THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

Alternatives for the treatment of ischemic heart

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Introduction

Cardiovascular diseases are one of the most common causes of death all over the world and in Hungary as well. These alarming statistical data make reasonable the detailed research of the subject so that we can better understand the mechanism of ischemia/reperfusion injury of the heart. In the past three decades, a number of studies have been devoted to understand the mechanisms of ischemic/reperfusion-induced damages and extensive research has been conducted to find effective treatment for ischemic failure. Accordingly, the experimental techniques have been developed considerably, but until recently the complex treatment of ischemic diseases are still unsolved.

Although, natural and endogenous compounds have been taken off, to our knowledge, relatively little attention has been paid on the effects of adrenocorticotropic hormone and its fragments, especially ACTH (4-10) on the recovery of postischemic cardiac function and infarct size. Melanocortins (including α –, β - and γ-melanocyte-stimulating hormone are derived from a larger precursor molecule, the pro-opiomelanocortin peptide (POMC). The adrenocorticotropic hormone is a peptide, which contains 39 amino acids (1-39). Its active fragment contains 13 amino acides, namely the α-MSH (1-13) and within this fragment the ACTH (4-10). Melanocortins exert their effects by activating seven-transmembrane domain G-protein-coupled receptors. These receptors have a wide and varied distribution and are found in several organs. A few years ago α-melanocyte-stimulating hormone (α-MSH) was described to have a protective effect on renal cells, gut, lung, and brain tissue against ischemia/reperfusion-induced injury. Furthermore, melanocortins including α-MSH are able to exert protective effects in a model of permanent coronary artery occlusion. Moreover, melanocortin peptides have a peculiar, adrenal-independent anti-inflammatory activity. In our previous study, we demonstrated that melanocortin peptides, i.e. α-MSH, significantly attenuated the harmful consequences of myocardial ischemia, including arrhythmias, apoptotic and necrotic cell death, and impaired cardiac function. Yet, α-MSH, as an endogenous derivate is a double edge sword due to the unexpected hormonal side-effects. Therefore, the finding of a similar hormone or derivate without any adrenocorticotropic effect would be an optimal aim.

Sudden cardiac death most frequently results from chronic heart failure and malignant ventricular arrhythmias related to the short- and long-term effects of ischemic heart disease.

These pathophysiological changes result from electrophysiological and metabolic alterations due to acute ischemia, as well as structural alterations related to previous infarction. Since the discovery by Murry *et al.*, cardiac preconditioning (PC) of the myocardium has come to be emphasized as one of the most consistently powerful and reproducible interventions of delaying the development of ischemic injury. The cardiac protection associated with PC can be seen immediately, the so-called "first window of protection," but disappears rapidly. A new concept has been later discovered, introduced, and termed as the "second window of cardiac protection". Since the discovery of the first and second window of cardiac protection, a considerable body of evidence has been accumulated indicating that PC indeed offers a substantial cardiac protection against ischemia-induced damage in intact myocardium. Concerning the underlying mechanisms, the PC has been suggested to promote the formation and release of various endogenous protective substances, such as nitric oxide, bradykinin, calcium, adenosine, TRX-1, that are supposed to mediate the antiischemic and antiarrhythmic effects in ischemic/reperfused myocardium. However, PC and the great majority of animal studies, as well as the proposed PC mechanisms, have been done in intact and healthy myocardium instead of a diseased heart. Therefore, in the present study, we investigated whether PC could protect the myocardium in previously diseased, hypercholesterolemic hearts.

Recently, a role of the intracellular messenger ceramide has been indicated in PC. Ceramide generated from sphingomyelin during ischemia/reperfusion can induce cardiomyocyte death. Ischemia/reperfusion can also generate another second messenger sn-1,2-diacylglycerol (DAG) that tends to inhibit the effects of ceramide. In a recent study, PC was found to enhance DAG, which in turn neutralized the damaging effects of ceramide. In another study, TNFa-mediated PC occurred through ceramide signaling. Cell permeable exogenous ceramide reduced the infarct size of the brain supporting the role of ceramide in PC. Evidence is rapidly accumulating suggesting that ceramide performs its signaling function from within the lipid rafts, ordered sphingolipid and cholesterol-rich lipid domains, which can function as an ordered support for receptormediated signaling events. Caveolae, 50–100 nm invaginations of the plasma membrane, are subset of lipid rafts enriched in sphingolipids and cholesterol. The caveolae can selectively sequestrate membrane-targeted proteins and create a unique signaling microdomain, thereby controlling transmembrane signaling. Caveolae are characterized by the presence caveolins, which distinguishes caveolae from other lipid raft domains.At least three caveolins isoforms ofmolecular weights between 22 and 24 kDa have been identified of which caveolin-1

and caveolin-2 are abundant in most cell types while caveolin-3 is specific to muscle cells. Caveolin-1, a substrate for nonreceptor tyrosine kinases including Fyn, Abl and Src, acts as a scaffolding protein and can be phosphorylated on tyrosine 14 by these kinases in response to external stimuli such as oxidative stress. Such tyrosine phosphorylation activates the downstream signaling targets, and thus, serves as a crucial step for intracellular signaling occurring within caveolae. Nitric oxide plays a crucial role in myocardial PC. Both iNOS and eNOS have been found to be activated in the preconditioned heart. Because ceramide signaling may occur through NO and NOS can localize in lipid raft/caveolinrich microdomains, we sought to determine if lipid rafts play any role in ceramide-NO signaling in the ischemic and preconditioned heart.

It is a proven fact that antioxidants are generally highly effective in reducing ischemia/reperfusion injury of the heart by removing the toxic oxygen metabolites. In our study we examined the antioxidant and cardioprotective effects of Euryale ferox, also known as Makhana in East Indies. Euryale ferox (Family Nymphaeaceae) plant is a seasonal or perennial giant water lily having flat leaf surface. The leaves, petioles, sepals and fruits of gorgon nuts are covered with semi-delicate bent prickles. The seeds are sweet and sour astringent herb, which are native to the East Indies, but has been cultivated in China for several thousand years. Makhana has been widely used in traditional oriental medicine to cure a variety of diseases including kidney problems for leakage of fluids, chronic diarrhea, excessive leucorrhea and hypofunction of the spleen. However, very little is known about the mechanisms of action by which this fruit exerts its therapeutic effects. A recent paper described antioxidant activity of extracts from Euryale ferox seed. This study showed relatively high level of radical scavenging activity toward 1,1-diphenyl-2-picrylhydrazyl (DPPH) and demonstrated enhanced viability of Chinese hamster lung fibroblast cells under exposure to oxidative agents. In another related study, the glycolipid components of Euryale ferox were identified by using nuclear magnetic resonance and mass spectroscopic methods.

Objectives

I. We studied the effects of various cycles of preconditioning (PC) (one cycle, 1XPC; two cycles, 2XPC; three cycles, 3XPC; and four cycles, 4XC) on infarct size in isolated hearts obtained from rabbits with hypercholesterolemia.

II. We studied the role of lipd rafts in ceramide and nitric oxide signaling in the ischemic and preconditioned rat hearts.

III. We sought to determine if Euryale ferox seeds (Makhana) could reduce myocardial ischemic reperfusion injury in rat hearts.

IV. The aim of our fourth study was to investigate the contribution of adrenocorticotrope hormone's fragment, ACTH (4-10), on the recovery of postischemic cardiac function. The effects of ACTH (4-10) on caspase-3 activity, cardiomyocyte and endothelial apoptosis, and HO-1 protein expression were studied.

Materials and methods

All animals (rabbits and rats) received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985).

Induction of HC in rabbits

Age-matched control rabbits were fed ordinary laboratory chow, whereas in the hypercholesterolemic groups, rabbits received laboratory chow enriched with 1.0% cholesterol for 8 weeks *ad libitum*. Serum cholesterol levels were measured after 0, 2, 4, 6, and 8 weeks of the cholesterol-enriched diet by an automatic analyzer using Boehringer cholesterol kits (Ingelheim, Germany).

Ischemia and reperfusion in preconditioned hearts

In noncholesterolemic age-matched controls, and 8-week hypercholesterolemic groups (n = 6 in each group), one, two, three, and four cycles of PC (1XPC, 2XPC, 3XPC, and 4XPC, respectively), each consisting of 5 min of global ischemia followed by 5 min of reperfusion, were carried out before the induction of 30 min of normothermic global ischemia followed by 120 min of reperfusion. One to four cycles of ischemic PC were selected for our studies in the isolated working rabbit heart, because generally one to four PC cycles protected the ischemic myocardium, in various degrees, against the incidence of reperfusion-induced damage in the models of PC in intact myocardium

Examination of the role of lipid rafts

The study used two different protocols using isolated working rat heart model: i) isolated working rat hearts preperfused with the Krebs Henseleit buffer (KHB) for 15 min in the absence or presence of desipramine followed by 30 min ischemia and 2 h of reperfusion and (ii) ischemic preconditioning protocol by subjecting the hearts to four cyclic episodes of 5 min ischemia each followed by another 10 min reperfusion. The preconditioned hearts were also subjected to 30 min ischemia and 2 h of reperfusion.

Pre-treatment with Makhana extract

Fruits of Makhana were obtained directly from the cultivator in Bihar, India. Makhana was made into a slurry-like preparation with water by using a blender. The final slurry was sonicated and dissolved either in alcohol (alcohol extract) or in water (water extract), and used directly either for feeding experiments or for acute perfusion experiments. Sprague Dawley male rats were randomly assigned to one of the control or fed groups. For the acute model the isolated hearts were perfused either with KHB buffer only (control) or with Makhana extracts [25 μg/ml, 125 μg/ml or 250 μg/ml concentration]. For the chronic model, a group of animals were fed makhana by gavaging in two different doses, 250 mg /kg/day and 500 mg /kg/day.

Pre-treatment with ACTH (4-10)

Sprague–Dawley rats weighing 275–300 g were subcutaneously injected with 0, 50, 200 μg/kg of ACTH (4-10) (Sigma, Budapest, Hungary) in saline buffer, and after 12 h later, hearts were isolated.

Isolated working heart preparation

Rabbits were anesthetized with ketamine/xylazine (40/5 mg/kg i.v.), rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p) and both were anticoagulated with heparin sodium (rabbit: 1000 IU/kg, rat: 500 IU/kg., i.v.). After ensuring sufficient depth of anaesthesia, thoracotomy was performed; the hearts were perfused in the retrograde Langendorff mode at 37 ◦C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mM: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulphate 1.2, and glucose 10). The Langendorff preparation was switched to the working mode following the washout period as previously described. At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The circuit was then switched back to the retrograde mode and the hearts were perfused either with KHB buffer only (control) or with Makhana extracts [25μg/ml, 125μg/ml or 250μg/ml concentration] for duration of 15 min. This was followed by a 5-min washout with KHB buffer, and then the hearts were subjected to global ischemia for 30 min and then 2 h of reperfusion. The first 10 min of reperfusion was in the

retrograde mode to allow for post ischemic stabilization and there after, in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 10-, 30-, 60- and 120- min reperfusion.

Cardiac function assessment

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc.,ValleyView, OH, USA) connected to a side arm of the aortic cannula, the signal was amplified using a Gould 6600 series signal conditioner and monitored on aCORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA, USA) (6.7). Heart Rate (HR), Left Ventricular developed pressure (LVDP) and the first derivative of developed pressure (dP/dT) were all derived or calculated from the continuously obtained pressure signal. Aortic flow (AF) was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL, USA) and coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart.

Determination of infarct size

Hearts for infarct size measurement were perfused, at the end of each experiment, with 15 ml of 1 % triphenyl tetrazolium (TTC) solution in phosphate buffer (88 mM Na2HPO4, 1.8 mM NaH2PO4) via the side arm of the aortic cannula and then stored at -70 °C for later analysis. Frozen hearts were sliced transversely, in a plane perpendicular to the apico-basal axis into 2-3 mm thick sections, weighted, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). With the use of the NIH Image 1.61 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels. The areas were measured by computerized planimetry software, these areas were multiplied by the weight of each slice, and then the results were summed up to obtain the weight of the risk zone (total weight of the left ventricle, mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Isolation of caveolin-rich membrane fractions

The hearts were homogenized in sodium carbonate buffer containing protease inhibitor cocktail, pH 11.0 using a Polytron homogenizer (three 10 s bursts) (Brinkman Instruments, Westbury, NY). The homogenate was sonicated (three 20 s bursts), and adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube as described previously. A 35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 35% sucrose—both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm in an SW41 rotor (Beckman Instruments, Palo Alto, CA) The light scattering band confined to the 5–35% sucrose interface was collected, which was enriched in caveolin without inclusion of most cellular proteins.

Immunoprecipitation with caveolin 1

From the top of each gradient, 1ml gradient fractions (from fractions 4–6) were collected to yield a total of 11 fractions as described elsewhere. Caveolin migrates mainly in fractions 5 and 6 of these sucrose density gradients. Immunoprecipitation was performed with an Immunoprecipitation starter Pack obtained from Amersham Pharmacia using a polyclonal antibody against caveolin-1 (Santa Cruz Biotechnology). Incubation conditions were maintained as instructed by the supplier. Western blot analysis was then performed with eNOS antibody (Santa Cruz Biotechnology) according to established method.

Estimation of ceramide and sphingosine-1-phosphatein caveolin-rich membrane fraction

Ceramide and its breakdown products were analyzed in the caveolin-rich membrane by HPTLC method as described previously.

Antibody array

Antibody array slides (BD Biosciences Clontech, Paolo Alto, CA) consisting of over 500 individual antibodies spotted in duplicate upon a glass slide with two such slides making up one experiment. The heart tissue from the left ventricle (100 mg) was homogenized in 1 ml extraction/labeling buffer (supplied with the kit, BD Biosciences). Protein concentration was measured using Pierce BCA Protein Assay reagent kit and the samples were diluted to 1.1 mg protein per ml Cy3 and Cy5 dyes (Amersham Biosciences, Piscataway, NJ) were each diluted

with 110 ml extraction/labeling buffer. Aliquots of each protein sample were separately labelled with 50 ml each of the dyes, and the unbound dye was removed by filtration of the samples through PD-10 columns (Amersham Biosciences), Cy-5 labelled proteins from each group of sample (100 mg each) were mixed with Cy3 proteins from the other group of sample. From each of these mixtures, 20 mg protein was incubated independently with antibody micro array slides for 30 min and washed in buffers. The slides were dried and scanned immediately using Perkin-Elmer Life Sciences Scanarray Express. The images for each slide scanned separately for Cy3 and Cy5 labels were merged and analyzed using Imagene software (BioDiscovery, Inc) to produce text files with signal intensities. The replicate values within each slide were averaged and an internally normalized ratio (INR) was calculated using Microsoft Excel sheet corresponding to our lot number downloaded from the BD Biosciences website. A scatter plot was drawn using Silicon Genetics GeneSpring software. INR is defined as ratio1/ratio2 and ratios 1 and 2 correspond to the ratios Cy5 and Cy3 relative fluorescence units in slide 1 and slide 2.

In vitro ROS scavenging activities

The amount of free radicals scavenged by Makhana extracts was determined by comparing with known rate of oxygen radical generation using SOD (for O_2^-) and DMTU (for OH^-). Superoxide radicals were generated by the action of xanthine (100μM) on xanthine oxidase (8 mU) in a reaction mixture containing 10 mM phosphate buffer (pH 7.4) and 10 μM EDTA in a total volume of 1 ml. To generate OH^- radical, 100 μM FeCl3 and 100 μM EDTA were added to the O_2^- generating system.

Determination of cardiomyocyte and endothelial cell apoptosis

The formaldehyde-fixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick) and deparaffinized with a graded series of xylene and ethanol solutions. Immunohistochemical detection of apoptotic cells was carried out using TUNEL in which residues of digoxigenin-labelled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme which catalyzes a template-independent addition of nucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA. The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody as described by the manufacturer (Apop Tag Plus, Oncor Inc., Gaithersburg, MD). The sections (n = 5) were washed in PBS three

times, blocked with normal rabbit serum and incubated with mouse monoclonal antibody recognizing-α-sarcomeric actin (Sigma-Aldrich Biotec Inc.) followed by staining with TRITC-conjugated rabbit anti-mouse IgG (200:1 dilution). For detection of apoptosis in endothelial cells the sections were first stained with TUNEL (FITC staining). The sections were then incubated with rabbit polyclonal anti-von Willebrand factor (Sigma-Aldrich Biotec Inc., St. Louis, MO, USA) as a primary antibody followed by incubation with tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG as a secondary antibody. The fluorescence staining was viewed with laser confocal microscopy (Fluoview, Olympas, Tokio, Japan). For the quantitative purpose, the number of TUNEL-positive cardiomyocytes and endothelial cells were counted on 100 high power fields (HPF, magnification × 600) from the endocardium through the epicardium of the mid portion of the left ventricular free wall in five sections from each heart. Representative confocal images show von Willebrand factor-positive endothelial cells (strong red staining of the cytosol) which are negative for TUNEL staining (absence of green staining in the nucleus) as well as those positive for TUNEL staining (magnification ×1200).

Measurement of caspase III activity by immunohystochemistry

The free-floating sections of the heart were first incubated with biotinylated goat anti-caspase-3 antibody (Sigma, St. Louis, MO, USA; diluted 1:1000) for overnight at 4 ^{o}C. The immunological and immunocytochemical characteristics of antibody have been published earlier. Sections were then transferred into a solution of biotinylated rabbit antibody (Vector Laboratories, Burlingame, CA, USA; diluted 1:200) for 50 min at room temperature, than avidin-biotinylated-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA; diluted 1:100) for 4 h at room temperature, and were completed with a diaminobenzidine chromogen reaction. Prior to the antibody treatments, sections were kept in 10 % normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 50 min. All incubations were performed under continuous gentle agitation, and all of antibodies were diluted in 10 mM phosphate-buffered saline (PBS, pH 7.4) to which 0.1 % Triton X-100 and 1 % normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) were added. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium (Fluka, Buchs, Switzerland).

Western blot method

Total protein (50 μg or 100 μg) in the Clontech Extraction buffer was added to an equal volume of sodium dodecyl sulphate (SDS) buffer and boiled for 10 min before being separated on 12 % SDS polyacrylamide gels in running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3) at 200 V. The Precision plus Protein Kaleidoscope standards (10 μl) (Bio-Rad Laboratories, CA, USA) were used as molecular weight standards. The gel was transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA) at 100 V for 1 h in transfer buffer (25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol, pH 8.3). After blocking the membranes for 1 h in Tris-buffered saline (TBS-T) (50 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1 % (v/v) Tween-20 and 5 % (w/v) non-fat dry milk, blots were incubated overnight at 4 °C with the primary antibody. Membranes were washed three times in TBS-T prior to incubation for 1 h with horseradish peroxide (HRP)-conjugated secondary antibody diluted 1:2000 in TBS-T and 5 % (w/v) non-fat dry milk. Western blots were developed with the ECL Detection Reagents 1 and 2 (Amersham Biosciences, NJ, USA) and exposed to Kodak X-OMAT film.

Statistical analysis

In the ACTH (4-10) and preconditioning project, HR, CF, AF, LVDP, infarct size, caspase-3 and HO-1 activities were expressed as mean value ± SEM. A two-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free group by Bonferroni correction. A different procedure, because of the nonparametric distribution, was used for the distribution of discrete variables, such as the incidence of VF and VT. Thus, the chi-square test was used to compare individual groups.

In case of examination of the role of lipid rafts, the values for number of apoptotic cardiomyocytes and infarct sizes as well as ceramide and sphingosine-1-phosphatewere all expressed as the mean ± standard error of mean (S.E.M.). The statistical analysis was performed by analysis of variance followed by Bonferroni's correction for any differences between the mean values of all groups. Differences between data were analyzed for significance by performing a Student's *t*-test. The results were considered significant if $P < 0.05$.

In the Makhana project, the values for myocardial functional parameters, total and infarct volumes and infarct sizes are all expressed as the mean ± standard error of mean (SEM). Analysis of variance test was first carried out to test for any differences between the mean values of all

groups followed by Bonferroni's correction. If differences between established, the values of the treated groups were compared with those of the control group by a modified *t*-test. The results were considered significant if $p < 0.05$.

Results

I. Effects of PC in nonhypercholesterolemic and hypercholesterolemic hearts

The experimental condition defined for PC studies, 30 min of ischemia followed by 120 min of reperfusion, for infarct size measurement in both nonhypercholesterolemic and hypercholesterolemic hearts was selected. The results demonstrate that in hearts subjected to 1XPC, 2XPC, 3XPC, and 4XPC followed by 30 min of ischemia and 120 min of reperfusion, the infarct size was "cycle-dependently" reduced from its nonhypercholesterolemic control value of 39 ± 6% to 37 ± 5%, 25 ± 5%, 21 ± 7% ($p < 0.05$), and 20.0 ± 6% ($p < 0.05$), respectively. With HC (after 8 weeks), this PC-induced cardiac protection in infarct size was abolished, and a significant "cycle-dependent" increase in infarct size was observed in comparison with the nonpreconditioned hypercholesterolemic control group. Thus, it is reasonable to assume that the lack of PC-induced protective effect in the 8-week hypercholesterolemic ischemic/reperfused groups is dependent on the state of myocardium (previously diseased or not) and serum cholesterol levels.

II. Role of lipid rafts in ceramide and NO signaling

1. Effects of ischemia/reperfusion and PC on ceramide and sphingosine-1-phosphate accumulation in caveolae

We estimated the amount of ceramide and sphingosine-1-phosphate content in the isolated caveolae fraction. Significant amount of ceramide was associated with caveolae after ischemia/reperfusion. The amount of ceramide in caveolae fractionwas significantly lower (threefold less) when the heart was subjected to PC. In the preconditioned heart, the reduction of ceramide content was associated with an enhancement in sphingosine-1-phosphate content in the caveolae, suggesting that preconditioning triggers breakdown of ceramide in the caveolae with corresponding accumulation of sphingosine-1-phosphate. In contrast, there was no accumulation of sphingosine-1-phosphatein the ischemic/reperfused heart pre-perfused with desipramine.

2. Effects of ischemia/reperfusion and PC on the association of eNOS with caveolin-1

The examination of the expression of eNOS protein in the caveolin-1 immunoprecipitated fraction by Western blot analysis revealed that eNOS was increasingly associated with caveolin-1 fraction after ischemia/reperfusion. The association of eNOSwas reduced significantly when the heart was subjected to PC, suggesting that PC potentiated the release eNOS from the caveolae. The similar result was found when ceramide formation was blocked by preperfusing the heart with desipramine.

3. Effects of ischemia/reperfusion and PC on cardioprotection

As expected, ischemia/reperfusion caused significant amount of tissue injury as reflected by increased amount of myocardial infarct size and cardiomyocyte apoptosis. Both desipramine and PC reduced the tissue injury as evidenced by decreased amount of infarct size and apoptosis. Post-ischemic ventricular function was significantly higher in the PC hearts compared to non-PC hearts. LVDP and aortic flow were reduced during the postischemic reperfusion in all groups. However, the reduction in recovery was significantly less for the PC groups, which reached significance compared to non-PC group at 60 and 120 min of reperfusion. The reduction of ischemia/reperfusion-mediated contractile function was also partially restored with desipramine.

III. Effects Of Makhana

1. In vitro ROS scavenging activity

Two different concentrations of Makhana extracts were used for this purpose. At 0.005% concentration, water extracts scavenged 39% of O_2^- and alcohol extract scavenged 52% of O_2^- when compared with SOD [100%]. At 0.01% concentration, water extracts and alcohol extracts of Makhana scavenged 93% and 100%, respectively, of O_2^-. At 0.005% concentration, both water and alcohol extracts of Makhana scavenged 60% and 63%, respectively, of OH·when compared with DMTU [100%]. At 0.01% concentration, both water and alcohol extract scavenged almost 100% of OH.

2. Effects of Makhana on myocardial function

2/a Acute model

There were no differences in baseline function between all the four groups. In general, there were no significant differences between the three Makhana treated groups vs. control, on heart rate and coronary flow. As was expected, upon reperfusion, the absolute values of all functional parameters were decreased in all the groups as compared with the respective baseline values. The cardioprotective effects of makhana were evidenced by the noticeable differences in the aortic flow after 60 min of reperfusion. At 60 min and 120 min of reperfusion, aortic flow was significantly higher for both 125μg/ml and 250μg/ml doses compared to control. For LVDP and LVmaxdp/dt, Makhana treated group showed higher recover only after 120 min of reperfusion for both concentrations.

2/b Feeding model

Again, for baseline cardiac function, there were no differences in between all the three groups. Like acute model, the heart rate or coronary flow did not change significantly with any of the concentrations of Makhana compared to the control animals. Upon reperfusion all the cardiac parameter was decreased compare to their baseline values. The cardioprotective effects of Makhana were evident only during the end of the reperfusion. For example, significant recovery of aortic flow was evident at 60 min and 120 min of reperfusion for both 250 mg/kg and 500 mg/kg of Makhana. For LVDP, Makhana showed improved recovery only at 120 min of reperfusion for 500 mg/kg concentrations. At 120 min of reperfusion, Makhana improved LVmaxdp/dt at higher concentration only.

3. Effects of Makhana on myocardial infarct size

Infarct size (percent of infarct vs. total area at risk) was reduced in makhana-treated groups as compared to the control in both the acute as well as in the feeding models. In the acute model, Makhana reduced the infract size at both 125 µg/ml and 250 µg/ml concentrations. Although at 25 µg/ml, relatively lower infract size was obtained, but the differences between the two groups were not significant. In the feeding model, both at 250 mg/kg and 500 mg/kg concentrations, Makhana reduced myocardial infract size significantly compared to the control group.

4. Antibody array and western blot analysis

The set of proteins differentially expressed in the Makhana treated hearts was studied using public protein databases including SwissProt and LocusLink for their known biological functions and two proteins known to have an important role in cardioprotection were chosen to be validated through the Western blot technique. Analysis of antibody array data showed the upregulation of TRP32 [thioredoxin-related protein-32] by about 1.3-fold obtained from scatter plot of the signal intensity data. Western blot analysis was performed for TRP32 and thioredoxin according to the Methods described in the Methods Section. Makhana treat hearts showed about 2.8-fold increase in TRP- 32 and 4.5-fold activation of thioredoxin proteins.

IV. Effects of ACTH (4-10)

1. Effect of ACTH (4-10) on cardiac arrhythmias

The incidence of reperfusion induced VF in isolated hearts obtained from rats subcutaneously treated with 0 μg/kg (untreated control), 50 μg/kg and 200 μg/kg of ACTH (4-10), 12 hours before the isolation of hearts and induction of ischemia and reperfusion. Thus, our results show that the incidence of reperfusion-induced ventricular fibrillation was significantly reduced from its untreated control value of 100 % to 90 %, and 40 % (*$p<0.05$), respectively. In the reduction of the incidence of reperfusion-induced VT followed the same pattern. The incidence of reperfusion-induced ventricular tachycardia was significantly reduced from its untreated control value of 100 % to 100 % and 50 % (*$p<0.05$) with the doses of 50 and 200 μg/kg of ACTH (4-10). These data and our previous results represent that higher dose of ACTH (4-10) was able to reduce the incidence of VF and VT, however it was not so effective than 200 μg/kg of α-MSH (ACTH 1-13).

2. Effect of ACTH (4-10) on cardiac function

In rats treated with various doses of sc. ACTH (4-10) significantly improved postischemic contractile function in compared to the untreated controls. It can be one of the consequences of the VF or VT prevention. Interestingly, the ACTH (4-10) at any doses had no effect on heart rate. However, aortic flow was increased from its drug-free ischemic/reperfused control value of 6.5 ± 0.9 ml/min to 6.5 ±1 ml/min (NS) and 24.8 ± 1.8 ml/min (*$p<0.05$) with the concentrations of 50 and 200 μg/kg of ACTH (4-10), respectively. Postischemic CF and LVDP also showed

significant improvement in hearts obtained from rats treated with 50 and 200 μg/kg of ACTH (4-10). As it is shown, the lower dose of ACTH (4-10) failed to improve the postischemic recovery in HR, CF, AF, and LVDP.

3. Extent of infarct size

Infarct size in the control hearts (35.3 ± 6 %). In hearts treated with 200 μg/kg of ACTH a marked reduction in infarct size (20.16 ± 1.5 % (*p<0,05)) at the end of the reperfusion was observed, suggesting that ACTH (4-10) can prevent reperfusion-induced injury. The lower concentration (50 μg/kg) of the ACTH (4-10) failed to reduce infarct size (33.0 ± 2.6 %).

4. Measurement of caspase-3 activity

Visualization of the caspase-3 protein after immunoperoxidase reaction revealed a decreased activity of these proteins in treated hearts subjected to 30 min of ischemia followed by 2 h of reperfusion. Immunoreactive caspase-3 was localized mainly in cytoplasm of cardiomyocytes and vascular smooth muscle cells. Caspase-3 activity in hearts obtained from rats treated with 0 μg/kg (untreated control) 50 μg/kg and 200 μg/kg ACTH (4-10), and subjected to ischemia/reperfusion. Caspase activity, using immunohistochemistry, was reduced in treated subjects indicating by a reduction in brown staining intensity in the myocardium. The results of caspase-3 activity were confirmed by Western blot analysis (see below).

5. Cardiomyocyte and endothelial apoptosis

The results show that TUNEL-positive nuclei were condensed; representing apoptotic cells. Similar to myocardial infarct size, the numbers of apoptotic cardiomyocytes and endothelial cells were reduced significantly when hearts were pretreated by the higher dose of ACTH (4-10). Total numbers of cardiomyocytes at 100 high power field, which cover almost all the mid portion of left ventricular free wall, were examined for detecting apoptotic cells. The data were expressed in counts/100 high power field and not in percent of apoptotic cells. Treatment with 200 μg/kg of ACTH (4-10), the numbers of endothelial and cardiomyocyte apoptotic cells were also significantly reduced. However, the lower dose of ACTH (4-10) did not cause a significant change.

6. Effects of ACTH (4-10) on the expression of caspase-3 and HO-1

The ischemia/reperfusion induced caspase-3 expression was decreased by the higher dose of ACTH (4-10), and Western blot analysis confirmed the semi-quantitative immunohistochemistry results. In addition, we examined whether HO-1 signaling is involved in ACTH (4-10) treatment. Pretreatment with the higher dose (200 μg/kg) of ACTH (4-10) increased the expression of HO-1, while the housekeeping gene expression (GAPDH) was unchanged.

Discussion

a.) Effects of PC in nonhypercholesterolemic and hypercholesterolemic hearts

About two decades after the discovery and intensive investigation of the "first" and after a decade of the observation of the "second window protection" of PC in intact myocardium, relative little attention has been paid to the PC phenomenon in previously diseased hearts. Indeed, thousands of studies are now available and indicate the powerful protective effect of PC against sustained myocardial ischemia- and reperfusion-induced damage in various models of intact myocardium. Thus, as a consequence, many pathways have been investigated and suggested as a potential mechanism responsible for the adaptation of myocardium to anaerobe conditions in healthy myocardium. However, it is highly probable that a number of interacting mechanisms, rather than a single mechanism, combine to determine the outcome of PC to ischemia/reperfusion-induced damage, and a variety of such triggers have been postulated, including a-adrenoceptor, protein tyrosine kinase signaling related to nitric oxide production, phospholipase D, bradykinin, opioid receptors, prostanoids, and angiotensin receptors.

It was not the aim of our experiments to study and determine what could be the most important PC mechanism(s), if there is any, in hypercholesterolemic myocardium, but we studied the final outcome of PC on infarct size. To determine how the HC affects PC-induced protection to reperfusion-induced injury, we have compared infarct size in isolated hearts obtained from hypercholesterolemic and nonhypercholesterolemic age-matched control rabbits. The results of our study show that HC resulted in an increased infarct size in the isolated rabbit heart. With progressive HC (after 8 weeks), the development of cardiac failure was observed before the induction of PC and ischemia/reperfusion in our model.

The mechanism by which hyperlipidemia could influence the outcome of cardiac ischemia and PC is currently not known; however, the accumulation and redistribution of tissue/membrane cholesterol and the resulting changes in mitochondrial and sarcolemmal membrane microviscosity, rather than a direct effect of high serum lipoprotein levels and coronary atherosclerosis, may account for this phenomena. Our results clearly show that the protective effect of ischemic PC cannot be observed in hypercholesterolemic hearts, indicating that the PC might be an "intact heart" phenomenon.

b.) Role of lipid rafts in ceramide and nitric oxide signaling

The results of the present study demonstrate that significant amount of ceramide is accumulated in the caveolin-rich membrane as a result of ischemia/reperfusion. An increased amount of eNOS becomes associated with caveolin-1 in these hearts. PC results in a reduction of ceramide with corresponding increase in its metabolite, sphingosine-1-phosphate. The amount of caveolin-1-associated eNOS is reduced when the heart is subjected to PC. Inhibition of ceramide formation with desipramine also resulted in a reduced accumulation of ceramide and eNOS association with caveolin-1, but without the accumulation of sphingosine-1-phosphate. Both PC and desipramine improved ischemia reperfused mediated cardiac dysfunction and reduced myocardial infarct size and cardiomyocyte apoptosis. This raises an interesting possibility that although ischemia/reperfusion increases the amount of eNOS, these proteins becomes associated with caveolin-1, and may not be available to the heart. PC lowers the association of eNOS with caveolin-1 and the releases NOS, so that NO may be available to the heart.

The role of NO in cardioprotection has been a controversial issue for a long time. Despite ischemia/reperfusioninduced increase in the amount of NO in the heart, it has been found that exogenous NO is cardioprotective. This has been explained by the fact that reperfusion causes a reduction of NO release. Our results tend to provide an explanation for the paradoxical role of NO in cardioprotection. As shown in our study, ischemia/reperfusion-mediated increase in NO is unavailable to the reperfused myocardium because eNOS becomes associated with caveolin-1. It appears that PC triggers the release of eNOS, at least partially, so that the released NO can be utilized by the reperfused myocardium. Sphingomyeline, the major phospholipids class located in the cellular membranes of myocytes, is broken down during ischemia and reperfusion, generating intracellular ceramide and sphingosine in the heart, the former being instrumental for ischemic

injury. A previous study from our laboratory demonstrated sphingomyeline breakdown in the ischemic reperfused heart resulting in ceramide formation. Sphingomyeline hydrolysis is catalyzed by sphingomyelinases that hydrolyze the phosphodiester bond of sphingomyeline yielding ceramide and phosphocholine. The present study also showed an increased formation of ceramide after reperfusion in the ischemic myocardium. Apparently, desipramine by inhibiting ceramide accumulation in the ischemic reperfused heart provided cardioprotection. Under normal conditions, ceramide can be rapidly metabolized into sphingosine and sphingosine-1-phosphate, the later being cardioprotective. Shingosine-1-phosphate transmits survival signal by activating PI-3-kinase and Akt as well as by inducing Bcl-2. Ischemia/reperfusion has been shown to be associated with the phospholipids metabolism and phosphoinositide response. The present study confirms these previous reports and further demonstrates that degradation of sphingomyeline results in the accumulation of ceramide, which becomes located in the caveolae. In the ischemic heart, ceramide, being pro-apoptotic, results in cardiac dysfunction. In fact, sphingomyeline breakdown products are now recognized as important players in apoptotic cell death. It should be noted that although both desipramine and PC provided cardioprotection, their mechanisms of cardioprotection appeared to be different from each other. Cardiac performance was depressed following ischemia as expected, due to reduction of contractile function and cell death. Both desipramine and PC improved cardiac performance, but through different mechanisms. Desipramine reduced the formation of ceramide, which is likely to reduce the ability to form the lipid raft, whereas PC stimulated the breakdown of ceramide resulting in the formation of cardioprotective shingosine-1-phosphate. Shingosine-1-phosphate is a metabolite of ceramide produced after phosphorylation of sphingosine by sphingosine kinase; but unlike ceramide, it transmits survival signal and protects the cell from ceramide-mediate apoptosis.

Both PC and ceramide inhibited the association of eNOS with caveolin-1. Recently, ceramide has been found to be localized within the lipid raft. Ceramide can induce the formation of raft platform domains by both stabilizing and associating with the lipid rafts. A recent study showed that stress-induced changes in raft microdomains led to altered receptor tyrosine kinase signal transduction through the modulation of caveolin-1 by ceramide. Ceramide-caveolin-1 interaction is believed to occur within lipid raft membrane, and the results of our study certainly support this notion.

c.) Effects of Makhana

There are several salient features of the study. First, Makhana either given acutely by preperfusing isolated hearts with makhana extract or chronically by feeding the animals for 21 days, provided cardioprotection as evidenced by the improved postischemic ventricular performance and reduction of the infarct size. The extracts of Makhana dose-dependently scavenged superoxide anions and hydroxyl radicals. Most interestingly, the two redox-regulated proteins TRP-32 and Trx-1 were induced in the Makhana-treated hearts.

Since, massive generation of ROS in concert with reduced antioxidant reserve play a crucial role in the pathophysiology of myocardial ischemic reperfusion injury, we sought to determine if Makhana could render the heart resistant to ischemic reperfusion injury. *In vitro* experiments demonstrated that even at extremely low concentrations (0.005% wt/vol), Makhana is capable of scavenging O–2 and OH· radicals 0.005%, (wt/vol); at 0.01% (wt/vol) concentration, it can scavenge these ROS to nearly 100%. The presence of high amount of OH·has been shown in the ischemic reperfused myocardium. It is likely that Makhana reduced the cellular injury by scavenging ROS.

Several reports exist in the literature to indicate cardioprotective roles of redox proteins such as thioredoxin, which is ubiquitously present and is evolutionarily conserved from prokaryotes to eukaryotes. A recent study demonstrated a reduction of thioredoxin-1 (Trx-1) protein in the ischemic reperfused myocardium. When the same heart was adapted to ischemic stress by preconditioning with repeated cyclic episodes of small duration of ischemia and reperfusion, there was an increased induction of Trx-1 expression. Inhibition of Trx-1 expression resulted in reduced post-ischemic ventricular recovery and increased myocardial infarct size in the preconditioned heart. Corroborating with these findings, transgenic mouse hearts overexpressing Trx-1 were resistant to ischemic reperfusion injury as compared to the hearts from wild-type mice.

TRP32, also ubiquitously expressed in mammalian cells, is a 32-kDa protein with an N-terminal Trx domain. The precise function of TRP32 remains unknown, but it is likely to be responsible for redox regulation of cytoplasmic proteins and transcription factors. The DNA binding activity of redox-regulated transcription factor NFκB is known to be regulated by a thiol-redox control mechanism, and TRP32 augments the DNA binding and transcription activities of the p50 subunit of NFκB. An essential role of NFκB in cardioprotection has been established.

d.) Effects of ACTH (4-10)

After the ischemic period without any doubt reperfusion, although a prerequisite for the survival of ischemic cells, is not without risk. Thus, components of the reperfusion process might temporary increase the presumption of ventricular arrhythmias, and heart failure decreasing the chance of the myocardial recovery to steady-state contractile function. In view of the above statement, the utmost importance is to protect the tissue from ischemia-reperfusion-induced damage. Currently it is also well known that many factors play a critical role in ischemia and reperfusion, but the relative importance of these factors is uncertain and controversial.

In our previous study we have described that melanocortin peptides, i.e. α-MSH, significantly attenuated the life threatening consequences of myocardial ischemia. Thus, in the present study we investigated whether ACTH (4-10) pretreatment could afford cardiac protection against ischemia/reperfusion-induced cardiac damage. Thus we investigated that ACTH (4-10) is able (i) to improve postischemic cardiac function, (ii) to reduce the incidence of reperfusion induced ventricular fibrillation and tachycardia, (iii) to decrease the myocardial infarct size, (iv) to attenuate the apoptotic endothelial and myocardial cell death, and (v) to influence the caspase-3 activity and HO-1 signaling. The action mechanisms of melanocortins remain on speculation and a possible explanation is based on previous investigations. One of the protective effects of adrenocorticotropic hormones in myocardial infarct seems to be due to the capacity of these peptides to inhibit the overproduction of free radicals and to their anti-inflammatory activity. The antiinflammatory action of melanocortins is related to the melanocortin-1 and melanocortin-3 receptors (MC1-R, MC3-R). The anti-inflammatory effects of melanocortin peptides are also associated with a reduced production of proinflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, tumor necrosis factor (TNF), and with an enhanced genesis of the anti-inflammatory IL-10 and of the angiogenic factor IL-8. The role of melanocortin peptides on the immune system appears to be complex and depends upon several concerted action. Thus, exogenously applied α-MSH was found to inhibit the lipopolysaccharide (LPS)-induced release of proinflammatory TNF-α in whole blood under in vitro condition. Furthermore, the POMC peptides are widely expressed in the body. Thus, POMC and POMC-derived ACTH-peptides have been shown to be produced by monocytes cells, melanocytes and endothelial cells. Therefore, it is reasonable to believe that endogenous mediators such as α-MSH or ACTH fragments could contribute to the reduced release of inflammatory mediators in the heart and other organs.

Programmed cell death in the myocardium has been also linked to ischemia/reperfusion injury as well as to extreme mechanical forces associated with an increase in ventricular loading. Moreover, hypoxia and ischemia activate the suicide program of cardiomyocytes and endothelial cells under in vitro and in vivo conditions. Apoptosis also frequently occur as a harmful mechanism in both acute and chronic tissue injury, yet the signal transduction is responsible for this action is not precisely known. The endpoints of our previous study showed that higher doses of α-MSH reduced the apoptotic cell death (38). In the present study, we investigated whether ACTH (4-10) which also contains the His-Phe-Arg-Trp "core" could influence the apoptotic capability of cardiomyocyte and endothelial cells.

Caspase-3 is an important apoptotic marker in the cellular and suicide cascade which is well known and documented. Caspase-3 is a downstream effector of caspase-9 which is activated by cytochrome-c by mitochondria or by caspase-8. Caspase-3 protein is also related to the Bcl-2 family, Bax-like, Fas/FasL, TNF-α, TNF−α receptor, and right ventricular dysplasia and end-stage heart failure. Therefore, it is important to consider, whether cardiac function and infarct size can be modified by caspase-3 down/up regulation. However, blockade of caspase activation with a specific inhibitor was reported to decrease infarct size after coronary artery ligation in rats, but the cause-effect relationship between caspase-3 and cardiac function has not been established yet. The results of our study showed, for the first time, that the beneficial effect of ACTH (4-10) is due to its ability to reduce caspase-3 activation, which is linked, at least in part to the reduction of apoptotic cell death. Caspase-3 activity, shown by immunohistochemistry and Western blot analysis, was reduced in the ACTH (4-10) treated group. Since caspase-3 activity was diminished, accordingly the number of death cells of cardiomyocytes and endothelial cells was also reduced in the ACTH (4-10) treated group. Moreover, one of the reasons for decreased apoptosis could be the overexpression of the HO-1 protein. HO-1 induction appears to be an important factor in ischemic/reperfused protection of the myocardium. The role of heme oxygenase signaling in various disorders (ischemia/reperfusion, hypertension, cardiomyopathy, organ transplantation, endotoxemia, lung disease, and immunsuppression) has already been well documented. Zou et al have shown that treatment with α-MSH at 1 hour of reperfusion led to an increased HO-1 protein expression in gut ischemia/reperfusion model. The exact antiapoptotic mechanism(s) of HO is (are) not clearly known, therefore it is under speculation. One possible mechanism could be via the bilirubin system, because bilirubin has been shown to protect

cardiomyocytes against oxidative damage. Therefore, we investigated whether ACTH (4-10) is also able to affect the HO-1 protein level in our model. Without doubt, it is reasonable to believe that a reduction in programmed cell death limits infarct size in ACTH treated rats.

List of publications used for the dissertation

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