

Alpha-MSH induces vasodilatation and exerts cardioprotection via the heme-oxygenase pathway in rat hearts

Miklos Vecsernyes¹, Miklos Szokol², Mariann Bombicz^{3,4}, Daniel Priksz^{3,4}, Rudolf Gesztelyi^{3,4}, Gabor Aron Fulop⁵, Balazs Varga^{3,4}, Bela Juhasz^{3,4}, David Haines³, Arpad Tosaki³

¹ Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Debrecen, Debrecen H-4032, Hungary; vecsernyes.miklos@pharm.unideb.hu (Miklos Vecsernyes, Ph.D.)

² Department of Cardiology, Faculty of Medicine, University of Debrecen, Debrecen H-4032, Hungary; szokol.miklos@med.unideb.hu (Miklos Szokol, M.D.)

³ Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, Debrecen H-4032, Hungary; bombicz.mariann@pharm.unideb.hu (Mariann Bombicz, Pharm.D.); priksz.daniel@pharm.unideb.hu (Daniel Priksz, Pharm.D.); gesztelyi.rudolf@pharm.unideb.hu (Rudolf Gesztelyi, Ph.D.); varga.balazs@pharm.unideb.hu (Balazs Varga, Ph.D.); juhasz.bela@pharm.unideb.hu (Bela Juhasz, Ph.D.); tosaki.arpad@pharm.unideb.hu (Arpad Tosaki, Ph.D., D.Sc.) ddhaines2002@yahoo.com (David Haines, Ph.D.)

⁴ Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen, Debrecen H-4032, Hungary

⁵ Department of Cardiology, Faculty of Medicine, Division of Clinical Physiology, University of Debrecen, Debrecen H-4032, Hungary; fulop.gabor@med.unideb.hu (Gabor Aron Fulop, M.D.)

Correspondence: Bela Juhasz, Ph.D.

Institute of Pharmacology and Pharmacotherapy
University of Debrecen, Faculty of Medicine
H-4032, Debrecen, Nagyerdei krt. 98, HUNGARY
E-Mail: juhasz.bela@pharm.unideb.hu
Telephone: +3652427899/56109
Fascimile: +3652427899/56109

Short running title: Alpha-MSH exerts cardioprotection via HO-1

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ABSTRACT

Alpha-melanocyte-stimulating hormone (α -MSH) is a protein with known capacity for protection against cardiovascular ischemia-reperfusion (I/R) injury. The present investigation evaluates the capacity of α -MSH to mitigate I/R effects in an isolated working rat heart model and determine the dependency of these alterations on the activity of heme oxygenase-1 (HO-1, hsp-32), a heat shock protein that functions as a major antioxidant defense molecule. Healthy male Sprague-Dawley rats were used for all experiments. Following treatment with selected doses of α -MSH, echocardiographic examinations were carried out on live, anesthetized animals. Hearts were harvested from anesthetized rats pretreated with α -MSH and/or the HO-1 inhibitor SnPP, followed by cardiac function assessment on isolated working hearts, which were prepared using the Langendorff protocol. Induction of global ischaemia was performed, followed by during-reperfusion assessment of cardiac functions. Determination of incidence of cardiac arrhythmias was made by ECG. Major outcomes include echocardiographic data, suggesting that α -MSH has mild effects on systolic parameters, along with potent antiarrhythmic effects. Of particular significance was the specificity of dilatative effects on coronary vasculature, and similar outcomes of aortic ring experiments, which potentially allow different doses of the compound to be used to selectively target various portions of the vasculature for dilation.

Keywords: alpha-melanocyte-stimulating hormone; alpha-MSH; isolated working heart; echocardiography, vasodilatation

1. INTRODUCTION

Alpha-melanocyte-stimulating hormone (α -MSH) is a melanocortin family neuropeptide, expressed ubiquitously at various levels by a wide variety of cells (1). The biologically active protein is produced by proteolytic cleavage of adrenocorticotrophic hormone (ACTH), the cleavage product of proopiomelanocortin (POMC) (2). The compound exerts its effect by interacting non-selectively 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 (3). The most significant of its functions in the context of the present investigation is its role as an endogenous countermeasure to ischemia-reperfusion (I/R) injury (4, 5). For example, rat studies of cardiac and hepatic responses in melanocortin-induced modulation of 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- α (TNF- α) and apoptotic cell depletion. There are protective effects mediated substantially via α -MSH-induced HO-1 protein expression and enzyme activity (6, 7). Related

studies have underscored the role of HO-1 in particular as an essential component of protection against I/R injury to cardiovascular tissue (8-11). Based on these findings, and in the context of ongoing efforts by authors of the present report to characterize HO-1-dependent processes with applications in human medicine (12), the present study was undertaken to evaluate the role of HO-1 in alpha-MSH-mediated cardiovascular effects. Here, isolated working hearts harvested from pretreated rats were subjected to I/R injury and evaluated for changes in cardiac physiological outcomes indicative of levels of adaptation to I/R-mediated disruption of normal cardiovascular function. The dependency of these changes on HO-1 activity was determined by selective use of the HO-1 inhibitor, SnPP.

2. METHODS

2.1. Animals.

Male Sprague-Dawley rats weighing 300-350 grams (Charles River International, Inc., USA) were used for all experiments in the present study. The animals were housed at room temperature (approximately 25°C), with lighting set to alternating dark and light periods of 12 hours. The rats were maintained on normal rodent chow and tap water *ad libitum*. 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).

2.2. Chemical reagents.

Alpha-Melanocyte stimulating hormone (alpha-MSH, CAS Number 581-05-5) was obtained from Sigma-Aldrich Ltd. (St. Louis, MO, USA), and Sn(IV) protoporphyrin IX dichloride (SnPP, MFCD#: MFCD00673909) was provided by Frontier Scientific Inc. (Logan, USA). All other chemicals and buffer solutions were obtained from Sigma-Aldrich Ltd. (Budapest, Hungary). Reagents used for isolated aortic ring experiments included: phenylephrine, a selective alpha adrenergic receptor agonist; α -melanocyte-stimulating hormone (α -MSH); N^ω-nitro-L-arginine (NOARG), a nitric oxide 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, while α -MSH was dissolved in Krebs solution after dispersed in 20 μ L acetic acid:water (1:9) solution (v/v). NOARG and INDO were dissolved in 96% ethanol, then NOARG solution was further diluted in physiological salt solution.

2.3. Echocardiography.

Echocardiographic examinations were carried out on animals anesthetized with a mixture of ketamine and xylazine (50 mg/kg, 5 mg/kg), administered intramuscularly (IM). Rats were positioned in a dorsal decubitus position and the chest of each was shaved. Echocardiographic measurements were performed using Vivid E9 ultrasound system (GE Healthcare, UK), 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. Conventional measurements

were made in 2 dimensions and M-mode, using standard views. Parasternal long axis views were recorded to ensure that the mitral and aortic valves, as well as the apexes were visualized. The parasternal short axis views were recorded at the mid-papillary muscle level. M-mode acquisitions were performed at the mid-papillary muscle level, either in parasternal long or short axis views. Measurements included assessment of interventricular and left ventricular free-wall thickness in diastole and systole (IVSs, IVSd), left ventricular internal diameter at end-diastole (LVIDd), and end-systole (LVIDs). End-systolic volume (ESV), end-diastolic volume (EDV), stroke volume (SV), and left ventricular mass (LVmass) were calculated. Fractional shortening was computed by using the equation $FS = [(LVIDd - LVIDs) / LVIDd] \times 100$, and ejection fraction was calculated by using the formula (Teiholz): $EF = (LVEDD^2 - LVESD^2) / LVIDD^2$. All measurements were averaged over three to five consecutive cardiac cycles. Analyses of data were carried out by a trained cardiologist, who was blinded to experimental conditions to ensure objective interpretations.

2.4. Isolated working heart preparation.

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 following the washout period as previously described (13). Baseline cardiac functions (as described below) were then assessed. In Protocol I and II these procedures were followed by 30 minutes of global ischaemia 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 ischaemia/reperfusion, 1 ml alpha-MSH solution (in concentration of 1 μmol/L) was injected directly into the system, via the aortic cannula of the apparatus, to monitor direct effect of alpha-MSH administration (see also section 2.11 Experimental Design).

2.5. Induction of global ischemia and reperfusion in isolated hearts.

Induction of global ischaemia was performed during isolated working heart procedures of Protocols I and II (see sections 2.4 and 2.11). Following 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 (MASTERFLEX, Vernon Hills, Illinois, USA) 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 (ADInstruments, PowerLab, Castle Hill, Australia). Before ischemia and during reperfusion, the values of heart rate (HR), coronary flow (CF) and aortic flow (AF) were registered. Aortic flow was measured by a timed collection of the buffer pumped out from the heart, and coronary flow rate was measured by a timed collection of the coronary perfusate, dripped from the heart. Pre-selected exclusion criteria for these experiments were conducted to exclude isolated working hearts if ventricular arrhythmias occurred before the induction of ischemia.

2.6. Determination of cardiac arrhythmia incidence.

During evaluation of isolated working hearts, as described above, epicardial electrocardiogram (ECG) measurements were monitored using two silver electrodes attached directly to the heart. ECG readings were analyzed to determine the incidence of cardiac arrhythmias. Arrhythmias were analysed based on their occurrence using a quantal approach: whether there was at least one arrhythmic event or not. Arrhythmias (i.e. at least one arrhythmic event) were further classified into two groups defined as follows: (I) if the pump function of the heart recovered spontaneously from the arrhythmic event(s), it was considered to have “non-severe” arrhythmia and (II) 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.

2.7. 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 six, 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 millinewtons, using a wire instrument, in a 10 ml vertical organ chamber (Experimetria TSZ-04) containing Krebs solution, oxygenated with 95% O₂ and 5% CO₂ (36°C; 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).

2.8. Determination of infarcted areas of isolated hearts.

Triphenyl-tetrazolium chloride (TTC) staining was used to determine infarcted areas of each heart, as a direct, post-sacrifice approach. 100 ml of 1 % triphenyl tetrazolium chloride (TTC) solution in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM) was administered directly via the side arm of the aortic cannula at the endpoint of isolated working heart procedure. TTC stained viable myocardium deep red, while potentially infarcted areas stayed pale as it was seen during the analysis. Hearts were stored at -80°C 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 flat bed scanner (Hewlett-Packard, Palo Alto, California, USA). 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 area (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, Maryland, USA). Infarct size was expressed as a percentage ratio of the infarcted zone to the total area in each heart (percentage of pixels).

2.9. 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 homogenised in a solution containing HEPES 10mM, sucrose 32mM, DTT 1mM, EDTA 0.1mM, soybean trypsin inhibitor 10µg/ml, Leupeptin 10µg/ml, Aprotinin 2µg/ml, and pH 7.4 (homogenisate buffer). All reagents were purchased from Sigma-Aldrich, St. Louis, Missouri, U.S.A. The supernatant was collected by centrifugation of the homogenate for 30 minutes at 20,000g at 4°C. Assessment of heme oxygenase activity was done on each sample of supernatant, according to procedures used by Tenhunen et al (14). Briefly, HO-1 enzyme activity was measured using a computer-based, spectrophotometric analysis of bilirubin formation from heme. This HO-1 enzymatic measurement protocol utilized a reaction mixture containing an aliquot of the supernatant, plus glucose-6-phosphate 2mM, glucose-6-phosphate dehydrogenase 0.14 U/ml, heme 15µM, NADPH 150µM, rat liver cytosol as a source of biliverdin reductase 120µg/ml, MgCl₂ 2mM, and KH₂PO₄ 100mM. 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 530nm. The HO-1 activities were expressed in nanomols (nmol) of bilirubin formed per milligram protein per hour.

2.10. Western blot analysis for expression of HO-1 protein in heart tissue.

300 mg of frozen tissue from the left ventricular myocardium of each rat was homogenized in 800 µl homogenization buffer (25 mM Tris-HCl, pH 8, 25 mM NaCl, 4 mM Na-Orthovanadate, 10 mM NaF, 10 mM Na-Pyrophosphate, 10 nM Okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1x Protease inhibitor cocktail), using a Polytron-homogenizer. Homogenates were centrifuged at 2000 rpm at 4°C for 10 minutes. Supernatants were further centrifuged at 10,000 rpm at 4°C for 20 minutes. The resultant supernatants are cytosolic extracts that were used experimentally to evaluate this cell fraction. Samples (50µg each) were next separated with SDS-PAGE (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 non-specific binding of probing antibody, using 5% dry milk powder, and then incubated overnight at 4°C with primary antibodies (1:1000 dilution; anti-GAPDH; anti-HO-1 rabbit monoclonal antibodies, SIGMA-Aldrich). Following overnight incubation, membranes were treated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:3000 dilution) secondary antibody. Chemiluminescent detection (ECL, Litmus Scientific, Advansta Inc., Menlo Park, CA, USA) was used to identify bands. Detection was made by using autoradiography for variable lengths of time with Medical X-Ray Film (Agfa-Gevaert N.V., Belgium). Quantitative analysis of scanned blots were performed using the Scion for Windows Densitometry Image program (version Alpha 4.0.3.2, Scion Corporation, Maryland, USA).

2.11. Experimental design.

The experimental strategies used to analyze effects of alpha-MSH are summarized in Figure 1. For the present work, 5 basic protocols were designed:

2.11.1. Protocol I: Investigation of effect of alpha-MSH pre-treatment on cardiac HO-1 expression in ischaemic-reperfusion injury.

16 healthy male Sprague-Dawley rats weighing 300-330 g were divided into two groups (n=8/group): I-a group received 0.5 ml saline subcutaneously 24 hours before the isolated working heart procedure (described in section 2.4); I-b group received 250 µg/body weight alpha-MSH solution (dissolved in saline) subcutaneously 24 hours before the isolated working heart operation. During isolated working heart method hearts were exposed to ischaemia/reperfusion (described in section 2.5). Samples from myocardial tissues were frozen on liquid nitrogen and stored at -80°C for further western blot analysis (Figure 1A).

2.11.2. Protocol II: Evaluation of significance of the HO-1 pathway in cardioprotective effects of alpha-MSH.

In Protocol II. (Figure 1B), another population of male Sprague-Dawley rats (50 rats) were subdivided into 4 groups based on the pre-treatments 24 hours before the isolated heart procedure (described in section 2.4): group II-a animals (n = 15) received subcutaneous saline injection of 0.5 ml; group II-b animals (n=14) were injected with 250 µg/body weight alpha-MSH solution (diffused in saline) subcutaneously; group II-c animals (n=11) received 50 µg/body weight SnPP diffused in 0.5 ml saline intraperitoneally together with 250 µg/body weight alpha-MSH solution subcutaneously; group II-d animals (n = 10) were injected only with 50 µg/body weight intraperitoneal SnPP solution. During isolated working heart method hearts were exposed to ischaemia/reperfusion (described in section 2.5). At the endpoint of the experiments, samples from myocardial tissues were either frozen on liquid nitrogen and stored at -80°C for further analysis of HO-1 activity or stained for determination of infarcted areas (TTC-staining) and then frozen on liquid nitrogen and stored at -80°C for further analysis.

2.11.3. Protocol III: Estimation of direct cardiac effects of alpha-MSH on isolated hearts.

Hearts harvested from Sprague-Dawley rats without any pre-treatment (n = 8) were subjected to isolated working heart procedure (described in section 2.4) without ischaemia/reperfusion. 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, HR) were recorded. After measuring baseline cardiac function, 1 ml 1 µmol/L alpha-MSH solution was injected directly via 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 alpha-MSH (Figure 1C).

2.11.4. Protocol IV: Analysis of direct effects of alpha-MSH on echocardiographic heart functions.

As shown on Figure 1D, rats without any pre-treatment (n = 8) were anaesthetized 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 carried out (described in section 2.3). After recording baseline parameters, three different doses of alpha-MSH (10 µg/kg, 100 µg/kg, 250 µg/kg, respectively) were

injected intravenously via the catheter and cardiac parameters were estimated and recorded again under the influence of the different (and cumulative) doses of alpha-MSH.

2.11.5. Protocol V: Evaluation of vascular response of isolated aortic rings to alpha-MSH administration.

For each isolated aortic ring experiment ($n = 8$), a rat without any pre-treatment was guillotined and then the abdominal aorta rapidly excised (described in section 2.5). Following a 90-min incubation in Krebs solution, $1 \mu\text{M}$ phenylephrine was administered to five of the six aortic rings, including the intima-deprived (ET-) specimen, for 2 minutes, followed by a 45-minute washout period. Next, these five rings were subjected to one of the following treatments: (i) Krebs solution alone for one of the intact rings (naïve) and (ii) for the intima-deprived ring (naïve ET-), (iii) $200 \mu\text{M}$ NOARG, (iv) $3 \mu\text{M}$ INDO, and (v) $200 \mu\text{M}$ NOARG together with $3 \mu\text{M}$ INDO at the same time. Immediately following these treatments, these five preparations received $1 \mu\text{M}$ phenylephrine. After 30 minutes, during which the contractile force reached a plateau, an α -MSH concentration-response (E/c) curve was constructed (from 1 nM to $1 \mu\text{M}$) for these aortic rings. In the case of the sixth aortic ring, (vi) after the 90-minute incubation in Krebs solution, an α -MSH E/c curve was generated (from 1 nM to $1 \mu\text{M}$). Protocol V ($n = 8$ animals) is illustrated on Figure 1E.

2.12. Data analysis and statistics.

All data are presented as average magnitudes of each outcome in a group \pm standard error of the mean (SEM). Normality of data sets was verified with the D'Agostino-Pearson omnibus test. A Student's t-test was used to determine differences between two sets of data. More than two data sets were compared with one-way ANOVA followed by Tukey post-testing. Difference of means was considered significant at $p < 0.05$. Statistical analysis was carried out with GraphPad Prism 6.07. (GraphPad Software Inc., CA, USA).

3. RESULTS

3.1. Echocardiographic outcomes: fractional shortening and ejection fraction (Protocol IV).

Echocardiographic examinations shown in Figure 2, include ejection fraction (EF, 2A) and fractional shortening (FS, 2B). Heart rates and respiratory frequencies of each animal remained stable throughout the experiments. Moreover, no significant differences were noted between the independent measurements obtained by two blinded readers. FS and EF data correlated strongly with measurements of both the parasternal long and short axis views. FS following administration of $100 \mu\text{g/kg}$ and $250 \mu\text{g/kg}$ alpha-MSH was significantly increased in comparison with this outcome evaluated before the injection (Control) of the material ($\text{FS}_{100\mu\text{g/kg}}: 53.79 \pm 2.497$ and $\text{FS}_{250\mu\text{g/kg}}: 55.00 \pm 2.688$ versus $\text{FS}_{\text{Control}}: 45.69 \pm 2.241$). The same pattern was observed in EF results ($\text{EF}_{100\mu\text{g/kg}}: 88.31 \pm 1.745$, and $\text{EF}_{250\mu\text{g/kg}}: 89.21 \pm 1.527$ versus $\text{EF}_{\text{Control}}: 81.52 \pm 2.296$). Alpha-MSH at the dose of $10 \mu\text{g/kg}$ was observed to result in trends for increased EF and FS, but these increases did not reach statistical significance ($\text{FS}_{10\mu\text{g/kg}}: 52.50 \pm 2.773$ versus $\text{FS}_{\text{Control}}: 45.69 \pm 2.241$; and $\text{EF}_{10\mu\text{g/kg}}: 87.04 \pm 1.963$ versus $\text{EF}_{\text{Control}}: 81.52 \pm 2.296$). There were no significant changes observed in interventricular and

left ventricular free-wall thicknesses and LV mass after administration of a single dose of alpha-MSH.

3.2. Isolated working heart results (Protocol II).

Data from isolated working heart procedure of Protocol I is not shown, since similar outcomes observed by using the same experimental circumstances has already been published by the authors of this present report (15). Novel Western blot results of Protocol I, which were produced by analization of frozen-stored myocardial tissue samples gained at the end of isolated working heart procedure of Protocol I are shown in section 3.8. The data presented in Figure 3 provides outcomes of cardiac function evaluations on isolated hearts using Protocol II, to determine the effect of SnPP-mediated inhibition of HO-1 on heart rate, coronary flow, aortic flow, cardiac output and stroke volume.

3.2.1. Alpha-MSH and SnPP effects on heart rate (HR).

Results of ECG heart rate analyses, recorded during the isolated working heart procedure, are shown in Figure 3A. Here it was observed that alpha-MSH-treatment increased heart rate, especially at the first part of reperfusion period ($p < 0.05$), compared to the control group. SnPP treatment counteracted the heart rate-elevating effect of alpha-MSH ($p < 0.05$) throughout the whole reperfusion period (e.g. 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).

3.2.2. Alpha-MSH and SnPP effects on coronary flow (CF).

Coronary flow values during the isolated working heart method, shown in Figure 3B, were also measured before ischaemia (baseline), and at 30, 60, 90 and 120 minutes during reperfusion. As shown, alpha-MSH-treatment improved preischaemic coronary flow values compared to controls (28.14 ± 0.99 ml/min vs. 21.93 ± 1.39 ml/min). Coronary flow values of alpha-MSH-treated animals, measured during reperfusion, were elevated at every timepoint, and differences were significant compared to any other groups ($p < 0.05$). Coronary flow values measured in MSH-treated animals following 120 minutes of reperfusion (25.45 ± 1.60 ml/min), was 16% higher than preischaemic values of the control group (21.93 ± 1.39 ml/min), and decreased only by 10% versus preischaemic levels observed in the alpha MSH-treated group (28.14 ± 0.99 ml/min). The HO-1 inhibitor, SnPP, significantly counteracted the coronary flow-elevating effect of MSH, as seen from the results of SnPP+MSH-treated group at every timepoint of the reperfusion period ($p < 0.05$).

3.2.3. Alpha-MSH and SnPP effects on aortic flow (AF).

Aortic flow (AF) outcomes under the influence of alpha-MSH and modulation of HO-1 are shown in Figure 3C. Using Protocol II for these experiments, aortic flow was significantly increased in the α -MSH-treated group (52.07 ± 1.93 ml/min) compared to controls (39.57 ± 2.44 ml/min, $p < 0.001$) before the ischaemic insult. Preischaemic aortic flow values in SnPP-treated and SnPP+MSH-treated groups were decreased compared to the MSH-group, but increased compared to the control group values (48.00 ± 2.16 ml/min and 43.36 ± 3.42 ml/min, respectively). Aortic flow values in the α -MSH-treated group were significantly elevated at any timepoints of reperfusion compared to the paired control values ($p < 0.01$). Even compared to preischaemic controls AF values in the MSH-treated group were elevated in all timepoints, except at 120 minutes of reperfusion. Additionally, significant differences in AF between the α -MSH-treated group versus all other groups were measured during the reperfusion period ($p < 0.01$). While baseline AF values of SnPP-treated animals were increased compared to

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.

3.2.4. Alpha-MSH and SnPP effects on cardiac output, (CO) and stroke volume (SV).

Cardiac output (Figure 3D) and stroke volume values (Figure 3E) in alpha-MSH pre-treated animals were significantly elevated at all timepoints compared to their matched controls. However, SnPP failed to significantly counteract this effect.

3.3. Effect of alpha-MSH administration directly onto isolated working hearts (Protocol III).

Figure 4 shows the effects on cardiac function resulting from addition of $1 \mu\text{mol/L}$ alpha-MSH directly to isolated working hearts (MSH+) through the aortic cannula of the Langendorff apparatus on which the hearts are mounted, compared with baseline values (MSH-). As shown, alpha-MSH treatment resulted in significantly elevated CF (4B) and CF/CO% (4D) in comparison to baseline values ($p < 0,05$). No significant effects of alpha-MSH treatment were observed in AF, CO, HR or SV in comparison to baseline values.

3.4. Determination of incidence and severity of ventricular arrhythmias (Protocol II).

Outcomes of ECG analyses, shown in Figure 5, demonstrate that no arrhythmic events were detected in 80% of the control rats. However, in 6,7% and in 13,3%, non-severe 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, while in 27,2% of cases non-severe 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, while in 60,0% non-severe and in 10,0% severe arrhythmic cases were detected.

3.5. NOARG and INDO effect on α -MSH-mediated vascular tone response (Protocol V).

3.5.1. Vascular response to phenylephrine (precontraction).

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

3.5.2. Vascular response to α -MSH.

Treatment with α -MSH did not directly result in significant alteration of the resting vascular tone (data not shown). Conversely, the precontracted aortic rings exhibited significant relaxation in response to α -MSH (Figure 6). The maximal relaxing effect induced by $1 \mu\text{M}$ α -MSH concentration (in percentage of the initial contractile force, mean \pm SEM) did not differ significantly among the naïve, naïve ET-, 200 μM NOARG, 3 μM INDO and 3 μM INDO + 200 μM NOARG groups (20.01 ± 4.64 , 21.65 ± 4.53 , 16.75 ± 5.41 , 21.24 ± 6.73 , 23.71 ± 5.7 ,

respectively). Thus, the effect of α -MSH on the increased vascular tone (due to $\alpha 1$ 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 nitric oxide (NO).

3.6. Effects of Alpha-MSH and HO-1 inhibition on ischemia-reperfusion-induced cardiac infarct zone extent (Protocol II).

As shown in Figure 7, significant changes were observed in the extent of infarcted areas (expressed in % of total area) of hearts from Protocol II-treated animals, after ischaemia/reperfusion injury. TTC staining of hearts harvested from alpha-MSH-pretreated groups revealed significantly lower infarct zone extent, in comparison to tissue sections from hearts from vehicle-treated Control animals (19.74 ± 1.044 % versus 41.91 ± 3.922 %) ($p < 0.05$). This cardioprotective effect was completely counteracted by SnPP pre-treatment of alpha-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 %).

3.7. Effects of Alpha-MSH and SnPP on tissue HO-1 activity (Protocol II).

HO-1 enzyme activity was measured in myocardial tissue samples obtained from Protocol II (pre-treated) animals. No significant differences in activity of the enzyme were noted (data not shown). Nevertheless, HO-1-activity of MSH-treated animals were slightly increased compared to controls, SnPP pre-treatment decreased the activity of the enzyme, while this reduction seemed to be less intense in the SnPP+MSH treated group compared to 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.

3.8. Western blot analysis (Protocol I).

Western blot method was carried out by analization of frozen-stored myocardial tissue samples gained at the end of isolated working heart procedure of Protocol I. Results of western blot analyses, shown in Figure 8, demonstrate that expression of HO-1 protein in myocardial tissue harvested from post-ischemic/reperfused, isolated working hearts excised from rats treated with 250 ug/kg Alpha-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. DISCUSSION

Echocardiographic assessment of alpha-MSH effects on ejection fraction (EF) and fractional shortening (FS), shown in Figures 2A and 2B respectively, reveal that administration of the hormone correlated with significant increases in both parameters. These effects may reflect increased activation of sympathetic tone, since it is well-known that alpha-MSH and its analogues are associated with mild sympathetic activation in the cardiovascular system, including increased heart rate, and thus may influence systolic activity (16). This increase of ejection fraction upon alpha-MSH administration has also been shown by other authors in systemic inflammatory response syndrome in pigs (17). Effects on cardiac function in isolated hearts shown in Figure 3, include results shown in Figure 3A, demonstrating that alpha-MSH pre-treatment according to Protocol II increased the beat rate of isolated working hearts during the initial reperfusion period, as measured by ECG. Such heart rate elevating effects of alpha-MSH has been confirmed on other animal models (19, 20). The contribution of HO-1 activity to this effect was demonstrated by the observation that increased heart rate stimulated by the hormone was counteracted by SnPP, an inhibitor of HO-1. Nevertheless, it should be noted that the conspicuous low heart rates in the SnPP+MSH group are partly due to the observed many severe arrhythmic events, as described in present report. Likewise, alpha-MSH pretreatment significantly increased coronary flow (CF, Figure 3B) and aortic flow (AF, Figure 3C), both in the preischemic periods and during reperfusion (Figure 3B). This effect was also demonstrated to be significantly HO-1-dependent, since 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 (21) and improved clinical prognoses for patients who have experienced ischemic events (22, 23). These and related observations suggest possible use of alpha-MSH and agents that enhance expression of HO-1 in prevention of and therapy for ischemia/reperfusion-associated pathologies. Conversely, although alpha-MSH treatment significantly increased magnitude of cardiac output (Figure 3D) and stroke volume (Figure 3E), this effect was not or only partially HO-1-dependent, as evidenced by inability of SnPP to significantly counteract these increases.

Additional suggestion that Alpha-MSH holds potential for improvement of cardiovascular function through its vasoactive influence on cardiac function, is shown by the effects of direct administration of hormone directly to healthy rat hearts in Figure 4. Administration of the hormone induced significant increases in coronary flow (CF) by treated hearts versus controls. These outcomes reveal that alpha-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. Such measures fall under the broad category of “biotherapeutic” interventions – which are strategies that target specific, root causes of a disease, ultimately resulting in true “cures” rather than providing only transitory palliative relief of symptoms (24).

The present investigation further revealed potential uses of agents such as alpha-MSH that modulate HO-1, in reducing susceptibility to ventricular arrhythmias. Here, ECG analysis was conducted to evaluate the involvement of alpha-MSH-induced HO-1 activity in antiarrhythmic cardioprotection. These results, shown in Figure 5, 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 finding that coincides with results of previous investigations on mice conducted by authors of the present report (25). 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 heart rate-increasing effect of alpha-MSH. This property of the hormone, combined with the pro-arrhythmic effect of SnPP observed in the SnPP-treated group, may promote higher incidence of severe arrhythmic events. These outcomes are particularly intriguing in the context of previous work by the authors demonstrating that naturally occurring ginkgolide terpenes interact with platelet activating factor receptors, calcineurin and the macrolide immunosuppressant FK506, to synergistically ameliorate ventricular arrhythmias in the same model as used in the present report (26). Thus, it is likely that alpha-MSH may also exhibit clinically relevant synergism with these agents in management of cardiac arrhythmias.

Figure 6 shows the outcomes of experiments using an isolated, phenylephrine-precontracted aortic ring model conducted according to Protocol V, to evaluate the anti-vasoconstrictive capacity of alpha-MSH. Results of these procedures demonstrate that treatment with α -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 α -MSH. The maximal relaxing effect induced by 1 μ M α -MSH concentration (in percentage of the initial contractile force, mean \pm SEM) did not differ significantly among the naïve, naïve ET-, 200 μ M NOARG, 3 μ M INDO and 3 μ M INDO + 200 μ M NOARG-treated groups. Thus, the effect of α -MSH on the increased vascular tone (due to α_1 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 nitric oxide (NO). These findings, along with the significant levels of relaxation induced by treatment with the hormone in phenylephrine-treated rings, demonstrates that alpha-MSH has potential for clinical use in treatment regimens where vasodilation is a desired outcome for improvement of patient prognosis (27, 28). Moreover the endothelium-independent α_1 -adrenoceptor-mediated effects on vascular tone, suggested by these outcomes, provide insight for future drug discovery efforts in which specific therapeutic targets may be identified and exploited (29).

The capacity of alpha-MSH pretreatment to significantly inhibit the extent of ischaemia/reperfusion-induced infarct zones shown in Figure 7, provides additional evidence of the range of cardioprotective effects mediated by the hormone. As shown in Figure 7, the mechanistic basis for this effect is substantially due to the cytoprotective action of HO-1, since inhibition of the enzyme with SnPP suppressed IR-injury-associated infarct area increases, not only in alpha-MSH-treated hearts, but also in the control groups. Authors of the present report have extensively characterized processes by which ischemia/reperfusion injury promotes infarction-associated tissue damage and methods by which it may be counteracted by increasing HO-1 expression (30, 31). Exploration by the authors, of mechanisms contributing to mechanisms by which infarcted zone expansion is augmented, strongly suggests that the antioxidant capacity of HO-1 reinforces compartmentalization of ionic species within cardiomyocytes and other cells composing cardiovascular tissue the integrity of which is compromised by elevated levels of reactive oxygen molecules induced during the re-oxygenation period associated with post-ischemic reperfusion (32, 33).

The significant increases in both protein expression, shown in Figure 8, and activity of HO-1 in myocardial tissue stimulated by pretreatment with alpha-MSH, demonstrate that the hormone is capable of amplifying this major adaptive response at levels that further underscore the therapeutic value of its use. Pharmacological amplification of HO-1 has been demonstrated to powerfully remediate cardiovascular disease, along with disorders of the lung, kidney, and central nervous system (8).

5. CONCLUSIONS.

The present investigation is consistent with results of other studies demonstrating a diverse range of cardioprotective effects mediated by alpha-MSH. The results provide insight as to the relevance of these findings in the "biotherapeutic" management of cardiovascular diseases through specific effects on aspects of vasodilation, which may be manipulated to improve patient prognoses in treatment regimens that utilize the compound. Major findings of the present study include echocardiographic outcomes that suggest its effects on systolic parameters of the heart (ejection fraction), along with outcomes of cardiac function experiments demonstrating favorable influences on heart rate, coronary flow, aortic flow and aortic flow. The hormone showed potent antiarrhythmic properties and further promotes intensive dilatative effects on coronary vasculature – a finding that will potentially allow development of interventions that specifically target selected blood vessels for dilation. Such precise targeting of underlying causes of a particular disease process is the core concept of "biotherapeutic" approaches which have emerged as a major policy current emphasis by the U.S. National Institutes of Health (NIH) on developing medical strategies that attack disease at its roots, rather than merely providing analgesic and palliative relief from symptoms. This encouraging trend is reflected by the NIH's current policy directive (34). Also a major finding of this investigation is that most of the cellular and tissue processes studied required the activity of HO-1 as a downstream effector of alpha-MSH. Nevertheless, some of the potentially beneficial effects, such as changes in cardiac output and stroke volume, were observed to occur independently of HO-1 activity, as evidenced by insensitivity of these processes to treatment with the HO-1 inhibitor, SnPP. These findings are particularly significant to ongoing work by the authors, who have developed numerous preventive and therapeutic applications for phytochemical HO-1 inducers (24) (18).

Acknowledgments: This work was supported in part by the TÁMOP-4.2.2.D-15/1/KONV-2015-0016 project, implemented through the New Széchenyi Plan, and co-financed by the European Social Fund, and in part by KUTEKY (Bela Juhasz), University of Debrecen (Bela Juhasz), OTKA PD-78223, NKFI-K—104017, GINOP-2.3.2-1. Further support is derived from AGR-PIAC-13-1-2013-0008 (D. P., M. B., R. G., B. V., B. J.) and the TÁMOP-4.2.2.A-11/1/KONV-2012-0045 and TÁMOP-4.2.6.-15/1-2015-0001 (D. P., M. B., R. G., B. V., B. J., A. T.) projects co-financed by the European Union and the European Social Fund. This research was also supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11/1-2012-0001 'National Excellence Program' (M. B., B. V.). The authors are sincerely grateful to Stephanie C. Fox, of *QueenBeeEdit* in Bloomfield, Connecticut, USA, for her hard work in organizing, formatting, and editing this article.

Author Contributions: Miklos Vecsernyes: study design, isolated working heart preparations, isolated aortic ring, treatments, data analysis, manuscript preparation. Miklos

Szokol: study design, treatments, echocardiography. Mariann Bombicz: isolated working heart method, protein isolation, enzyme activity measurements, Western blot analysis. Daniel Priksz: echocardiography, isolated working heart method, manuscript preparation; Balazs Varga: treatments, isolated working heart preparations, manuscript preparation; Rudolf Gesztelyi: isolated aortic ring experiments, data analysis, statistical analysis; Gabor Aron Fulop: echocardiography, data analysis; David Haines: data interpretation and manuscript preparation; Arpad Tosaki: isolated working heart preparations, manuscript preparation, Bela Juhasz: study design and set up, echocardiography, corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Experimental design. Protocols shown here were used for different parts of the study. 1A: Protocol I: investigation of effect of alpha-MSH pre-treatment on cardiac HO-1 expression in ischaemic-reperfusion injury; 1B: Protocol II.: evaluation of significance of the HO-1 pathway in cardioprotective effects of alpha-MSH; 1C: Protocol III.: estimation of direct cardiac effects of alpha-MSH on isolated hearts; 1D: Protocol IV.: analysis of direct effects of alpha-MSH on echocardiographic heart functions; 1E: Protocol V.: Evaluation of vascular response of isolated aortic rings to alpha-MSH administration.

Figure 2. Echocardiographic outcomes. Effect of selected doses of alpha-MSH on Ejection Fraction (2A) and Fractional Shortening (2B). Echocardiographic examination was carried out after ketamine-xylazine anaesthesia. After recording baseline parameters (Control), different doses of alpha-MSH (10 µg/kg, 100 µg/kg, 250 µg/kg, respectively) were injected intravenously via catheter and cardiac parameters were estimated and recorded again. Results are shown as average values from each group of rats ±SEM of Ejection Fraction (EF, %, 2A) and of Fractional Shortening (FS, %, 2B).

* $P < 0.05$ compared with Control.

Figure 3. Effects of pretreatment of animals with alpha-MSH and/or the HO-1 inhibitor SnPP on cardiac functions in isolated working hearts (Protocol II.). Working hearts were isolated from 4 groups of rats which had been pretreated with injections of alpha-MSH and/or saline - and selected animals receiving injections of the HO-1 inhibitor SnPP, 24 hours prior to sacrifice and heart isolation. Treatments were as follows: 0.5 ml saline subcutaneously (group II-a, Control); 250 µg/kg body weight alpha-MSH subcutaneously (group II-b, MSH); 50 µg/kg body weight SnPP intraperitoneally, plus 250 µg/kg body weight alpha-MSH subcutaneously (group II-c, SnPP+MSH); or 50 µg/kg body weight SnPP, intraperitoneally (group II-d, SnPP). Cardiac function parameters were recorded before ischaemia (baseline), and at 30, 60, 90, and 120 minutes of reperfusion. Results are shown as average values from each group of rat hearts ± SEM of heart rate (HR, beats.min⁻¹, 3A); coronary flow (CF, ml.min⁻¹, 3B); aortic flow (AF, ml.min⁻¹, 3C); cardiac output (CO, ml.min⁻¹, 3D); and stroke volume (SV, ml, 3E).

* $p < 0.05$, significant differences between groups.

** $p < 0.01$, significant differences between groups.

*** $p < 0.001$, significant differences between groups.

Figure 4. Influence of direct administration of alpha-MSH-addition to isolated working heart functions (Protocol III). Isolated working hearts excised from groups of ketamine-xylazine-anesthetized rats, were treated by 1.0 ml 1 µmol/L alpha-MSH (MSH+) directly into the Langendorff system in which the hearts were mounted. Baseline values are designated as MSH-. Administration of alpha-MSH solutions to hearts was accomplished via the aortic cannula of the apparatus. Results are shown as average values from each group of rats ±SEM of aortic flow (AF, ml.min⁻¹, 4A); coronary flow (CF, ml.min⁻¹, 4B); Cardiac output (CO, ml.min⁻¹, 4C); the ratio of coronary flow to cardiac output in % (CF/CO, %, 4D); heart rate (HR, beats.min⁻¹, 4E); and stroke volume (SV, ml, 4F).

* $p < 0.05$, significant differences between groups as shown.

Figure 5. Effects of pretreatment of animals with alpha-MSH and the HO-1 inhibitor SnPP on cardiac arrhythmias in isolated working hearts (Protocol II.). Working hearts were isolated from 4 groups of rats which had been pretreated with 0.5 ml saline

subcutaneously (Control); 250 µg/kg body weight alpha-MSH subcutaneously (MSH); 50 µg/kg body weight SnPP intraperitoneally, plus 250 µg/kg body weight alpha-MSH subcutaneously (SnPP+MSH); or 50 µg/kg body weight SnPP, intraperitoneally (SnPP). Arrhythmic events in which hearts failed to recover their pump function are classed as Severe, whereas those hearts in which pump function was spontaneously restored following the arrhythmia, were classed as Non-severe.

Figure 6. Alpha-MSH effects on vascular tone. Effects of alpha-MSH on tone of rat abdominal aortic rings precontracted with 1 µM phenylephrine, in rings from which intimal endothelium had been debrided (Naive ET-); and rings from which endothelium remained intact (Naive). Effects of alpha-MSH on contractile force of Naive, versus Naive ET- rings are shown in the left panel, with influence of NOARG, INDO and NOARG + INDO on alpha-MSH-treated Naive rings are shown in the right panel. For both diagrams, the x-axis denotes the common logarithm of the molar concentration of α-MSH, and the y-axis indicates the effect as a percentage decrease of the initial contractile force. The symbols show the responses to α-MSH averaged within the groups (± SEM).

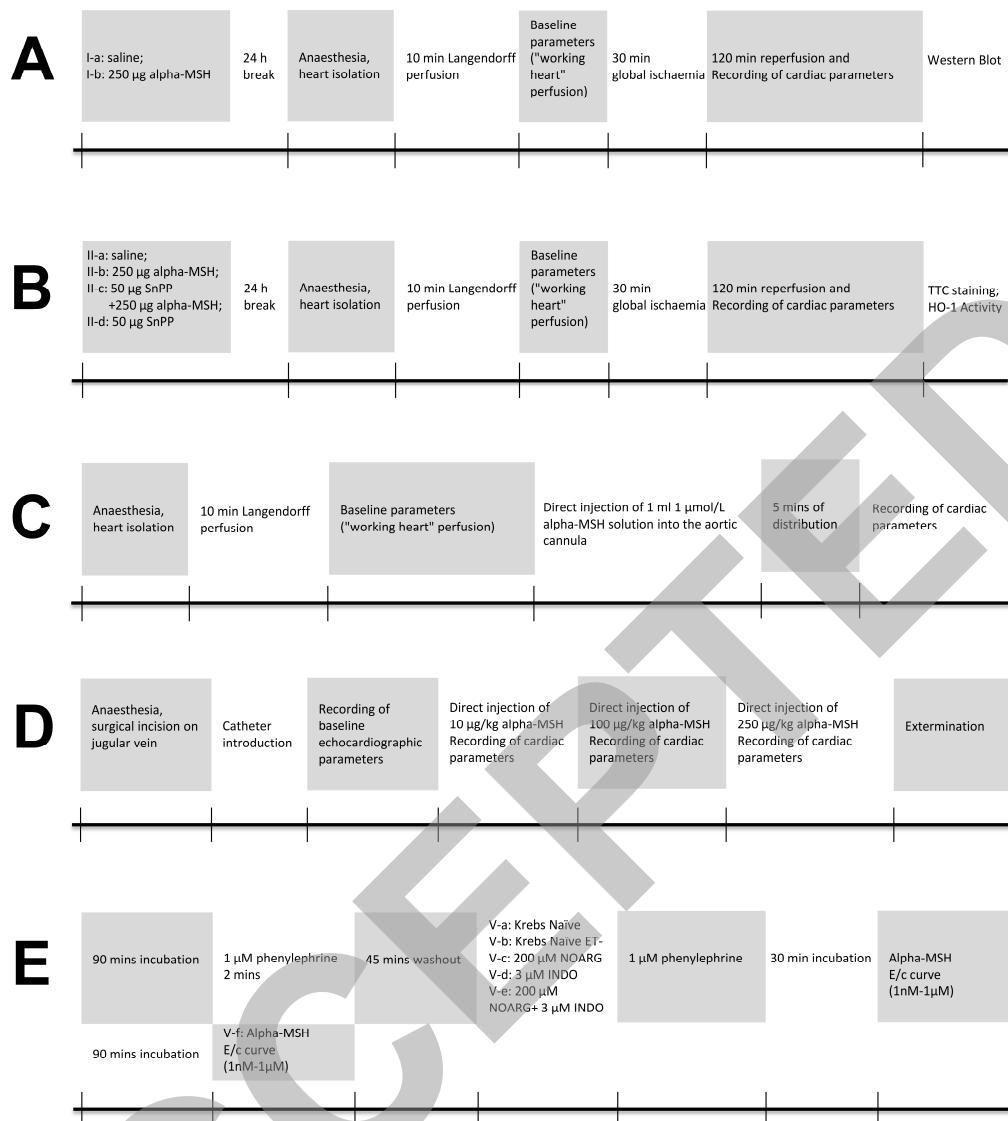
Figure 7. Effect of Alpha-MSH and HO-1 inhibition on infarcted zone magnitude (Protocol II). Rats administered subcutaneous saline (Group II-a), or subcutaneous 250 µg/kg body weight alpha-MSH (group II-b), or intraperitoneal injections of 50 µg/kg body weight SnPP plus 250 µg/kg body weight subcutaneous alpha-MSH (group II-c), or 50 µg/body weight SnPP (group II-d), followed by sacrifice and ischaemia/reperfusion injury during isolated working hearts according to Protocol II. Staining of infarcted tissue was accomplished by administration to working hearts of 1 % triphenyl tetrazolium chloride (TTC) solution in phosphate buffer, via the side arm of an aortic cannula. Hearts were sliced transversely, blotted dry, placed in between microscope slides, and scanned. Infarct area of each slice were traced and the respective areas were calculated by pixel density analysis.

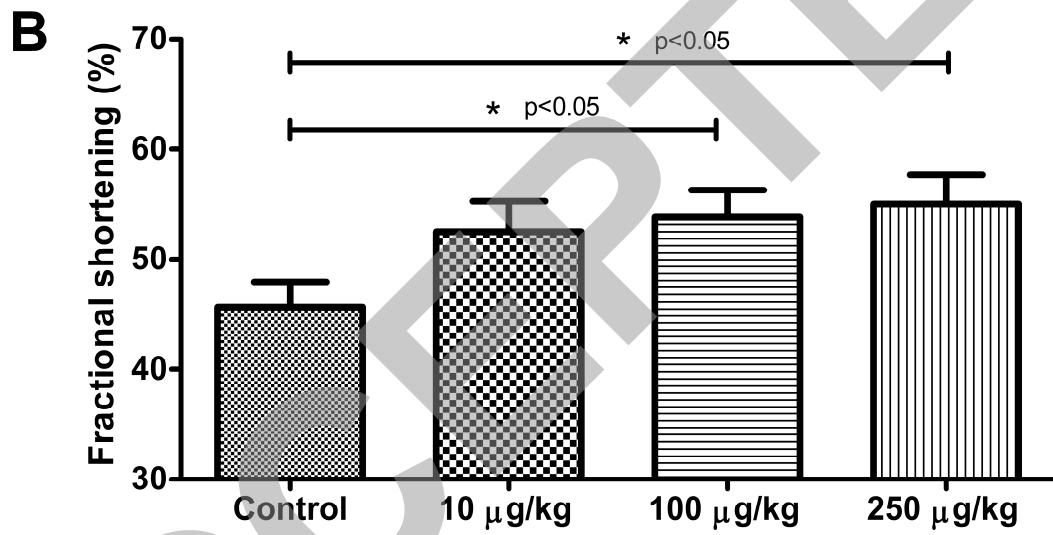
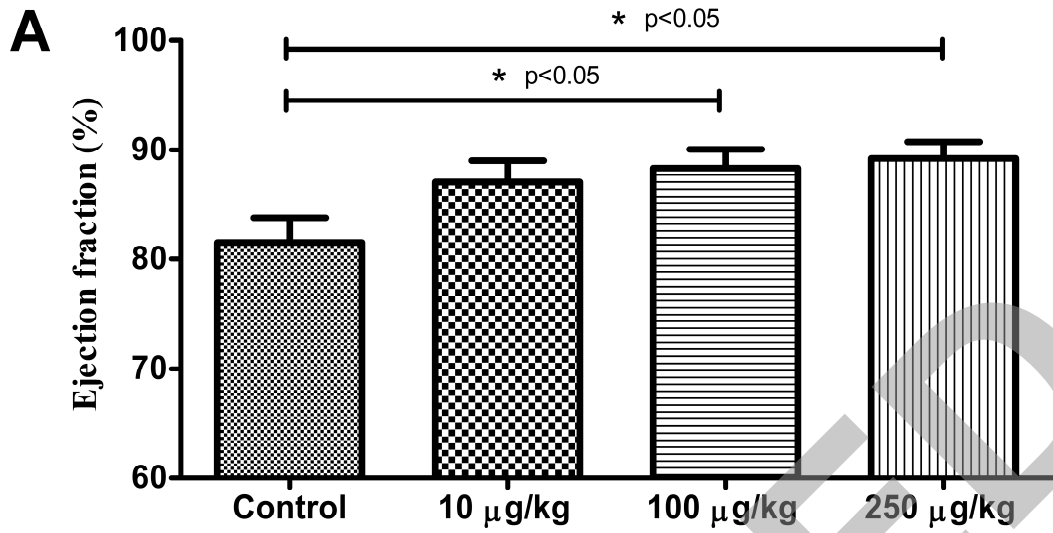
* $P < 0.05$ for comparison of infarcted areas to Control.

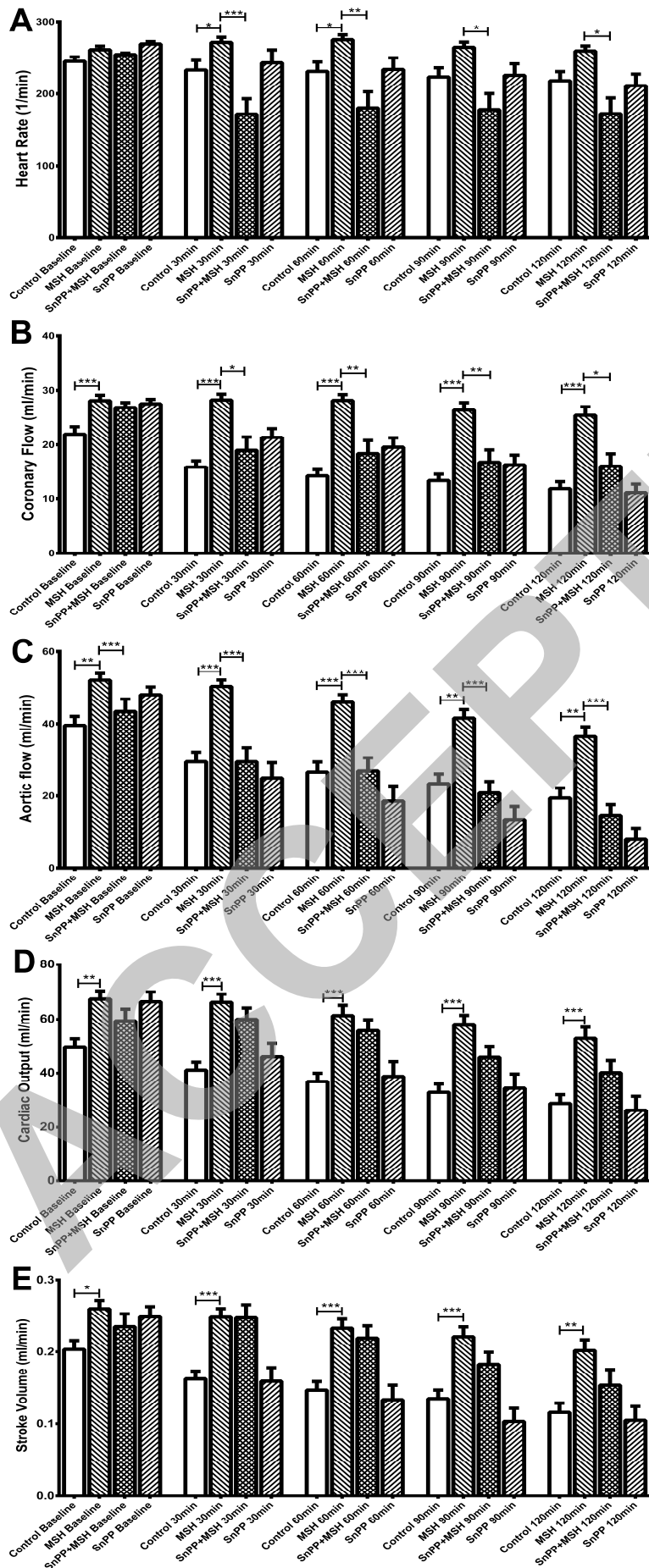
$P < 0.05$ for comparison of infarcted areas to alpha-MSH pretreated (250µg/kg) group.

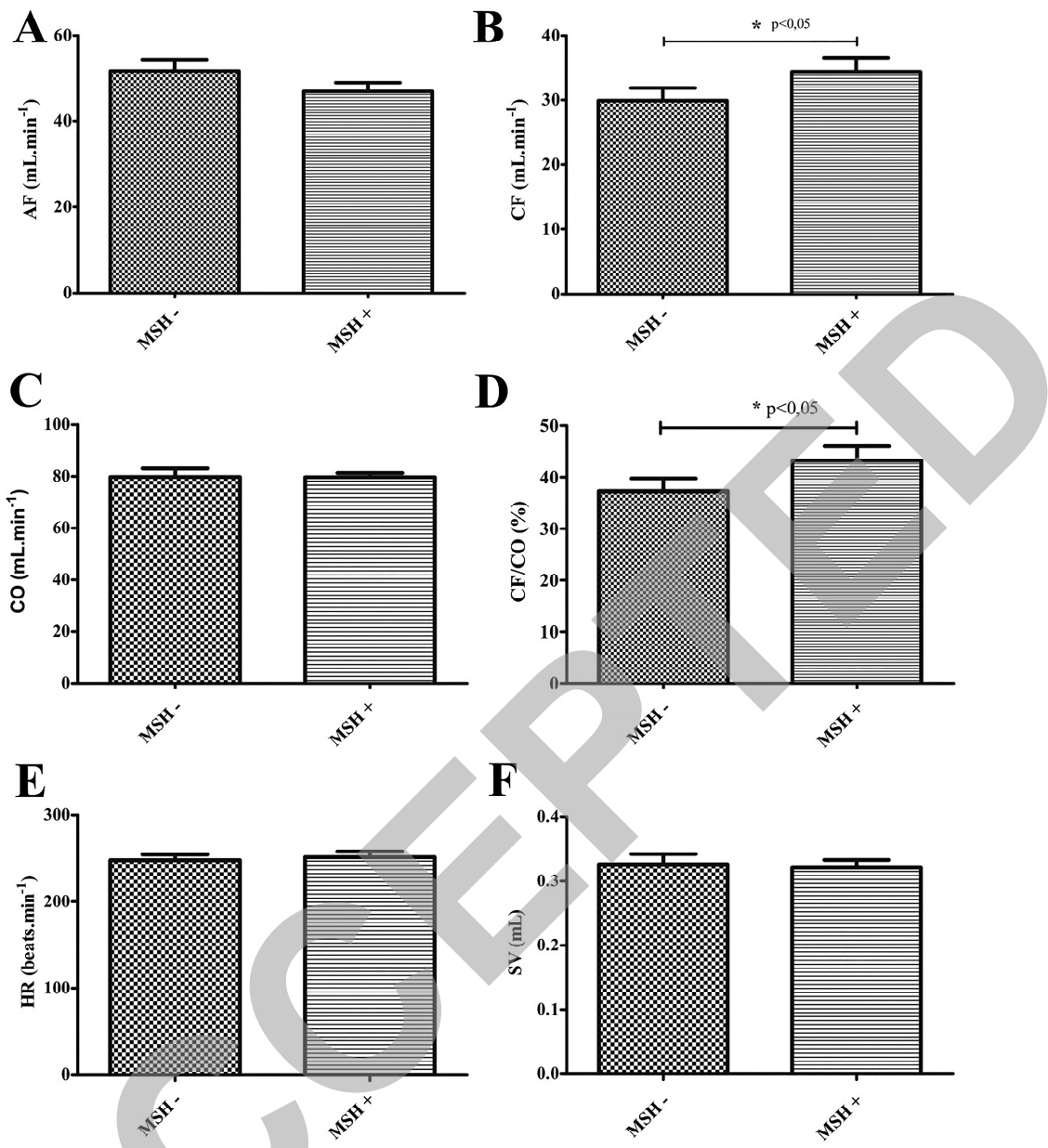
Figure 8. Western blot analysis for HO-1 protein expression of myocardial tissue from isolated working hearts (Protocol I). Expression of HO-1 protein in rat myocardial tissue was measured in homogenized left ventricular cardiac tissue samples drawn from 2 test groups of rats, defined as follows: Rats in the control group were treated with 0.5 ml physiologic saline vehicle; and a second group of animals were treated with 250 µg/kg body weight alpha-MSH. Treatments were administered by subcutaneous injection 24 hours prior to sacrifice and excision of hearts. Working hearts were mounted in a Langendorf apparatus and subjected to ischaemia/reperfusion injury as described by Protocol I. Homogenized myocardial tissue was evaluated for expression of HO-1 protein by Western blot analysis. The signal intensity of resulting bands corresponding to proteins of interest was evaluated as densitometry image intensity. Tissue content of HO-1 is shown in arbitrary units as the mean for each group of animals ±SEM.

* $P < 0.05$ for comparison of average expression levels of HO-1 in myocardium to Control.

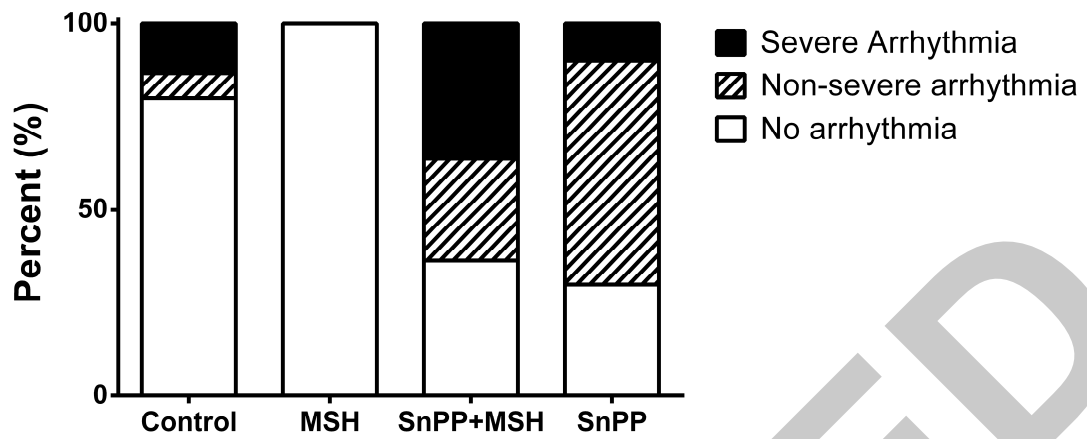




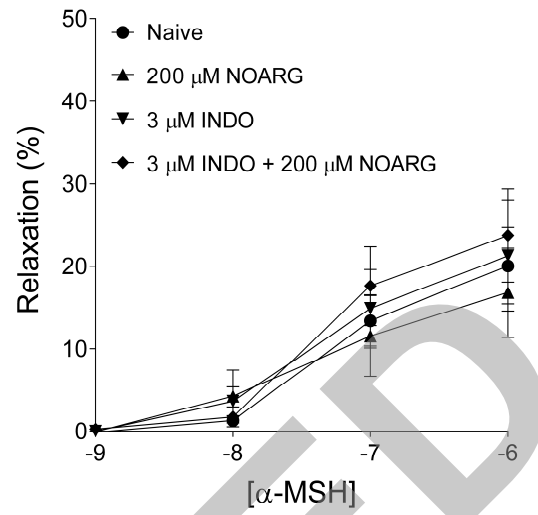
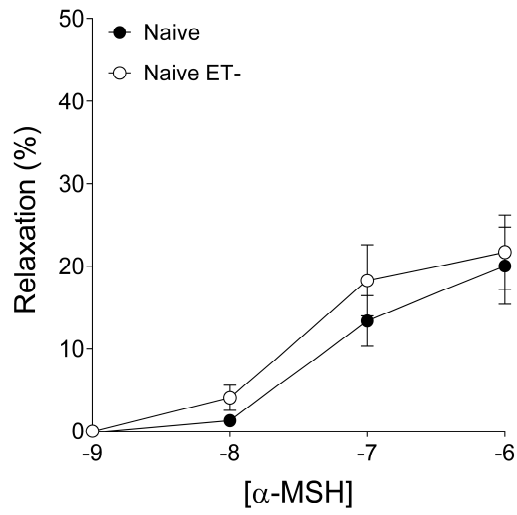




Incidence of Arrhythmic Events



ACCEPTED



ACCEPTED

