Chronic Therapy with Elamipretide (MTP-131), a Novel Mitochondria-Targeting Peptide, Improves Left Ventricular and Mitochondrial Function in Dogs with Advanced Heart Failure

SUPPLEMENTAL MATERIAL

Methods

In this study, 14 healthy mongrel dogs, underwent serial intracoronary microembolizations performed 1 to 2 weeks apart, to produce HF. Embolizations were discontinued when LV ejection fraction (EF), determined angiographically, was ~30%. Nearly 6 weeks after the last microembolizations, dogs were randomized to 3 months of therapy with subcutaneous injections with elamipretide (0.5 mg/kg per day, n=7) or saline (Control, n=7). All the procedures were performed during cardiac catheterization under general anesthesia and sterile conditions. Induction of anesthesia was initiated with intravenous hydromorphone (0.22 mg/kg) and diazepam (0.17 mg/kg). Plane of anesthesia was maintained with 1-2% isofluorane.

Hemodynamic, Ventriculographic and Echocardiographic/Doppler Measurements

Aortic and LV pressures were measured with catheter-tip micromanometers (Millar Instruments, Houston, TX). Left ventriculograms were obtained with the dog placed on its right side and recorded on digital media at 30 frame/sec during the injection of 20 ml of contrast material (ISOVU-300, Bracco Diagnostics, Inc., Princeton, NJ). Correction for image magnification was made with a radiopaque calibrated markers imbedded in the shaft of the LV ventriculography catheter. LV end-systolic volume (ESV), end-diastolic

volume (EDV) and LV EF were calculated using the area-length method (1). Stroke volume was calculated as the difference between EDV and ESV. Cardiac output (CO) was calculated as the product of stroke volume (SV) and heart rate (HR). Systemic vascular resistance (SVR) was calculated as previously described (2). Cardiac index (CI) was measured as the ratio of CO to body surface area.

Echocardiographic and Doppler studies were performed using a General Electric VIVID-7 ultrasound system with a 3.5 MHZ transducer and recorded on digital media for off-line analysis. Trans-mitral inflow velocity waveforms, measured using pulsed-wave Doppler echocardiography, were used to calculate indexes of LV diastolic function namely the time-velocity integral of mitral inflow velocity waveform representing early filling (Ei), the time-velocity integral representing LA contraction (Ai), the ratio Ei/Ai, and deceleration time (DT) of early mitral inflow velocity as previously described (2, 3). LV end-diastolic circumferential wall stress (EDWS) was calculated as previously described (2).

Determination of Plasma Biomarkers and Reactive Oxygen Species

Assay kits for TNFα and IL-6 were purchased from R&D Systems, Inc, Minneapolis, MN; nt-pro-BNP kits from Kamiya Biomedical Company, Seattle, WA, and CRP kits from ALPCO, Salem, NH. Detection method for all these kits were colorimrtric and plasma volume used in TNF□ and IL6 assays were 150 µl, in nt-proBNP 35 µl, and CRP 100 µl after 1000 fold dilution. Incubation time with primary antibody was overnight at 4^{o}C except 10 min at room temperature in case of CRP. All the values calculated for the

biomarkers were from the standard curve generated using a software MasterPlex ReaderFit 2010. All the values of biomarkers were expressed per ml plasma without any normalization.

Total burden of reactive oxygen species (ROS) in plasma was determined in an aliquot of 100 µl of 10-folds diluted EDTA-plasma with PBS buffer using the luminol-dependent chemiluminescence assay and expressed as relative luminescence units (RLU)/ml (4). Total ROS in LV tissue was determined using 100 µl of 30 fold-diluted cytosol of 0.05% LV tissue using homogenate in PBS buffer with the same assay as plasma and expressed as RLU/mg protein (4).

Histomorphometric Measurements

The volume fraction of replacement fibrosis (VFRF), interstitial fibrosis (VFIF), myocyte cross-sectional area (MCSA), a measure of cardiomyocyte hypertrophy, capillary density (CD), and oxygen diffusion distance (ODD) were assessed histomorphometrically as previously described (5, 6). Briefly, from each heart, 3 transverse slices (3 mm thick), 1 each from the basal, middle, and apical thirds of the LV, were obtained. From each slice, transmural tissue blocks were obtained and embedded in paraffin blocks. From each block, 6 µm-thick sections were prepared and stained with Gomori trichrome to identify fibrous tissue. The VFRF, namely, the proportion of scar tissue to viable tissue in all 3 transverse LV slices, was calculated as the percent total surface area occupied by fibrous tissue by use of computer-based video densitometry (MOCHA, Jandel Scientific). LV free-wall tissue blocks were obtained from a second midventricular transverse slice,

mounted on cork with Tissue-Tek embedding medium (Sakura), and rapidly frozen in isopentane precooled in liquid nitrogen and stored at -70°C until used. Cryostat sections were prepared and stained with fluorescein-labeled peanut agglutinin (Vector Laboratories Inc) after pretreatment with 3.3 U/mL neuraminidase type V (Sigma Chemical Co) to delineate the myocyte border and the interstitial space, including capillaries. Sections were double stained with rhodamine-labeled Griffonia Simplicifolia lectin I (GSL-I) to identify capillaries. Average MCSA area was calculated by computer-assisted planimetry. The VFIF was calculated as the percent total surface area occupied by interstitial space minus the percent total area occupied by capillaries. CD density was calculated as the number of capillaries per square millimeter and ODD was calculated as half the distance between 2 adjoining capillaries.

Determination of Mitochondrial Function

Isolation of Cardiomyocytes: At the end of 3 months of therapy, cardiomyocytes were freshly isolated from LV free wall using collagenase as previously described (7, 8). The yield of rod-shaped cardiomyocytes that exclude tryptan blue was in the range of 80% to 90%.

Mitochondrial Respiration: Oxygen consumption was measured in freshly isolated, digitonin-permeabilized, cardiomyocytes using a Clark-type electrode (Strathklein Respirometer) in a final volume of 0.5 mL of respiration buffer as previously described (9). Mitochondrial (MITO) state-4 respiration was measured by adding substrate

(pyruvate and malate) and state 3 by adding 2 mM ADP and expressed as ng Atom of O_2 consumed/mg protein/min.

***Mitochondrial Permeability Transition Pore (mPTP)*:** mPTP opening was assessed using calcein studies performed in freshly isolated cardiomyocytes. The rate of calcein exit through mPTP was measured by recording the fluorescence signal every 2 min using a Turner Quantech Digital Filter Fluorometer with excitation filter NB490 and Emission filter SC515. The rate of calcein exit was calculated as a percent change from maximal fluorescence signal.

Measurement of Mitochondrial Membrane Potential

The membrane-permeant JC-1 dye, used as an indicator of MITO membrane potential ($\Delta\psi m$) in cardiomyocytes, exhibits potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green (495ex/534em) to red (495ex/590em). Briefly, using a commercially available kit (Sigma-Aldrich, St. Louis, MO), 10 µl of 2 fold diluted gravity settled cardiomyocytes were added in 90 µl of 0.2 mg/ml JC-1 dye with and without valinomycin (final concentration 0.1 mg/ml) working solution and incubated at room temperature for 2 minutes followed by measurement of fluorescence in microplate using a Tecan Safire fluorometer. The ratio of red to green was calculated after substracting valinomycin-sensitive fluorescence.

ATP Synthesis and ATP/ADP Ratio: MITO maximal rate of ATP Synthesis and the ratio ATP/ADP were determined in freshly isolated cardiomyocytes using the ApoSENSOR™

ADP/ATP ratio bioluminescent assay kit (BioVision, Milpitas, CA). The assay utilizes the enzyme luciferase to catalyze the formation of light from ATP and luciferin. The level of ADP was measured by its conversion to ATP that is subsequently detected using the same reaction. The maximal rate of ATP synthesis was expressed in relative light units (RLU)/mg protein.

Determination of Mitochondrial Complex I and Complex IV Activities

Complex I (NADH: ubiquinone oxidoreductase) Activity: The complex I activity was measured in frozen MITO fractions (at -80^{o}C) isolated from LV tissue. Approximately, 50 μg MITO fraction was treated with 1% cholate at 4^{o}C for 10 minutes to solubilize the macro complex I. The activity of solubilized MITO complex-I was assayed spectrophotometrically in MITO membrane fractions isolated from LV tissue as previously described (8) by following the oxidation of NADH (0.25 mM) at 340 nm at 30°C in an assay buffer containing 62.5 μM ubiquinone, 0.25% BSA, antimycin A (2 μg/ml), and mitochondria in the absence and presence of rotenone (10 μg/ml). Considering molecular absorptivity of NADH as 6.22, Complex-I activity was calculated as the rotenone-sensitive NADH:ubiquinone oxidoreductase activity and expressed as nmoles/min/mg protein.

Complex-IV (Cytochrome c Oxidase) Activity: The activity of MITO complex-IV was determined polarographically as previously described (10) in MITO membrane fractions isolated from LV tissue. Briefly, using Strathkelvin respirometer, approximately 5 μg MITO protein extract (obtained after treating approximately 50 μg frozen MITO fraction

in 1% sodium deoxycholate in a 100 µl volume by incubating in ice bath for 10 minutes) was added to oxidize reduced 40 µM cytochrome c containing 1 mM N'N'N'N' tetramethyl phenylene diamine and 10 mM ascorbate, with and without 2 mM sodium azide. Sodium-azide-sensitive oxidation rate of cytochrome c was calculated and expressed as nmoles molecular O_2/min/mg protein.

Western Blotting

Western blotting was used to quantify changes in LV tissue levels of proteins involved in specific MITO functions/dynamics and signaling. Equal amounts of LV protein lysate was loaded for each of the 3 study groups for every protein measured. After separating proteins on 4%-20% SDS-PAGE and transferring on PVDF membrane, blots were treated with specific primary antibodies followed by the corresponding secondary antibody coupled with horse raddish peroxidase. The bands on PVDF membrane were developed by Chemiluminescence and band intensity was quantified using a BioRad Model GS-670 imaging densitometer and expressed as densitometric units (du). The following protein groups were evaluated:

Regulators of Mitochondrial Biogenesis and Respiration: Antibodies for endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) were obtained from Life Span Biosciences, Seattle, WA. The antibody for MITO biogenesis regulating transcriptional factor peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) was purchased from Life Span Biosciences, Seattle, WA. Cardiolipin (CL) in LV myocardium was also quantified on a per milligram of mitochondrial protein. Briefly,

mitochondria protein was isolated from LV tissue and quantified as mitochondria protein/mg of noncollagen protein. Cardiolipin/mg of noncollagen protein in LV tissue was then normalized to mitochondria protein/mg noncollagen protein in LV tissue.

Regulators of Mitochondria-Based Programmed Cell Death or Apoptosis: Protein Levels of cytosolic cytochrome c was determined in an SDS extract of 10,000 g supernatant of LV extract. Specific antibody for cytochrome c was purchased from Santa Cruz, Dallas, TX.

Specific antibody for active caspase 3 was obtained from Sigma-Aldrich, St. Louis, MO.

Sarcoplasmic Reticulum Calcium ATPase: Protein levels of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA-2a) were measured in LV homogenate.

Results

None of the study dogs developed acute decompensation or died during the study and none developed ventricular or atrial arrhythmias. There were no significant differences in serum electrolytes (Na+, K+, Creatinine, Glucose, chloride, and blood urea nitrogen) in either study group between samples obtained at baseline, pre-treatment and post treatment time points. All electrolytes at all time points were within normal limits for all dogs.

References

1. Dodge HT, Sandler H, Baxley WA, Hawley RR. Usefulness and limitations of radiographic methods for determining left ventricular volume. Am J Cardiol. 1966;18:10-24.
2. Sabbah HN, Imai M, Cowart D, Amato A, Carminati P, Gheorghiade M. Hemodynamic properties of a new-generation positive luso-inotropic agent for the acute treatment of advanced heart failure. Am J Cardiol. 2007;99:41A-46A.
3. Rastogi S, Guerrero M, Wang M, Ilsar I, Sabbah MS, Gupta RC, Sabbah HN. Myocardial transfection with naked DNA plasmid encoding hepatocyte growth factor prevents the progression of heart failure in dogs. Am J Physiol. 2011;300:H1501-H1509.
4. Hiroshi Kobayashi, Enrique Gil-Guzman, Ayman M. Mahran, Rakesh K. Sharma, David R. Nelson, Anthony J. Thomas Jr., Askok Agarwal. Quality Control of Reactive Oxygen Species Measurement by Luminol-Dependent Chemiluminescence Assay. J Androl. 2001;22:568-574.
5. Liu YH, Yang XP, Sharov VG, Nass O, Sabbah HN, Peterson E, Carretero OA. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. J Clin Invest. 1997; 99:1926-1935.
6. Sabbah HN, Stanley WC, Sharov VG, Mishima T, Tanimura M, Benedict CR, Hegde S, Goldstein S. Effects of dopamine beta-hydroxylase inhibition with nepicastat on the progression of left ventricular dysfunction and remodeling in dogs with chronic heart failure. Circulation. 2000; 102:1990-1995.
7. Sharov VG, Todor AV, Imai M, Sabbah HN: Inhibition of mitochondrial permeability transition pores by cyclosporine A improves cytochrome C oxidase function and increases rate of ATP synthesis in failing cardiomyocytes. Heart Fail Rev. 2005;10:305-310.
8. Sharov VG, Todor A, Khanal S, Imai M, Sabbah HN: Cyclosporine A attenuates mitochondrial permeability transition and improves mitochondrial respiratory function in cardiomyocytes isolated from dogs with heart failure. J Mol Cell Cardiol. 2007;42:150-158.
9. Sharov VG, Goussev A, Lesch M, Goldstein S, Sabbah HN. Abnormal mitochondrial function in myocardium of dogs with chronic heart failure. J Mol Cell Cardiol. 1998;30:1757-1762.
10. O'Toole JF, Patel HV, Naples CJ, Fujioka H, Hoppel CL. Decreased cytochrome c mediates an age-related decline of oxidative phosphorylation in rat kidney mitochondria. Biochem J. 2010;427:105-112.