissolved in anhydrous acetonitrile (100 mL) and methyl iodide (0.86 mL, 13.80 mmol) was added. The resulting reaction mixture was refluxed for 1 h and the solvent was evaporated. Obtained crude was purified with silica gel column using dichloromethane and methanol as eluents to get (*S*)-4-(3-methoxy-3-oxo-2-(2,2,2-trifluoroacetamido) propyl)-3-methyl-1-trityl-1*H*-imidazol-3-ium iodide (**5**, 4.0 g, 89% yield) as a yellow solid compound.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.26 – 8.25 (d, 1H), 7.48 – 7.38 (m, 7H), 7.35 – 7.20 (m, 10H), 4.86 - 4.79 (m, 1H), 4.03 (s, 3H), 3.78 (s, 3H), 3.56 – 3.41 (m, 2H) ppm.

(S)-1-Carboxy-2-(1-methyl-1H-imidazol-5-yl)ethanaminium chloride (6):

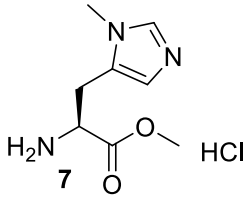


6

In a round bottom flask, we added (*S*)-4-(3-methoxy-3-oxo-2-(2,2,2-trifluoroacetamido) propyl)-3-methyl-1-trityl-1*H*-imidazol-3-ium iodide (**5**, 4.0 g, 6.16 mmol) and 4N HCl (100 mL). The reaction mixture was refluxed for 6 h, and evaporated to dryness, and the solid obtained was washed with hexanes and dried to get (*S*)-1-carboxy-2-(1-methyl-1*H*-imidazol-5-yl) ethanaminium chloride, (**6**, 1.10 g, 87% yield) as a yellow solid compound.

^{1}H NMR (D_2O, 400 MHz): δ 8.62 (s, 1H), 7.36 (s, 1H), 4.26 – 4.19 (m, 1H), 3.78 (s, 3H), 3.41 – 3.25 (m, 2H) ppm.

(S)-Methyl 2-amino-3-(1-methyl-1H-imidazol-4-yl) propanoate hydrochloride (7):

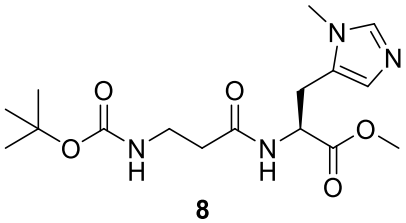


7

In 300 mL of methanolic hydrochloric acid was added (*S*)-2-amino-3-(1-methyl-1*H*-imidazol-5-yl) propanoic acid hydrochloride (2.60 g, 12.64 mmol) at room temperature. The reaction mixture was then refluxed for 48 h, and evaporated to dryness to get (*S*)-methyl 2-amino-3-(1-methyl-1*H*-imidazol-4-yl) propanoate hydrochloride, (**7**, 2.5 g, 90% yield) as a white solid. (Note: Methanolic-HCl was made by purging HCl gas into required methanol for 5 min.)

^{1}H NMR (D_2O, 400 MHz): δ 8.62 (s, 1H), 7.39 (s, 1H), 4.45 – 4.19 (m, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 3.47 - 3.26 (m, 2H) ppm.

(*S*)-Methyl 2-(3-(*tert*-butoxycarbonylamino) propanamido)-3-(1-methyl-1H-imidazol-5-yl) propanoate (8):

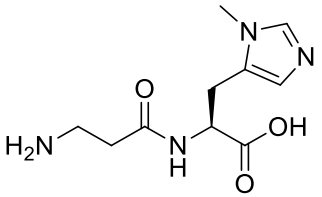


8

Added sequentially to a reaction flask under nitrogen were (*S*)-methyl 2-amino-3-(1-methyl-1*H*-imidazol-5-yl)propanoate hydrochloride (**7**, 2.0 g, 9.10 mmol), boc-b-alanine (1.89 g, 10.02 mmol), EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide) (1.92 g, 10.02 mmol) followed by DCM (100 mL) and then *N*-methylmorpholine (4.00 mL, 36.4 mmol via syringe). The contents were stirred at room temperature under nitrogen and the solids gradually dissolved. The contents were stirred at room temperature for 24 h, and then slowly diluted into iced water and extracted with DCM. The organic phase was dried evaporated and chromatographed using dichloromethane and methanol as eluents to get (*S*)-methyl 2-(3-(*tert*-butoxycarbonylamino) propanamido)-3-(1-methyl-1*H*-imidazol-5-yl) propanoate (**8**, 2.0 g, 62% yield) as an off-white solid compound.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37 (s, 1H), 6.75 (s, 1H), 6.66 – 6.50 (m, 1H), 5.15 – 5.08 (m, 1H), 4.82 – 4.77 (m, 1H), 3.74 (s, 3H), 3.57 (s, 3H), 3.45 – 3.30 (m, 3H), 3.16 – 3.02 (m, 1H), 2.50 – 2.35 (m, 2H), 1.42 (s, 9H) ppm.

L-Anserine (9):



L-Anserine

To (*S*)-methyl 2-(3-(tert-butoxycarbonylamino) propanamido)-3-(1-methyl-1*H*-imidazol-4-yl) propanoate (**8,** 2.2 g, 6.2 mmol) was added 2N NaOH (18.6 mL) in THF (50 mL) and methanol (50 mL) and the reaction was stirred for the 24 h at RT. Solvent was evaporated and the reaction mixture

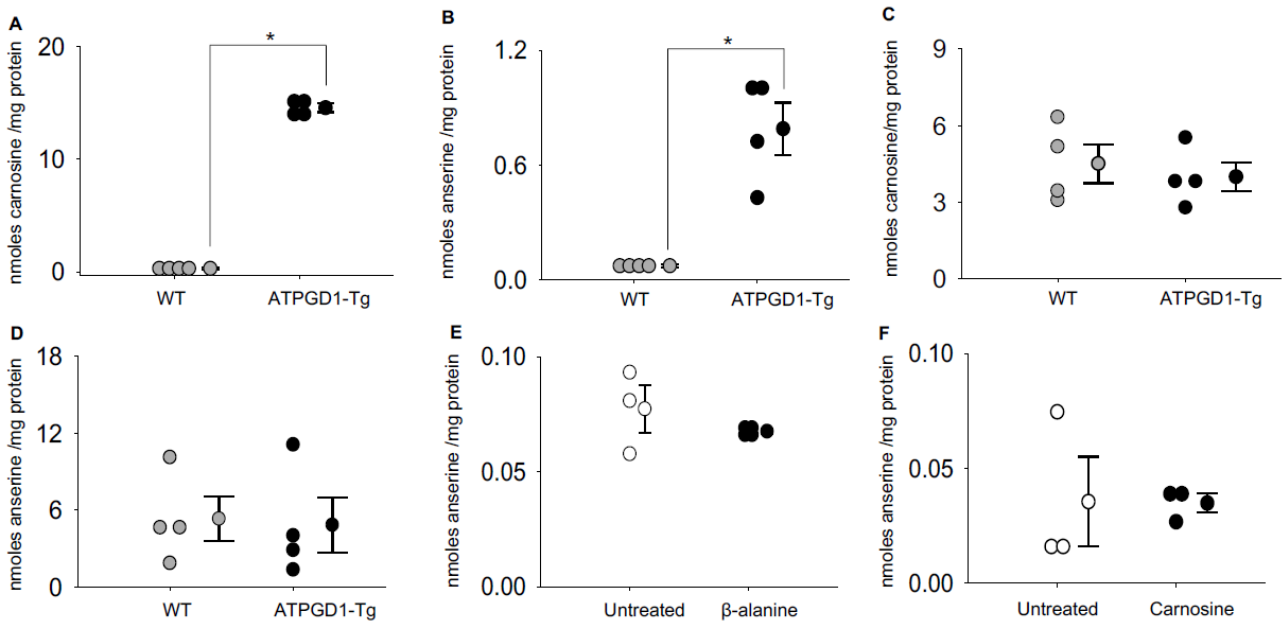
was neutralized by 1N HCl to pH 3. It was further stirred and neutralized by aqueous saturated sodium bicarbonate solution. All the water was evaporated to dryness and the obtained solid was triturated with DCM and dried again. The obtained solid was dissolved in hot ethanol and passed through the celite plug, twice. Filtrate was evaporated and dried under high vacuum to get the L-anserine (**9**, 1.30 g, 87% yield) as a white solid compound.

^{1}H NMR (D_2O, 400 MHz): δ 8.61 (s, 1H), 7.27 (s, 1H), 4.54 – 4.50 (m, 1H), 3.86 (s, 3H), 3.31 – 3.19 (m, 3H), 3.14 – 3.07 (m, 1H), 2.80 – 2.64 (m, 2H) ppm; ^{13}C NMR (D_2O, 100 MHz) *δ* 176.3, 171.7, 135.1, 131.3, 117.5, 53.2, 35.2, 33.1, 31.9, 25.8.

Echocardiographic analysis of cardiac function

Transthoracic echocardiography of the left ventricle was performed using a Vevo 770 echocardiography system as described previously. [79] Briefly the body temperature of the mice was maintained (36.5 -37.5°C) using a rectal thermometer interfaced with a servo-controlled heat lamp. Mice were anesthetized with 2% isoflurane, maintained under anesthesia with 1.5% isoflurane. The chest was shaved and the mouse was placed chest up on an examination board interfaced with the Vevo 770. The 707-B (30 MHZ) scan head was used to obtain 2D images of the parasternal long axis. M-modes were taken from the same anatomical position. The probe was then rotated to acquire a short axis view of the heart. Stroke volume (SV) was calculated as: Diastolic Volume-Systolic Volume. Ejection fraction was calculated as (SV/Diastolic Volume) *100%. Cardiac output was determined by: SV*HR. All images were acquired with the Vevo 770's rail system to maintain probe placement and allow for precise adjustments of position. Left ventricular diameters during diastole (LVIDd) and left ventricular diameter during systole (LVIDs) and heart rate (HR) were determined from long axis M-modes. Left ventricular fractional shortening (%FS) was calculated as ((LVIDd-LVIDs)/LVIDd) *100%.

Figure S1. Histidyl dipeptide levels are enhanced in the carnosine synthase (ATPGD1-Tg) heart.



Levels of carnosine in the (**A**) heart and (**C**) gastrocnemius skeletal muscle of WT and ATPGD1-Tg mice. Levels of anserine in the (**B**) heart and (**D**) skeletal muscle of WT and ATPGD1-Tg mice. Levels of anserine in the hearts of (**E**) β-alanine and (**F**) carnosine fed mice. Data is presented as mean±SEM, n=4 mice in each group, *p<0.05 vs WT heart.

Figure S2. Synthesis of anserine.

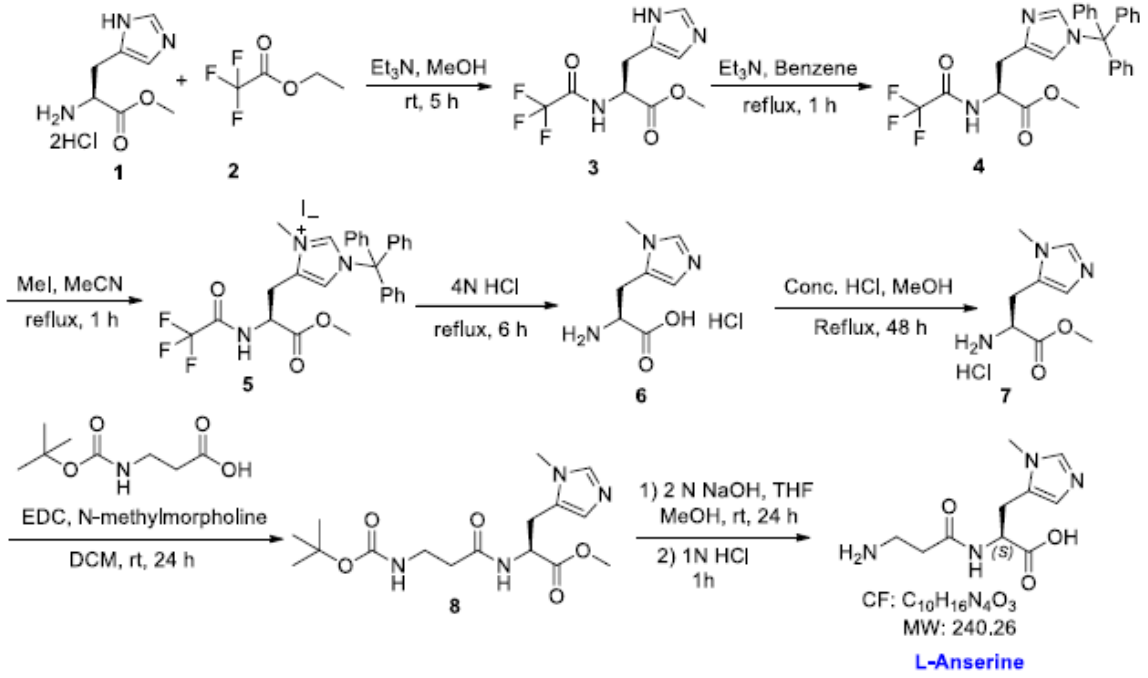


Table S1. Echocardiographic parameters of the wild type and ATPGD1-Tg hearts, age 12-14-week, n=7 in each group.

	Baseline	
	WT	**ATPGD1-Tg**
LVIDd(mm)	3.95±0.05	3.98±0.07
LVIDs(mm)	2.27±0.07	2.48±0.09
EF(%)	63±1	60±1
Heart rate	580±5	540±5
FS	41±1	38±2

Data are presented as S.D. in each group.