THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

THE RECOVERY OF ISCHEMIC MYOCARDIUM: PHARMACOLOGICAL INTERVENTIONS AND MECHANISMS

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Introduction

Myocardial reperfusion by transluminal angioplasty, thrombolytic therapy, and bypass surgery has emerged the fundamental strategy in the management of acute ischemic episodes. Furthermore, it is well known that spontaneous reperfusion after coronary artery spasm or thrombosis is a common event in humans with coronary artery diseases. In the past three decades, intensive research was done, and the results confirmed that reperfusion, restoration of flow, nutrition, and oxygen to the previously ischemic tissue, triggers sudden metabolic, electrophysiological, morphological, and functional changes. The mechanisms underlying the genesis of ischemia/reperfusion-induced myocardial damages are complex and not clearly understood. It is highly possible that a number of interacting mechanisms combine to determine the extent of reperfusion-induced damage including α and β receptors, ions and their exchangers, fatty acids and phospholipids, free radicals, various gene regulations, and apoptotic signals. Recently, the concept has been introduced that α-melanocyte-stimulating hormone (α-MSH) may protect renal cells and tissues against ischemia/reperfusion-induced injury. α-MSH (1-13 amino acids) is an active fragment of adrenocorticotropic hormone (ACTH, 1-39 amino acids), and it has been reported that ACTH and its fragments (in various degree) including α-MSH have a prompt and sustained resuscitating effect in conditions of severe tissue hypoxia, either due to hypoperfusion including hemorrhage shock, cardiogenic shock, splanchnic artery occlusion-induced shock, or to prolonged respiratory arrest. Relatively little attention, to our knowledge, has been paid on the effects of ACTH and its fragments, especially α-MSH, on the recovery of postischemic cardiac function. The aim of our study was to analyze (i) the effects of α-MSH on postischemic cardiac function, (ii) on myocardial infarct size and the incidence of reperfusion-induced ventricular fibrillation, and (iii) on apoptotic cell death.

Another aim of our work was to study the effect of cardiac protection associated with preconditioning (PC) in diseased myocardium. The so-called “first window of protection,”

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. However, PC and the great majority of animal studies, as well as the proposed PC mechanisms, have been done, without any preliminary coronary or myocardial diseases, in intact rabbit, rat, dog, and pig myocardium. The present study was designed to examine whether an impaired capability of the myocardium to adapt to repeated ischemic insults, the PC, was involved in the mechanisms responsible for the increased severity of ischemic attacks in rabbit hearts with hypercholesterolemia (HC) induced by exposure to a cholesterol-enriched diet.

In addition, recently, a number of studies have been devoted to understanding the cause of the so-called French paradox, the observation that in France and other Mediterranean countries the morbidity and mortality of coronary heart disease in absolute value and in consideration of their rate to other manners of death are significantly lower than in other developed countries, despite high consumption of fat and saturated fatty acids. Wines, especially reds, incorporate about 1,800–3,000 mg/l of polyphenolic compounds, many of which are potent anti-oxidants capable of scavenging free radicals and inhibiting lipid peroxidation in vitro and in vivo. Recent studies from our laboratory demonstrated that red wine and its polyphenolic constituents could provide cardioprotection against ischemic reperfusion injury. Because both red and white wines are made from the grapes, and grapes contain such polyphenolics as resveratrol, catechins, procyanidins, flavonols, and anthocyanins that are also present in red wine, therefore, we sought to determine whether grapes could provide cardioprotection.

Materials and methods

Isolated working heart preparation:

Sprague Dawley rats (320-350 g) were anesthetized with ether then intravenous heparin (500 IU/kg) was injected. Additional experiments were carried out with male, adult New Zealand white rabbits weighing 2.2 – 2.6 kg were anesthetized intravenous heparin (1000 IU/kg) and ketamine/xylazine (40/5 mg/kg) were intravenously injected. After thoracotomy, the heart was excised, the aorta was cannulated, and the heart was perfused (at 37 ^{o}C) according to the Langendorff method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The Langendorff preparation was switched to the 'working' mode following the washout period.

Induction of regional and global ischemia:

After a 10-min aerobic perfusion of the heart, the left main coronary artery was occluded, a suture mounted on a curved needle was placed under the origin of the main descending coronary artery and the ends of the suture were passed through a small plastic tube. Myocardial ischemia was induced by clamping the plastic tube onto the surface of the heart with a surgical clamp. Reperfusion was initiated by releasing the snare. Infarct size was measured in hearts subjected to coronary artery occlusion (regional ischemia) in drug-free and α-MSH treated groups. α-MSH was subcutaneously injected 12 h before the isolation of hearts and the onset of ischemia/reperfusion.

For the measurement of cardiac function (heart rate, coronary flow, aortic flow, left ventricular developed pressure), the incidence of reperfusion-induced ventricular fibrillation (VF), and DNA laddering for apoptosis, a model of global cardiac ischemia was used. Thus, after 10 min aerobic perfusion of the heart, the atrial inflow and aortic outflow lines were totally clamped at a point close to the origin of the aortic cannula. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. An epicardial electrocardiogram (ECG) was recorded by two silver electrodes attached directly to the heart. ECGs were

analyzed to determine the incidence of ventricular fibrillation. Before ischemia and during reperfusion, the values of heart rate, coronary flow, and aortic flow were registered.

Determination of infarct size:

Hearts for infarct size measurement were perfused, at the end of each experiment, with 30 ml of 1% triphenyl tetrazolium solution in phosphate buffer via the side arm of the aortic cannula then stored at -70˚C for later analysis.

Ischemia and reperfusion in preconditioned hearts:

In noncholesterolemic age-matched controls, and eight-week hypercholesterolemic groups (n=6 in each group), one, two, three, and four cycles of preconditioning, each consisting of 5 min global ischemia followed by 5 min reperfusion, respectively, have been carried out before the induction of 30 min of normothermic global ischemia followed by 120 min of reperfusion. Preconditioning studies have been carried out after eight-week of cholesterin diet and comparisons were made with the age-matched noncholesterolemic groups. Cardiac function was registered before the induction of global ischemia, and after 60 and 120 min of reperfusion.

DNA fragmentation:

Apoptosis is very well characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or multiples therefore that are readily detected as a DNA ladder by gel electrophoresis. Genomic DNA was isolated from untreated and α-MSH treated hearts to carry out DNA laddering. Briefly, cardiac tissues (about 60 mg) were pelletted in eppendorf tubes using 1,000 *g* for 5 min, and supernatants were removed. Three hundred microliteres of lysis buffer were added and vortexed, then sixty µl of proteinase K (from a stock

solution) were added to each sample. Mixtures were vortexed and incubated for 2 h at 55 ^{o}C, then binding buffer (300 µl) was used and samples were further incubated for 20 min at 72 ^{o}C in the presence of 150 µl of isopropanol. Gel loading buffer (5 microliters) was used, and DNA samples were electrophoresed on a 1.8% agarose gel with ethidium bromide. DNA laddering was visualized and photographed under ultraviolet transillumination.

Induction of HC:

The experiments were carried out with male, adult New Zealand white rabbits weighing 2.2 – 2.6 kg. Rabbits were continued to feed ordinary laboratory chow (age-matched controls), whereas in hypercholresterolaemic 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 cholesterol enriched diet, respectively.

Estimation of malonaldehyde:

Malonaldehyde was assayed in the heart as to monitor the development of oxidative stress. The malonaldehyde was derivatized using 2,4- dinitrophenylhydrazine. Aliquots of 25 µl of derivatized malonaldehyde in acetonitrile were injected onto a Beckman Ultrasphere C18 (3 mm) column in a Waters high-performance liquid chromatographer. The products were eluted isocratically and detected at 307, 325, and 356 nm. The amount of malonaldehyde was quantified using a Maxima software program.

Evaluation of standardized grape extract for free radical scavenging activities:

Free radical scavenging activities were determined by chemiluminescence technique using a Lumat LB 9507 luminometer. Superoxide anions were generated by the action of xanthine oxidase (7 mU) on xanthine (100 µ*M*) in a 500-µl reaction mixture containing 10 m*M* of

sodium carbonate, pH 9.0, and 28 μ*M* of luminol. The reaction was activated by xanthine and detected by luminometer. The hydroxyl radical assay mixture contained xanthine oxidase (0.6 mU), 10 μ*M* sodium carbonate, pH 9.0, 28 μ*M* luminol, 100 μ*M* xanthine, 100 μ*M* ethylenediamine tetra-acetic acid, and 100 μ*M* ferric chloride. The scavenging activities were compared with 10 mU/ml superoxide dismutase for superoxide anion and dimethyl thiourea for hydroxyl radical.

Statistical analysis:

The values for myocardial function, infarct sizes, (n=6 in each group) were all expressed as the mean ± standard error of the mean (S.E.M.). Two-way analysis of variance (ANOVA) was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of α-MSH treated groups were compared with those of the drug-free control group by multiple t-test followed by Bonferroni correction. If differences were established the values of SGE treated groups compared with those of the drug free control group by multiple t-test followed by Scheffe test, and Dunnett's correction was used in PC studies. For the distribution of discrete variables such as the incidence of VF, which follows a nonparametric distribution (non-Gaussian distribution), an overall chi-square test for a 2xn table was constructed followed by a sequence of 2x2 chi-square tests to compare individual groups. A change of $p<0.05$ between the drug-free control and treated groups was considered to be significant.

Results

I. Effects of standardized grape extract:

Effects of SGE on myocardial function:

The hearts of the rats given SGE orally significantly improved postischemic contractile function as compared with those given control diets. Three different doses were used. SGE had no effect on heart rate and coronary flow. Aortic flow was reduced in all groups during the postischemic reperfusion. However, SGE at 100-mg/kg and 200-mg/kg doses significantly improved the aortic flow during the reperfusion as compared with control and 50 mg/kg dose groups. Stroke volume and cardiac output exhibited significant improvement for SGE-fed rat hearts. Postischemic LVDP and its first derivative (LVmaxdP/dt) also showed significant improvement for the hearts of the rats given 100 mg/kg and 200 mg/kg of SGE.

Effects of SGE on myocardial infarct size:

Myocardial infarct size expressed as the percent infarct of the entire risk area was 33.8% for the control hearts subjected to 30 min of ischemia followed by 2 h of reperfusion. There was no change in the infarct size for the hearts of the rats fed 50 mg/kg of SGE. However, there was a significant reduction in the infarct size for the hearts of the animals given either 100 mg/kg or 200 mg/kg SGE.

Effects of SGE on malonaldehyde formation in the heart:

Malonaldehyde is the presumptive marker for the development of the oxidative stress in the heart. Malonaldehyde content of the heart was significantly decreased in the hearts of the animals fed SGE; i.e., the amount of malonaldehyde in the heart was 20% less in the hearts of the rats fed 50 mg/kg SGE and about 50 and 60% less for the hearts of those given 100 mg/kg and 200 mg/kg SGE, respectively.

In vitro oxygen free radical scavenging by SGE:

To further confirm that lowering of oxidative stress in the SGE-fed rat hearts was due to oxygen free radical scavenging activities of SGE, the scavenging activities were measured in vitro. The results for the chemiluminescence response of the chemically generated superoxide and hydroxyl radicals in the presence of SGE and luminol. Inhibition efficiency was calculated as compared with that of superoxide dismutase for superoxide anion and of dimethyl thiourea for hydroxyl radicals. The results demonstrate that SGE scavenged both superoxide and hydroxyl radicals efficiently.

II. Effects of alpha-meloanocyte-stimulating hormone:

Effects of α-MSH on DNA fragmentation:

DNA fragmentation was not seen under aerobic control perfusion. However, in hearts subjected to 30 min of global ischemia followed by 120 min of reperfusion, DNA laddering was detected in the drug-free myocardium. In rats treated with 40, 200, and 400 μg/kg of α-MSH, and hearts were excised, isolated, and subjected to ischemia/reperfusion, DNA fragmentation was reduced indicating by the intensity of lanes. Thus, with the use of 200 and 400 μg/kg of α-MSH, the fragmentation of DNA was completely abolished in the ischemic/reperfused myocardium.

Effects of α–MSH on myocardial infarct size:

The does of 200 and 400 μg/kg of α-MSH, infarct size was significantly reduced from its control value of 38 ± 5 % to 17 ± 3 % and 19 ± 4 %, respectively. The lowest concentration of α-MSH (40 μg/kg) failed to significantly reduce the infarct size in comparison with the drug-free control value.

Effects of α-MSH on myocardial function:

Our results show that 40 μg/kg of α-MSH did not significantly change the preischemic or postischemic cardiac function (heart rate, coronary and aortic flow rates, and left ventricular

developed pressure). However, the increase of α-MSH dose from 40 μg/kg to 200 μg/kg and 400 μg/kg, a significant increase was observed in aortic flow and left ventricular developed pressure before the induction of ischemia. During reperfusion, the postischemic cardiac recovery, including coronary flow, aortic flow, and left ventricular developed pressure, was significantly improved in hearts treated with 200 μg/kg and 400 μg/kg of α-MSH, respectively. Interestingly, heart rate was not significantly changed either before the onset of ischemia or during reperfusion in α-MSH treated groups in comparison with the drug-free control values.

Effects of a-MSH on ventricular fibrillation:

It is reasonable to believe that the improvement in postischemic cardiac function in α-MSH treated groups, reflected in a significant reduction in the development of reperfusion-induced ventricular fibrillation. Thus, our results show that the incidence of reperfusion-induced ventricular fibrillation was significantly reduced from its drug-free control value of 92% to 83%, 17% ($p<.005$), and 25% ($p<0.05$) with the concentrations of 40 μg/kg, 200 μg/kg, and 400 μg/kg of α-MSH, respectively.

III. Effects of preconditioning:

Effects of PC on serum cholesterol:

Serum cholesterol was monitored and significantly increased (about six-fold) after 2-week of cholesterin diet, and reached a constant level (about 12-fold) after 4-, 6-, and 8-week periods in all groups.

Effects of PC on myocardial function:

Statistically significant differences were found in cardiac function between the nonhypercholesterolemic age-matched controls and in rabbits subjected to 8-week cholesterin diet (hypercholesterolemic groups). Under aerobic conditions, all preischemic values in HR, CF, AF, and LVDP were significantly reduced in the 8-week hypercholesterolemic groups in

comparison with the corresponding nonhypercholesterolemic age-matched control groups indicating the development of HC-induced cardiac failure.

The 8-week HC reduced the recovery of myocardial function upon reperfusion compared to the 8-week age-matched nonhypercholesterolemics showing a reduced resistance to ischemia/reperfusion injury in the previously diseased myocardium.

Further question was whether 1xPC, 2xPC, 3xPC, and 4xPC could protect the ischemic/reperfused myocardium obtained from previously diseased (hypercholesterolemia) animals in comparison with the well known protection afforded by PC in intact myocardium? Hearts from age-matched nonhypercholesterolemic controls and 8-week hypercholesterolemics were subjected to one, two, three, and four cycles of preconditioning, respectively, before the induction of 30 min of ischemia followed by 120 min of reperfusion, and cardiac function was monitored. Our result shows, in agreement with many previous studies that preconditioning, independent of the numbers of preconditioning cycles, does attenuate the ischemia/reperfusion-induced damage during reperfusion in the hearts obtained from intact animals. The 3xPC and 4xPC significantly improved ($p<0.05$) the recovery of CF, AF, and LVDP in isolated hearts obtained from intact animals and subjected to ischemia/reperfusion. However, in our studies, PC-induced cardiac protection was not detected during reperfusion in hearts obtained from intact rabbits and subjected to 1xPC or 2xPC. Furthermore, in contrast with the positive results obtained from previously intact myocardium subjected to various cycles of PC and ischemia/reperfusion, in the 8-week hypercholesterolemic hearts, 3- or 4-cycle of PC significantly reduced ($p<0.05$) the recovery of postischemic cardiac function (AF and LVDP) in comparison with the nonhypercholesterolemic or hypercholesterolemic age-matched preconditioned (3xPC and 4xPC) myocardium. Thus, our results clearly show that 1xPC and 2xPC did not result in any

cardiac protection, while 3xPC and 4xPC increased the extent of ischemia/reperfusion-induced injury in the hypercholesterolemic rabbit myocardium.

Effects of PC on myocardial infarct size:

The experimental condition defined for preconditioning 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 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 preconditioning-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 preconditioning-induced protective effect in the 8-week hypercholesterolemic ischemic/reperfused groups is depending on the state of myocardium (previously diseased or not), and serum cholesterol levels.

Effects of PC on arrhythmias:

The incidence of arrhythmias, including VT and VF, after 30 min ischemia followed by 120 min of reperfusion in nonhypercholesterolemic and hypercholesterolemic rabbit hearts. A very low incidence of VT (17%) and VF (33%) was observed in control nonhypercholesterolemic and nonpreconditioned hearts in our model, respectively. In hypercholesterolemic and nonpreconditioned myocardium, the incidence of VT (50%) and VF (50%) was increased indicating a higher vulnerability of the myocardium to arrhythmias in hypercholesterolemic subjects. The lack of statistical significance between the nonhypercholesterolemic and hypercholesterolemic values can be explained, because of the nonparametric distribution, by the low numbers of animals (n=6) used in each group. In the

4xPC nonhypercholesterolemic group, the incidence of VT (17%) and VF (8%) was at a low level, while in the 4xPC hypercholesterolemic group, the incidence of reperfusion-induced VT (67%) and VF (67%) was at a relatively high level.

Discussion

a.) Effects of SGE

The results of the current study demonstrate that the hearts of the rats fed SGE (100 mg/kg and 200 mg/kg) were resistant to myocardial ischemic reperfusion injury. The SGE-fed rat hearts displayed significant improvement in postischemic left ventricular function and reduced myocardial infarct size compared with those of control animals fed a mixture of glucose and fructose. Control studies were performed by feeding the rats 45 μg/100 g body weight of glucose and fructose, because 200 μg of SGE contained this amount of glucose and fructose. Rats fed with 45 μg/100 g of glucose and fructose did not exhibit differences in contractile function or myocardial infarct size compared with control. The hearts of SGE-fed rats contained reduced amount of malonaldehyde, suggesting that SGE functioned as an in vivo anti-oxidant after consumption. Oxygen-derived free radicals or oxidative stress play a significant role in a variety of cardiovascular diseases including congestive heart failure, valvular heart disease, cardiomyopathy, hypertrophy, atherosclerosis, and ischemic heart disease. Since the implication of oxygen free radicals in the pathogenesis of myocardial ischemia/reperfusion injury more than two decades ago, the role of these reactive oxygen species in many other cardiovascular diseases has become increasingly apparent. Under normal conditions there is a balance between the formation of prooxidants (oxygen free radicals) and the amount of anti-oxidants present. This steady-state condition is interrupted in pathophysiologic conditions because of the excessive production of free radicals, decrease in anti-oxidants, or both. Substantial evidence supports the notion that ischemia and reperfusion

generate superoxide and hydroxyl radicals among other cytotoxic free radicals. The presence of reactive oxygen species was confirmed directly by estimating free radical formation and indirectly by assessing lipid peroxidation and DNA breakdown products Among the oxygen free radicals, superoxide anion ($\mathbf{O_2^-}$) is the most innocent free radical whereas the hydroxyl radical (**OH·**) is the most detrimental to the cells. Virtually all biomolecules including lipids, proteins, and DNA are potential targets for **OH·** radical attack. Our study demonstrated that SGE can directly scavenge both superoxide and hydroxyl radicals. These results were further supported by a significant reduction of malonaldehyde content in the SGE-treated hearts. Anti-oxidants have long been known to protect against the damaging effects of free radical–mediated tissue injury, especially ischemia/reperfusion injury of the heart and other organs. Amidst the intense interest generated in light of the various findings that support red wine as being a plausible preventive intervention against coronary heart disease, we were interested in learning whether grapes possess the same cardioprotective properties. Grapes as opposed to other fruits and vegetables as sources of polyphenols and anti-oxidants are unique in a number of ways. First of all, resveratrol and a few other polyphenols are present in grapes but are virtually absent from commonly consumed fruits and vegetables. Resveratrol, a stilbene polyphenol, protects heart from ischemic reperfusion injury by reducing both necrosis and apoptosis. Resveratrol also has cancer chemopreventive activity, inhibits platelet aggregation, and reduces oxidative stress in PC 12 cells. In addition, proanthocyanidins are cardioprotective and reduce ventricular arrhythmias and cardiomyocyte apoptosis. Finally, grape seed proanthocyanidins inhibit the proapoptotic genes *Jnk* and *Jun.*

In summary, grapes appear to be cardioprotective. Because of the presence of several unique polyphenolic compounds, grapes not only may function as in vivo anti-oxidants but also may trigger intracellular signaling cascades that strengthen the anti-oxidant defenses of the heart.

b.) Effects of a-MSH

The goal of our experiments was to study whether α-MSH treatment could afford cardiac protection against ischemia/reperfusion-induced injury. Thus, we studied that α-MSH is able to improve postischemic cardiac function, to reduce myocardial infarct size and the incidence of reperfusion-induced ventricular fibrillation, and to attenuate apoptotic cell death. However, the primary aim of our investigation was not to study the action mechanism(s) in rats treated with α-MSH, but to clarify the outcome and final endpoints (cardiac function, infarct size, ventricular fibrillation, and apoptotic cell death) of reperfusion-induced injury in the myocardium. In our study, thus, the action mechanism(s) of α-MSH remains on speculation and the possible explanation is based on previous investigations. It is important to note that drugs, which can slow down heart rate, are able to protect the ischemic-reperfused myocardium. The results of our study have shown that α-MSH inhibits reperfusion-induced damage, and the protective effect of α-MSH was unrelated to any change in heart rate. Although not conclusive in our studies, α-MSH was given before the induction of ischemia, and this would lead us to suggest that a major component of the protection of α-MSH may occur as a result of its action during the preceding period of ischemia. It is often justified on the grounds that time must be allowed for the intervention to reach the tissue and must be available to act before the critical early moments of ischemia and reperfusion. Thus, acceptable evidence for ischemia/reperfusion injury could only be obtained from additional studies in which α-MSH is given at the onset of reperfusion. This conclusion could be supported by the fact that in hearts obtained from rats treated with 200 μg/kg or 400 μg/kg of α-MSH, the preischemic values of aortic flow and left ventricular developed pressure were significantly increased in comparison with the corresponding drug-free control values.

The data, as one of the endpoints of our study, show that α-MSH at concentrations of 200 μ/kg and 400 μ/kg reduced the apoptotic cell death. It is reasonable to assume that a reduction in apoptotic cell death limits infarct size in α-MSH treated subjects because apoptotic cell death

was detected, beside the necrotic cells, in the infarcted area. However, it is not clear and is not the aim of our present study to what extent of apoptosis and necrosis individually contribute to the development of myocardial infarction, and probably both of them, a "necro-apoptotic" mechanism contributes to the development of reperfusion-induced injury and cell death. On the other hand, α-MSH and melanocortin peptides have been shown to inhibit inflammation under many experimental conditions, such as arthritis, inflammation, and ischemia. The anti-inflammatory effects of α-MSH and melanocortin peptides are often associated with a reduced production of proinflammatory cytokines, such as interleukin-1α, interleukin-1β, interleukin-6, and tumor necrosis factor-α and with an enhanced genesis of the anti-inflammatory interleukin-10 and of the angiogenic factor interleukin-8. Thus, it is reasonable to believe that endogenous anti-inflammatory mediators such as α-MSH could contribute to the reduced release of inflammatory mediators in the heart and other organs in humans. Furthermore, it is also possible that other action mechanisms of α-MSH and melanocortin peptides could be involved in the cardioprotective activity, as is the case for various cardiovascular effects of ACTH fragments, particularly under conditions of circulatory shock and hypoxia/reoxygenation induced by prolonged respiratory arrest.

One of the objectives of our study was to ascertain whether the antifibrillatory effect observed during reperfusion was a direct consequence of the actions of α-MSH during the reperfusion period or whether they were secondary to some effects operative during ischemia. The drug was subcutaneously injected to rats 12 h prior to the isolation of hearts and the induction of ischemia/reperfusion, therefore, this would lead us to support that antifibrillatory effect of α-MSH during reperfusion may occur, at least in part, as a result of its action during the preceding period of cardiac ischemia.

In conclusion, the finding of the present study demonstrates that melanocortin peptides, i.e., α-MSH, significantly attenuate the consequences of myocardial ischemia/reperfusion. However,

there is a clear need for further extensive and careful investigation in the field of ischemia/reperfusion-induced injury in order to precisely clarify the action mechanism(s) of α-MSH, an active fragment of ACTH, including arrhythmias, apoptotic and necrotic cell death, and cardiac function. Furthermore, these results are rather exciting, because they could disclose a new therapeutic approach in the treatment of ischemia/reperfusion-induced injury.

c.) Effects of PC

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 preconditioning in intact myocardium, relative little attention has been paid on the preconditioning phenomenon in previously diseased hearts. Indeed, thousands of studies are now available and indicate the powerful protective effect of preconditioning 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.

Probably, the most two important mechanisms involved in preconditioning are ATP-sensitive K^+ channels and adenosine or adenosine receptors. However, the roles and functions of ATP-sensitive K^+ channels and adenosine in preconditioning are somehow controversial. Thus, it has been suggested and stressed in many studies that ATP-sensitive K^+ channels may play or not a central role in the mechanism of preconditioning-induced cardiac protection. The same conclusion (play or not, or no evidence) was reached with the studies of adenosine and adenosine receptors in intact preconditioned myocardium. Thus, it has been initially proposed that adenosine and adenosine receptors could or partially play a significant role in preconditioning). However, later it was stressed by the same investigators that adenosine and adenosine receptors do not play an important role in this phenomenon, and finally it was

found that adenosine pathway is critically depending on the time of initiation of an adenosine antagonist.

An important point could be that all aforementioned preconditioning studies have been done in various models of ischemia/reperfusion in intact and healthy myocardium instead of a diseased heart. However, a previously diseased and preconditioned heart may respond to ischemia/reperfusion on a completely different way in comparison with the intact myocardium. Thus, the clinical implication of preconditioning, results obtained in previously diseased and preconditioned myocardium, could be much more important in comparison with the data obtained in intact hearts. Therefore, in the present study, we investigated whether preconditioning could protect the myocardium in previously diseased, hypercholesterolemic, hearts. It was not the aim of our experiments to study and determine what could be the most important preconditioning mechanism(s), if there is any, in hypercholesterolemic myocardium, but we studied the final outcome of preconditioning including cardiac function (HR, CF, AF, and LVDP), infarct size, and arrhythmias as functional endpoints.

Although the effects and mechanisms of preconditioning on cardiac performance, in various aspects, have been extensively investigated in intact myocardium, relatively little information is available of this phenomenon on diseased, e.g., diabetic, ageing, arteriosclerotic, hypertrophic, and failing ischemic/reperfused hearts. For instance, the prognosis following infarction appears to be worse in diabetic patients, who exhibit a higher incidence of congestive heart failure and death compared to non-diabetics. It has been found that the progress of diabetes increases myocardial infarct size and abolishes preconditioning-induced cardiac protection in both rats and dogs. Another important factor in preconditioning could be the ageing because the pharmacotherapy and dosages of various drugs are different in elderly patients in comparison with the young and middle age subjects. Therefore, it is also reasonable to investigate the preconditioning phenomenon in senescent patients. It has been found and assessed by clinical,

ECG, and metabolic evidence that ischemic preconditioning failed to provide any protection in elderly patients. It has been also found that the protective effect of ischemic preconditioning is diminished in aged patients undergoing coronary bypass surgery and suffered from angina in senescent subjects.

In order to determine how the HC affects preconditioning-induced protection to reperfusion-induced injury we have compared heart function, infarct size, and the incidence of reperfusion-induced VT and VF in isolated hearts obtained from hypercholesterolemic and nonhypercholesterolemic age-matched control rabbits. The results of our study show that HC resulted in a low recovery in postischemic cardiac function, and 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 preconditioning and ischemia/reperfusion in our model. Although the incidence of reperfusion-induced VF and VT was very low in our isolated rabbit heart studies, HC increased the incidence of VF and VT from their nonhypercholesterolemic control values of 17% and 33% to 50% and 50%, respectively. In the 4xPC hypercholesterolemic ischemic/reperfused group, the incidence of reperfusion-induced arrhythmias was further increased. The effects of HC on cardiac performance have been extensively investigated, but relatively little efforts have been made on infarct size and cardiac function of preconditioning in hypercholesterolemic ischemic and reperfused myocardium.

Thus, with progressive HC, the preconditioning-induced protection is abolished and cardiac failure with reduced function of the heart to ischemia/reperfusion was observed. Furthermore, our results clearly show that the protective effect of ischemic preconditioning cannot be observed in hypercholesterolemic hearts, indicating that the preconditioning might be an "intact heart phenomenon".

In summary, the mechanisms of preconditioning, even the first and second window protection and their implication to an actual clinical situation, were mainly studied in intact myocardium. Despite this, the effects of preconditioning or its mechanisms appear currently to be controversial and a dilemma especially in previously diseased myocardium. The solution to the problem of preconditioning in intact and diseased myocardium requires further data from two various sources: (i) in diseased animal from laboratories, and (ii) diseased or aging human myocardium from the clinic. Once the data are available in previously diseased human or animal subjects then the conditions and mechanisms under which preconditioning will be beneficial rather than harmful could be established and the dilemma could be solved.

Publication (the thesis based on)

Original articles

1. **Juhasz B,** Der P, Turoczi T, Bacskay I, Varga E, Tosaki A. (2004) Preconditioning in Intact and Previously Diseased Myocardium: Laboratory or Clinical Dilemma? *Antioxid. Redox Signal*. 6, 325-333**. IF: 3.027**

2. Vecsernyes M, **Juhasz B,** Der P, Kocsan R, Feher P, Bacskay I, Kovacs P, Tosaki A. (2003) The administration of α-melanocyte-stimulating hormone protects the ischemic/reperfused myocardium. *Eur. J. Pharmacol*. 3, 177-83. **IF:2.352**

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