1. INTRODUCTION

Cardiovascular diseases as a whole and ischemic heart disease are the most frequent causes of morbidity and mortality in the developed countries putting considerable medical and economic burden on the society. The availability of invasive diagnostic and therapeutic procedures is constrained by their extreme financial and personal requirements. Thus, the development of novel agents for the treatment of ischemic heart disease has been long in the limelight of scientific interest. Despite the intensive research on this field of medicine, the pathomechanism of these illnesses still holds many unexplored areas. The impact of primary prevention strategies is limited, especially in the developing countries. Since the infarct size is directly related to the subsequent morbidity and mortality, there is an urgent need for effective treatments, which can limit the extent of an evolving myocardial infarction during the acute phase. Prolonged and unresolved regional myocardial ischemia without reperfusion inescapably causes myocyte death. At present, timely reperfusion is the only means of saving ischemic myocardium resulting in limited cardiomyocyte loss. The two major clinical approaches to reperfusion are the use of thrombolytics and coronary angioplasty. Although reperfusion is clearly beneficial and is a prerequisite for salvage, it comes, paradoxically, at a price; a phenomenon called reperfusion injury.

Albeit the early restoration of blood flow to the jeopardized myocardium is necessary to save myocytes from eventual death, abundant evidence indicates that reperfusion has additional deleterious effects on the ischemic myocardium, which can be modified by interventions given at the onset of reperfusion. The manifestations of reperfusion injury include arrhythmias, reversible contractile dysfunction, myocardial stunning, endothelial dysfunction, and cell death. Principal contributors to myocardial reperfusion injury are oxygen free radical formation, calcium overload, neutrophil-mediated myocardial and endothelial damage, progressive decline in microvascular flow to the reperfused myocardium, and depletion of high-energy phosphate stores. A variety of pharmacological compounds have been investigated to fight against reperfusion injury including free radical scavengers, antioxidants, calcium channel blockers, inhibitors of neutrophils, nitric oxide, adenosine-related agents, inhibitors of the renin-angiotensin system, endothelin receptor antagonists, Na^+/H^+ exchange inhibitors, and antiapoptotic agents.

In the present work, we studied the cardioprotective properties of a group of free radical-entrapping antioxidant molecules under conditions of myocardial ischemia-

reperfusion in an isolated heart perfusion system. Interestingly, studies of the molecular mechanisms involved in combating oxidative and nitrosative stress have recently shown that the classification of molecules as antioxidants is not as simple as demonstrating a direct chemical or scavenging reaction with free radicals, but additional roles can be attributed to them such as modulators of cell signaling pathways. The modulation of cellular survival signaling pathways versus cell death pathways by any pharmacological intervention has significant biological consequences. Therefore, our additional objective was to focus on how the applied antioxidant compounds affected the oxidative stress-related intracellular signal transduction pathways.

Free radicals and antioxidants

Oxygen free radicals as highly reactive chemical species containing an unpaired electron are generated in biological systems during numerous physiological and pathophysiological processes. In physiological circumstances they play a role in cellular metabolism and cellular defense systems, on the other hand, large amount of oxygen free radicals is highly toxic for tissues and cells, because they can oxidatively modify and injure a variety of biological systems. Although cells have various defense systems against free radical damages including scavenger enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic molecules (e.g. glutathione, ubiquinone [coenzyme Q], ascorbate, vitamin E), in case of impaired antioxidant defense or increased production of free radicals, these reactive agents can take part in formation of serious disorders. The past decade has seen an explosion of knowledge regarding the role of oxidative stress in the pathogenesis of a wide variety of diseases such as atherosclerosis, ischemia-reperfusion injury, diabetes mellitus, inflammatory diseases, cancer, immunological disorders, and aging.

Reactive oxygen species (ROS) in living cells are mainly formed by physiological enzymatic mechanisms. They are generated along the aerobic metabolism that utilizes life-sustaining oxygen to oxidize fuels. Being the major producers of ROS, mitochondrial structures are exposed to high concentrations of ROS and, therefore, are particularly susceptible to their attack. However, various cytosolic and membrane-bound oxidases and dehydrogenases (e.g. xanthine oxidase, lipoxygenase, and NADH oxidase) are also known to produce free radicals.

As mentioned above, cells have developed numerous enzymatic and nonenzymatic systems against continuously generated free radicals. Superoxide anions can be scavenged by

superoxide dismutase, which catalyzes the conversion of two anions into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is neutralized by catalase that furthers the conversion of hydrogen peroxide into water and oxygen. Peroxidases catalyze an analogous reaction, in which hydrogen peroxide is reduced to water by a reductant. Glutathione, which is present in high concentrations (4-5mM) in animal cells, has a key role in detoxification by reacting with hydrogen peroxide and organic peroxides, and serves as a sulfhydryl buffer. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG). GSSG is reduced to GSH by glutathione reductase. Other important physiological antioxidants are vitamin E and reduced coenzyme Q. Ascorbate and reduced glutathione are water-soluble antioxidants that can regenerate alpha-tocopherol. Nevertheless, the protective effect of ascorbate and GSH against oxidative attack can be attenuated or even reversed in the absence of vitamin E within the membranes. Physiological compounds such as urate, bilirubin, and ceruloplasmin can also protect against oxidative attack.

In spite of the complexity of the above described antioxidant system, free radicals generated excessively under certain circumstances can break this defense barrier and cause oxidative stress to the cell. During ischemia-reperfusion cycle reactive oxygen species are generated mainly along the mitochondrial respiratory chain and they trigger the oxidative damage of several cellular components including lipid peroxidation, protein oxidation, enzyme inactivation as well as DNA strand breaks, and impair an ample of physiological functions, e.g. blocking of ionic pumps, restricting glycolysis or promoting mitochondrial calcium release. Intracellular sodium and calcium accumulation is the consequence of not only the injury of ion channels, but also the decrease of myocardial high-energy phosphate levels. High levels of intracellular calcium activate the proteolytic enzymes. Additionally, high intracellular calcium and inorganic phosphate concentrations induce the opening of mitochondrial permeability transition pores. As a consequence, the mitochondrial membrane potential will collapse and the mitochondrial energy production will be ceased. Mitochondrial respiratory complexes play a central role in the development of postischemic myocardial damage. They are the main sources of ROS during re-oxygenation, but they are also injured and inactivated by ROS. One possible approach to diminish those ischemia-reperfusion-induced processes that compromise the structural and functional integrity of cardiomyocytes is the delivery of small scavenger molecules to the site of free radical formation in spatial and temporal containment. Research activity on this field is nourished by the fact that the administration of the aforementioned natural scavenger molecules and antioxidant enzyme systems failed to give substantial benefit. Though antioxidant molecules and enzymes have

been thought to potentially limit the oxidative injury, they are not readily internalized within myocardial cells or cannot reach the right cell compartment to exert their protective effect.

Application of scavenger compounds renders cardioprotection by gathering and neutralizing free radicals and, in turn, limiting the time-span during which radicals can exert their deleterious effects. As a consequence, the extent of cellular oxidative injury is reduced, which is manifested in lower level of lipid peroxidation, protein oxidation and DNA damage as well as the preservation of mitochondrial enzymatic energy metabolism. According to our previous data, heterocyclic nitroxide precursor compounds containing a 2,2,5,5-tetramethyl-pyrroline ring were able to scavenge ROS and proved to be protective during ischemia-reperfusion in an isolated heart perfusion system as well as in a model for red blood cell filterability. The amine moiety of these molecules is transformed in vitro and in vivo into nitroxide form, which can be reduced to hydroxylamine. This is a cyclic process enabling the scavenging of radicals (superoxide anion and hydroxyl radical, respectively) in each cycle. After the oxidation of the amine compound, the formed stable nitroxide radical can be reduced by ascorbic acid to hydroxylamine, which is reactive enough to undergo reversible one-electron oxidation to a nitroxide together with the scavenging of another ROS.

Under our experimental conditions, the examined heterocyclic compounds exerted cardioprotection in a strikingly low concentration, i.e. 5μM. According to literature data, superoxide dismutase-mimetic and hydroxyl radical-scavenging five- or six-membered cyclic molecules, for instance Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), are efficacious in milimolar concentration range in various models of oxidative stress. In the contrary, our applied compounds being able to entrap ROS delivered protection under 10^{-5} M concentration. As it is questionable that, by taking together the above mentioned issues, these molecules are capable of harvesting the majority of free radicals produced during ischemia-reperfusion in such a low concentration, it raises the possibility that they may interfere with alternative cellular pathways. The molecules applied in our study are substituted mexiletine derivates. Although mexiletine is a class I/b antiarrhythmic agent with the ability of blocking sodium channels, it does not exhibit significant protection on myocardium under conditions of ischemia and reperfusion. Therefore, it is implausible that the eventual antiarrhythmic property of these compounds appreciably conduces to their cardioprotection.

Signal transduction pathways during ischemia-reperfusion

Several protein kinase cascades and inflammatory reactions have recently become established as part and parcel of any external stress-related tissue injury such as heat, ischemia-re-oxygenation, and other oxidative, metabolic, toxic as well as infectious insults. Oxidative challenge of the myocardium influences among others the functioning of the mitogen-activated protein kinases (MAPK), phospholipase C, protein kinase C, p53, ATM (ataxia-telangiectasia mutated) kinase, nuclear factor-κB, and heat shock proteins. Furthermore, ischemia-reperfusion in cardiomyocytes expedites the phosphorylation of the growth-factor-associated kinase Akt (also known as protein kinase B) mostly in a phosphatidylinositol-3-kinase (PI3-kinase)-dependent manner. The PI3-kinase/Akt pathway is one of several signal transmission pathways brought into connection with cell survival. Under conditions of ischemia and reperfusion nitric oxide and superoxide anion can form peroxynitrite, which features as a "ligand" of receptor tyrosine kinases and leads to the nitration of tyrosine residues igniting the above mentioned machinery. Akt, in turn, targets a wide variety of substrates by phosphorylation: inactivates the proapoptotic Bcl-2 family member Bad, the apoptotic effector enzyme caspase-9 and Forkhead transcription factor; as a consequence, apoptosis is suppressed. In addition, p70 ribosomal S6 kinase is activated promoting mRNA translation and cell cycle progression as well as phosphorylating Bad. Akt also induces endothelial nitric oxide synthase (eNOS) as well as IκB kinase-α (IKKα) activity, but blocks glycogen synthase kinase-3 (GSK-3).

Inactivation of GSK-3 will allow glycogen synthase to build up cellular glycogen stores and eukaryotic initiation factor 2b as well as cyclin D_1 to facilitate cell cycle progression. Two Bad molecules constituting a homodimer can contribute to the release of cytochrome c from the external side of the inner mitochondrial membrane into the cytoplasm, where the free cytochrome c triggers apoptotic cell death with the participation of caspase-9. Bad homodimer formation is prevented either through the dimerization with Bcl-2 antiapoptotic molecule or by Bad phosphorylation, for instance by Akt, directing it toward degradation. Moreover, endothelial nitric oxide synthase activated by Akt forms nitric oxide causing vasorelaxation. The overall impact of Akt action is thus a remarkable antiapoptotic effect, metabolic adjustment, and vasodilation, each of which inevitably promotes cell survival. Activation of the Akt signaling route can be blocked by two commonly used, but not completely specific inhibitors of PI3-kinase enzyme, i.e. wortmannin and LY 294002, which enables the more accurate analysis of this pathway.

Insulin treatment-induced, eNOS mediated low concentrations of nitric oxide have been reported to exhibit antiapoptotic impacts by nitrosating caspase-3, 6, 7, and 8, inhibiting caspase-dependent Bcl-2 cleavage, and eventually downregulating MAPK phosphatase-3 mRNA levels resulting in prolonged phosphorylation of extracellular signal-regulated kinase (ERK), another prosurvival factor, or by NO inhibition of neutrophil infiltration. Akt also influences glucose uptake by recruiting GLUT-4 to the cell membrane, which propagates the more favorable bioenergetics of glycolytic metabolism. PI3-kinase can also regulate protein kinase C (PKC) during ischemic preconditioning, namely through initiating its phosphorylation by PDK1 and PI3-kinase lipid products, which altogether will potentiate the allosteric regulation of PKC by diacylglycerol and NO (formed by eNOS) giving rise to the remarkable finding called second window of protection.

Apoptosis, or programmed cell death, is a naturally occurring cell death process, essential for the normal development and homeostasis of all multicellular organisms. Considerable research activity has been devoted to the question of to which extent apoptotic cell death is responsible for myocardial tissue damage and dysfunction after ischemia-reperfusion. The depletion of intracellular ATP levels during ischemia blocks the activation of the downstream proapoptotic genes, which prevents the typical apoptotic changes from taking place. However, reperfusion rapidly restores the intracellular energy stores, thereby providing the ATP necessary to allow the apoptotic pathway to proceed. Moreover, experimental data suggest that necrosis and apoptosis occur simultaneously during reperfusion, with a relatively rapidly developing necrotic cell death during the early phase of reperfusion followed by a slower appearance of apoptosis during the late phase of reperfusion. Albeit apoptotic cardiomyocyte loss merely amounts to around 6% during postischemic reperfusion, remarkable percentage (up to 40%) of vascular endothelial cells may decease via apoptosis, which can compromise the coronary flow and magnify the proportion of cells destined for necrotic death.

Mitogen-activated protein kinase (MAPK) cascades constitute an integrated part of stress-related signaling routes and include three distinct but interlinked pathways: extracellular signal-regulated kinase (ERK 1/2), c-jun N-terminal kinase (JNK) and p38-MAPK. ERK is generally involved in the transmission of mitogen signals through Ras/Raf-1/MEK/ERK pathway. However, sustained activation of ERK during re-oxygenation was shown to render delayed cytoprotection. Also hydrogen peroxide injury is limited by an elevated prostacyclin formation through ERK-mediated cyclooxygenase-2 expression. The fact that ERK can block caspase-3 enzyme activity and downregulate c-jun N-terminal kinase

(JNK) lends additional support to the notion that ERK possesses cytoprotective properties during oxidative insult. A downstream substrate of ERK, p90 ribosomal S6 kinase (RSK) can phosphorylate GSK-3β thereby reinforcing the action of Akt on this kinase. There are data indicating that ERK is only active if the mitochondrial electron chain and substrate oxidation are intact, otherwise ERK becomes inactivated.

Several researchers conveyed evidences that underpin the proapoptotic nature of the concerted activation of stress-responsive kinases, i.e. JNK as well as p38-MAPK, as a result of oxidative or cytokine (TNF, Fas ligand) stress. It is assumed that reactive oxygen species can trigger the dissociation of thioredoxin and ASK1 (apoptosis signal-regulating kinase-1), and, in turn, JNK and p38-MAPK activation ensues. JNK can phosphorylate as well as expedite the expression of c-jun and ATF-2 (activator transcription factor-2), the heterodimer of which (along with c-fos, among others) constitutes AP-1 (activator protein-1) transcription factor that participates in departing proinflammatory and proapoptotic gene programs. Additionally, JNK and p38-MAPK can precipitate apoptosis by mitochondria-dependent caspase activation. ATF-2 may be phosphorylated and induced by p38-MAPK, as well.

Appreciating the extreme complexity of signal transduction pathways, we have to admit that JNK more or less features as a proapoptotic factor, on the other hand, p38-MAPK deserves further attention. Two isoforms (α and β) of p38-MAPK are expressed in the heart and while p38α transmits proapototic signals, p38β is a candidate for delivering hypertrophic stimuli and as so may indeed protect against apoptosis. Furthermore, a downstream substrate of p38-MAPK is MAPK-activated protein kinase-2 (MAPKAPK2), which can modulate heat shock protein-27 functioning, a protein required for cytoskeletal integrity. This p38/MAPKAPK2/hsp27 route is implicated in delayed cytoprotection following ischemic preconditioning.

Modulation of pathways involved in mediating cellular responses to oxidant injury offer unique opportunities for therapeutic interventions aimed at treatment of diseases or conditions where oxidative stress is a relevant player. In this study, we investigated the impact of two antioxidant cardioprotective compounds on the ischemia-reperfusion-induced activation of phosphatidylinositol-3-kinase/Akt signaling cascade.

2. STUDY OBJECTIVES

1. We planned to investigate the impact of a group of free radical-entrapping mexiletine derivates on the myocardial energy metabolism during ischemia-reperfusion cycle in Langendorff perfused rat hearts. In turn, the molecular structure was selected that delivered the most optimal cardioprotection in our isolated heart perfusion experiments.

2. We intended to characterize the cardioprotective properties of the best two molecules from various standpoints. Therefore, the effect of these compounds on postischemic myocardial energetics, functional recovery, and infarct size was evaluated, then the capability of the compounds to scavenge superoxide anions and hydroxyl radicals as well as to diminish oxidative myocardial damage was determined.

3. We presumed that these molecules were not capable of harvesting the majority of free radicals produced during ischemia-reperfusion in such a low concentration (5μM), but rather they may interfere with alternative cellular pathways. To gain further insight into the molecular mechanism of their action, the current study focused on how the two agents influenced the prosurvival phosphatidylinositol-3-kinase/Akt intracellular signal transduction pathway.

3. MATERIALS AND METHODS

Chemicals. The synthesis of heterocyclic compounds was (HO-2, HO-2434, HO-3144, and H-2693) or will be (HO-3073) published elsewhere. Wortmannin was purchased from Calbiochem (Darmstadt, Germany). All other reagents were of the highest purity commercially available.

Animals. The hearts of adult male Wistar rats weighing 300-350 g were used for the Langendorff heart perfusion experiments. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pécs Medical School.

Heart perfusion. Rats were anesthetized with 200mg/kg ketamine intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70mmHg, at 37°C as described previously. The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118mM NaCl, 5mM KCl, 1.25mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 11mM glucose and 0.6mM octanoic acid and, in the treated group, mexiletine, HO-2, HO-2434, HO-3144 (each in 5μM concentration), H-2693, or HO-3073 (in 2, 5, and 10μM concentrations), and/or wortmannin (100nM). The perfusate was adjusted to pH 7.40 and bubbled with 95% O_2 and 5% CO_2 through a glass oxygenator. After a washout, non-recirculating period of 10 minutes, hearts were either perfused under normoxic conditions for 10 minutes, or were subjected to a 25-minute global ischemia by closing the aortic influx and reperfused for either 5, 15, 30, or 45 minutes. The above listed compounds were administered into the perfusate at the beginning of normoxic perfusion. During ischemia hearts were submerged into perfusion buffer at 37°C. Hearts were freeze-clamped at the end of each perfusion.

NMR spectroscopy. NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z•SPEC® 20-mm broadband probe (Nalorac Co., Martinez, CA, USA), applying GARP-1 proton decoupling ($\gamma B2$=1.2 kHz) during acquisition. Field homogeneity was adjusted by following the ^{1}H signal (w1/2=10-15 Hz). Spectra were collected with a time resolution of 3 minutes by accumulating 120 transients in each FID. 45° flip angle pulses were employed after a 1.25 s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16K. Under the above circumstances the relative concentrations of the species can be taken proportional to the peak areas, because interpulse delays exceeded 4-5×T1 values of the metabolites to be analyzed in ^{31}P experiments. The pH value in the myocardium was calculated by the inorganic phosphate-chemical shift (δ) from the creatine phosphate peak according to the following equation: $pH = 6.77 + \log [(\delta-3.23)/(5.70-\delta)]$.

Measurement of hydroxyl radical scavenging. Hydroxyl radical formation was detected by using the oxidant-sensitive non-fluorescent probe benzoic acid, which is hydroxylated to 2, 3, or 4-hydroxy-benzoic acid in the presence of hydroxyl radicals. Hydroxylation of benzoic acid results in the appearance of intensive fluorescence, which enables the monitoring of the hydroxylation reactions by a Perkin Elmer fluorescence spectroscope at an excitation wavelength of 305nm and an emission wavelength of 407nm. The reaction was studied in quadruplicate for 60 seconds in 3 ml reaction volume containing 20mM sodium phosphate buffer (pH 6.8), 0.1mM

benzoic acid, 0.02mM hydrogen peroxide, and 0.04mM Fe(II)-EDTA as well as the amine and hydroxylamine forms of H-2693 and HO-3073 in different concentrations. This technique allowed us to calculate the concentration at which the rate of hydroxyl radical-induced hydroxylation was inhibited by 50%.

Measurement of superoxide anion scavenging. Superoxide anion generation by hypoxanthine/xanthine oxidase system was determined using the ferricytochrome c reduction technique by measuring the increase in absorbance at 555nm. The reaction was performed in quadruplicate in one ml reaction volume containing 50mM Tris buffer (pH 8.50), 200μM hypoxantine, 10-fold diluted xantine oxidase enzyme, 40μM cytochrome c as well as the nitroxide radical form of H-2693, HO-3073, Tempol, and superoxide dismutase (as reference agents) in different concentrations. This method made us possible to calculate the concentration at which the rate of superoxide anion-induced cytochrome c reduction was inhibited by 50%.

Determination of heart function. A latex balloon was inserted into the left ventricle through the mitral valve and filled to achieve an end-diastolic pressure of 8-12mmHg. All measurements were performed at the same balloon volume. Hearts were selected on the basis of the stability of high-energy phosphates (assessed by NMR) during a control period of 15 minutes before the experiment. The length of normoxia, ischemia and reperfusion were 15, 25, and 45 minutes, respectively. The experimental drugs (H-2693, HO-3073) were added to the perfusion medium after the 15-minute control period. Functional data of rat hearts (LVEDP – left ventricular end-diastolic pressure, LVDP – left ventricular developed pressure, RPP – rate-pressure product, HR – heart rate, and dP/dt) were monitored during the entire perfusion. The administration of both H-2693 and HO-3073 during normoxia caused a slight, but statistically insignificant ($p<0.11$ and $p<0.18$, respectively) reduction in heart rate, while having no effect on the remaining hemodynamic parameters.

Infarct size measurement. For infarct size measurements 90-minute postischemic reperfusion was employed either untreated or treated with 5μM H-2693 or HO-3073 and/or 100nM wortmannin. After removing from the Langendorff perfusion apparatus, ventricles were cut out and kept overnight at –4°C. Frozen ventricles were sliced into 2-3 mm thick sections, then incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C in 0.2M Tris buffer (pH 7.4) for 30 minutes. While the normal myocardium was stained brick red, the infarcted areas remained unstained. Size of the infarcted area was estimated by the volume and weight method.

Lipid peroxidation. Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a described method. Cardiac tissue was homogenized in 6.5% trichloroacetic acid (TCA) and a reagent containing 15% TCA, 0.375% thiobarbituric acid (TBA) and 0.25% HCl was added, mixed thoroughly, heated for 15 minutes in a boiling water bath, cooled, centrifuged and the absorbance of the supernatant was measured at 535nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nmol/g wet tissue.

Determination of protein carbonyl content. Fifty mg of freeze-clamped perfused heart tissue were homogenized with 1 ml 4% perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined by means of the 2,4-dinitrophenyl-hydrazine-method.

Western blot analysis. Fifty mg of heart samples (perfused for 5, 10, and 30 minutes under normoxic conditions or reperfused for 5, 15, 30, and 45 minutes) were homogenized in ice-cold Tris buffer (50mM, pH=8.0) and harvested in 2x concentrated SDS-polyacrylamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking (two hours with 3% non-fat milk in Tris buffered saline) membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-1/protein kinase B-α Ser^{473} (1:1000 dilution), non-phosphorylated C-terminal domain of Akt/PKB (1:1000), and phospho-specific glycogen synthase kinase (GSK)-3β Ser^{9} (1:1000; Cell Signaling Technology, Beverly, USA). Those amounts of protein samples were employed that contained equal amount of non-phosphorylated Akt/PKB, which allowed the assessment of differences in the phosphorylation states of Akt-1 and GSK-3β. Membranes were washed six times for 5 minutes in Tris buffered saline (pH 7.5) containing 0.2% Tween (TBST) prior to addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; BioRad, Budapest, Hungary). Membranes were washed six times for 5 minutes in TBST and the antibody-antigen complexes were visualized by means of enhanced chemiluminescence on conventional films. After scanning, results were quantified by means of Scion Image Beta 4.02 program.

Measurement of caspase-3 activity. 100-mg samples of normoxic (perfused for 10 min), control, and treated hearts (with H-2693 or HO-3073 and/or wortmannin) reperfused for 45-minutes, were homogenized with lysis buffer (20mM Tris, pH 7.5, 150mM NaCl, 2mM

EDTA, 2mM EGTA, 100μM phenylmethylsulfonyl fluoride, and 0.2% Tween-20). The lysates were collected in microcentrifuge tubes, sonicated, and centrifuged. Protein concentrations were determined using the bicinchoninic acid (BCA) method. The lysates were stored at –80°C until used for measuring caspase activity. Fluorometric assays were conducted in 96-well clear-bottom plates, and all measurements were carried out in triplicate wells. To each well 200μl of assay buffer (20mM HEPES, pH 7.5, 10% glycerol, 2mM dithiothreitol) was added. Peptide substrate for caspase-3 (Acetyl-DEVD-amidomethylcoumarin; Sigma-Aldrich Inc., St. Louis, USA) was added to each well in a final concentration of 25ng/μl. In case the caspase-3 inhibitor was used, it was added at a concentration of 2.5ng/μl immediately before the addition of caspase-3 substrate. Tissue lysates (20μg of protein) were added to start the reaction. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer without the tissue lysate. Assay plates were incubated at 37°C for one hour. Fluorescence was measured on a fluorescence plate reader set at 360nm excitation and 460nm emission wavelength. Caspase activity was calculated as ((mean amidomethylcoumarin fluorescence) – (background fluorescence))/μg of protein.

Statistical analysis. Statistical analysis was performed by analysis of variance and all of the data were expressed as the mean ± S.E.M. Significant differences were evaluated by use of unpaired Student`s *t* test and *p* values below 0.05 were considered to be significant.

4. CONCLUSIONS

1. Our NMR spectroscopic studies demonstrated that from the selected modified mexiletine derivates the cardioprotective effects could be best optimized by substituting with a 2,2,5,5-tetramethylpyrroline-3-carboxamide (H-2693) or a 2,2,6,6-tetramethyl-tetrahydropyridin-4-carboxamide (HO-3073) moiety. These two compounds significantly promoted the better postischemic recovery of creatine phosphate, ATP as well as intracellular pH and, in the meantime, triggered the faster and more complete reutilization of inorganic phosphate during reperfusion. In the contrary, the sole pyrroline ring present in H-2693 and mexiletine itself had no positive effect on ischemia-reperfusion-related energy metabolism compared to untreated postischemic hearts.

2. The applied two experimental antioxidant agents also had beneficial impact on the postischemic contractile function of the myocardium as well as on the size of the ischemia-reperfusion-induced infarcted area. We have shown that the different forms of H-2693 and HO-3073 were effective in scavenging superoxide as well as hydroxyl radicals in vitro. Furthermore, the two compounds were able to significantly reduce two indirect indices of myocardial oxidative damage, i.e. lipid peroxidation and protein oxidation. These findings underpin that the examined antioxidant molecules are definitely capable of entrapping free radicals and as so can limit the extent of oxidative stress-related adverse reactions.

3. We identified that H-2693 and HO-3073 administration was accompanied by increased ischemia-reperfusion-induced phosphorylation of Akt kinase, the central component of the prosurvival phosphatidylinositol-3-kinase/Akt signaling cascade. Akt phosphorylation presumably resulted in its activation because one of its substrates, glycogen synthase kinase-3β also became phosphorylated (meaning its inactivation). Both H-2693 and HO-3073 brought about Akt phosphorylation during normoxic perfusion, as well. The two compounds diminished the ischemia-reperfusion-induced enhanced caspase-3 activation.

4. By the parallel application of the PI3-kinase inhibitor wortmannin, we explored that the PI3-kinase/Akt cascade probably played an important role in the mechanism of action of both compounds. As a matter of fact, wortmannin could neutralize the favorable influences of both agents on myocardial energetics, cardiac functional performance, infarct size, Akt activation as well as caspase-3 activity.

Taken together, our data provide the first insight into how free radical-entrapping mexiletine derivates can influence the intracellular signal transduction pathways. The protective effects of the examined sterically hindered secondary amine molecules (nitroxide precursors) may be attributable not only to their oxygen radical-scavenging activity but also to their ability to upregulate the prosurvival Akt protein kinase cascade. These promising results further add to our understanding of the executive mechanisms of how these compounds may confer their remarkable cardioprotection and attract additional efforts to elucidate the precise interplay between the molecules and the signaling elements.

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