SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Acute and chronic effects of alpha melanocyte stimulating hormone on heme-oxygenase signal transduction, in healthy and Zucker Diabetic Fatty (ZDF) rat models

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1. INTRODUCTION

Diabetes mellitus type 2 (T2DM) is a long term, life-threatening metabolic disorder characterized by high systemic glucose levels, insulin resistance and damage to many tissues due to dysregulated inflammation. Unfortunately for those afflicted, this disease persists for a lifetime and progressively causes a wide range of debilitating effects, prominently: retinopathy, nephropathy. myocardial infarction, stroke, and damage to extremities severe enough to require amputation. Pharmacotherapy for T2DM includes insulin analogues, biguanids, sulfonylureas, α-glucosidase inhibitors, thiazolidinediones, SGLT-2-inhibitors, GLP-1 analogues, DPP-4-inhibitors, amylin analogues. These agents may partly normalize blood glucose levels and attenuate the severe downstream consequences, allowing management of the disease to some degree in many patients. These therapeutic strategies are nevertheless palliative and generally fail to restore type 2 diabetics to full health. Moreover, epidemiological studies show that incidence of this disorder has dramatically increased to epidemic proportions. Numerous factors have contributed to this phenomenon, principally caused by sedentary lifestyle-related factors such as over-nutrition, lack of exercise and particularly obesity. Efforts to develop countermeasures to onset and pathogenesis of T2DM, have focused particularly on physiological energy utilization mechanisms. Within this domain, the melanocortin system plays a crucial role regulating whole body energy homeostasis since it functions as a potent anorexigenic influence by controlling appetite resulting in lower food intake.

Melanocortins are one of the most important regulators of human food intake behaviour and thus strongly influence development of obesity, with resulting increased risk of T2DM onset or exacerbation. It has also been shown that both peripheral and central injection of melanocortin analogue caused a dramatic alteration in food intake and body weight. Furthermore, genetic studies have revealed that mutations in genes coding for components of the melanocortin system correlate with obesity and occurrence of T2DM in both animals and humans. Mechanistic studies reveal that alpha-MSH activates the MC5R signalling pathway via cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and mitogen-activated protein kinase/extracellular signal-regulated (MAPK/ERK1/2) and significantly decreases the fat content of adipocyte. In addition to these effects, the beneficial properties of α-MSH have been characterized in many other animal disease models, in which this system exhibits antiapoptotic, anti-inflammatory, antiischemic, antioxidant features. The compound exerts its effect by interacting nonselectively as a full agonist with melanocortin receptors. Its major effect in normal homeostasis includes hair and skin pigmentation (through MC1 receptor activation), reproductive function, food intake regulation, and regulation of energy metabolism. The most significant of its functions in the context of this investigation is its role as an endogenous countermeasure to ischemia–reperfusion (I/R) injury. For example, rat studies of cardiac and hepatic responses in melanocortin induced modulation of Janus kinase/ extracellular signal-regulated kinase/transcription signal transducer (JAK/ERK/STAT) signaling in animals subjected to extended myocardial ischemia/reperfusion revealed

left ventricular cardioprotective abatement of inflammatory tissue damage by tumor necrosis factor–a and apoptotic cell depletion. There are protective effects mediated substantially through a-MSH–induced HO-1 protein expression and enzyme activity. Related studies have underscored the role of HO-1 in particular as an essential component of protection against I/R injury to cardiovascular tissue. Nevertheless, greater insight into the fact how these molecules may affect cardiomyocyte force during long-term treatment regimens and the molecular changes induced by α-MSH/HO-1relationship would clearly be beneficial .

Based on these findings, and in the context of ongoing efforts by authors of this report to characterize HO-1–dependent processes with applications in human medicine, this study was undertaken to evaluate the role of HO-1 in α-MSH–mediated cardiovascular effects and to determine underlying cellular processes in diabetic circumstances.

2. AIMS

1) Examining the effects of I/R on physiologic cardiometabolic parameters in isolated working hearts of α-MSH-treated animals.
2) Determining the dependency of these changes on HO-1 activity with the help of selective HO-1 inhibitor, SnPP.
3) Demonstrating the effects of α-MSH stimulation sustained over a fairly long time period (6 weeks) on cardio metabolic parameters, diastolic cardiac function, and myofilament co-operation using an osmotic mini pump in the Zucker diabetic fatty rat model.

3. MATERIALS AND METHODS

3.1. Animal models

Male Sprague Dawley rats weighing 300–350 g were used in the first phase, while Male Zucker Diabetic Fatty (ZDF-Leprfa) rats weighing 300–350 g were used in the second phase of our study. All the animals were housed between 20-24°C, with lighting set to alternating dark and light periods of 12 hours. Sprague Dawley rats were maintained on normal rodent chow and tap water, while ZDF rats were kept on Purina 5008 special chow and tap water ad libitum.

3.2. Ethical guidelines

All animals received humane care, in compliance with the "Principles of Laboratory Animal Care" according to the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, formulated by the National Academy of Sciences, and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985). All protocols involving animal use were approved by the Committee of Animal Research, University of Debrecen, Hungary (DE MÁB 45/ 2001 and DE MÁB 35/2007). A 4-week adaptation period was provided to animals before the start point of the study.

3.3. Study design

3.3.1. First phase

For this work, 5 basic protocols were designed.

Protocol I: Investigation of Effect of a-MSH Pretreatment on Cardiac HO-1 Expression in Ischaemic–Reperfusion Injury

Sixteen healthy male Sprague Dawley rats weighing 300–330 g were divided into 2 groups (n = 8/group): I-a group received 0.5 mL saline subcutaneously 24 hours before the isolated working heart procedure, I-b group received 250 mg/body weight a-MSH solution (dissolved in saline) subcutaneously 24 hours before the isolated working heart operation. During isolated working heart method, hearts were exposed to ischemia/reperfusion (described in Induction of Global Ischemia and Reperfusion in Isolated Hearts). Samples from myocardial tissues were frozen on liquid nitrogen and stored at 2808C for further Western blot analysis.

Protocol II: Evaluation of Significance of the HO-1 Pathway in Cardioprotective Effects of α-MSH

In protocol II, another population of male Sprague Dawley rats (50 rats) was subdivided into 4 groups based on the pretreatments 24 hours before the isolated heart procedure (described in Isolated Working Heart Preparation): group II-a animals (n = 15) received subcutaneous saline injection of 0.5 mL; group II-b animals (n = 14) were injected with 250 mg/body weight a-MSH solution (diffused in saline) subcutaneously; group II-c animals (n = 11) received 50 mg/ body weight SnPP diffused in 0.5 mL

saline intraperitoneally together with 250 mg/body weight a-MSH solution subcutaneously; group II-d animals (n = 10) were injected only with 50 mg/body weight intraperitoneal SnPP solution. During isolated working heart method, hearts were exposed to ischemia/ reperfusion (described in Induction of Global Ischemia and Reperfusion in Isolated Hearts). At the endpoint of the experiments, samples from myocardial tissues were either frozen in liquid nitrogen and stored at 2808C for further analysis of HO-1 activity or stained for determination of infarcted areas (TTC staining) and then frozen in liquid nitrogen and stored at 2808C for further analysis.

Protocol III: Estimation of Direct Cardiac Effects of a-MSH on Isolated Hearts

Hearts harvested from Sprague Dawley rats without any pretreatment (n = 8) were subjected to isolated working heart procedure without I/R. After 10 minutes of retrograde perfusion using modified Krebs–Henseleit buffer (washout period), the set-up was switched to anterograde-mode (working heart), and baseline parameters (AF, CF, AoP (aortic pressure), and HR) were recorded. After measuring baseline cardiac function, 1 mL alpha-MSH solution (containing 1 mmol/L alpha-MSH) was injected directly through the side arm of the aortic cannula into the system. After 5 minutes of distribution, cardiac parameters were recorded again to estimate direct effects of a-MSH.

Protocol IV: Analysis of Direct Effects of a-MSH on Echocardiographic Heart Functions

Rats without any pretreatment (n = 8) were anesthetized using ketamine–xylazine combination, and the chest of each animal was shaved. The right jugular vein was exposed and cleaned from the adhering connective tissue, and a small incision was made on the wall of the vein, then a polyethylene catheter containing saline was introduced into it. The animal was placed into dorsal decubitus position and echocardiographic examination was performed. After recording baseline parameters, 3 different doses of a-MSH (10, 100, 250 mg/kg, respectively) were injected intravenously through the catheter, and cardiac parameters were estimated and recorded again under the influence of the different (and cumulative) doses of a-MSH.

Protocol V: Evaluation of Vascular Response of Isolated Aortic Rings to a-MSH Administration

For each isolated aortic ring experiment (n = 8), a rat without any pretreatment was guillotined and then the abdominal aorta rapidly excised. After a 90-minute incubation in Krebs solution, 1 mM phenylephrine was administered to 5 of the 6 aortic rings, including the intima-deprived [ET-(endothelium-deprived)] specimen, for 2 minutes, followed by a 45-minute washout period. Next, these 5 rings were subjected to one of the following treatments: (1) Krebs solution alone for one of the intact rings (naive) and (2) for the intima-deprived ring (naive ET-), (3) 200 mM NOARG, (4) 3 mM INDO, and (5) 200 mM NOARG together with 3 mM INDO at the same time. Immediately after these treatments, these 5 preparations received 1 mM phenylephrine. After 30 minutes, during which the contractile force reached a plateau, an a-MSH concentration–response (E/c) curve was constructed (from

1 nM to 1 mM) for these aortic rings. In the case of the sixth aortic ring, (6) after the 90-minute incubation in Krebs solution, an a-MSH E/c curve was generated (from 1 nM to 1 mM).

3.3.2. Second phase

After recording baseline data (n = 12), including weight, OGT test and echocardiographic recordings, rats were randomly divided into 2 subgroups as follows: untreated animals (Control; n = 6) receiving vehicle (saline solution), and animals (MSH; n = 6) receiving alpha-MSH solution, both subcutaneously administered by mini-osmotic pumps, for 6 weeks. A limitation of this current report is that we do not present data about lean control animals. Alpha-MSH concentration was adjusted to 4.8 mg/mL, and the pump delivered 0.15 μL/h (0.72 μg alpha-MSH in each hour). After six weeks, study was terminated, endpoint data (weight, OGTT, echocardiography, serum parameters and blood pressure) was recorded, and rats were sacrificed by thoracotomy under deep anaesthesia by ketamine/xylazine combination. Hearts and basilar arteries were excised. Isolated working heart method was carried out, and cardiac tissue samples were rapidly frozen for NADPH oxidase activity and for myocyte force measurements. Basilar arteries were subjected to contractile force studies.

3.4. Chemicals

α-MSH was obtained from Sigma-Aldrich Ltd (St. Louis, MO), and Sn(IV) protoporphyrin IX dichloride was provided by Frontier Scientific Inc (Logan, UT). All other chemicals and buffer solutions were obtained from Sigma Aldrich Ltd (Budapest, Hungary). Reagents used for isolated aortic ring experiments included the following: phenylephrine, a selective alpha adrenergic receptor agonist; a-MSH; Nvnitro-L-arginine (NOARG), a nitric oxide (NO) synthase inhibitor; indomethacin (INDO), a cyclooxygenase inhibitor; and salts for Krebs–Henseleit buffer (Krebs solution). The Krebs solution used in these experiments was composed of NaCl: 118 mM, KCl: 4.7 mM, CaCl2: 2.5 mM, NaH2PO4: 1 mM, MgCl2: 1.2 mM, NaHCO3: 24.9 mM, glucose: 11.5 mM, and ascorbic acid: 0.1 mM, dissolved in redistilled water. Phenylephrine was dissolved in Krebs solution, whereas a-MSH was dissolved in Krebs solution after dispersed in 20 mL acetic acid:water (1:9) solution (vol/vol). NOARG and INDO were dissolved in 96% ethanol, and then NOARG solution was further diluted in physiological salt solution.

3.5. Echocardiography

Echocardiographic measurementes were carried out in oth phases of our study on animals anesthetized with a mixture of ketamine and xylazine (50, 5 mg/kg), administered intramuscularly. Rats were positioned in a dorsal decubitus position, and the chest of each was shaved. Echocardiographic measurements were performed using Vivid E9 ultrasound system, with an i13L type probe designed for rodent models at a working frequency of 14 MHz. Each examination was completed during a 20-minute time interval. Complete 2-dimensional, M-mode (at papillary muscle levels), Doppler (PW), and tissue Doppler (TVI) echocardiograms were acquired and digitally stored for further analysis as recommended

by the American Society of Echocardiography. ECG was continously monitored during echocardiographic examinations in all cases. Obtained M-mode, Doppler and tissue Doppler parameters included the followings: aortic root diameter (Ao), left atrial diameter (LA), interventricular septum thickness in diastole and systole (IVSs, IVSd), left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs), left ventricular ejection fraction (EF), fractional shortening (FS), stroke volume (SV, calculated), cardiac output (CO, calculated), heart rate (HR), mitral annular peak systolic excursion (MAPSE), left ventricular peak E and peak A waves (mitral early and late filling velocities), the E to A ratio (E/A), ejection time (ET), isovolumic contraction- and relaxation time (IVCT and IVRT), myocardial performance index (MPI or Tei-index), lateral e' and a' wave velocities, lateral e'/a' ratio, E/e' ratio. Left ventricular outflow tract (LVOT) maximal and mean velocity and pressure parameters: LVOT Vmax, Vmean, LVOT maxPG and meanPG. LV mass was calculated by echocardiography, using the following formula: LV Mass (g) = 0.8{1.04[([LVIDd + IVSd + PWd]3 − LVIDd3)]} + 0.6; where LVIDd is the diameter of the left ventricle in end-diastole; IVSd is thickness of the interventricular septum in diastole; PWd is the thickness of the posterior wall in diastole. All measurements were averaged over 3–5 consecutive cardiac cycles. Analyses of data were conducted by a trained cardiologist, who was blinded to experimental conditions to ensure objective interpretations.

3.6. Isolated working heart method

We applied isolated working heart method in both phases of our study to determine different cardiometabolic parameters. Rats were anesthetized with a mixture of ketamine– xylazine (50/5 mg/kg) and administered heparin (1000 IU/kg) intravenously through the dorsal penile vein. After thoracotomy, hearts were excised, and placed into ice-cold perfusion buffer followed by aortic cannulation. Perfusion of hearts was conducted according to the Langendorff protocol for a 10- minute washout period at a constant perfusion pressure equivalent to 100 kPa. The perfusion medium was a modified Krebs–Henseleit bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl2, 25 mM NaHCO3, 0.36 mM KH2PO4, 1.2 mM MgSO4, and 10 mM glucose). The Langendorff apparatus was switched to "working" mode after the washout period as previously described. Baseline cardiac functions (as described below) were then assessed. In protocols I and II, these procedures were followed by 30 minutes of global ischemia and 120 minutes of reperfusion, during which cardiac parameters were measured at the 30-, 60-, 90-, and 120-minute timepoints. In protocol III, after recording baseline heart functions, instead of ischemia/reperfusion, 1 mL a-MSH solution (in concentration of 1 mmol/L) was injected directly into the system, through the aortic cannula of the apparatus, to monitor direct effect of a-MSH administration. In the second phase of our study isolated working heart method was carried out at the end of the 6 week treatment period following extermination. Immediately after 120 min reperfusion small myocardial biopsies from LV heart tissue were removed and frozen for further analysis.

3.7. Induction of Global Ischemia and Reperfusion in Isolated Hearts

Induction of global ischemia was performed during isolated working heart procedures of protocols I and II. After measurement of baseline cardiac functions, the atrial inflow and aortic outflow lines were clamped at a point close to the origin of the atrial and aortic cannulas, respectively, and the peristaltic pump was halted. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines, and starting the peristaltic pump. To prevent the myocardium from drying out during normothermic global ischemia, the thermostated glassware (in which hearts were suspended) was covered and the vapor content was kept at a constant level. Cardiac function was recorded and monitored throughout the experimental period using a computerized array consisting of silver electrodes and pressure transducers connected directly to isolated hearts. Before ischemia and during reperfusion, the values of heart rate (HR), coronary flow (CF), and aortic flow (AF) were registered. AF was measured by a timed collection of the buffer pumped out from the heart, and CF rate was measured by a timed collection of the coronary perfusate, dripped from the heart. Preselected exclusion criteria for these experiments were conducted to exclude isolated working hearts if ventricular arrhythmias occurred before the induction of ischemia.

3.8. Determination of Cardiac Arrhythmia Incidence

During evaluation of isolated working hearts, epicardial electrocardiogram (ECG) measurements were monitored using 2 silver electrodes attached directly to the heart. ECG readings were analyzed to determine the incidence of cardiac arrhythmias. Arrhythmias were analyzed based on their occurrence using a quantal approach: whether there was at least 1 arrhythmic event or not. Arrhythmias (ie, at least 1 arrhythmic event) were further classified into 2 groups defined as follows: (1) if the pump function of the heart recovered spontaneously from the arrhythmic event(s), it was considered to have “nonsevere” arrhythmia and (2) if the heart failed to recover its pump function, it was considered to have “severe” arrhythmia, regardless of the type or mechanism of the arrhythmia or the number of arrhythmic events.

3.9. Isolated Aortic Ring Experiments

For each experiment, a rat was guillotined and then the abdominal aorta rapidly excised. Aortas from each animal were processed by sectioning 6, approximately 2-mm-wide ring segments from the vessels. One of the rings was deprived from its intimal layer using a small cotton swab. All rings were mounted horizontally at a resting tension of 10 mN, using a wire instrument, in a 10 mL vertical organ chamber (Experimetria TSZ-04) containing Krebs solution, oxygenated with 95% O2 and 5% CO2 (368C; pH 7.4). The isometric contractile force of the circulatory muscle layers was measured by a transducer (Experimetria SD-01) and strain gauge (Experimetria SG-01D), and recorded by a polygraph (Medicor R-61 6CH Recorder).

3.10. Determination of Infarcted Areas of Isolated Hearts

Triphenyl-tetrazolium chloride (TTC) staining was used to determine infarcted areas of each heart, as a direct, postsacrifice approach. One hundred milliliters of 1% TTC solution in phosphate buffer (Na2HPO4 88 mM, NaH2PO4 1.8 mM) was administered directly through the side arm of the aortic

cannula at the endpoint of isolated working heart procedure. TTC stained viable myocardium deep red, whereas potentially infarcted areas stayed pale as it was seen during the analysis. Hearts were stored at 2808C for a week and then sliced transversely to the apico-basal axis into 2–3-mm thick sections, weighed, blotted dry, and placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet single pass flatbed scanner (Hewlett-Packard, Palo Alto, CA). Using NIH 1.61 image processing software, each image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Infarcted areas (pale areas, white pixels) of each slice were traced, and the respective areas were calculated by pixel density analysis. Infarcted areas and total area were measured by computerized planometry software: Scion for Windows Densitometry Image program (version Alpha 4.0.3.2, Scion Corporation, Meyer Instruments, Inc, Houston, TX). Infarct size was expressed as a percentage ratio of the infarcted zone to the total area in each heart (percentage of pixels).

3.11. Measurement of HO-1 Enzyme Activity in Heart Tissue

Activity of heme oxygenase-1 enzyme in myocardial tissue was measured according to the following general protocol: Tissue samples were homogenized in a solution containing HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10 mM, sucrose 32 mM, DTT (Dithiothreitol) 1 mM, EDTA 0.1 mM, soybean trypsin inhibitor 10 mg/mL, leupeptin 10 mg/mL, aprotinin 2 mg/mL, and pH 7.4 (homogentisate buffer). All reagents were purchased from SigmaAldrich, St. Louis, MO. The supernatant was collected by centrifugation of the homogenate for 30 minutes at 20,000g at 48C. Assessment of heme oxygenase activity was done on each sample of supernatant, according to the procedures used by Tenhunen et al. Briefly, HO-1 enzyme activity was measured using a computer-based, spectrophotometric analysis of bilirubin formation from heme. This HO-1 enzymatic measurement protocol used a reaction mixture containing an aliquot of the supernatant, plus glucose-6-phosphate 2 mM, glucose-6-phosphate dehydrogenase 0.14 U/mL, heme 15 mM, NADPH 150 mM, rat liver cytosol as a source of biliverdin reductase 120 mg/mL, MgCl2 2 mM, and KH2PO4 100 mM. Each reaction mixture was incubated for 1 hour in the dark. The reactions were arrested by placing the samples on ice. Bilirubin formation was calculated on the basis of difference between optical densities obtained at 460 and 530 nm. The HO-1 activities were expressed in nanomols (nmol) of bilirubin formed per milligram protein per hour.

3.12. Western Blot Analysis for Expression of HO-1 Protein in Heart Tissue

Three hundred milligrams of frozen tissue from the left ventricular myocardium of each rat was homogenized in 800 mL homogenization buffer (25 mM Tris-HCl, pH 8, 25 mM sodium chloride, 4 mM sodium orthovanadate, 10 mM NaF (sodium fluoride), 10 mM sodium pyrophosphate, 10 nM Okadaic acid, 0.5 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 1x protease inhibitor cocktail), using a Polytron homogenizer. Homogenates were centrifuged at 2000 rpm at 48C for 10

minutes. Supernatants were further centrifuged at 10,000 rpm at 48C for 20 minutes. The resultant supernatants are cytosolic extracts that were used experimentally to evaluate this cell fraction. Samples (50 mg each) were next separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% tris-glycine gel at 120 V for 90 minutes. Then, proteins were transferred onto nitrocellulose membranes at 100 V for 1 hour. Membranes were blocked as a countermeasure to nonspecific binding of probing antibody, using 5% dry milk powder, and then incubated overnight at 48C with primary antibodies (1:1000 dilution; anti-GAPDH; anti–HO-1 rabbit monoclonal antibodies, Sigma-Aldrich). After overnight incubation, membranes were treated with horseradish peroxidase–conjugated goat anti–rabbit IgG (1:3000 dilution) secondary antibody. Chemiluminescent detection was used to identify bands. Detection was made using autoradiography for variable lengths of time with medical xray film. Quantitative analysis of scanned blots was performed using the Scion for Windows Densitometry Image program.

3.13. Osmotic Pump Implant Surgery

Alzet® osmotic mini-pumps were surgically implanted into a 1 cm opening in the nape skin of ZDF rats under ketamine/xylazine (100/5 mg/kg) deep anaesthesia. Before implantation, the pumps were primed by injecting them with alpha-MSH solution (200 μL solution at the concentration of 4.8 mg/mL) and placing them into 37 ◦C physiological SAL for a minimum of 4 h. Following insertion of the mini-pumps, the skin was closed with surgical sutures, and the wound was disinfected with Betadine® solution. Surgery time was 10–15 min, and the rats received postoperative care. The pump provided continuous administration of 0.15 μL content per hour. The mini-pumps of the control group were filled with physiological SAL and were implanted with the same methods.

3.14. Oral Glucose Tolerance Test (OGTT)

OGTT for ZDF rats was performed according to the standard method. Briefly, all animals were selected for OGT test at baseline and at the endpoint, after starving at water for 12 h. 3 g/kg glucose was administered in 1 g/mL solution for each animal via gavage technique. The serum blood glucose levels were obtained by pricking the tail vein and using glucometer at time 0 (baseline), 30, 60, 90, 120 and 180 min.

3.15. Blood Pressure Measurement of Conscious Rats

At the endpoint, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by a non-invasive tail-cuff blood pressure system utilizes Volume Pressure Recording (VPR) sensor technology. Measurements were obtained in conscious rats restrained in a thermal plastic chamber as described elsewhere . Five consecutive recordings were evaluated in each animal to ensure results.

3.16. Analysis of Serum Parameters

At the endpoint of the treatment, after 14-h fasting, rat blood samples were collected from the tail vein in EDTA-K2 evacuated tubes. The samples were collected and processed aseptically to minimize haemolytic activity. Serum glucose, cholesterol and triglyceride were detected by Department of Laboratory Medicine at the University of Debrecen.

3.17. Force Measurements of Isolated Cardiomyocytes

Contractile function of skinned left ventricular (LV) cardiomyocytes (n = 12 per group) from isolated working hearts (n = 3–4) after the protocol of ischemia/reperfusion (I/R) was measured as it described previously. Briefly, deep-frozen (−80 ◦C) tissue samples were mechanically disrupted and demembranated by 0.5% Triton X-100 detergent for 5 min in isolating solution (MgCl2: 1.0 mM; KCl: 100.0 mM; EGTA: 2.0 mM; ATP: 4.0 mM; imidazole: 10.0 mM; pH 7.0) at 4 ◦C. Each subjected cell was attached with silicone adhesive at one end to a stainless steel insect needle connecting to a high-speed length controller, while at the other end to a stainless steel insect needle connecting to a sensitive force transducer at 15 ◦C. Subsequent cardiomyocyte isometric force generation was recorded at sarcomere length of 2.3 µm and analysed by LabVIEW software. Ca2+-dependent force production of a single cardiac cell was induced by transferring the preparation from relaxing (BES: 10.0 mM; KCl: 37.11 mM; MgCl2: 6.41 mM; EGTA: 7.0 mM; ATP: 6.94 mM; creatine phosphate: 15.0 mM; pH7.2) to activating solutions (same composition as relaxing solution aside from containing Ca2+-EGTA instead of EGTA). Ca2+ concentrations were indicated as −log10[Ca2+] units (pCa). Protease inhibitors were added to all solutions freshly: phenylmethylsulfonyl fluoride: 0.5 mM; leupeptin: 40 µM and E-64: 10 µM. All chemicals were purchased from Sigma-Aldrich Corp. Cardiomyocyte Ca2+-activated force generation was registered by using maximal activating solution (pCa 4.75) and activating solutions with different pCa (5.4–7.0). Submaximal forces were normalized to maximal force and fitted to a modified Hill equation in Origin 6.0 analysis program. The pCa value for the half-maximal contraction indicated by pCa50 defines per se the Ca2+ sensitivity of force generation of the contractile machinery, while the steepness of the Ca2+ sensitivity curve expressed as a coefficient (nHill) reflects the co-operation between the myofilaments. Original active forces were normalized to myocyte cross-sectional area indicating active tension (expressed in kN/mCa2). Statistical analysis was performed by GraphPad Prism 5.02 software. Data were compared with unpaired Student's t-test.

3.18. Arterial Contractile Force Measurement

During the isolated heart experiments, after thoracotomies performed under ketamine/xylazine anaesthesia, the brain was removed and placed into a silicone containing petri dish, filled with 0–4 ◦C Krebs solution (composition in mmol: 110 NaCl, 5.0 KCl, 1.0 MgSO4, 1.0 KH2PO4, 5.0 glucose and 24.0 NaHCO3, obtained from Sigma-Aldrich, St. Louis, MO, USA). The solution was equilibrated previously with a gaseous mixture of 5% CO2, 10% O2 and 85% N2 at pH 7.4. Basilar arteries were isolated with microsurgical tools. The arteries were equally cut into 4 mm long rings, which were then

mounted in an isometric contraction measurement system. The Ca2+-free Krebs solution was changed to Ca2+-containing one (composition in mM: 110 NaCl, 2.5 CaCl2, 5.0 KCl, 1.0 MgSO4, 1.0 KH2PO4, 5.0 glucose and 24.0 NaHCO3, obtained from Sigma-Aldrich, St. Louis, MO, USA). Before every experiment, a normalization protocol was performed, by stretching the preparations with 1.5 mN force, which was increasing evenly every 15 s until the calculated intraluminar pressure reached 13.4 kPa. The experiments were then performed at this stretch level. Contractile responses for KCl (6–66 mM), serotonine (1 nM–10 μM) and angiotensin II (1 nM–100 μM) have been recorded.

3.19. NADPH Oxidase Activity Measurement

NADPH oxidase-derived superoxide production was measured using lucigenin-enhanced chemiluminescence, as described previously. Left ventricular (LV) samples were snap frozen immediately after termination of ex vivo perfusion experiments and were stored on −70 ◦C until subsequent analyses. Approximately 100 mg (wet tissue) of LV heart samples (n = 4 from both α-MSH treated and untreated) were homogenized in 10 volumes of ice cold 1× Ca2+-free Dulbecco's Phosphate Bufferred Saline containing 40 μM leupeptin, and 5 μM E64 protease inhibitors. After centrifuging, the pellet was discarded and Bicinchoninic Acid Assay was used to determine protein concentration in the supernatant using bovine serum albumin (BSA) as a standard. Protein concentrations were ~20 mg/mL. The reaction mixture for the measurement of NADPH oxidase enzyme activity contained 50 μL heart homogenate in a buffer of Krebs- (4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES) (110 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM KH2PO4, 5 mM glucose, 24 mM NaHCO3, 20 mM HEPES, 50 μM lucigenin). The scintillation tubes went through dark adaptation, and basal luminescence of samples were recorded via PerkinElmer Tricarb 2800 tr liquid scintillation counter. Enzymatic reaction was stimulated by adding 100 μM NADPH to the reaction mixture. Luminescence was recorded directly after for 2 min. Differences between basal and stimulated luminescence were calculated and values were normalized to protein concentration.

3.20. Statistical Analyses

All data are presented as the average magnitudes of each outcome in a group ± standard error of the mean (SEM). The D'Agostino & Pearson omnibus normality test was used to estimate Gaussian distribution. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni post-testing (when normality test was passed) or by Kruskal-Wallis test with Dunn's post-testing (if the normality test was not passed). Student's t-test was used to determine of significance in the case of comparing significance of two groups. Statistical analyses were conducted using GraphPad Prism software for Windows, version 6.07. Probability values (p) less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Results of the first phase

4.1.1. Echocardiography: Fractional shortening and ejection fraction (Protocol IV)

HRs and respiratory frequencies of each animal remained stable throughout the experiments. Moreover, no significant differences were noted between the independent measurements obtained by 2 blinded readers. FS and EF data correlated strongly with measurements of both the parasternal long and short axis views. FS after administration of 100 and 250 mg/kg α-MSH was significantly increased in comparison with this outcome evaluated before the injection (Control) of the material (FS100mg/kg: 53.79 ± 2.497 and FS250mg/kg: 55.00 ± 2.688 vs. FSControl: 45.69 ± 2.241). The same pattern was observed in EF results (EF100mg/kg: 88.31 ± 1.745, and EF250mg/kg: 89.21 ± 1.527 vs. EFControl: 81.52 ± 2.296). α-MSH at the dose of 10 mg/kg was observed to result in trends for increased EF and FS, but these increases did not reach statistical significance (FS10mg/kg: 52.50 ± 2.773 vs. FSControl: 45.69 ± 2.241; and EF10mg/kg: 87.04 ± 1.963 vs. EFControl: 81.52 ± 2.296). There were no significant changes observed in interventricular and left ventricular free-wall thicknesses and LV mass after the administration of a single dose of a-MSH.

4.1.2. Isolated working heart method (Protocol II)

4.1.2.1. *α-MSH and SnPP Effects on HR*

Here, it was observed that a-MSH treatment increased HR, especially at the first part of reperfusion period ($p < 0.05$), compared with the control group. SnPP treatment counteracted the HR-elevating effect of a-MSH ($p < 0.05$) throughout the whole reperfusion period (eg, at a 30-minute timepoint: 172.0 ± 21.22 bpm SnPP + MSH vs. 271.4 ± 7.117 bpm MSH; and at 60 minutes: SnPP + MSH 180.5 ± 22.28 bpm vs. 275.1 ± 6.698 bpm MSH).

4.1.2.2. *α-MSH and SnPP Effects on CF*

α-MSH treatment improved preischemic CF values compared with controls (28.14 ± 0.99 vs. 21.93 ± 1.39 mL/min). CF values of a-MSH–treated animals, measured during reperfusion, were elevated at every timepoint, and differences were significant compared with any other groups ($p < 0.05$). CF values measured in MSH-treated animals after 120 minutes of reperfusion (25.45 ± 1.60 mL/min) were 16% higher than preischemic values of the control group (21.93 ± 1.39 mL/min), and decreased only by 10% versus preischemic levels observed in the alpha-MSH–treated group (28.14 ± 0.99 mL/min). The HO-1 inhibitor, SnPP, significantly counteracted the CF-elevating effect of MSH, as seen from the results of SnPP + MSH-treated group at every timepoint of the reperfusion period ($p<0.05$).

4.1.2.3. *a-MSH and SnPP Effects on AF*

Using protocol II for these experiments, AF was significantly increased in the α-MSH–treated group (52.07 ± 1.93 mL/min) compared with controls (39.57 ± 2.44 mL/min, $p < 0.001$) before the ischemic

insult. Preischemic AF values in SnPP-treated and SnPP + MSH-treated groups were decreased compared with the MSH-group, but increased compared with the control group values (48.00 ± 2.16 and 43.36 ± 3.42 mL/min, respectively). AF values in the α-MSH–treated group were significantly elevated at any timepoints of reperfusion compared with the paired control values ($p < 0.01$). Even compared with preischemic controls, AF values in the MSH-treated group were elevated in all timepoints, except at 120 minutes of reperfusion. In addition, significant differences in AF between the a-MSH–treated group versus all other groups were measured during the reperfusion period ($p < 0.01$). Although baseline AF values of SnPP-treated animals were increased compared with control, they declined by the end of the reperfusion period (8.00 ± 2.94 mL/min) to 20% of control baseline values. Similar trends were observed in the SnPP + MSH-treated group, although these values remained higher at any timepoints than values of SnPP-only treated group, and over the whole reperfusion period.

4.1.2.4. *a-MSH and SnPP Effects on CO and SV*

CO and SV values in α-MSH– pretreated animals were significantly elevated at all timepoints compared with their matched controls. However, SnPP failed to significantly counteract this effect.

4.1.3. Effect of a-MSH Administration Directly Onto Isolated Working Hearts (Protocol III)

a-MSH treatment resulted in significantly elevated CF and CF/CO% in comparison with baseline values ($p < 0.05$). No significant effects of a-MSH treatment were observed in AF, CO, HR, or SV in comparison with baseline values.

4.1.4. Determination of Incidence and Severity of Ventricular Arrhythmias (Protocol II)

No arrhythmic events were detected in 80% of the control rats. However in 6.7% and in 13.3%, nonsevere and severe arrhythmias were found in this group, respectively. No arrhythmic events were determined in the MSH-treated group. In the SnPP + MSH-treated animals, 36.4% of isolated hearts showed no signs of arrhythmia, whereas in 27.2% of cases nonsevere and in further 36.4% of cases severe arrhythmic events were observed. In the SnPP-only treated group, 30.0% of isolated hearts showed no arrhythmia, whereas in 60.0% nonsevere and in 10.0% severe arrhythmic cases were detected.

4.1.5. NOARG and INDO Effect on a-MSH–Mediated Vascular Tone Response (Protocol V)

4.1.5.1. *Vascular Response to Phenylephrine (Precontraction)*

In the naive, naive ET-, 200 mM NOARG, 3 mM INDO, and 3 mM INDO + 200 mM NOARG animals, the contractile force of aortic rings before dosing with a-MSH E/c curve (in mN, mean ± SEM) were 3.47 6 0.37, 4.21 6 0.41, 4.46 6 0.39, 1.98 6 0.26, and 3.68 6 0.4, respectively, with a significant difference between the naive and 3 mM INDO groups ($p < 0.01$).

4.1.5.2. *Vascular Response to a-MSH*

Treatment with a-MSH did not directly result in significant alteration of the resting vascular tone. Conversely, the precontracted aortic rings exhibited significant relaxation in response to a-MSH. The maximal relaxing effect induced by 1 mM α-MSH concentration (in percentage of the initial contractile force, mean ± SEM) did not differ significantly among the naive, naive ET-, 200 mM NOARG, 3 mM INDO, and 3 mM INDO + 200 mM NOARG groups (20.01 ± 4.64, 21.65 ± 4.53, 16.75 ± 5.41, 21.24 ± 6.73, and 23.71 ± 5.7, respectively). Thus, the effect of α-MSH on the increased vascular tone (due to α1 adrenoceptor stimulation) proved to be independent from the presence or absence of endothelium as well as from the ability of endothelium to produce prostacyclin (PGI2) or NO.

4.1.6. *Effects of a-MSH and HO-1 Inhibition on Ischemia-Reperfusion–Induced Cardiac Infarct Zone Extent (Protocol II)*

Significant changes were observed in the extent of infarcted areas (expressed in % of total area) of hearts from protocol II-treated animals, after ischemia/reperfusion injury. TTC staining of hearts harvested from a-MSH–pretreated groups revealed significantly lower infarct zone extent, in comparison with tissue sections from hearts from vehicle-treated control animals (19.74 ± 1.044% vs. 41.91 ± 3.922%) ($p < 0.05$). This cardioprotective effect was completely counteracted by SnPP pretreatment of α-MSH–treated animals (42.15 ± 3.235% vs. 19.74 ± 1.044%) ($p < 0.05$). Moreover, SnPP treatment alone was found to increase infarcted areas (53.17 ± 2.347%).

4.1.7. Effects of a-MSH and SnPP on Tissue HO-1 Activity (Protocol II)

No significant differences in activity of the enzyme were noted (data not shown). Nevertheless, HO-1 activity of MSH-treated animals was slightly increased compared with controls; SnPP pretreatment decreased the activity of the enzyme, whereas this reduction seemed to be less intense in the SnPP + MSH-treated group compared with SnPP treatment. Although measurement of the HO-activity corresponds to the results of the formerly mentioned methods, differences between the groups did not reach the level of statistical significance.

4.1.8. Western Blot Analysis (Protocol I)

Western blot method was performed by analyzation of frozen-stored myocardial tissue samples gained at the end of isolated working heart procedure of protocol I. Results of Western blot analyses demonstrate that the expression of HO-1 protein in myocardial tissue harvested from postischemic/reperfused, isolated working hearts excised from rats treated with 250 mg/kg a-MSH was significantly elevated relative to content of HO-1 protein in heart tissue from control rats treated with physiologic saline ($p < 0.05$).

4.2. Results of the second phase

4.2.1. Weight Gain, Serum Parameters, Blood Pressure and LV Mass/Whole Body Mass Ratio

No significant differences were found in the abovementioned parameters. On the contrary, significant change was observable between the control and alpha-MSH treated groups in the ratio of left ventricle (LV) mass to whole body mass at the endpoint.

4.2.2. Results of OGT Tests

Values of ZDF control and alpha-MSH-treated animals were elevated compared to the baseline (BASE), but no significant changes were observed in MSH group compared to Control at any time points.

4.2.3. Echocardiography

parameters (EF, FS, MAPSE) and diastolic values (E wave velocities, E/e' ratio, and IVRT) were found to be deteriorated in ZDF Control group compared to baseline (BASE) data. Mild but significant increase in Tei-index (0.491 ± 0.014 vs. 0.305 ± 0.012) shows worsened global heart function. Systolic function of MSH group animals showed a mild improvement in comparison to Control group, demonstrated by fractional shortening (FS), ejection fraction (EF) and mitral annular plane systolic excursion (MAPSE) parameters. FS and EF of alpha-MSH-treated animals were significantly increased in comparison with values of ZDF control animals (FS: 32.33 ± 0.421% vs. 36.83 ± 0.703%; and EF: 66.50 ± 0.067% vs. 72.00 ± 0.774%, respectively). MAPSE values of MSH rats were maintained at the normal range, however, MAPSE was significantly deteriorated in ZDF control rats (2.268 ± 0.010 mm vs. 1.602 ± 0.045 mm). Diastolic function of the left ventricle was slightly improved in alpha-MSH-treated animals compared to ZDF Controls, demonstrated by a decrease in isovolumic relaxation time (58.00 ± 1.826 ms vs. 43.00 ± 1.125 ms). Diameter of the left atrium was increased in ZDF controls compared to MSH animals showed by left atrium to aortic (LA/Ao) ratios (1.104 ± 0.043 vs. 0.945 ± 0.029). E/A and E/e' ratios, as well as lateral e' parameters were found to be unaffected by the treatment. Tei index (Myocardial Performance Index, MPI) was elevated in Control animals when compared to MSH group, showing deteriorated global heart function in Control rats (0.491 ± 0.014 vs. 0.392 ± 0.013). Left ventricle outflow tract (LVOT) parameters were also found to be significantly increased in MSH group compared to ZDF Controls. Alpha-MSH treatment slightly elevates blood flow velocities (V) and pressure gradient (PG) (LVOTV mean: 0.441 ± 0.024 m/s vs. 0.553 ± 0.019 m/s; and LVOT mean PG: 1.095 ± 0.088 mmHg vs. 1.592 ± 0.106 mmHg). Consequently, stroke volume (SV) and cardiac output (CO) were found to be elevated in treated animals (SV: 0.406 ± 0.046 mL vs. 0.581 ± 0.030 mL; and CO: 77.55 ± 7.763 mL/min vs. 112.30 ± 6.110 mL/min, respectively). Heart rate values did not show any difference among groups when measured on anaesthetized animals by echocardiography.

4.2.4. Isolated Working Heart Results

Six weeks of treatment had no effect on pre-ischemic parameters of contractile function including aortic flow, coronary flow, heart rate, cardiac output, stroke volume and aortic pressure in hearts isolated from either Control or MSH-treated rats. However, although nearly all parameters showed no differences in pre-ischemic state, in time derivative of developed pressure MSH group featured significantly lower

compared to Control group. Interestingly, in 60 and 120 min of recovery the dp/dt was significantly higher in MSH group than Control parameters in the same time points. Furthermore, 30 min after global ischemia AF and SV were increased in MSH group compared to Control. In addition, at the end of post-ischemic recovery, AF (1.667 ± 0.711), CO (11.500 ± 3.708), SV (0.072 ± 0.024), AoP (40.500 ± 12.230) and dp/dt (341.700 ± 71.830) were significantly suppressed in hearts isolated from Control rats compared to their pre-ischemic state (AFpre: 23.170 ± 4.554; COpre: 43.500 ± 5.054; dp/dtpre : 1650 ± 96.120; AoPpre: 93.330 ± 9.482 and SVpre: 0.268 ± 0.021). In contrast, only the post-ischemic AF (2.400 ± 0.778), AoP (68.800 ± 3.952) and dp/dt (558.300 ± 55.630) showed significant reduction in MSH treated group compared to pre-ischemic values (AFpre: 22.800 ± 2.480; dp/dtpre: 1133 ± 127.200; AoPpre: 98.200 ± 2.760). In other words, despite the fact that there were few remarkable differences between the two groups at parallel time points of measurement, in MSH group post-ischemic CO (19.400 ± 2.849) and SV (0.128 ± 0.025) values did not reach statistical significance in reduction compared to their pre-ischemic state (COpre: 42.000 ± 4.195; SVpre: 0.249 ± 0.021). No significance was found in coronary flow and heart rate values, neither between groups, nor between different time points of the same group.

4.2.5. Enhancement of Cardiomyocyte Contractile Performance after Alpha-MSH Treatment

Active tension-pCa relationships of LV cardiomyocytes apparently showed alpha-MSH-induced changes in cellular mechanical performance of ZDF rats. LV cardiomyocytes from ZDF animals after vs. without alpha-MSH treatment had a trend towards higher active tension (at pCa 5.6: 31.04 ± 3.44 kN/m2 vs. 23.38 ± 2.46 kN/m2 , $p = 0.08$; at pCa 5.8: 25.50 ± 3.43 kN/m2 vs. 18.13 ± 2.15 kN/m2 , $p = 0.08$). Normalized force-pCa relationships of LV cardiomyocytes from treated vs. Control ZDF rats showed similar Ca2+ sensitivity (pCa50: 5.87 ± 0.03 vs. 5.82 ± 0.02), but significantly higher Hill coefficient (nHill: 2.87 ± 0.19 vs. 2.17 ± 0.08) as indicative for better myofilament co-operation in the alpha-MSH-treated group.

4.2.6. Vascular Status Brain Arteries

Significantly higher hyperpolarization induced relaxation in the ZDF Control group compared to alpha-MSH treated group (5.52 ± 0.56 mN in ZDF vs. 2.73 ± 1.05 mN in alpha-MSH treated ZDF $p < 0.05$ at 16 mM KCl) although no difference in the maximal contractile force evoked by 66 mM KCl (1.06 ± 0.466 mN in the Control group vs. 2.49 ± 0.77 mN in the alpha-MSH treated group) was seen during contractile force measurement experiments. No difference was found between the 5HT responses of the two groups (7.31 ± 0.85 mN in Control group vs. 7.95 ± 1.51 mN in the alpha-MSH treated group for the maximal 10 μM dose of 5HT). There was also no difference in the ATII evoked contractions between the two groups (1.99 ± 0.55 mN in the Control group vs. 1.53 ± 0.188 mN in the alpha-MSH treated group for the maximal 100 μM dose of ATII).

4.2.7. NADPH Oxidase Activity

NADPH oxidase activities of left ventricle samples did not differ significantly in the α-MSH treated and untreated Control groups.

5. DISCUSSION

Alpha-MSH (α-MSH), a neuropeptide derivative of proopiomelanocortin, is a melanotropin (melanocyte-stimulating hormone) secreted to bind with melanocortin receptors, with resulting downstream physiologic effects, principally food-intake regulation, with resulting weight loss and prevention of obesity—and corollary reduction of diabetes risk. Previous studies have demonstrated that the hormone also has a positive effect on the cardiovascular system, because it plays a role in decreasing or even in the prevention of ischaemia/reperfusion induced damages. In our study we aimed to examine the effects of I/R on physiologic cardiometabolic parameters in isolated working hearts of α-MSH-treated animals and also to determine the dependency of these changes on HO-1 activity with the help of selective HO-1 inhibitor, SnPP. Moreover, we wanted to demonstrate the effects of chronic α-MSH stimulation on cardiometabolic parameters in diabetic conditions.

Echocardiographic assessment of a-MSH effects on EF and FS reveals that administration of the hormone correlated with significant increases in both parameters. These effects may reflect increased activation of sympathetic tone because it is well-known that α-MSH and its analogs are associated with mild sympathetic activation in the cardiovascular system, including increased HR, and thus may influence systolic activity. This increase of ejection fraction on a-MSH administration has also been shown by other authors in systemic inflammatory response syndrome in pigs. Effects on cardiac function in isolated hearts demonstrate that α-MSH pretreatment according to protocol II increased the beat rate of isolated working hearts during the initial reperfusion period. The contribution of HO-1 activity to this effect was demonstrated by the observation that increased HR stimulated by the hormone was counteracted by SnPP, an inhibitor of HO-1. Nevertheless, it should be noted that the conspicuous low HRs in the SnPP + MSH group are partly due to the observed many severe arrhythmic events, as described in this report. Likewise, a-MSH pretreatment significantly increased CF and AF, both in the preischemic periods and during reperfusion. This effect was also demonstrated to be significantly HO-1 dependent because CF and AF increases induced by the hormone were counteracted by SnPP. Increases in both of these outcome measures have been observed to correlate with improved cardiac function in isolated working heart studies and improved clinical prognoses for patients who have experienced ischemic events. These and related observations suggest possible use of a-MSH and agents that enhance expression of HO-1 in prevention of and therapy for ischemia/reperfusion-associated pathologies. Conversely, although a-MSH treatment significantly increased magnitude of CO and SV, this effect was not or only partially HO-1 dependent, as evidenced by inability of SnPP to significantly counteract these increases.

Administration of the hormone induced significant increases in CF by treated hearts versus controls. These outcomes reveal that a-MSH mediates an intensive dilatative effect on coronary vasculature, pointing to an additional physiologic effect that may be used by clinicians to design treatment strategies

targeted specifically to pathological conditions that might be ameliorated by dilation of selected blood vessels.

This investigation further revealed potential uses of agents such as α-MSH that modulate HO-1, in reducing susceptibility to ventricular arrhythmias. Here, ECG analysis was conducted to evaluate the involvement of α-MSH–induced HO-1 activity in antiarrhythmic cardioprotection. These results demonstrate that MSH treatment— possibly through HO-1 induction, inhibited the occurrence of arrhythmic events in comparison with a vehicle-treated control group. Treatment with SnPP in addition to the hormone resulted in a possible increase in susceptibility to ventricular arrhythmias, suggesting that HO-1 is cardioprotective in this respect. A possible major contributor to the biological basis for the higher occurrence of severe arrhythmias in hearts of animals receiving SnPP + MSH than in the SnPP-only group may be the HR-increasing effect of α-MSH. This property of the hormone, combined with the proarrhythmic effect of SnPP observed in the SnPP-treated group, may promote higher incidence of severe arrhythmic events.

The outcomes of experiments using an isolated, phenylephrine-precontracted aortic ring model demonstrate that treatment with a-MSH did not directly result in significant alteration of the resting vascular tone. Nevertheless conversely, the precontracted aortic rings exhibited significant relaxation in response to a-MSH. The maximal relaxing effect induced by 1 mM a-MSH concentration (in percentage of the initial contractile force, mean 6 SEM) did not differ significantly among the naive, naive ET-, 200 mM NOARG, 3 mM INDO, and 3 mM INDO + 200 mM NOARG-treated groups. Thus, the effect of a-MSH on the increased vascular tone (due to a1 adrenoceptor stimulation) proved to be independent from the presence or absence of endothelium as well as from the ability of endothelium to produce prostacyclin (PGI2) or NO. These findings, along with the significant levels of relaxation induced by treatment with the hormone in phenylephrinetreated rings, demonstrate that a-MSH has potential for clinical use in treatment regimens where vasodilation is a desired outcome for improvement of patient prognosis.

The capacity of a-MSH pretreatment to significantly inhibit the extent of ischemia/reperfusion-induced infarct zones provides additional evidence of the range of cardioprotective effects mediated by the hormone. The mechanistic basis for this effect is substantially due to the cytoprotective action of HO-1 – reinforces compartmentalization of ionic species within cardiomyocytes and other cells composing cardiovascular tissue – because inhibition of the enzyme with SnPP suppressed IR injury–associated infarct area increases, not only in α-MSH-treated hearts, but also in the control groups.

The significant increases in both protein expression and activity of HO-1 in myocardial tissue stimulated by pretreatment with a-MSH demonstrate that the hormone is capable of amplifying this major adaptive response at levels that further underscore the therapeutic value of its use.

The result of the second phase of our study show that alpha-MSH-treated animals exhibited lower weight gain than vehicle-treated control rats, however this difference was non-significant for the sample size studied. This outcome notwithstanding, significant differences were noted between alpha-MSH-treated versus control animals in the ratios of left ventricle to whole body mass at the end of the experiment. A major implication of this result is that there was greater thickening in the left ventricular walls of control animals versus those treated with the hormone. Since this phenomenon, known as ventricular wall hypertrophy, is associated with increased risk of ischemic heart failure, treatment with alpha-MSH was for these experiments, assessed as beneficial—with potential for human clinical applications.

Echocardiographic outcomes reveal the protective effects of long-term alpha-MSH treatment on cardiovascular systolic and diastolic function. This result is relevant to previous work by authors of the present report, in which echocardiographic parameters were monitored using single acute administration of alpha-MSH doses (10, 100 and 250 μg/kg), with resulting significant enhancement of systolic function (EF, FS). In the present study, the chronic alpha-MSH treatment engendered a similar pattern. Specifically, in animals receiving the hormone, fractional shortening, ejection fraction, stroke volume and cardiac output values were significantly elevated in comparison with control animals (FS: 32.33 ± 0.421% vs. 36.83 ± 0.703%; EF: 66.50 ± 0.067% vs. 72.00 ± 0.774%, SV: 0.41 ± 0.046 mL vs. 0.58 ± 0.030 mL, CO: 77.55 ± 7.763 mL/min vs. 112.30 ± 6.110 mL/min), respectively. Left ventricular outflow tract (LVOT) values are also increased in treated group, demonstrating direct correlation with elevated EF, FS, SV and CO as indicators of systolic function.

Previous investigations have shown that typically alpha-MSH does not affect blood pressure or may only slightly elevate it. Moreover, other effects of the hormone on other echocardiographic outcomes shown here, bear consideration. For example, increasing left atrial aortic root ratio (LA/Ao) value indicates means left atrial dilation. Here, alpha-MSH treatment decreased these values, although, both remained in healthy range. MV Deceleration Time (ms), a powerful prognostic marker of LV remodelling and diastolic dysfunction was also considered. α-MSH osmotic pump treatment was observed to significantly improve the diabetes-induced diastolic dysfunction. MV Deceleration Time: 66.67 ± 3.201 vs. 85.50 ± 5.258 ms, and IVRT: 58 ± 1.826 vs. 43 ± 1.125 ms standards were enhanced for hormone-treated animals in comparison with control rats. Other experiments in the present study considered mitral annular plane systolic excursion (MAPSE) also known as left atrioventricular plane displacement (AVPD). In previous studies reduced MAPSE has been shown to correlate with age, and LV function in patients with myocardial infarction, heart failure and atrial fibrillation and to be more sensitive than conventional echocardiographic markers in detecting abnormalities of LV systolic function at an early stage. Another measure of heart function considered here, the myocardial performance index (MPI) is an easily performable, recordable and reproducible parameter that may be determined by flow Doppler. MPI as a single prognostic variable, may be used for assessment of diabetic cardiac dysfunction. The present investigation showed that long-term alpha-MSH treatment

significantly improved both parameters in our present study; MAPSE: 1.602 ± 0.045 vs. 2.268 ± 0.010 mm, MPI: 0.491 ± 0.014 vs. 0.392 ± 0.01.

Evaluation of cardiac functions in Langendorff-mounted isolated working hearts revealed significantly increased pre-ischemic pressure change rate (dp/dt) value in MSH group compared to untreated Control group. Also, ischemic-reperfusion injury-associated decreases in AF, dp/dt, AoP versus pre-ischemic values were observed in both groups by the end of recovery. Despite the fact that nearly all outcome parameters decreased after 120 min of reperfusion, elevated AF and SV were measured at the 30 min recovery time point in the α-MSH-treated group, relative to control animals. Moreover, post-ischemic CO and SV values in animals receiving the hormone did not reach statistical significance in magnitude of suppression, versus their pre-ischemic state. These findings are consistent with previous studies by the authors, in which alpha-MSH treatment significantly inhibited the extent of ischemia/reperfusion-induced infarct zones, increased the magnitude of CO and SV, therefore provides additional evidence of the range of cardio-protective effects mediated by the hormone. Recent work by authors of the present report and others, demonstrate that melanocortins protect against tissue damage in response to prolonged myocardial ischemia/reperfusion via activation of pro-survival HO-1 protein, JAK/ERK/STAT signalling and decreased expression of the pro-inflammatory mediator TNF-α and pro-inflammatory/pro-apoptotic factor pJNK and also by vagus nerve-mediated cholinergic anti-inflammatory pathway. Analysis of results of cardiomyocyte contractile performance data, showed improved actin-myosin co-operation and strong trends toward a higher active tension—suggesting improved cardiomyocyte mechanical performance that may contribute to the beneficial effects of alpha-MSH on global cardiac contractility. In contrast, it appears that MSH had no effect on Ca2+ sensitivity of the contractile apparatus. Moreover, it was also noted that the effects of hormone treatment on cerebral vascular smooth muscle, produced no deleterious effects. Specifically, the treatment did not alter values for KCl, serotonin, or responses evoked by angiotensin II—indicating an apparent lack of vascular response to α-MSH stimulation.

In accordance with previous studies, one of the major hypotheses which may account for the protective effect of the hormone was its immunomodulatory, anti-inflammatory and antioxidant properties. These phenomena were a major rationale for aspects of experimental design used in the present study, involving assessment of NADPH oxidase enzyme activity. Nevertheless, a limitation of the current study is that we only investigated the hormone's antioxidant capacity by measuring NADPH oxidase activity. Other possible mechanisms are under intensive research to clarify beneficial effects of the hormone.

6. CONCLUSIONS

1) A major finding of this study is the echocardiographic outcomes that suggest favorable influences of α-MSH on EF, HR, CF, and AF of the heart even in IR conditions, the fact that HO-1 might play a huge role in these mechanisms, and that α-MSH treatment was able to increase both the expression and activity of the enzyme.

2) α-MSH treatment— possibly through HO-1 induction, inhibited the occurrence of arrhythmic events in comparison with a vehicle-treated control group. Treatment with SnPP in addition to the hormone resulted in a possible increase in susceptibility to ventricular arrhythmias, suggesting that HO-1 is cardioprotective in this respect.

3) We demonstrated the capacity of α-MSH pretreatment to significantly inhibit the extent of ischemia/reperfusion-induced infarct zones. The mechanistic basis for this effect is substantially due to the cytoprotective action of HO-1, because inhibition of the enzyme with SnPP suppressed IR injury–associated infarct area increases, not only in α-MSH-treated hearts, but also in the control groups.

4) An outstanding result of the present study is that longterm treatment with α-MSH decreased diabetic left ventricular hypertrophy, and significantly improved the systolic (FS, EF, SV, CO) and diastolic (mitral valve deceleration time, MAPSE, MPI) heart function of diabetic animals.

Our results suggest that α-MSH treatment can be protective both for healthy and diabetic myocardium, that protection involves systolic and diastolic functions as well, either in normal or in IR conditions. Elevated HO-1 expression and increased myocardial enzyme activity by the hormone suggest that α-MSH might be able to improve the antioxidant capacity of the myocardium, that further emphasize its potential therapeutic role, regarding that in many pathologic conditions (e.g. diabetes, coronary artery disease, myocarditis) chronic inflammatory reactions are responsible for myocardial damage. Overall, outcomes of our study raise the possibility of incorporating α-MSH treatment into human cardiological practice. Nevertheless, other possible mechanisms are under intensive research to clarify beneficial effects of the hormone.

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List of publications related to the dissertation

1. Vecsernyés, M., **Szokol, M.**, Bombicz, M., Priksz, D., Gesztelyi, R., Fülöp, G. Á., Varga, B., Juhász, B., Haines, D. D., Tósaki, Á.: Alpha-MSH induces vasodilatation and exerts cardioprotection via the heme-oxygenase pathway in rat hearts.
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