ANTIOXIDANT CARDIOPROTECTIVE COMPOUNDS INFLUENCING THE PHOSPHATIDYLINOSITOL-3-KINASE/AKT SIGNALING CASCADE

Ph.D. thesis

Author: Ambrus Tóth, M.D.

Project leader: Prof. Kálmán Tóth, M.D., D.Sc.

First Department of Medicine, Division of Cardiology
University of Pécs Medical School
Pécs, Hungary

2003

LIST OF ABBREVIATIONS

ASK-1: apoptosis signal-regulating kinase-1

ATF-2: activator transcription factor-2

ATP: adenosine triphosphate

CAD: coronary artery disease

ERK: extracellular signal-regulated kinase

eIF2B: eukaryotic initiation factor 2B

eNOS: endothelial nitric oxide synthase

FKHR: Forkhead transcription factor

GSK-3β: glycogen synthase kinase-3β

HR: heart rate

hsp 27: heat shock protein 27

IHD: ischemic heart disease

IKK: IκB kinase

IR: ischemia-reperfusion

JNK: c-jun N-terminal kinase

LVDP: left ventricular developed pressure

MAPK: mitogen-activated protein kinase

NF-κB: nuclear factor-κB

NMR: nuclear magnetic resonance

NO: nitric oxide

PARP: poly(ADP-ribose) polymerase

PDK1: phosphatidylinositol-dependent kinase-1

PI3K: phosphatidylinositol-3-kinase

RSK: p90 ribosomal S6 kinase

ROS: reactive oxygen species

RPP: rate-pressure product

TBARS: thiobarbituric acid reactive substances.

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 (such as coronary angiography, percutaneous transluminal coronary angioplasty, stent implantation, coronary artery bypass grafting) 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 (Catapano *et al.*, 2000). 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 (Yellon & Baxter, 1999).

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 (Zhao & Vinten-Johansen, 2002). 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 (Wang *et al.*, 2002).

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 (Azzi *et al.*, 1993; Patel *et al.*, 2000). 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.

1.1. 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 (Figure 1). 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-repertusion injury, diabetes mellitus, inflammatory diseases, cancer, immunological disorders, and aging (Beckler *et al.*, 1999; Coghlan *et al.*, 1991; Downey, 1990; Halliwell & Gutteridge, 1999; Weiss *et al.*, 1993).

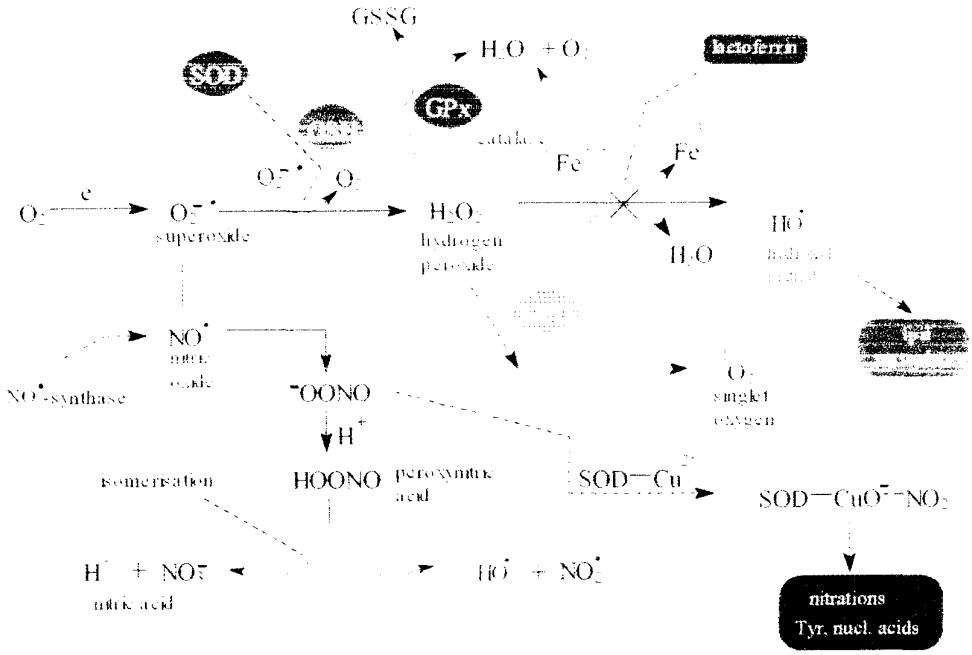


Figure 1. Biological redox systems *(after K. Hideg).*

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 (Halliwell & Gutteridge, 1999).

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 (Halliwell & Gutteridge, 1999).

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 (Halliwell & Gutteridge, 1999; Habon *et al.*, 2001; Halmosi *et al.*, 2001; Halmosi *et al.*, 2002; Szabados *et al.*, 1999a and 1999b), and impair an ample of physiological functions, e.g. blocking of ionic pumps, restricting glycolysis or promoting mitochondrial calcium release (Depre & Taegtmeyer, 2000). 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 (Halliwell & Gutteridge, 1999; Halmosi *et al.*, 2001; Halmosi *et al.*, 2002). 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 they cannot reach the right cell compartment to exert their protective effect (Halmosi *et al.*, 2002; Krishna *et al.*, 1998).

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 (Halmosi *et al.*, 2002; Marton *et al.*, 2001). 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 (see Figure 2 for HO-3073 containing a 2,2,6,6-tetramethyl-tetrahydropyridin ring) (Krishna *et al.*, 1998; Li *et al.*, 2000; Marton *et al.*, 2001; Shankar *et al.*, 2000; Twomey *et al.*, 1997). The hypothetical free radical-entrapping mechanism of these molecules is

delineated on Figure 3. 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.

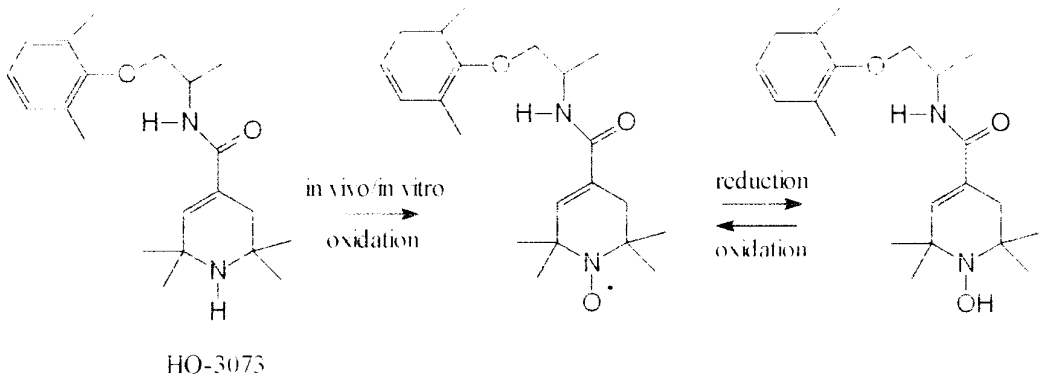


Figure 2. Chemical structure and possible conversions of HO-3073.
HO-3073: 2,2,6,6-tetramethyl-N-[1-methyl-2-(2,6-dimethylphenoxy)ethyl]-1,2,3,6-tetrahydropyridin-4-carboxamide.

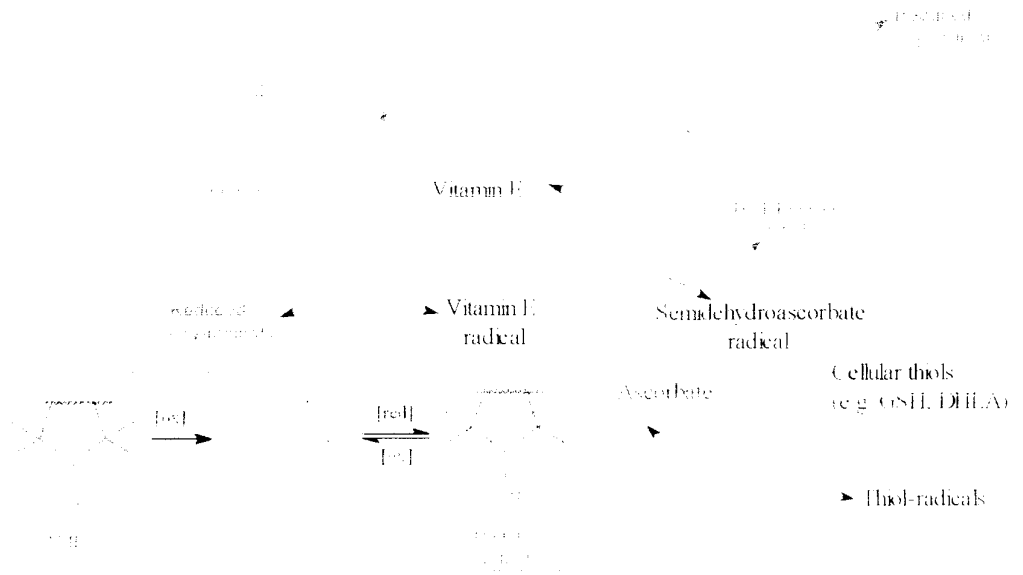


***Figure 3. Scavenging mechanism of heterocyclic amine compounds and their metabolites** (after K. Hideg).*

Under our experimental conditions, the examined heterocyclic compounds exerted cardioprotection in a strikingly low concentration, i.e. 5nM. 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 millimolar concentration range in various models of oxidative stress (Monti *et al.*, 1996, Park *et al.*, 2002). 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 (Halmosi *et al.*, 2002; Li *et al.*, 2000). Therefore, it is implausible that the eventual antiarrhythmic property of these compounds appreciably conduces to their cardioprotection.

1.2. 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 (Kannan & Jain, 2000; Martindale & Holbrook, 2002; Piacentini & Karliner, 1999; Yang *et al.*, 2003). 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 (Mockridge *et al.*, 2000). The PI3-kinase/Akt pathway is one of several signal transmission pathways brought into connection with cell survival (Fujio *et al.*, 2000; Scheid & Woodgett, 2001). 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 (Klotz *et al.*, 2000; Yamashita *et al.*, 2001). 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 (Brazil & Hemmings, 2001; Klotz *et al.*, 2000). In addition, p70 ribosomal S6 kinase is activated promoting mRNA translation and cell cycle progression as well as phosphorylating Bad (Jonassen *et al.*, 2001). Akt also induces endothelial nitric oxide synthase (eNOS) (Dimmeler *et al.*, 1999; Gao *et al.*, 2002) as well as IκB kinase-α (IKKα) activity (Romashkova & Makarov, 1999), but blocks glycogen synthase kinase-3 (GSK-3) (Brazil & Hemmings, 2001; Scheid & Woodgett, 2001).

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 (Pap & Cooper, 2002; Scheid & Woodgett, 2001). 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 (Kroemer & Reed, 2000). Moreover, endothelial nitric oxide synthase activated by Akt forms nitric oxide causing vasorelaxation (Dimmeler *et al.*, 1999; Gao *et al.*, 2002). 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 (Davies *et al.*, 2000).

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 pro-survival factor (Gao *et al.*, 2002), or by NO inhibition of neutrophil infiltration (Matsui *et al.*, 2001). Akt also influences glucose uptake by recruiting GLUT-4 to the cell membrane, which propagates the more favorable bioenergetics of glycolytic metabolism (Matsui *et al.*, 2001). 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 (Tong *et al.*, 2000).

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 (Ashe & Berry, 2003; Kannan & Jain, 2000; Zhao *et al.*, 2002). 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 (Yellon & Baxter, 1999).

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 (Clerk *et al.*, 1998; Seger & Krebs, 1995). ERK is generally involved in the transmission of mitogen signals through Ras-Raf-1/MEK-ERK pathway (Punn *et al.*, 2000). However, sustained activation of ERK during re-oxygenation was shown to render delayed cytoprotection (Punn *et al.*, 2000). Also hydrogen peroxide injury is limited by an elevated prostacyclin formation through ERK-mediated cyclooxygenase-2 expression (Adderley & Fitzgerald, 1999). The fact that ERK can block caspase-3 enzyme activity (Yellon & Baxter, 1999) and downregulate c-jun N-terminal kinase (JNK) (Hong *et al.*, 2001) lends additional support to the notion that ERK possesses cytoprotective properties during oxidative insult (Depre & Taegtmeyer, 2000). A downstream substrate of ERK, p90 ribosomal S6 kinase (RSK) can phosphorylate GSK-3β thereby reinforcing the action of Akt on this kinase (Seger & Krebs, 1995). There are data indicating that ERK is only active if the mitochondrial electron chain and substrate oxidation are intact, otherwise ERK becomes inactivated (Bogoyevitch *et al.*, 2000).

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 (Berra *et al.*, 1998; Clerk *et al.*, 1998; Punn *et al.*, 2000; Tobiume *et al.*, 2001). 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 (Tobiume *et al.*, 2001). 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 (Piacentini & Karliner, 1999). Additionally, JNK and p38-MAPK can precipitate apoptosis by mitochondria-dependent caspase activation (Tobiume *et al.*, 2001). ATF-2 may be phosphorylated and induced by p38-MAPK, as well (Piacentini & Karliner, 1999).

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 (Rakhit *et al.*, 2001). 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 (Benjamin & McMillan, 1998; Rakhit *et al.*, 2001). 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. Figure 4 gives an outline of the aforementioned signal transduction pathways that may participate in the mediation of ischemia-reperfusion-related oxidative injury. 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.

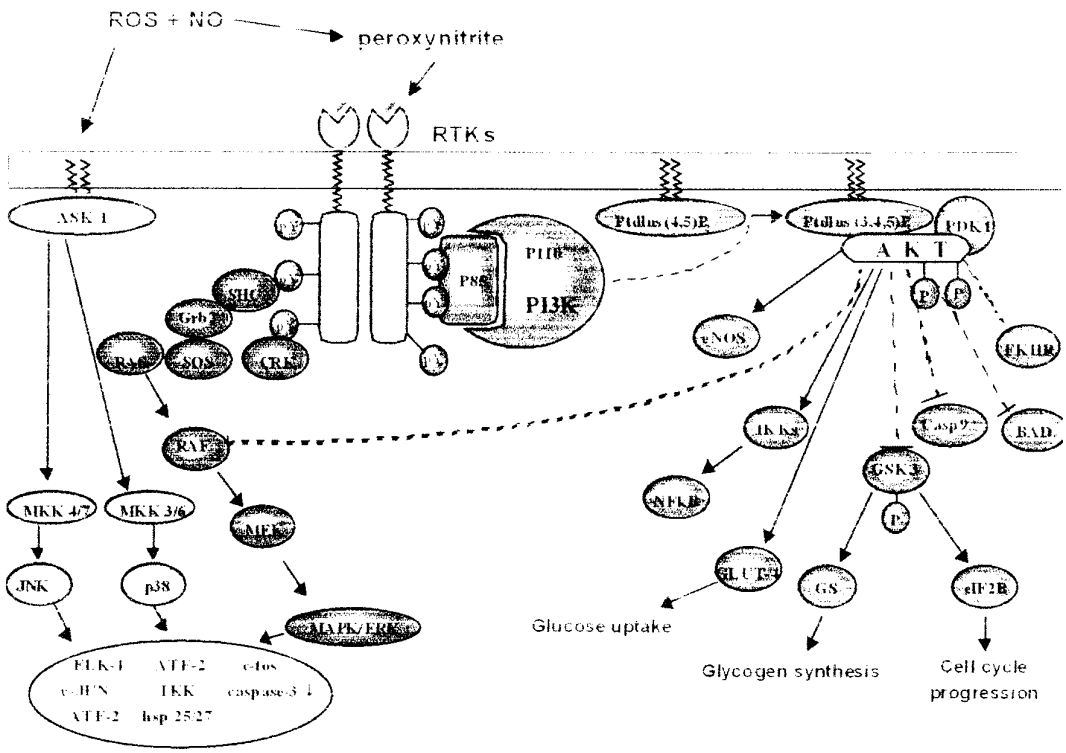


Figure 4. Illustration of the complexity of some ischemia-reperfusion-related signal transmission pathways (JNK, p38-MAPK, ERK, and Akt) and their possible connection to reactive oxygen species formation. *Peroxynitrite formed from superoxide anion and nitric oxide can activate receptor tyrosine kinases (RTKs), which, in turn, leads to the activation of phosphatidylinositol-3-kinase (PI3K) recruiting Akt to the proximity of cell membrane and facilitating Akt phosphorylation by phosphatidylinositol-dependent kinase-1 (PDK-1). As a consequence, Akt phosphorylates a number of substrates resulting in antiapoptotic effects, metabolic adjustment, and nitric oxide formation by eNOS. On the other hand, active receptor tyrosine kinases may also culminate in ERK activation (leading to caspase-3 and JNK downregulation). Reactive oxygen species can induce p38-MAPK and JNK activation through apoptosis signal-regulating kinase-1 (ASK-1), as well, having basically proapoptotic impacts.*

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

3.1. Chemicals

The synthesis of heterocyclic compounds was (HO-2, HO-2434, HO-3144, and H-2693) (their structures are shown on Figure 5) or will be (HO-3073) published elsewhere (structure of HO-3073 is shown on Figure 2) (Hankovszky *et al.*, 1986). Wortmannin was purchased from Calbiochem (Darmstadt, Germany). All other reagents were of the highest purity commercially available.

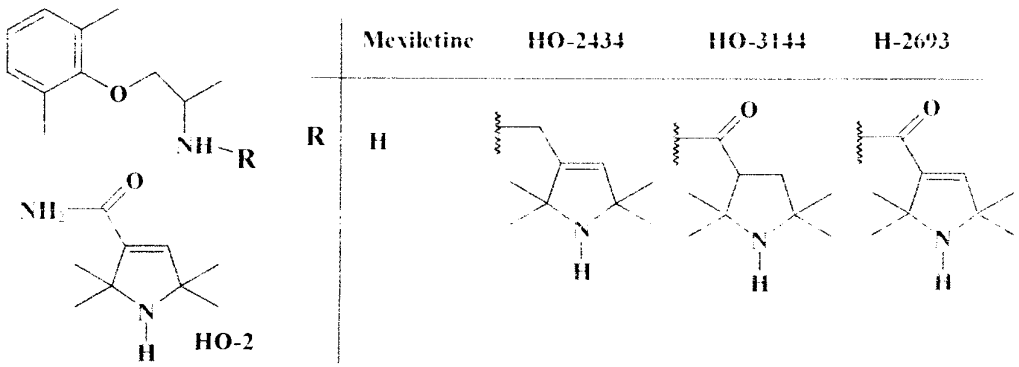


Figure 5. Chemical structure of mexiletine and the examined substituted mexiletine derivates *(except for HO-3073).*

HO-2: *2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-carboxamide.*

HO-2434: *N-[(1-methyl-2-(2,6-dimethylphenoxy)ethyl]-N-[(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl]amine;*

HO-3144: *2,2,5,5-tetramethyl-N-[1-methyl-2-(2,6-dimethylphenoxy)ethyl]pyrrolidine-3-carboxamide;*

H-2693: *2,2,5,5-tetramethyl-N-[1-methyl-2-(2,6-dimethylphenoxy)ethyl]-2,5-dihydro-1H-pyrrole-3-carboxamide.*

3.2. 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 Pecs Medical School.

3.3. 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 (Szabados *et al.*, 1999b; Varbiro *et al.*, 2003a; Varbiro *et al.*, 2003b). 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.

3.4. NMR spectroscopy

NMR spectra were recorded with a Varian UNITYINOVA 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 ^1H 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

possible to calculate the concentration at which the rate of superoxide anion-induced cytochrome c reduction was inhibited by 50%.

3.7. 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.

3.8. 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 (Sharma & Singh, 2000).

3.9. 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 (Serbinova *et al*., 1992). 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.

3.10. 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 (Butterfield *et al.*, 1997).

3.11. 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.

3.12. Measurement of caspase-3 activity

100-mg samples of normoxic (perfused for 10 min), control and treated hearts (with H-2093 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 (Bijur *et al.*, 2000).

3.13. 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. RESULTS

4.1. Effect of the compounds on the energy metabolism of perfused hearts during IR

Energy metabolism of Langendorff perfused hearts was monitored in the magnet of a ^{31}P NMR spectroscope making possible to detect changes in high-energy phosphate intermediates (representative spectra are shown on Figure 6).

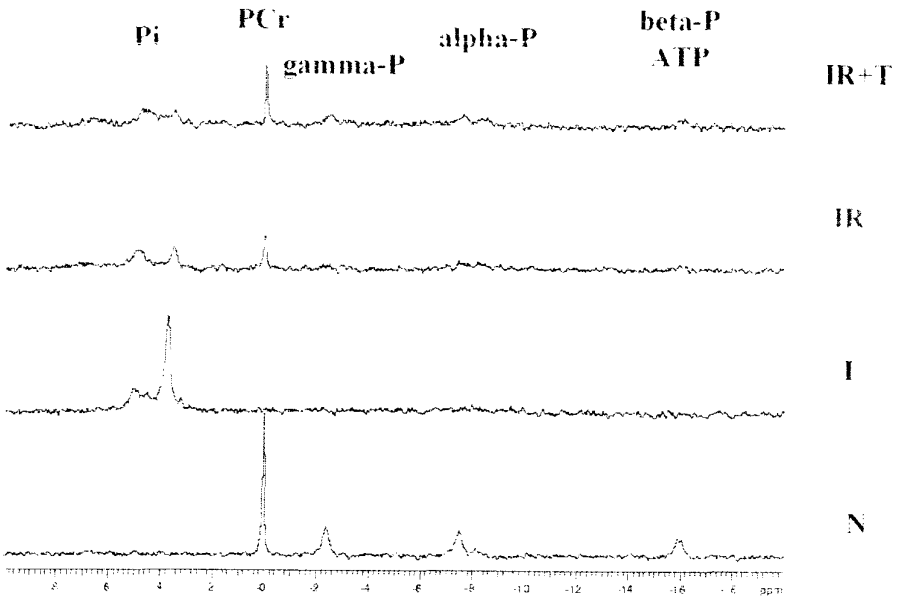


Figure 6. Representative ^{31}P NMR spectra of high-energy and inorganic phosphates. *The area under the curves is proportional to the quantity of the given phosphate groups in the heart. During normoxic (N) perfusion there are ample of creatine phosphate (PCr) and ATP (indicated by beta-phosphate), but no detectable inorganic phosphate (Pi). By the end of ischemia (I) all high-energy phosphates have been catabolized to inorganic phosphate. In the following period of reperfusion (IR) creatine phosphate and ATP partially recovered, and inorganic phosphates were partly consumed in untreated hearts, while administration of antioxidant agents (IR+T) facilitated the recovery of high-energy phosphates as well as the reutilization of inorganic phosphate.*

Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates recovered only partially in untreated and mexiletine-treated hearts during the 15-minute reperfusion phase, on the other hand substituted mexiletine derivates facilitated the recovery of creatine phosphate and ATP. The time dependence of creatine phosphate (Figure 7) shows that mexiletine did not affect notably the energy state of postischemic hearts, while its derivates (HO-2434 and HO-3144) improved the recovery of cardiac energy metabolism. However, beneficial effects of H-2693 as well as HO-3073 clearly and significantly surpassed those of the other examined compounds.

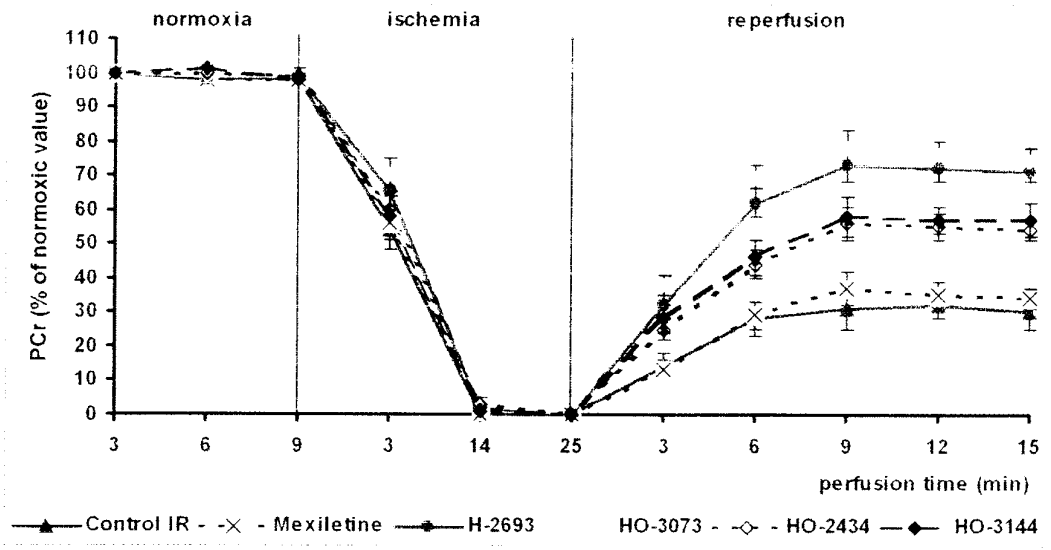


Figure 7. Effect of mexiletine and the substituted mexiletine derivates on the recovery of creatine phosphate (PCr) after ischemia-reperfusion in Langendorff perfused hearts. *Each compound was administered in 5μM concentration. Values are given as means ± SEM for five experiments. Recovery of HO-2434-, HO-3144-, H-2693- and HO-3073-treated hearts significantly differed from that of mexiletine-treated and untreated control IR hearts (p 0.01).*

This was a consequent finding in respect to the recovery of creatine phosphate (Figure 7) and ATP, as well as to the reutilization of inorganic phosphate. As a consequence, in our further experiments we investigated the effects of H-2693 and HO-3073, as best agents, on the parameters of heart function, infarct size, oxidative cell damage as well as signal transduction processes. Figure 7 demonstrates that H-2693 and HO-3073 significantly improved the final recovery of creatine phosphate ($p < 0.01$). This effect was also obvious when the ATP level was concerned ($p < 0.01$) (Table 1). Moreover, H-2693 as well as HO-3073 promoted the faster and more complete reutilization of inorganic phosphate during reperfusion ($p < 0.01$) (Table 1). The intracellular pH markedly decreased by the end of the ischemic period from 7.41±0.04 preischemic value. In the contrary, 15-minute reperfusion brought about a slight elevation in pH in untreated hearts, while this tendency proved to be more explicit in case of H-2693 and HO-3073 administration ($p < 0.01$) (Table 1). The impact of HO-2, the sole pyrroline ring present in H-2693, on ischemia-reperfusion-related energy metabolism did not differ at all from the energetics of untreated postischemic hearts (data not shown).

	Untreated IR	***IR+H-2693 (5μM)***	***IR+HO-3073 (5μM)***
ATP recovery (% of normoxic value)	22±4%	43±5%*	47±4%*
Inorganic phosphate reutilization (% of end-ischemic value)	54±6%	31±4%*	30±4%*
pH – end of ischemia	5.87±0.07	6.12±0.06*	6.16±0.07*
pH – end of reperfusion	6.01±0.07	6.70±0.09*	6.72±0.08*

Table 1. Percentage recovery of ATP and reutilization of inorganic phosphate as well as pH values for untreated, H-2693-, and HO-3073-treated hearts reperfused for 15 minutes. *Difference from the corresponding values of untreated IR hearts: $p < 0.01$.

In the same experimental setting 100nM wortmannin did not alter the poor recovery of high-energy phosphates and the evolution of inorganic phosphate when added by itself. On the other hand, wortmannin was able to neutralize the cardioprotective impacts of H-2693 and HO-3073 when administered together with

those compounds at the beginning of normoxic perfusion. So combined application of H-2693/HO-3073 and wortmannin yielded comparable creatine phosphate and ATP recoveries to that of untreated control hearts (Figure 8) (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

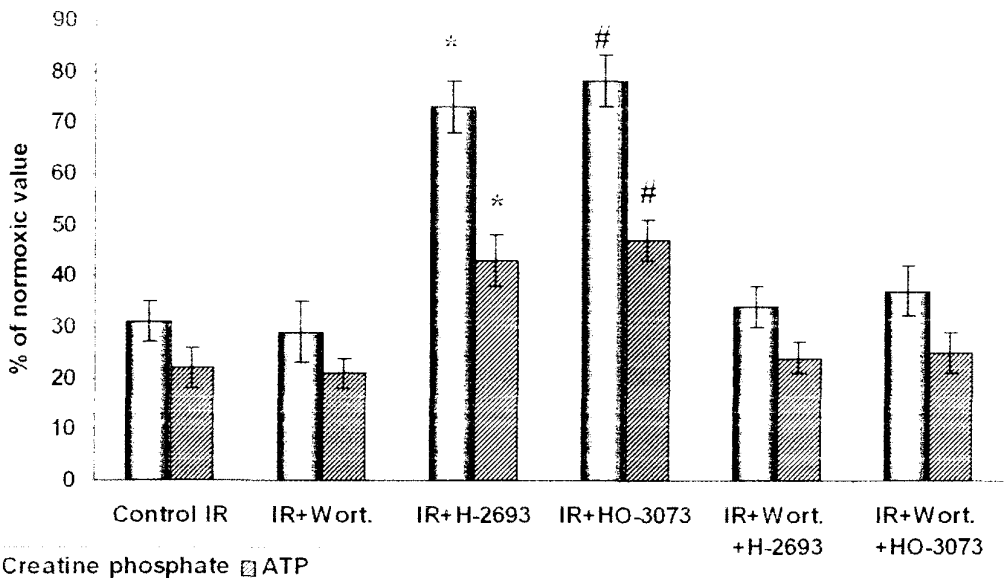


Figure 8. Creatine phosphate and ATP recovery peaks compared to untreated control IR in the reperfusion period of hearts treated with wortmannin, H-2693, HO-3073, or wotmannin plus H-2693/HO-3073. *H-2693 and HO-3073 were administered in 5μM, while wortmannin was given in 100nM, respectively. Values are given as means ± SEM for five experiments. *Difference from control IR, IR+Wort., and IR+Wort.+H-2693 values: p 0.01. #Difference from control IR, IR+Wort., and IR+Wort.+HO-3073 values: p 0.01.*

4.2. Hydroxyl radical scavenging capability

We measured the concentration of the amine as well as hydroxylamine moieties of H-2693 and HO-3073 at which the rate of hydroxyl radical-induced benzoic acid hydroxylation was inhibited by 50%. IC_{50} of 63±6μM was obtained for the amine and of 49±5μM for the hydroxylamine form of H-2693. IC_{50} of 52±5μM was measured for the amine and of 44±7μM for the hydroxylamine form of HO-3073. Thus the amine

and hydroxylamine forms of both compounds could effectively entrap hydroxyl radicals, while they were oxidized to the nitroxide moiety (Toth *et al.*, 2003a).

4.3. Superoxide anion scavenging capability

The rate of superoxide anion-induced cytochrome c reduction was inhibited by 50% in the presence of 243.7μM and 197.8μM of the nitroxide forms of H-2693 and HO-3073, respectively. Therefore, the nitroxide moiety of both compounds was able to scavenge superoxide anions and, in the meantime, it was reduced to the hydroxylamine form. The IC_{50} of Tempol in the same experimental setting was 282±5μM. Moreover, 40 Units of superoxide dismutase enzyme could completely block the cytochrome c reduction in this assay (Toth *et al.*, 2003a).

4.4. Functional recovery of postischemic rat hearts during reperfusion

To evaluate the effect of our experimental compounds on the postischemic myocardial functional recovery, isolated hearts were perfused with either 5μM H-2693/HO-3073, 100nM wortmannin, or both. At the end of the equilibration period, left ventricular developed pressure (LVDP) was 142±19mmHg, rate-pressure product (RPP) was 3.3±0.13 x 10^4mmHg/min, dP/dt was 1233±202mmHg/s and the average heart rate was 236±16 beats/min. Figure 9 shows the percentage recovery of LVDP, RPP, and dP/dt during reperfusion compared to the initial values. Both experimental agents significantly improved the recovery of all parameters ($p<0.01$), which was prevented by the co-administration of wortmannin. Table 2 summarizes the development of left ventricular end-diastolic pressure (LVEDP) at different time points in each experimental condition. H-2693 and HO-3073 had beneficial impact on the IR-triggered increase in LVEDP ($p<0.01$) (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

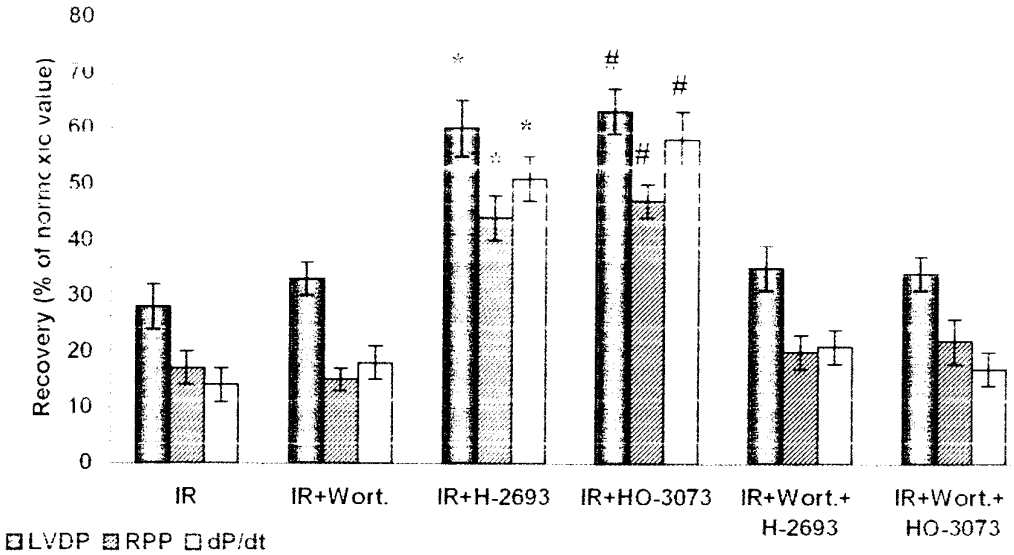


Figure 9. Maximal percentage recovery of left ventricular developed pressure (LVDP), rate-pressure product (RPP), and dP/dt during the 45-minute reperfusion period following ischemia in untreated (IR), wortmannin-, H-2693-, HO-3073-, and wortmannin plus H-2693/HO-3073-treated hearts compared to the normoxic values. *Wortmannin was given in 100nM, while H-2693 and HO-3073 were administered in 5μM. Values are given as means ± SEM for five experiments. *Recovery of H-2693-treated hearts significantly differed from that of the IR, IR+Wort., and IR+Wort.+H-2693 hearts for LVDP, RPP, and dP/dt ($p < 0.01$). #Recovery of HO-3073-treated hearts significantly differed from that of the IR, IR+Wort., and IR+Wort.+HO-3073 hearts for LVDP, RPP, and dP/dt ($p < 0.01$).*

Left ventricular end-diastolic pressure (mmHg)	Preischemic	Reperfusion 0 min.	Reperfusion 45 min.
Control IR	9±1.4	41±4*	29±3
IR+wortmannin	10±2	37±3*	30±2
IR+H-2693	10±1.1	19±2*#	12±2 #
IR+HO-3073	10±0.8	18±2*&	12±1 &
IR+wortmannin+H-2693	11±0.9	35±3*	26±3
IR+wortmannin+HO-3073	9±1.2	39±4*	27±3

Table 2. Absolute values of left ventricular end-diastolic pressure (LVEDP) at different time points throughout the perfusion. *Pressure values were measured at the end of the equilibration period (preischemic), at the end of ischemia (reperfusion 0 min.), and at the end of reperfusion (45 min.). *Difference from the corresponding preischemic values ($p < 0.01$). #Difference from the corresponding "reperfusion 0 min." values ($p < 0.05$). &Difference from the "Control IR", "IR+wortmannin", and "IR+wortmannin+H-2693" values at the same time point of reperfusion ($p < 0.01$). &Difference from the "Control IR", "IR+wortmannin", and "IR+wortmannin+HO-3073" values at the same time point of reperfusion ($p < 0.01$).*

4.5. Infarct size

Triphenyl tetrazolium chloride staining in five consecutive samples demonstrated that the ischemia followed by 90-minute reperfusion in untreated cases brought about the infarction of 64±7% of the ventricles. In the meantime, H-2693 and HO-3073 administration significantly reduced the infarct size to 35±7% and 29±6% of the heart samples, respectively ($p<0.01$) (representative sections are shown on Figure 10). The PI3-kinase inhibitor wortmannin did not alter the size of infarcted area when administered alone, on the other hand, it abrogated the beneficial influence of the two experimental agents on infarct size in the case of co-treatment. Control staining of "normoxic" hearts perfused for 10 minutes rendered no appreciable infarcted area (Toth *et al.*, 2003b).

Untreated heart sample **HO-3073-treated heart sample**

Figure 10. Representative sections stained by triphenyl tetrazolium chloride from untreated (left) and HO-3073-treated (right) hearts. *Normal myocardium was stained brick red, while the infarcted areas remained unstained (indicated by white arrows). As shown on the figure, during untreated ischemia-reperfusion sizable myocardial infarct developed (left), which was markedly reduced by the administration of HO-3073 (right).*

4.6. Ischemia-reperfusion-induced lipid peroxidation and protein oxidation

Lipid peroxidation induced by ischemia-reperfusion in Langendorff perfused hearts was characterized by the formation of thiobarbituric acid reactive substances (TBARS). In our current experiments, ischemia-reperfusion increased the amount of TBARS compared to the normoxic hearts ($p<0.01$) (Figure 11). When ischemia-reperfusion occurred in the presence of 5μM H-2693 or HO-3073, the formation of TBARS was significantly reduced ($p<0.01$) compared to untreated hearts (Figure 11), indicating that both compounds prevented the ischemia-reperfusion-related lipid peroxidation.

Reactive oxygen species formation in ischemia-reperfusion cycle can also trigger the oxidation of proteins in the cardiomyocytes, which can be determined by the quantity of protein-bound aldehyde groups. Figure 11 shows that ischemia reperfusion significantly elevated the level of protein oxidation ($p < 0.01$). However, the presence of H-2693 and HO-3073 during ischemia-reperfusion significantly attenuated ($p < 0.01$) the increase in the quantity of protein-bound aldehyde groups (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

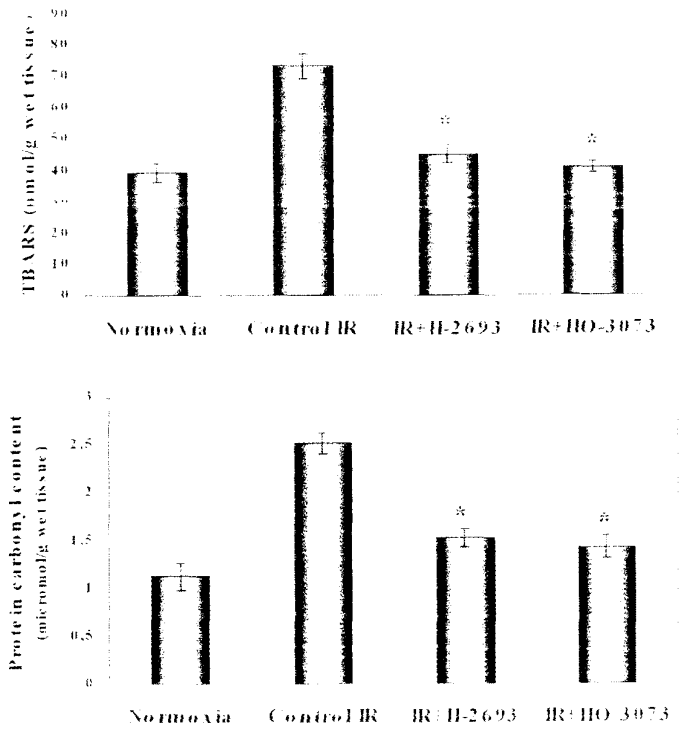
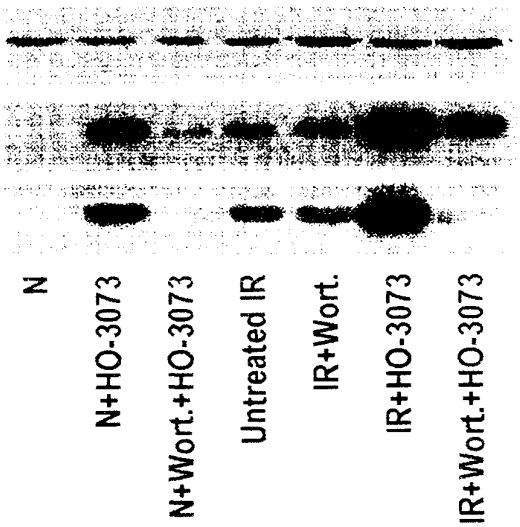
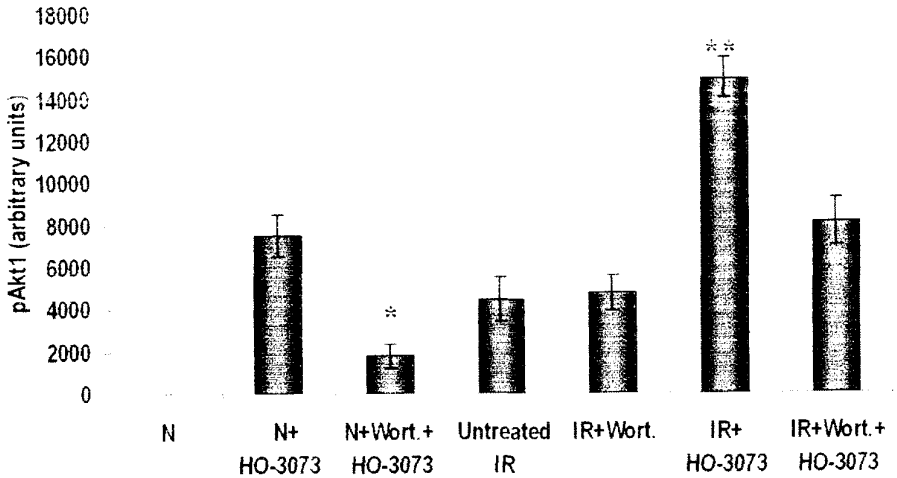


Figure 11. Effect of H-2693 and HO-3073 on the ischemia-reperfusion-induced lipid peroxidation (top) and protein oxidation (bottom) in Langendorff perfused hearts. *Values are given as means ± SEM for five experiments. *Difference from untreated control ischemia-reperfusion values: $p < 0.01$.*

4.7. Alterations in the phosphorylation state of Akt-1 and GSK-3β

Figure 12 demonstrates the characteristic changes in the phosphorylation of these kinases on the example of HO-3073 treatment. H-2693 administration triggered such changes that showed exactly the same pattern as in the case of HO-3073 treatment (thus those data are not shown). The undetectable phosphorylation of Akt-1 under normoxic conditions in our study increased after ischemia and 15-minute reperfusion, nevertheless, HO-3073/H-2693 administration further enhanced the activation of Akt-1 (Figure 12). GSK-3β was not phosphorylated during normoxia, but became moderately phosphorylated after ischemia-reperfusion, and strongly phosphorylated as a consequence of HO-3073/H-2693 treatment. As GSK-3β is phosphorylated by Akt-1 leading to its inactivation, the marked phosphorylation of GSK-3β in treated hearts is in accordance with the observed enhanced Akt-1 activation in the same tissue samples (Figure 12). Under the same experimental circumstances, we investigated the effect of the phosphatidylinositol-3-kinase inhibitor, wortmannin on the activation state of Akt-1 as well as GSK-3β. Wortmannin when administered alone caused about the same phosphorylation of Akt-1 and GSK-3β as ischemia-reperfusion itself in untreated hearts (Figure 12). However, wortmannin proved to be able to reduce the HO-3073/H-2693-induced Akt-1 (activation) and GSK-3β phosphorylation (inactivation) when the two compounds were given together (Figure 12). Interestingly, HO-3073/H-2693 also brought about Akt-1 as well as GSK-3β phosphorylation during the 10-minute baseline perfusion, which is in clear contrast with the untreated normoxic condition where no phosphorylation at all was observed (Figure 12). While wortmannin alone had no impact on the phosphorylation state of either of the kinases during the aforementioned baseline perfusion (data not shown), it did prevent the phosphorylation when was coadministered with HO-3073/H-2693 (Figure 12). Careful analysis of the time-course of Akt as well as GSK-3β phosphorylation revealed no differing phenomena at various time points during normoxia (5, 10, and 30 minutes) as well as reperfusion (5, 15, 30, and 45 minutes) (data not shown). This observation points out that both experimental compounds caused definite alterations in the examined signaling pathway, which was inhibited by wortmannin being reproducible at different time points of the perfusion, as well (Toth *et al.*, 2003a, Toth *et al.*, 2003b).


Akt
p-Akt1
p-GSK-3beta
N
N+HO-3073
N+Wort.+HO-3073
Untreated IR
IR+Wort.
IR+HO-3073
IR+Wort.+HO-3073


pAkt1 (arbitrary units)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
N
N+ HO-3073
N+Wort.+ HO-3073
Untreated IR
IR+Wort.
IR+ HO-3073
IR+Wort.+ HO-3073

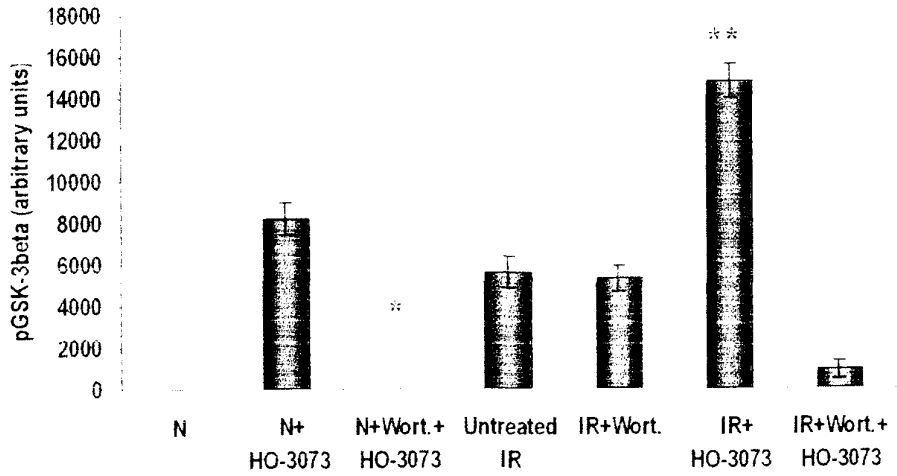

pGSK-3beta (arbitrary units)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
N
N+ HO-3073
N+Wort.+ HO-3073
Untreated IR
IR+Wort.
IR+ HO-3073
IR+Wort.+ HO-3073

Figure 12. Effect of HO-3073 and wortmannin on the phosphorylation state of Akt-1 as well as glycogen synthase kinase-3β. *Representative immunoblots from three experiments with similar results and densitometric evaluations are shown. Akt: non-phosphorylated Akt; p-Akt1: Akt-1 phosphorylated on Ser[473]; p-GSK-3beta: glycogen synthase kinase-3β phosphorylated on Ser[9]. N: "normoxia", i.e. baseline perfusion for 10 minutes; N+HO-3073: baseline perfusion for 10 minutes in the presence of 5μM HO-3073; N+Wort.+HO-3073: baseline perfusion for 10 minutes in the presence of 100nM wortmannin and 5μM HO-3073; Untreated IR: ischemia-reperfusion in the absence of any agent; IR+Wort.: ischemia-reperfusion in the presence of 100nM wortmannin; IR+HO-3073: ischemia-reperfusion in the presence of 5μM HO-3073; IR+Wort.+HO-3073: ischemia-reperfusion in the presence of 100nM wortmannin and 5μM HO-3073. *Difference of N+Wort.+HO-3073 hearts from N+HO-3073 samples: $p < 0.01$. **Difference of IR+HO-3073 hearts from the remaining samples (except for N): $p < 0.01$.*

4.8. Caspase-3 enzyme activity

Caspase-3 activity was measured to test whether H-2693 as well as HO-3073 treatment could influence this effector protease of the apoptotic cell death cascade. The specificity of the caspase-3 assay was confirmed by the use of a caspase-3 inhibitor, which resulted in the complete inhibition of caspase-3 activity (data not shown). Table 3 demonstrates that ischemia-reperfusion moderately enhanced caspase-3 activity when compared to normoxic conditions (by about 80%), but H-2693 and HO-3073 administration could significantly diminish this activation ($p<0.01$). Wortmannin did not affect caspase-3 activity during untreated ischemia-reperfusion, however, it prevented the enzyme activity-decreasing effect of H-2693 and HO-3073 when was administered along with one of the cardioprotective compounds (Toth *et al.*, 2003a).

	Caspase-3 activity in % of activity in normoxic hearts
Untreated control IR	179±7%
IR+wortmannin	171±8%
IR+H-2693	129±6%*
IR+HO-3073	125±5%
IR+wortmannin+H-2693	167±5%
IR+wortmannin+HO-3073	177±6%

Table 3. Caspase-3 enzyme activity in the percentage of activity in hearts under normoxic conditions. *The activity in H-2693-treated samples significantly differed from the "Untreated control IR", "IR+wortmannin", and "IR+wortmannin+H-2693" samples ($p<0.01$). The activity in HO-3073-treated samples significantly differed from the "Untreated control IR", "IR+wortmannin", and "IR+wortmannin+HO-3073" samples ($p<0.01$).

5. DISCUSSION

Oxygen is the most basic element necessary for all eukaryotic life, acting as the final electron acceptor in the respiratory chain. Insufficient oxygen (hypoxia) deprives the respiratory chain of its main electron acceptor. The less efficient anaerobic glycolytic pathway then supersedes oxidative phosphorylation as the principal source of ATP production, generating approximately one-fourth the amount of ATP produced by oxidative phosphorylation. Consequently, there are insufficient high-energy phosphates to maintain normal function. If hypoxia is reversed, the subsequent re-oxygenation can also produce cell-damaging effects through increased levels of radical oxygen species. These arise with partial reduction of oxygen to water and can induce damage to proteins (protein oxidation and enzyme inactivation), nucleic acids (DNA strand breaks), and cell membranes (lipid peroxidation) (Piacentini & Karliner, 1999).

The heart is an organ with particular susceptibility to hypoxia since only limited reserves of high-energy phosphates are maintained. The myocardium may be exposed to hypoxia or anoxia under a number of conditions such as myocardial ischemia after occlusion of a major coronary artery, high altitude, or anemia. The extent and duration of hypoxia, in addition to the presence of other confounding factors such as tissue ischemia, will determine the cardiac response to diminished oxygen supply. Both hypoxia and oxidative stress result in biochemical and functional changes as the heart attempts to maintain function in the face of perturbations in oxygen tension. As in other cell types, hypoxia and re-oxygenation alter the cardiac protein pattern, mainly through altered gene expression, but also through changes in mRNA stability, rates of translation, and protein degradation. Hypoxia induces up-regulation of specific proteins that mediate both protective and deleterious effects. These can be divided into two broad groups: those associated with maintenance of function increasing glycolysis, glucose uptake, and oxygen supply and those associated with recovery from stress, for example, pro- and antiinflammatory cytokines, antioxidants, and heat shock proteins. Increased oxygen levels or re-oxygenation generate pathophysiological quantities of reactive oxygen species. Recent experiments indicate that levels of superoxide anion increase rather

within the hypoxic cardiomyocyte, whilst hydrogen peroxide and hydroxyl radicals are generated mainly with re-oxygenation (Piacentini & Karliner, 1999).

In the current study, cardioprotective properties of heterocyclic nitroxide precursor compounds were tested in Langendorff perfused rat hearts during ischemia-reperfusion. The two most effective molecules (H-2693 and HO-3073) were able not only to promote the recovery of myocardial energy metabolism and mitigate the cardiac oxidative damage during postischemic reperfusion in a fairly low concentration (5μM), but also beneficially influenced the phosphatidylinositol-3-kinase/Akt signaling cascade.

5.1. Antioxidant context

Although reperfusion of ischemic tissue is the primary step in either preventing or eliminating cardiac damage, re-oxygenation itself can induce the formation of the above mentioned deleterious oxidants. Such oxidants can inflict significant biological damage and lead to inefficient cardiac contractility and/or malignant arrhythmias. Postischemic reperfusion injury is prevented by treatment with antioxidant enzymes such as superoxide dismutase (SOD) and catalase. However, since SOD and catalase, because of their molecular size, can penetrate only to a limited extent to all areas susceptible to oxidative damage, there have been intense efforts to employ small molecular weight antioxidant molecules (Krishna *et al.*, 1998; Li *et al.*, 2000; Marton *et al.*, 2001; Shankar *et al.*, 2000; Twomey *et al.*, 1997).

As these antioxidant molecules in our study were derived from mexiletine, a class I/b antiarrhythmic agent by substitution with a five-membered as well as a six-membered ring containing an amine group, their protective effects were related to those of untreated as well as mexiletine-treated hearts. Although mexiletine exhibited no significant protection, its substituted derivates facilitated the recovery of high-energy phosphate intermediates and the reutilization of inorganic phosphate during postischemic reperfusion. Evidences accumulated from ^{31}P nuclear magnetic resonance (NMR) spectroscopic studies suggested that the magnitude of protection

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 (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

As we put in the introductory chapter, this pyrroline or tetrahydropyridin ring has the potential to undergo oxidation at the secondary amine moiety to the corresponding stable free radical nitroxide. Nitroxides, on the other hand, have been shown to undergo one-electron reduction to the corresponding hydroxylamine (Shankar *et al.*, 2000; Twomey *et al.*, 1997). Thus, hydroxylamines and nitroxides can be in equlibrium based on the oxygen status of the tissue. In recent studies, nitroxides have been shown to possess antioxidant properties explained in terms of the following: SOD-mimetic activity, termination of free radical chain reactions, stimulation of catalase-like activity in heme proteins, participating in radical-radical recombination reactions, and oxidizing reduced transition metal complexes (Halmosi *et al.*, 2002; Krishna *et al.*, 1998; Li *et al.*, 2000; Marton *et al.*, 2001; Shankar *et al.*, 2000; Twomey *et al.*, 1997). Any or all of these pathways may account for the antioxidant properties exhibited by stable nitroxides. Our current experiments demonstrated that the amine and hydroxylamine forms of H-2693 and HO-3073 were able to scavenge hydroxyl radicals by being oxidized to the nitroxide form and the nitroxide moiety of H-2693 and HO-3073 was able to dismutase superoxide anions (at a degree comparable to that of Tempol) by being reduced to the hydroxylamine moiety of the experimental compound (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

Myocardial energy metabolism was preserved by the administration of 2, 5, and 10μM H-2693 as well as HO-3073 and we observed significant improvement in two indices of cellular oxidative injury, i.e. the level of lipid peroxidation and protein oxidation was attenuated compared to untreated hearts. Nevertheless, it cannot be excluded that these indirect evidences for myocardial free radical scavenging may have arisen from another, yet unknown, cardioprotective action of the selected compounds. As far as the cardiac energetics is concerned, the clear improvement in creatine phosphate and ATP levels during reperfusion was further underpinned by the rapid and more complete consumption of inorganic phosphate. This kinetics bears crucial importance because elevated concentrations of inorganic phosphate and calcium are the most potent triggers of mitochondrial permeability transition, a

process culminating in the permeabilization of the outer and inner mitochondrial membranes and the subsequent liberation of cytochrome c from the inner membrane, which may depart the apoptotic cell death cascade (Halmosi *et al.*, 2001; Kroemer & Reed, 2000).

In addition, the experimental compounds proved to be able to preserve cardiac contractile function in the ischemia-reperfusion cycle, as well. Both H-2693 and HO-3073 promoted the better recovery of left ventricular developed pressure, rate-pressure product as well as dP/dt and limited the elevation in left ventricular end-diastolic pressure during reperfusion compared to untreated hearts. The functional recovery of treated hearts consistently succeeded the energetic revival of the myocardium in the reperfusion period, i.e. cardiac function reached its approximate steady state level around 10 minutes after the reinstitution of the flow (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

An additional index of cardiac integrity was favorably affected by H-2693 and HO-3073 administration, as well. The extent of infarcted myocardial tissue diminished in case the experimental compounds were used during ischemia-reperfusion (Toth *et al.*, 2003b). The investigation of these three aspects of myocardial functioning convincingly supported the remarkable cardioprotective properties of the examined two mexiletine derivatives, which data are in accordance with the findings of other studies employing structurally resembling radical scavenging molecules (Halmosi et al., 2002; Li et al., 2000; Shankar et al., 2000). This notion and the rather low effective concentration of H-2693 and HO-3073 turned our attention toward the stress-related prosurvival intracellular signal transmission pathways.

5.2. Review of the protective phosphatidylinositol-3-kinase/Akt signal transduction pathway

Phosphatidylinositol-3-kinase (PI3-kinase) is a ubiquitous heterodimeric lipid-modifying enzyme consisting of a p85 regulatory and p110 catalytic subunit. Growth factors and oxidative stress can activate the PI3-kinase through the

autophosphorylation of receptor tyrosine residues. One or more of these phosphorylated tyrosine residues then serve as binding site for the Src homology 2 (SH2) domain of the p85 subunit. An increase in catalytic activity of PI3-kinase results from a combination of this docking of PI3-kinase in the proximity of the plasma membrane, where its lipid substrate is located, plus allosteric regulation of p110 activity by the receptor-bound p85 subunit. Activated p110 catalyzes the phosphorylation of membrane phosphatidylinositol-4,5-bisphosphate to generate phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate and its phospholipid phosphatase product, phosphatidylinositol-3,4-bisphosphate, accumulate in the membrane, creating docking sites for two lipid-binding protein kinases, namely phosphatidylinositol-dependent kinase-1 (PDK1) and protein kinase B, known as Akt, which bind to these lipids via their pleckstrin homology domains. Akt becomes fully activated as a result of this plasma membrane localization and by its phosphorylation on both Thr^{308} and Ser^{473}. These phosphorylation events are catalyzed by PDK1 and an unidentified but provisionally named phosphatidylinositol-dependent kinase-2, respectively (Brazil & Hemmings, 2001; Madge & Pober, 2000; Miao *et al.*, 2000).

Immediate biological effects of peroxynitrite formed from nitric oxide and superoxide anion include DNA damage, protein oxidation, lipid peroxidation and nitration, and nitrosation of biological molecules, such as purines or the tryptophan and tyrosine machinery (Klotz *et al.*, 2000). Under conditions of ischemia-reperfusion peroxynitrite features as a "ligand" of receptor tyrosine kinases and leads to the nitration of tyrosine residues departing the above mentioned PI3-kinase/Akt machinery (Klotz *et al.*, 2000; Yamashita *et al.*, 2001). Akt targets a wide range of substrates by phosphorylation: inactivates the proapoptotic protein Bad, the apoptotic effector enzyme caspase-9, Forkhead transcription factor; as a consequence, apoptosis is suppressed (Brazil & Hemmings, 2001; Klotz *et al.*, 2000). In addition, p70 ribosomal S6 kinase is activated promoting mRNA translation and cell cycle progression as well as phosphorylating Bad (Jonassen *et al.*, 2001). Akt also induces endothelial nitric oxide synthase (eNOS) activity (Dimmeler *et al.*, 1999; Gao *et al.*, 2002) as well as IκB kinase-α (IKKα) (Romashkova & Makarov, 1999), but blocks

glycogen synthase kinase (Brazil & Hemmings, 2001, Cohen & Frame, 2001, Scheid & Woodgett, 2001).

In our current study, H-2693 and HO-3073 were able to further enhance the ischemia-reperfusion-induced Akt activation and elicit GSK-3β phosphorylation promoting the inactivation of the latter one augmenting glycogen synthesis (through glycogen synthase) as well as cell cycle progression (through eukaryotic initiation factor 2b and cyclin D_1) (Toth *et al.*, 2003a, Toth *et al.*, 2003b, Pap & Cooper, 2002, Scheid & Woodgett, 2001).

The known PI3-kinase inhibitor wortmannin could neutralize the cardioprotective impacts of both H-2693 and HO-3073 as indicated by our ^{31}P NMR spectroscopic, left ventricular functional, and infarct size studies. The coadministration of the two antioxidant compounds with wortmannin yielded similarly low creatine phosphate, ATP, left ventricular developed pressure, rate-pressure product, and dP/dt (as well as high left ventricular end-diastolic pressure) recoveries during reperfusion as it was experienced in untreated control hearts. Parallel treatment with wortmannin also extended the size of infarcted myocardium that was markedly shrunk by the application of H-2693 and HO-3073. Thorough investigation was also conducted to reveal the impact of PI3-kinase blockade on the development of lipid peroxidation and protein oxidation. Surprisingly, the sole administration of both wortmannin and LY 294002 (another commonly used inhibitor of PI3-kinase applied in 10μM) could reduce the level of TBARS and protein carbonyl content back to the normoxic value (data not shown). This finding may be attributed to the limited specificity of these inhibitors towards the PI3-kinase enzyme (Davies *et al.*, 2000), which enables them to interfere with alternative pathways making unfeasible to judge their effect on the indirect markers of oxidative damage in the current experimental system. However, the H-2693- and HO-3073-induced enhanced phosphorylation of Akt as well as GSK-3β was mitigated by wortmannin treatment, which reinforces the findings of heart perfusion experiments, namely they facilitate the recovery of postischemic myocardium via at least two pathways. In addition to their free radical-entrapping properties, they can also somehow activate the protective Akt signaling route completing their cardioprotective features. This latter notion is further underpinned by our results that

H-2693 and HO-3073 were capable of bringing about the phosphorylation of Akt as well as GSK-3β when hearts were merely treated for 10 minutes during normoxia and then freeze-clamped. Nevertheless, wortmannin could not completely repress the ischemia-reperfusion- as well as H-2693- and HO-3073-triggered Akt and GSK-3β phosphorylation. This may be accounted for by the eventual limited delivery of this agent into all areas of myocardium, which notion was not supported by the time-course studies of the phosphorylation of the two kinases. Another possible explanation may lie in the hypothesis that Akt can potentially be activated by a yet undetermined PI3-kinase-independent manner, which is apparently not hindered by PI3-kinase inhibitors (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

Phosphorylation by Akt was also shown to negatively regulate apoptosis signal-regulating kinase 1 (ASK1). ASK1 is believed to be the mediator of oxygen free radical-associated activation of c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). JNK and p38-MAPK can precipitate apoptosis by mitochondria-dependent caspase activation (Tobiume *et al.*, 2001; Clerk *et al.*, 1998; Chang & Karin, 2001). As a consequence, Akt may also suppress apoptosis related to JNK and p38-MAPK activation through the inhibition of ASK1 (Kim *et al.*, 2001). The enzymes that ultimately carry out the command for apoptosis are the cysteine proteases known as caspases. Caspases, which are zymogens, are typically cleaved autocatalytically or by other caspases from inactive procaspase proteins to produce activated enzymes. Caspase-3 is activated by many proapoptotic stimuli and is an early step in the execution phase of apoptosis. The activation of caspase-3 commences after apoptotic signals induce the release of cytochrome c from the mitochondrial intermembrane space, which subsequently associates with apoptotic protease-activating factor-1 and procaspase-9 to form the "apoptosome". This complex formation stimulates the oligomerization of procaspase-9 and its autocatalytic activation. The effect of caspase-9 activity is the proteolytic activation of downstream caspases such as caspase-3, which in turn proteolyzes poly(ADP-ribose) polymerase and other proteins. Hence the measurement of caspase-3 activity can serve as a biochemical marker for the execution phase of apoptosis (Bijur *et al.*, 2000; Bishopric, *et al.*, 2001). Our caspase-3 activity measurements clearly demonstrated that H-2693 and HO-3073 could abrogate the enzyme activity during

45-minute reperfusion, which was prevented by parallel wortmannin treatment. This speaks for limited extent of apoptosis in H-2693 as well as HO-3073-treated hearts (Toth *et al.*, 2003a).

Albeit apoptotic cardiomyocyte loss merely amounts to around 6% under conditions of ischemia-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 (Yellon & Baxter, 1999). As we mentioned above, in case of oxidative challenge cardiomyocytes can tread on the way of apoptotis through different mechanisms. Since H-2693 and HO-3073 could entrap free radicals and promote the postischemic utilization of inorganic phosphate, they may have efficaciously prevented the mitochondrial permeability transition pore formation and the subsequent cytochrome c liberation. Moreover, by activating Akt H-2693 and HO-3073 could directly impede the proapoptotic Akt substrates, and indirectly abrogate the activity of various caspases (through eNOS produced NO) (Gao *et al.*, 2002) as well as eventually that of JNK and p38-MAPK (through ASK1) (Kim *et al.*, 2001). Based on this, it seems plausible that H-2693 and HO-3073 conferred their protective effects by limiting both necrosis related to energy depletion (through the inactivation of mitochondrial enzymes) and apoptotic cell death (Toth *et al.*, 2003a; Toth *et al.*, 2003b).

Our data provide the first insight into how free radical-entrapping mexiletine derivates can influence the intracellular signal transduction pathways. Two substituted mexiletine derivates, H-2693 and HO-3073 preserved cardiac energy metabolism as well as cardiac contractile function during ischemia-reperfusion, and also attenuated the oxidative injury and the infarct size of the myocardium. Moreover, H-2693 and HO-3073 administration prompted the activation of Akt and the subsequent inactivation of glycogen synthase kinase-3β both during normoxic perfusion and ischemia-reperfusion. The phosphatidylinositol-3-kinase inhibitor wortmannin abrogated the beneficial effects of H-2693 and HO-3073 concerning myocardial energetics, contractile function, infarct size, and Akt activation. H-2693 and HO-3073 could also diminish the activity of caspase-3, an effector enzyme of the

apoptotic cell death cascade, which was abolished by the co-administration of wortmannin implicating the eventual antiapoptotic effects of these antioxidant agents via Akt activation. Taken together, 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.

6. SUMMARY

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. In conclusion, the cardioprotective effects of the examined antioxidant nitroxide precursor molecules may be attributable not only to their free radical-entrapping properties but also to their ability to upregulate the prosurvival Akt kinase cascade.

7. REFERENCES

1. Adderley, S. R., Fitzgerald, D. J. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J. Biol. Chem. 274: 5038-46, 1999.

2. Ashe, P. C., Berry, M. D. Apoptotic signaling cascades. Prog. Neuro-Psychopharmacol. Biol. Psych. 27: 199-214; 2003.

3. Azzi, A.; Boscoboinik, D.; Chatelain, F.; Ozer, N. K., Stauble, B. D-alpha-tocopherol control of cell proliferation. Mol. Asp. Med. 14: 265-271; 1993.

4. Beckler, L. B.; Hoek, T. L., Shao, Z. H., Li, C. Q.; Schumacker, P. T. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. Am. J. Physiol. 27: H2240-2246; 1999.

5. Benjamin, I. J. & McMillan, D. R. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ. Res. 83: 117-32; 1998.

6. Berra, E.; Diaz-Meco, M. T.; Moscat, J. The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway. J. Biol. Chem. 273: 10792-97; 1998.

7. Bijur, G. N., De Sarno, P., Jope, R. S. Glycogen synthase kinase-3β facilitates staurosporine- and heat shock-induced apoptosis. J. Biol. Chem. 275: 7583-7590; 2000.

8. Bishopric, N. H.; Andreka, P.; Slepak, T., Webster, K. A. Molecular mechanisms of apoptosis in the cardiac myocyte. Curr. Op. Pharmacol. 1: 141-150; 2001.

9. Bogoyevitch, M. A.; Ng, D. C.; Court, N. W.; Draper, K. A., Dhillon, A., Abas, L. Intact mitochondrial electron transport function is essential for signalling by hydrogen peroxide in cardiac myocytes. J. Mol. Cell. Cardiol. 32: 1469-80, 2000.

10. Brazil, D. P.; Hemmings, B. A. Ten years of protein kinase B signalling: a hard Akt to follow. TiBS 26: 657-64, 2001.

11. Butterfield, D. A.; Howard, B. J.; Yatin, S.; Allen, K. L.; Carney, J. M. Free radical oxidation of brain proteins in accelerated senescence and its modulation by N-tert-butyl-alpha-phenylnitrone. Proc. Natl. Acad. Sci. 94: 674-678; 1997.

12. Catapano, A. L.; Maggi, F. M.; Tragni, E. Low density lipoprotein oxidation, antioxidants, and atherosclerosis. Curr. Opin. Cardiol. 15: 355-363, 2000.

13. Chang, L., Karin, M. Mammalian MAP kinase signalling cascades. Nature 410: 37-40; 2001.

14. Clerk, A.; Fuller, S. J.; Michael, A.; Sugden, P. H. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38- mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J. Biol. Chem. 273: 7228-34; 1998.

15. Coghlan, J. G.; Flitter, W. D.; Ilslev, C. D.; Ress, A.; Slater, T. F. Reperfusion of infarcted tissue and free radicals. Lancet 338: 1145-1146; 1991.

16. Cohen, P.; Frame, S. The renaissance of GSK3. Nature Rev. 2: 769-76; 2001.

17. Davies, S. P.; Reddy, H.; Caivano, M.; Cohen, P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. 351: 95-105; 2000.

18. De Graaf *In vivo NMR Spectroscopy*. Chichester: Wiley, 1998.

19. Depre, C.; Taegtmeyer, H. Metabolic aspects of programmed cell survival and cell death in the heart. Cardiovasc. Res. 45: 538-548; 2000.

20. Dimmeler, S.; Fleming, I.; Fisslthaler, B.; Hermann, C.; Busse, R.; Zeiher, A. M. Activation of nitric oxide synthase by Akt-dependent phosphorylation. Nature 399: 601-605; 1999.

21. Downey, J. M. Free radicals and their involvement during long-term myocardial ischemia and reperfusion. Annu. Rev. Physiol. 52: 487-504; 1990.

22. Fujio, Y.; Nguyen, T.; Wencker, D.; Kitsis, R. N.; Walsh, K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation 101: 660-67; 2000.

23. Gao, F.; Gao, E.; Yue, T. L.; Ohlstein, E. H.; Lopez, B. L., Christopher, T. A.; Ma, X. L. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation 105: 1497-502; 2002.

24. Habon, T.; Szabados, E.; Kesmarky, G.; Halmosi, R.; Past, T.; Sumegi, B.; Toth, K. The effect of carvedilol on enhanced ADP-ribosylation and red blood cell membrane damage caused by free radicals. Cardiovasc. Res. 52: 153-160; 2001.

25. Halliwell, B.; Gutteridge, J.M.C. eds. *Free radicals in biology and medicine*. New York, Oxford University Press; 1999.

26. Halmosi, R.; Berente, Z.; Osz, E.; Toth, K.; Literati-Nagy, P.; Sumegi, B. Effect of poly-ADP-ribose-polymerase inhibitors on the ischemia-reperfusion induced oxidative cardiac injury and mitochondrial metabolism in Langendorff heart perfusion system. Mol. Pharmacol. 59: 1497-1505; 2001.

27. Halmosi, R.; Deres, P.; Toth, A.; Berente, Z.; Kalai, T.; Sumegi, B.; Hideg, K.; Toth, K. 2,2,5,5-Tetramethylpyrroline-based compounds in prevention of oxyradical-induced myocardial damage. J. Cardiovasc. Pharmacol. 40: 854-867; 2002.

28. Hankovszky, O. H.; Hideg, K.; Baldi, L.; Frank, L. New antiarrhythmic agents. 2,2,5,5-Tetramethyl-3-pyrroline-3-carboxamides and 2,2,5,5-tetramethylpyrrolidine-3-carboxamides. J. Med. Chem. 29: 1138-52; 1986.

29. Hong, F.; Kwon, S. J.; Jhun, B. S.; Kim, S. S.; Ha, J.; Kim, S. J.; Sohn, N. W.; Kang, C.; Kang, I. Insulin-like growth factor-1 protects H9c2 cardiac myoblasts from oxidative stress-induced apoptosis via phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways. Life Sci. 68: 1095-1105; 2001.

30. Jonassen, A. K.; Sack, M. N.; Mjos, O. D.; Yellon, D. M. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ. Res. 89: 1191-98; 2001.

31. Kannan, K.; Jain, S. K. Oxidative stress and apoptosis. Pathophysiol. 7: 153-163; 2000.

32. Kim, A. H.; Khursigara, G.; Sun, X.; Franke, T. F.; Chao, M. V. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol. Cell. Biol. 21: 893-901; 2001.

33. Klotz, L. O.; Schieke, S. M.; Sies, H.; Holbrook, N. J. Peroxynitrite activates the phosphoinositide-3-kinase/Akt pathway in human skin primary fibroblasts. Biochem. J. 352: 219-225; 2000.

34. Krishna, M. C.; DeGraff, W.; Hankovszky, O. H.; Sar, C. P.; Kalai, T.; Jeko, J.; Russo, A.; Mitchell, J. B.; Hideg, K. Studies of structure-activity relationship of

nitroxide free radicals and their precursors as modifiers against oxidative damage. J. Med. Chem. 41: 3477-92; 1998.

35. Kroemer, G.; Reed, J. C. Mitochondrial control of cell death. Nature Med. 6: 513-19; 2000.

36. Li, H.; Xu, K. Y.; Zhou, L.; Kalai, T.; Zweier, J. L.; Hideg, K.; Kuppusamy, P. A pyrroline derivative of mexiletine offers marked protection against ischemia/reperfusion-induced myocardial contractile dysfunction. J. Pharmacol. Exp. Ther. 295: 563-71; 2000.

37. Madge, L. A.; Pober, J. S. A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFκB in human endothelial cells. J. Biol. Chem. 275: 15458-65; 2000.

38. Martindale, J. L.; Holbrook, N. J. Cellular response to oxidative stress: signaling for suicide and survival. J. Cell. Physiol. 192: 1-15; 2002.

39. Marton, Z.; Halmosi, R.; Horvath, B.; Alexy, T.; Kesmarky, G.; Vekasi, J.; Battyany, I.; Hideg, K.; Toth, K. Scavenger effect of experimental and clinically used cardiovascular drugs. J. Cardiovasc. Pharmacol. 38: 745-53; 2001.

40. Matsui, T.; Tao, J.; del Monte, F.; Lee, K. H.; Li, L.; Picard, M.; Force, T. L.; Franke, T. F.; Hajjar, R. J.; Rosenzweig, A. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation 104: 330-35; 2001.

41. Miao, W.; Luo, Z.; Kitsis, R. N.; Walsh, K. Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. J. Mol. Cell. Cardiol. 32: 2397-402; 2000.

42. Miles, A. M., Bohle, D. S., Glassbrenner, P. A., Hansert, B., Wink, D. A., Grisham, M. B. Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. J. Biol. Chem. 271: 40-47, 1996.

43. Mockridge, J. W.; Marber, M. S.; Heads, R. J. Activation of Akt during simulated ischemia / reperfusion in cardiac myocytes. Biochem. Biophys. Res. Comm. 270: 947-52; 2000.

44. Monti, E., Cova, D.; Guido, E.; Morelli, R., Oliva, C. Protective effect of the nitroxide tempol against the cardiotoxicity of adriamycin. Free Radic. Biol. Med. 21: 463-70; 1996.

45. Pap, M., Cooper, G. M. Role of translation initiation factor 2B in control of cell survival by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3β signaling pathway. Mol. Cell. Biol. 22: 578-86, 2002.

46. Park, J. B.; Touyz, R. M.; Chen, X.; Schiffrin, E. L. Chronic treatment with a superoxide dismutase mimetic prevents vascular remodeling and progression of hypertension in salt-loaded stroke-prone spontaneously hypertensive rats. Am. J. Hypertens. 15: 78-84; 2002.

47. Patel, R. P.; Levonen, A.-L.; Crawford, J. H.; Darley-Usmar, V. M. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. Cardiovasc. Res. 47: 465-474; 2000.

48. Piacentini, L.; Karliner, J. S. Altered gene expression during hypoxia and reoxygenation of the heart. Pharmacol. Therapeut. 83: 21-37, 1999.

49. Punn, A.; Mockridge, J. W.; Farooqui, S., Marber, M. S., Heads, R. J. Sustained activation of p42/p44 mitogen-activated protein kinase during recovery from simulated ischaemia mediates adaptive cytoprotection in cardiomyocytes. Biochem. J. 350: 891-99; 2000.

50. Rakhit, R. D.; Kabir, A. N. M.; Mockridge, J. W.; Saurin, A.; Marber, M. S. Role of G proteins and modulation of p38 MAPK activation in the protection by nitric oxide against ischemia-reoxygenation injury. Biochem. Biophys. Res. Comm. 286: 995-1002; 2001.

51. Romashkova, J. A.; Makarov, S. S. NF-κB is a target of Akt in anti-apoptotic PDGF signaling. Nature 401: 86-90; 1999.

52. Scheid, M. P.; Woodgett, J. R. PKB/Akt: functional insights from genetic models. Nature Rev. 2: 760-68; 2001.

53. Seger, R.; Krebs, E. G. The MAPK signaling cascade. FASEB J 9: 726-35; 1995.

54. Serbinova, E.; Khwaja, S.; Reznick, A. Z.; Packer, L. Thioctic acid protects against ischemia-reperfusion injury in the isolated perfused Langendorff heart. Free Radic. Res. Commun. 17: 49-58; 1992.

55. Shankar, R. A.; Hideg, K.; Zweier, J. L.; Kuppusamy, P. A. Targeted antioxidant properties of N-[(tetramethyl-3-pyrroline-3-carboxamido)propyl]phtalimide and its nitroxide metabolite in preventing postischemic myocardial injury. J. Pharmacol. Exp. Ther. 292: 838-845; 2000.

56. Sharma, A.; Singh, M. Effect of ethylisopropyl amiloride, a Na^+-H^+ exchange inhibitor, on cardioprotective effect of ischaemic and angiotensin preconditioning. Mol. Cell. Biochem. 214: 31-38; 2000.

57. Szabados, E.; Fischer, M. G.; Toth, K.; Csete, B.; Nemeti, B.; Trombitas, K.; Habon, T.; Endrei, D.; Sumegi, B. Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. Free Radic. Biol. Med. 26: 309-317; 1999a.

58. Szabados, E.; Fischer, M. G.; Gallyas, F. Jr.; Kispal, Gy.; Sumegi, B. Enhanced ADP-ribosylation and its diminution by lipoamide after ischemia-reperfusion in perfused rat heart. Free Radic. Biol. Med. 27: 1103-1113; 1999b.

59. Tobiume, K.; Matsuzawa, A.; Takahashi, T.; Nishitoh, H.; Morita, K.; Takeda, K.; Minowa, O.; Miyazono, K.; Noda, T.; Ichijo, H. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO 21: 222-28; 2001.

60. Tong, H.; Chen, W.; Steenbergen, C.; Murphy, E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. Circ. Res. 87: 309-315; 2000.

61. Toth, A.; Halmosi, R.; Kovacs, K.; Deres, P.; Kalai, T.; Hideg, K.; Toth, K.; Sumegi, B. Akt activation induced by an antioxidant compound during ischemia-reperfusion. Free Radic. Biol. Med., in press, 2003a.

62. Toth, A.; Kovacs, K.; Deres, P.; Halmosi, R.; Hanto, K.; Kalai, T.; Hideg, K.; Sumegi, B.; Toth, K. Impact of a novel cardioprotective agent on the ischaemia-reperfusion-induced Akt kinase activation. Biochem. Pharmacol., in press, 2003b.

63. Twomey, P.; Taira, J.; DeGraff, W.; Mitchell, J. B.; Russo, A.; Krishna, M. C.; Hankovszky, O. H.; Frank, L.; Hideg, K. Direct evidence for in vivo nitroxide free radical production from a new antiarrhythmic drug by EPR spectroscopy. Free Radic. Biol. Med. 22: 909-16; 1997.

64. Varbiro, G.; Toth, A.; Tapodi, A.; Veres, B.; Sumegi, B.; Gallyas, F., Jr. Concentration dependent mitochondrial effect of amiodarone. Biochem. Pharmacol., 65: 1115-1128; 2003a.

65. Varbiro, G.; Toth, A.; Tapodi, A.; Bognar, Z.; Veres, B.; Sumegi, B.; Gallyas, F., Jr. Protective effect of amiodarone but not N-desethyl-amiodarone on postischemic hearts through the inhibition of mitochondrial permeability transition. J. Pharmacol. Exp. Ther., in press, 2003b.

66. Wang, Q.-D.; Pernow, J.; Sjoquist, P.-O.; Ryden, L. Pharmacological possibilities for protection against myocardial reperfusion injury. Cardiovasc. Res. 55: 25-37; 2002.

67. Weiss, J. N.; Goldhaber, J. I.; Ji, S. Oxygen free radicals in the pathophysiology of myocardial ischemia/reperfusion. In: Tarr M and Samson F eds. *Oxygen free radicals in tissue damage*. Boston: Birkhauser Press, 250-266, 1993.

68. Weiss, R. H.; Flickinger, A. G.; Rivers, W. J.; Hardy, M. M.; Aston, K. W.; Ryan, U. S.; Riley, D. P. Evaluation of activity of putative superoxide dismutase mimics. Direct analysis by stopped-flow kinetics. J. Biol. Chem. 268: 23049-54; 1993.

69. Yamashita, K.; Kajstura, J.; Discher, D. J.; Wasserlauf, B. J.; Bishopric, N. H.; Anversa, P.; Webster, K. A. Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts overexpressing insulin-like growth factor-1. Circ. Res. 88: 609-14; 2001.

70. Yang, J.; Yu, Y.; Duerksen-Hughes, P. J. Protein kinases and their involvement in the cellular responses to genotoxic stress. Mutation Res. 543: 31-58; 2003.

71. Yellon, D. M.; Baxter, G. F. Reperfusion injury revisited: is there a role for growth factor signaling in limiting lethal reperfusion injury? Trends Cardiovasc. Med. 9: 245-49; 1999.

72. Zhao, Z.-Q.; Vinten-Johansen, J. Myocardial apoptosis and ischemic preconditioning. Cardiovasc. Res. 55: 438-455; 2002.

8. ACKNOWLEDGEMENTS

Our studies were carried out in a collaboration of the First Department of Medicine, Division of Cardiology and the Department of Biochemistry and Medical Chemistry, University of Pecs, Medical School, in Pecs, Hungary.

I am grateful for the help of my teacher and project leader, Professor Kalman Toth who suggested the theme and gave support and useful advises during my Ph.D. curriculum. I am indebted to Professor Balazs Sümegi who was my direct supervisor in the daily practice and gave me invaluable help in the understanding of the biochemical aspects of cardiovascular diseases. I thank to Professor Kalman Hideg that he taught me on free radical mediated processes and supported us to examine new compounds developed by his team.

I am grateful to my closest colleagues who assisted me in pursuing my experimental studies: Dr. Róbert Halmosi, Dr. Krisztina Kovacs, Dr. Peter Deres, Dr. Anita Pálfi; and the collaborating student researchers: Katalin Hantó, Gyöngyi Kiss, and Ágnes Reskó.

I would like to express my thanks to the technicians at the Department of Biochemistry and Medical Chemistry for their kind help.

I express my gratitude to my parents and girlfriend for their encouraging support during my studies and work.

9. PUBLICATIONS OF THE AUTHOR

I. Papers serving as basis for the Ph.D. thesis

1. HALMOSI, R., DERES, P., TOTH, A., BERENTE, Z., KALAI, T., SUMEGI, B., HIDEG, K., TOTH, K. 2,2,5,5-Tetramethylpyrroline-based compounds in the prevention of oxyradical-induced myocardial damage. J Cardiovasc Pharm, 40, 854-867, 2002.
Impact factor: 1.553

2. TOTH, A., HALMOSI, R., KOVACS, K., DERES, P., KALAI, T., HIDEG, K., TOTH, K., SUMEGI, B. Akt activation induced by an antioxidant compound during ischemia-reperfusion. Free Radic Biol Med, in press, 2003.
Impact factor: 5.082

3. TOTH, A., KOVACS, K., DERES, P., HALMOSI, R., HANTO, K., KALAI, T., HIDEG, K., SUMEGI, B., TOTH, K. Impact of a novel cardioprotective agent on the ischaemia-reperfusion-induced Akt kinase activation. Biochem Pharmacol, in press, 2003.
Impact factor: 3.340

II. Book chapters

1. HALMOSI, R., TOTH, A., OSZ, E., BERENTE, Z., SUMEGI, B., TOTH, K. Effect of poly(ADP-ribose) polymerase inhibitors on mitochondrial reactive oxygen species formation and on postischemic energy metabolism in rat heart. In: Advances in Coronary Artery Disease. Ed.: Lewis, B.S., Halon, D.A., Flugelman, M.Y., Hradec, J. Monduzzi Editore, 197-202, 2001.

2. TOTH, A., HALMOSI, R., SZABADOS, E., SUMEGI, B., TOTH, K. Effect of poly(ADP-ribose) polymerase inhibitors on the activation of ischemia-reperfusion-induced oxidative cell damage and mitochondrial metabolism in Langendorff perfused hearts. In: Proceedings of the 37th Congress of the European Society for Surgical Research (Szeged, Hungary, May 23-25, 2002) Ed. Boros, M., Monduzzi Editore, 85-90, 2002.

3. KOVACS, K., TOTH, A., DERES, P., HANTO, K., HIDEG, K., SUMEGI, B. Effect of poly(ADP-ribose) polymerase inhibitors on the activation of ischemia-reperfusion induced inflammatory processes in Langendorff perfused hearts. In: Proceedings of the 37th Congress of the European Society for Surgical Research (Szeged, Hungary, May 23-25, 2002) Ed. Boros, M., Monduzzi Editore, 63-68, 2002.

4. ALEXY, T., MARTON, Z., DERES, P., TOTH, A., TOTH, K. Biological rhythms of the circulatory system, blood pressure and heart rate variability. (accepted for publication), 2003.

III. Additional papers

1. TÓTH K., TÓTH A., MÁRTON ZS., CZOPF L., KÉSMÁRKY G., HALMOSI R., HABON T., JURICSKAY I., MÓZSIK GY. A terheléses EKG vizsgálat során bekovetkezo QRS amplitudó változások értékelése ischaemiás szívbetegségben. Magyar Belorv. Arch., 52, 73-80, 1999.

2. KARÁDI ZS., TÓTH A., SIMON G. Pajzsmirigyhormon rezisztencia szindróma: egy hatéves fiú esete. Gyermekgyógyászat, 51, 337-343, 2000.

3. KARÁDI ZS., MAYER G., KÁDÁR K., HARTYÁNSZKY I., MÁLITS E., TÓTH A., SIMON G. Sulyos nyelési zavart okozó arteria lusoria. Gyermekgyógyászat, 51, 485-489, 2000.

4. TOTH, A., MARTON, ZS., CZOPF, L., KESMARKY, G., HALMOSI, R., JURICSKAY, I., HABON, T., TOTH, K. QRS score: a composite index of exercise-induced changes in the Q-, R- and S-waves during exercise stress testing in patients with ischemic heart disease. Ann Noninv Electrocard, 6, 310-318, 2001

5. TOTH, A., HALMOSI, R., HABON, T., SZABADOS, E., DERES, P., SUMEGI, B., HIDEG, K., TOTH, K. Az antioxidáns kezeléstől a poli(ADP-ribóz) polimeráz gátlókig – a kardioprotekció uj lehetőségei ischaemia-reperfúzio soran. Magyar Belorv. Arch., 54, 107-111, 2001

6. VARBIRO, G., TOTH, A., TAPODI, A., VERES, B., SUMEGI, B., GALLYAS, F., JR. Concentration dependent mitochondrial effect of amiodarone. Biochem Pharmacol, 65, 1115-1128, 2003

Impact factor: 3.340

7. VARBIRO, G., TOTH, A., TAPODI, A., BOGNAR, Z., VERES, B., SUMEGI, B., GALLYAS, F., JR. Protective effect of amiodarone but not N-desethyl-amiodarone on postischemic hearts through the inhibition of mitochondrial permeability transition. J Pharmacol Exp Ther, in press, 2003.

Impact factor: 3.555

8. ALEXY, T., TOTH, A., HORVATH, B., MARTON, Z., KESMARKY, G., HIDEG, K., SUMEGI, B., TOTH, K. Inhibition of ADP-evoked platelet aggregation by selected poly(ADP-ribose) polymerase inhibitors. J Cardiovasc Pharmacol, under publication, 2003.

Impact factor: 1.553

IV. Published abstracts

1. CZOPF I., MARTON ZS., TOTH A., JURICSKAY I., TOTH K. A QRS amplitudók terhelésre bekövetkező változásainak értékelése ischaemiás szívbetegekben. Magyar Kardiologusok Társasága 1998. évi Tudományos Kongresszusa, 1998. május 15-16., Balatonfüred. Card. Hung. Suppl. 98/1, 47, 1998.

2. MÁRTON ZS., TÓTH A., CZOPF I., JURICSKAY I., HALMOSI R., KESMARKY G., TÓTH K. Ergometria során bekövetkező QRS amplitúdóváltozások értékelése ischaemiás szívbetegekben. XLV. Dunántúli Belgyógyász Vándorgyűlés, 1998. június 4-6., Bükfürdő. Magyar Belorv. Arch. Suppl., 51, 16, 1998.

3. KESMARKY, G., TOTH, A., MARTON, ZS., CZOPF, I., HALMOSI, R., JURICSKAY, I., HABON, T., TOTH, K. QRS score: a composite index of exercise-induced changes in the Q-, R- and S-waves during exercise stress testing in patients with ischemic heart disease. Eur. Heart J., 20, 695, 1999.

4. HALMOSI R., TÓTH A., DERES P., TÓTH K., SÜMEGI B., HIDEG K. Nitroxid prekurzorok protektiv hatása ischaemia-reperfúsio által kiváltott szívizom-károsodásban. Magyar Szabadgyök Kutató Társaság I. Kongresszusa, 2001. április 5-7., Pécs. Folia Hepatol. Suppl. 1/6., 27, 2001.

5. HALMOSI R., DERES P., TÓTH A., LITERÁTI-NAGY B., TÓTH K., SÜMEGI B. Poli (ADP-ribóz) polimeráz gátlók hatása postischaemiás myocardiumban az oxidatív sejtkárosodásra és a mitokondrium metabolismusára. Magyar Kardiologusok Társasága 2001. évi Tudományos Kongresszusa, 2001. május 9-12., Balatonfüred. Card. Hung. Suppl. 2001/2, 61, 2001.

6. SUMEGI, B., HALMOSI, R., TOTH, A., OSZ, E., BERENTE, Z., TOTH, K. Effect of poly(ADP-ribose) polymerase inhibitors on mitochondrial reactive oxygen species formation and on postischemic energy metabolism in rat heart. 1st International Congress on Coronary Artery Disease – From Prevention to Intervention, October 21-24, 2001, Prague, Czech Republic. J C M, 1, 1, 7, 2001.

7. TOTH, A., HALMOSI, R., SUMEGI, B., HIDEG, K., TOTH, K. Cardioprotective effects of heterocyclic nitroxide precursor compounds in ischemia-reperfusion-induced myocardial injury. XIVth World Congress of Cardiology, May 5-9, 2002, Sydney, Australia. J Am Coll Cardiol Suppl B, 39/9, 2227, 2002.

8. HALMOSI, R., TOTH, A., SZABADOS, E., OSZ, E., BERENTE, Z., SUMEGI, HIDEG, K., TOTH K. Cardioprotective effect of poly(ADP-ribose) polymerase inhibitors is partly due to their mitochondrial effect. XIVth World Congress of Cardiology, May 5-9, 2002, Sydney, Australia. J Am Coll Cardiol Suppl B, 39/9, 3447, 2002.

9. TÓTH, A., HALMOSI, R., DERES, P., KOVÁCS, K., HIDEG, K., SÜMEGI, B., TÓTH, K. Kardioprotektiv heterociklikus vegyületek hatása az intracelluláris jelátviteli folyamatokra ischaemia-reperfúzió során Langendorff perfundált szíveken. Magyar Kardiológusok Társasága 2002. évi Tudományos Kongresszusa, 2002. április 30-május 3., Balatonfüred. Card. Hung. Suppl. 2002/1, 72, 2002.

10. CZOPF, L., PILSKOG, S., TOTH, A., MARTON, ZS., KESMARKY, G., HALMOSI, R., KOVÁCS, L., HABON, T., TÓTH, K., MÓZSIK, GY. A terhelés során észlelt QRS- és ST-változások szerepe az ischaemiás szívbetegség diagnosztikájában. A Magyar Belgyógyász Társaság Dunántúli Szekciójának XLIX. Vándorgyűlése, Kaposvár, 2002. Junius 13-15., Magyar Belorv. Arch. Suppl. 1/2002, 43-44, 2002.

11. KOVACS, K., TOTH, A., DERES, P., HIDEG, K., SUMEGI, B. Effect of poly(ADP-ribose) polymerase inhibitors on the intracellular signal transduction during ischaemia-reperfusion (IR). 22nd Meeting of the International Society for Heart Research - European Section, July 3-6, 2002, Szeged, Hungary, J Mol Cell Cardiol, 34, A19, 2002.

12. DERES, P., HALMOSI, R., TOTH, A., KOVACS, K., BERENTE, Z., HIDEG, K., TOTH, K., SUMEGI, B. Protective effect of H-2545 on doxorubicin-induced acute cardiotoxicity. 22nd Meeting of the International Society for Heart Research - European Section, July 3-6, 2002, Szeged, Hungary, J Mol Cell Cardiol, 34, A35, 2002.

13. SUMEGI, B., KOVACS, K., TAPODI, A., DERES, P., TOTH, A., BERENTE, Z., OSZ, E., KALAI, T., HIDEG, K. Effect of PARP inhibitors on the activation of MAP kinases in Langendorff perfused hearts. 28th Meeting of the Federation of European Biochemical Societies, 20-25 October, 2002, Istanbul, Turkey, Eur J Biochem, 269 Suppl. 1, PS5-152, 2002.

14. TÓTH A., HALMOSI R., KOVÁCS K., DERES P., HANTÓ K., SZABADOS E., HABON T., SÜMEGI B., HIDEG K., TÓTH K. Antioxidánsok és a poli(ADP-ribóz) polimeráz gátlók aktiválják a protektív jelátviteli utakat myocardialis ischaemia-reperfúzio során - lehetséges klinikai vonatkozások. A Magyar Belgyógyász Társaság XXXIX. Nagygyülése, Budapest, 2002, november 21-23., Magyar Belorv. Arch., Suppl. 3/2002, 55, 132, 2002.

15. TÓTH A., KOVÁCS K., DERES P., PÁLFI A., HANTÓ K., HALMOSI R., HIDEG K., SÜMEGI B., TÓTH K. Antioxidáns vegyületek hatása az Akt protektív jelátviteli út aktivitására myocardialis ischaemia-reperfúzio során. Magyar Kardiológusok Társasága 2003. évi Tudományos Kongresszusa, 2003 május 14-17., Balatonfüred. Card Hung Suppl. 2003, 33, A29, 2003.

16. ALEXY T., TÓTH A., MÁRTON ZS., HORVÁTH B., KOLTAI K., PÁLFI A., KESMÁRKY G., HIDEG K., SÜMEGI B., TÓTH K. Poli(ADP-ribóz) polimeráz gátlók trombocita aggregáció gátló hatásának vizsgálata. Magyar Kardiológusok Társasága 2003. évi Tudományos Kongresszusa, 2003. május 14-17., Balatonfüred. Card Hung Suppl 2003, 33, A63, 2003.

17. DERES P., TÓTH A., HALMOSI R., KOVÁCS K., PÁLFI A., HÁBON T., SÜMEGI B., TÓTH K. Doxorubicin okozta akut kardiotoxicitás kivédése poli(ADP-ribóz) polimeráz gátló vegyülettel. Magyar Kardiológusok Társasága 2003. évi Tudományos Kongresszusa, 2003. május 14-17., Balatonfüred. Card. Hung. Suppl. 2003, 33, A63, 2003.

18. KOVÁCS, K., TÓTH, A., DERES, P., SÜMEGI, B. Differential effect of Metoprolol, Verapamil and 4-hydroxyquinazoline on the ischemia-reperfusion-induced myocardial processes. Heart Failure/International Society for Heart Research – European Section Meeting 2003, June 21-24, 2003, Strasbourg, France. Basic Res Cardiol, 98, 189, 2003.

19. TÓTH, A., KOVÁCS, K., DERES, P., PÁLFI, A., HANTÓ, K., HALMOSI, R., HIDEG, K., SÜMEGI, B., TÓTH, K. Impact of an antioxidant compound on the activity of the protective Akt pathway during myocardial ischemia-reperfusion. Heart Failure/International Society for Heart Research – European Section Meeting 2003, June 21-24, 2003, Strasbourg, France. Eur J Heart Failure, 2/1, 58-59, 2003.

20. SÜMEGI, B., KOVÁCS, K., TÓTH, A., DERES, P. Effect of poly(ADP-ribose) polymerase inhibitors on the activation of the protective Akt pathway in Langendorff perfused hearts. Heart Failure/International Society for Heart Research – European Section Meeting 2003, June 21-24, 2003, Strasbourg, France. Eur J Heart Failure, 2/1, 55-56, 2003.

21. TÓTH K., TÓTH A., HALMOSI R., SZABADOS E., HABON T., DERES P., PÁLFI A., SÜMEGI B., HIDEG K. Az oxidatív stressz szerepe a kardiovaszkuláris betegségekben – Az antioxidánsok és poli(ADP-ribóz) polimeráz gátlók lehetséges terápiás alkalmazása. Magyar Belgyógyász Társaság Dunántúli Szekciójának 50. Vándorgyűlése. 2003. június 26-28., Pécs. Magyar Belorv. Arch. Suppl. 2003/2, 110-111, 2003.

22. KOVACS, K., TOTH, A., DERES, P., OSZ, E., VERES, B., RADNAI, B., SUMEGI, B. Impact of poly(ADP-ribose) polymerase inhibitors on the activation of PI3-kinase/Akt and mitogen-activated protein kinase pathways in postischemic myocardium. SFRR-Europe Meeting 2003, June 26-29, 2003, Ioannina, Greece. Free Radic Res, 37, Suppl. 1, PP101, 2003.

23. SUMEGI, B., KOVACS, K., TOTH, A., DERES, P., PALFI, A. Impact of poly(ADP-ribose) polymerase inhibitors on the activation of PI3-kinase/Akt and mitogen-activated protein kinase pathways in postischemic myocardium. Special FEBS 2003 Meeting on Signal Transduction, July 3-8, 2003, Brussels, Belgium. Eur J Biochem, 270 Suppl. 1, PS01-0867, 2003.

24. KOVACS, K., TOTH, A., DERES, P., SUMEGI, B. Activation of the Akt kinase pathway by poly(ADP-ribose) polymerase inhibitors during myocardial ischemia-reperfusion. European Society of Cardiology Congress 2003, August 31-September 3, 2003, Vienna, Austria. Eur Heart J, 2003.

25. SUMEGI, B., KOVACS, K., TAPODI, A., DERES, P., BERENTE, Z., OSZ, E., TOTH, A. Role of poly(ADP-ribose) polymerase in the pathomechanism of oxidative cell damage. IV. International Symposium on Myocardial Cytoprotection, September 25-27, 2003, Pécs, Hungary. J Exp Clin Cardiol, 2003.

26. KOVACS, K., TOTH, A., DERES, P., SUMEGI, B. Differential effect of metoprolol, verapamil and 4-hydroxyquinazoline on the ischemia-reperfusion-induced myocardial processes. IV. International Symposium on Myocardial Cytoprotection, September 25-27, 2003, Pecs, Hungary. J Exp Clin Cardiol, 2003.

27. TOTH, A., ALEXY, T., MARTON, Z., HORVATH, B., KOLTAI, K., PALFI, A., KESMARKY, G., KALAI, T., HIDEG, K., SUMEGI, B., TOTH, K. Inhibition of platelet aggregation by poly(ADP-ribose) polymerase inhibitors. IV. International Symposium on Myocardial Cytoprotection, September 25-27, 2003, Pecs, Hungary. J Exp Clin Cardiol, 2003.