INVESTIGATION OF OXIDATIVE STRESS ON VASCULAR AND CARDIAC DAMAGE

IN ANIMAL MODEL AND HUMANS

PhD Thesis

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2012

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1. Abbreviations

ADMA: asymmetric dimethylarginine

Aix: augmentation index

Akt: phospho-specific Akt-1/protein kinase B-α Ser^{473}

Ang-II: angiotensin-II

ATP: adenosine triphosphate

BGP-15: O-(3-piperidino-2-hydroxy-1-propyl) nicotinic-amidoxime

CAT: catalase

DDAH: dimethylarginine dimethylaminohydrolase

eNOS: endothelial nitric oxide synthase

ERK ½: extracellular signal regulated kinase

ET-1 endothelin-1

GSK-3β: phospho-specific glycogen synthase kinase

JNK: Thr^{180}–Gly–Tyr^{182}, phospho-specific c-Jun N-terminal kinase

MCP-1: monocyte chemotactic protein-1

MDA: malondialdehyde

MEKK: mitogen-activated protein kinase kinase

NADPH-oxidase: nicotinamide adenine dinucleotide phosphate-oxidase

Nf-kappaB: nuclear factor kappa B

NMR: nuclear magnetic resonance

NO: nitric oxide

p38-MAPK: Thr^{183}–Tyr^{185}, phospho-specific p38 mitogen activated protein kinase

PARP: Poly (ADP-ribose) polymerase

PRMT: protein methyl transferase

PWV: pulse wave velocity

ROS: reactive oxygen species

SOD: supeoxide dismutase

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

TCA: trichloroacetic acid

UPR: unfolded protein response

VO2max, maximal oxygen uptake

VSMC: vascular smooth muscle cells

2. Introduction

Cardiovascular disease constitutes a major and increasing health burden in developed countries. Although treatments have progressed, the development of novel treatments for patients with cardiovascular diseases remains a major research goal. Among the cardiovascular risk factors arterial stiffness and endothelial dysfunction are believed to be most important. On the other hand the most common path physiologic causes of heart disease are ischemic-reperfusion injury and direct toxic effect leading to apoptosis or necrosis of cardiomyocytes. Among others the oxidative stress plays a key role in all of these different pathophysiological processes. Understanding the physiological and pathophysiological roles of reactive oxygen species and oxidative stress may help to investigate potential protective drugs decreasing the cardiovascular morbidity and mortality.

2.1 Oxidative stress and myocardial damage

2.1.1 Role of oxidative stress in development of myocardial cell damage

Oxidative stress is among major apoptotic stimuli in ischemic heart disease. During ischemia the respiratory cytochromes become redox-reduced, allowing them to directly transfer electrons to oxygen. Reactive oxygen species (ROS) are therefore excessively generated from a likely mitochondria source and then precipitate DNA damage, protein oxidation, lipid peroxidation, and other direct cellular injuries, consequently launching apoptosis in cells.

ROS are highly reactive oxygen moieties that arise from electrons from the mitochondrial respiratory chain or through the activity of intracellular oxidases, including NADPH oxidase (NOX) and xanthine oxidase. A number of antioxidants are in charge to remove excessive ROS and to maintain a physiological redox balance ((Halliwell et al 1985) (Figure 1). Various remodelling stimuli (e.g., neurohormones, growth factors, and cytokines) that are released in response to cardiac injury enhance ROS production by activating ROS-generating enzymes and/or decreasing the antioxidant defense capacities, which results in a net increase of ROS (oxidative stress) (Dhalla et al 1994, 1996, Hill et al 1996). Excessive ROS may directly induce cellular injury via oxidation of DNA, lipids, and proteins associated with cell death, different diseases and premature aging. ROS also participate in cell signaling through activation of redox-sensitive signaling cascades. Thereby, they initiate both protective

(adaptive) and damaging (maladaptive) cellular events. According to the redox-homeostasis model shown in Figure 1, the following three strategies to modify oxidative stress-associated processes can be delineated: (i) scavenging or neutralization of ROS by enhancing antioxidant capacities, (ii) inhibition of sources of ROS, and (iii) protection of potential targets from oxidation.

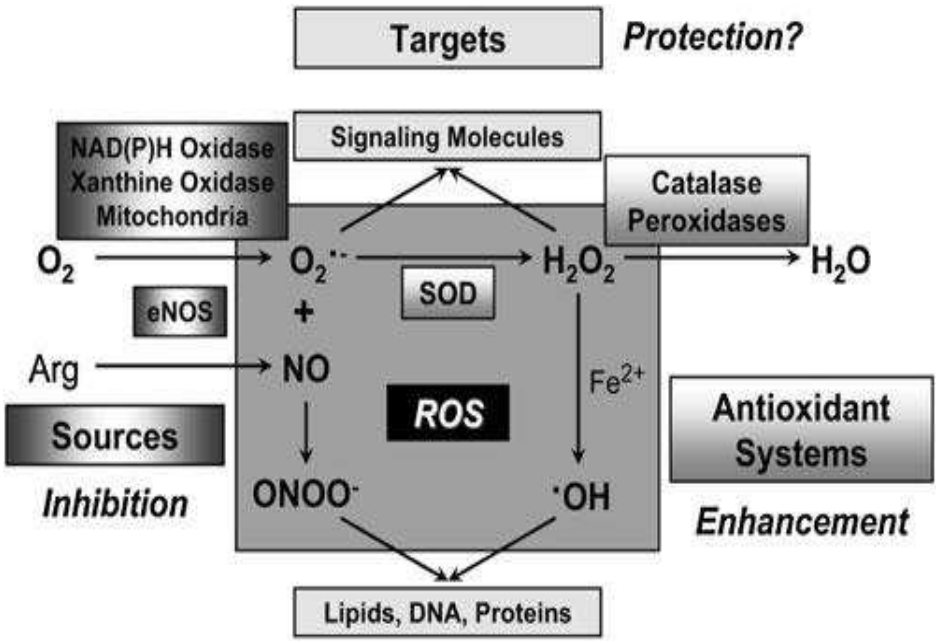


Fig.1. ANTIOXIDANTS & REDOX SIGNALING Volume 13, Number 12, 2010 From Gabriela M. Kuster

Markers of oxidative stress are elevated both systemically and locally in the myocardium in animal models and humans with heart failure. This notion has led to the hypothesis that ROS play an active role as mediators in myocardial remodelling. An array of studies showing that antioxidant treatment or inhibition of ROS-generating oxidases supported this hypothesis. In mice, for instance, dimethylthiourea mitigated oxidative stress and prevented postmyocardial infarction remodelling and heart failure (Kinugawa et al 2000), and the antioxidant N-2-mercaptopropionyl glycine diminished hypertrophic remodelling due to pressure overload in animals subjected to transverse aortic constriction.

Likewise inhibition of xanthine oxidase seemed to be effective against remodelling after myocardial infarction in mice and heart failure in rats. Unfortunately human clinical trials designed to prove beneficial effect of antioxidants in patients with cardiovascular disease didn’t live up to expectations. Great majority of studies yielded negative results. For

example the xanthine oxidase inhibitor oxypurinol or alfa-tocoferol and ß-carotene failed to decrease cardiovascular morbidity and mortality in large clinical trials. Several clinical factors may explain why the antioxidant therapeutic strategies were insufficient in these trials. Moreover biochemical explication exists. On the one hand ROS may be a two-edged sword, so may be more protective than destructive and on the other hand increased reductive stress can damage myocardial cells leading to cardiomyopathy or heart failure.

2.1.2 Role of signal mechanisms in the injured myocardial cells

ROS-associated proapoptotic effects

The role of ROS in cell damage and apoptotic cell death is well documented. Besides their nonselective effects associated with the oxidation of structural and functional molecules, including lipids, proteins, and nucleic acids, that lead to cell damage and death, ROS can selectively trigger intracellular signaling cascades that are linked to programmed cell death. This ROS-mediated proapoptotic signaling has been extensively reviewed by others (Filomeni et al 2006, Matsuzawa et al 2005). In brief, such signaling includes the ROS-dependent activation of apoptosis signalling kinase (ASK-1), p38, and c- Jun-N-terminal kinase (Ichijo et al 1997, Remondino et al 2003), which play a key role in stress-induced apoptosis in the heart (Yamaguchi et al 2003) and are responsible for the regulation of downstream targets such as the Bcl-2 family proteins, p53 and caspases (Matsuzawa et al 2005). Besides this activation of proapoptotic signaling, ROS can directly trigger the mitochondrial death pathway in a process referred to as ‘‘ROS-induced ROS release,’’ which describes the ROS-induced oxidative burst that rises from the mitochondrial electron transport chain and is associated with the dissipation of the mitochondrial membrane potential, an event generally believed to be a point-of-no-return in cell death (Gustafsson 2008).

In some cases, oxidative stress-dependent activation of ASK1 participates in pathogenesis through the induction of cell transformation but not of cell death. Angiotensin II, which plays an important role in cardiovascular diseases, is known to induce hypertension and hypertrophy. Analysis of ASK1-/- mice revealed that ASK1 is activated by angiotensin II in a ROS-dependent manner and thereby induces not only myocardial cell apoptosis but also myocardial remodelling including myocardial cell hypertrophy and fibrosis that are considered as one of the risk factors of heart failure (Izumiya et al., 2003). In addition, it is implicated that ASK1 activation may induce the proliferation and migration of vascular

smooth muscle cells, the ischemia-induced angiogenesis and the airway hyperplasia which is a characteristic pathology of asthma (Omura 2004; Kumasawa et al., 2005). These findings, thus, indicate that ASK1 plays a role in a wide range of cardiovascular pathogenesis from hypertension to heart failure. Nevertheless, further studies are required for the full explanation of the connection between ASK1 and these diseases. (Hiroaki Nagai et al 2007)

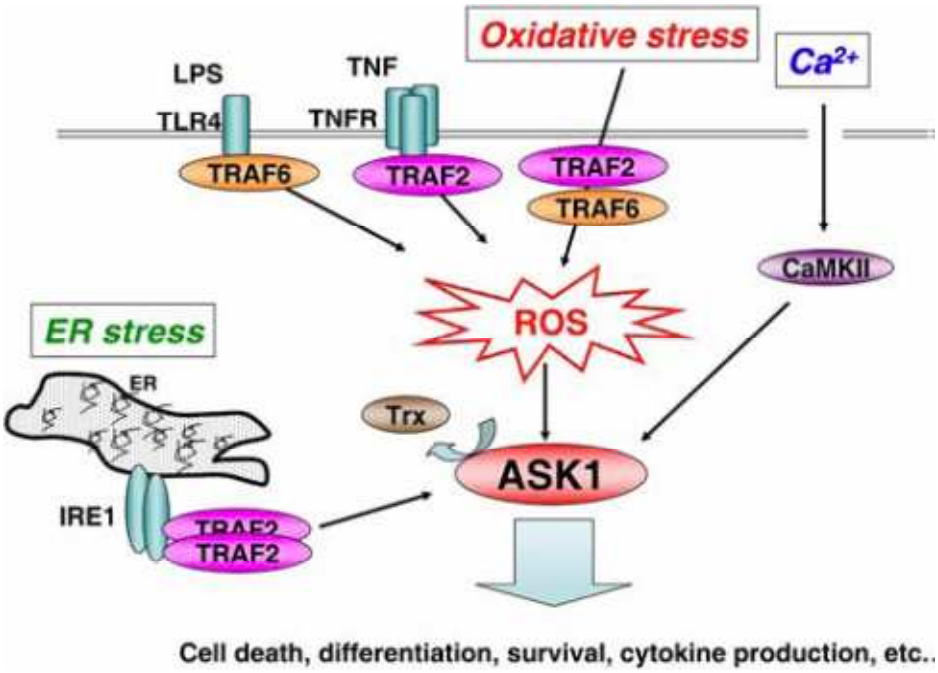


Fig.2. ASK1 in cellular stress responses. ASK1 is activated by various stresses such as oxidative stress (including TNF and LPS stimuli), ER stress and calcium influx. In response to oxidative stress, Trx (thioredoxin), a negative regulator of the ASK1-JNK/ p38 pathway, is dissociated from ASK1, and TRAF2 and TRAF6 are reciprocally recruited and thereby ASK1 is activated.In response to ER stress, TRAF2 is recruited to ASK1 and activates ASK1. In response to calcium influx, CaMKII activates ASK1 presumably by phosphorylation.
(From Hiroaki Nagai, et al, Signaling Pathways Journal of Biochemistry and Molecular Biology, Vol. 40, No. 1, January 2007)

Nowadays we have many evidences that the JNK and p38 are double-edged swords. JNK has both protective and pathological roles in ROS-induced processes in cardiomyocytes. Through the use of a JNK-specific inhibitor JNK activity was shown to play a pivotal role in myocyte apoptosis after ischemia/reperfusion. In contrast, many other studies demonstrated a critical role for JNK in myocyte survival and cardioprotection. JNK is reported to interact with proapoptotic Bax and Bad on mitochondrial membrane. However, other prosurvival pathways, including AKT, also are targeted by JNK. Therefore, it was not surprising that Kaiser et al reported enhanced myocyte survival after ischemia/reperfusion injury when JNK

activity was genetically activated or inhibited in the heart. These seemingly contradictory and confusing results underscore the complexity of the JNK pathway in cell death regulation in the heart.

The p38 pathway is involved in the regulation of myocyte contractility and cell death. Activation of p38 leads to suppressed contractility without affecting intracellular calcium cycling. The negative inotropic effect of p38 activity appears to be an epigenomic phenomenon because it is both rapid and reversible. Indeed, secondary modification of sarcomere proteins is observed in p38-activated heart associated with reduced force generation in isolated myofilaments. It is highly controversial whether p38 pathway is cardiac protective or prodeath in the heart. In many studies, inhibiting p38 activity in cultured myocytes or intact heart decreased apoptotic cell death under stress stimulation such as pressure overload or ischemia/reperfusion. Inhibition of p38 is consistently observed to improve cardiac function and to reduce remodelling in the heart after ischemia/reperfusion injury or infarction. However, a report also showed that such beneficial effect was not observed in pigs, suggesting a certain degree of species specificity. On the other hand, specific activation of p38 in the heart is not sufficient to induce myocyte apoptosis. Furthermore, a recent study from Martindale et al demonstrated that activation of p38 in the heart led to small heat-shock protein phosphorylation associated with enhanced protection against ischemia/reperfusion injury. Lastly, cardiac-specific inactivation of p38 leads to enhanced apoptosis in response to pressure overload. Therefore, p38 activity can be both protective and detrimental to myocyte survival in a stressor-specific manner.

ROS-associated prosurvival signaling

Generally in parallel to the beforementioned proapoptotic signaling, ROS mediate the activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways. Both of these pathways have been involved in cell growth and survival in various cell types, including cardiomyocytes. They exhibit a variety of individual and common activators such as cytokines and growth factors, including agonists of the Gq-protein-coupled receptors. Activation of the MEK/ERK signalling pathways initiated by the receptor-mediated activation of the small G-protein, Ras and translocation of Raf-1 to the plasma membrane. This Ras-Raf activation is followed by the downstream phosphorylation and activation of MEK1/2 and ERK1/2. ERK1/2, in turn, activate an array of transcription factors that are in control of the

expression of genes involved in the regulation of cell growth, proliferation, survival, and differentiation. Similar to other mitogen-activated protein kinase pathways, the MEK/ERK pathway can be activated by ROS of either exogenous (e. g., applied hydrogen peroxide) or endogenous origin (e.g. produced in response to receptor stimulation), with Ras acting as a direct ROS target. The MEK/ERK pathway is also an important mediator of cell survival as previously described in other cell types as well as more recently in cardiomyocytes (Bueno 2000, Kwon 2003). Kwon et al. showed that the ROS-dependent activation of ERK1/2 in response to exogenous hydrogen peroxide in cardiomyocytes is antiapoptotic, as hydrogen peroxide-associated cardiomyocyte apoptosis was markedly enhanced in the presence of the MEK1/2 inhibitor U0126. Altogether, these observations supply solid support for a role of ROS-mediated ERK1/2 activation in cardiomyocyte survival. The probably more classical mediator of cell survival is the PI3K/Akt pathway (Cantley 2002). Stimulation of different receptors in cardiomyocyte, included Gq-proteincoupled receptors, insulin- and insulin-like growth factor 1-linked tyrosine kinase receptors, could activate Akt which is associated with cardioprotection. Activation of PI3K/Akt inhibits apoptosis in myocardial cells exposed to hypoxia in vitro and in rodent hearts after ischemia-reperfusion in vivo. Similar to the MEK/ERK pathway, Akt can be activated by ROS, and ROS-mediated Akt activation has been implicated in cell survival. Wang et al. found that Akt was activated in response to exogenous hydrogen peroxide and that this activation mitigated oxidative stress-associated apoptosis in various nonmyocyte cell lines (Wang 2000). Similar observations were obtained by Kwon et al. in cultured rat myocardial cells. Whereas ROS-dependent activation of the MEK/ERK pathway occurs through direct interaction of ROS with Ras as described above, the mechanisms of ROS activation of the PI3K/Akt pathway are less clear. Additional mechanisms may include the oxidative modification of other upstream regulators of Akt, such as Src or other protein tyrosine kinases. Endothelial nitric oxide synthase (eNOS), NFkB, FOXO3a, and glycogen synthase kinase-3b have all been identified as downstream targets of Akt and their Akt dependent regulation represents an inherent feature of the progrowth and prosurvival properties of Akt. Namely, FOXO3a and glycogen synthase kinase-3b, which are both inhibited in response to Akt activation, have recently been characterized as downstream effectors of Akt in cardiomyocytes (Fig.3)

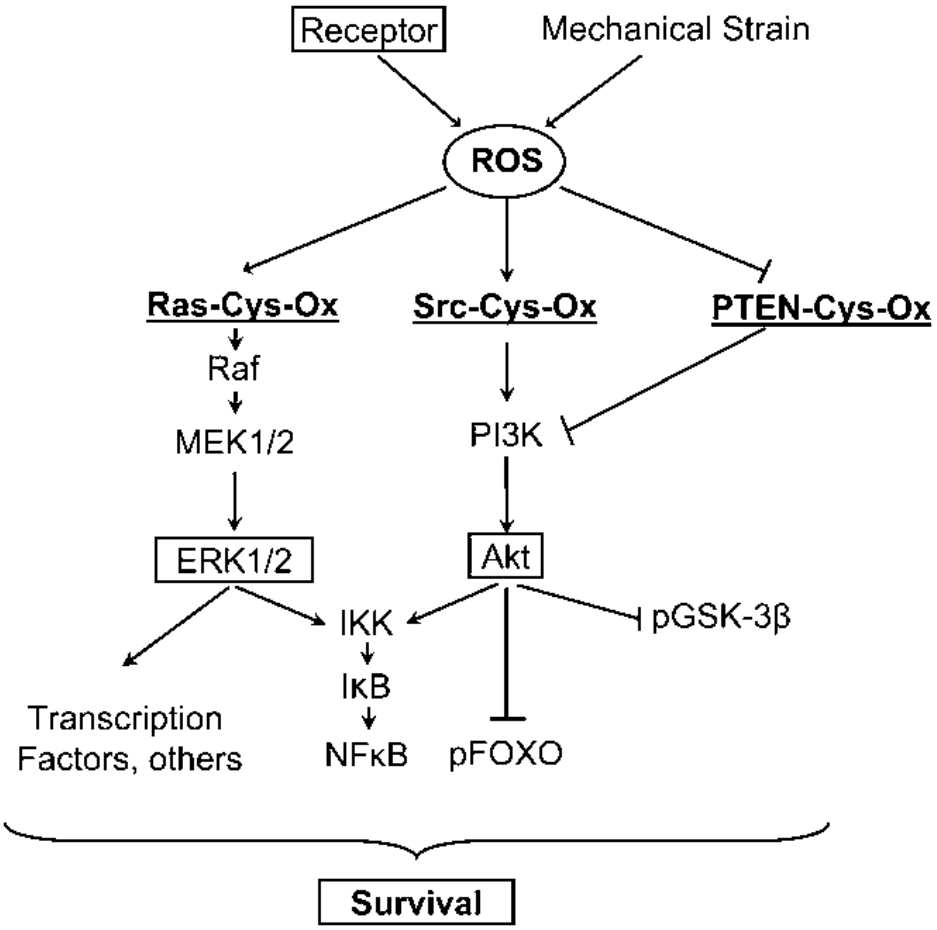


Fig.3. ANTIOXIDANTS & REDOX SIGNALING from Gabriela M. KusterVolume 13, Number 12, 2010

Glycogen synthase kinase-3β (GSK-3β) is a constitutively active Ser/Thr protein kinase which plays a key roles not only in glycogen metabolism but also in cell proliferation, growth and death In the cardiovascular system, GSK-3β has major roles in glucose metabolism, cardiomyocyte hypertrophy and cell death, particularly in ischemia/ reperfusion injury.

Both necrosis and apoptosis are involved in cardiomyocyte death after ischemia/ reperfusion. Ischemia/reperfusion enhances activation of caspase-3, -8, -9 and translocation of Bax and Bcl-2 in the myocardium. Apoptotic process can convert to necrotic process when essential co-factors for apoptosis are lacking. ATP is one of such co-factors, and switching from apoptosis to necrosis by inhibition of mitochondrial ATP production, which has been shown in non-cardiac cells. The mitochondrial permeability transition pore (mPTP) is a non-selective large conductance channel in the mitochondrial inner membrane, which is physiologically closed. Opening of mPTPs is involved in cell death induced by a variety of causes (for example, ischemia/reperfusion, alcohol, endotoxin, anti-cancer agents) In addition

to Ca2+, reactive oxygen species (ROS), accumulated inorganic phosphate and depletion of ATP encourage opening of mPTPs. Although the relative importance of each factor is not clear, all of these mPTP opening stimuli are induced in cardiomyocytes subjected to long-sustained ischemia and reperfusion. GSK-3β activity is a determinant of the threshold for mPTP opening in cardiomyocytes. It is confirmed that the threshold for ROS induced opening of mPTPs is elevated by inhibitory phosphorylation of GSK-3β, and pharmacological inhibitors of GSK-3β. These findings indicate that GSK-3β activity induces mPTP opening in response to ROS and/or Ca2+ overloading. Das et al showed that inhibition of GSK-3β suppressed ATP hydrolysis by reducing ATP transport from the cytosol to the mitochondria. GSK-3β is a substrate of multiple pro-survival protein kinases, including Akt, PKC-ε, extracellular signal-regulated kinase (ERK) and protein kinase G, and GSK-3β phosphorylation is therefore a step to which multiple protective signaling pathways converge. Nevertheless, these findings support the notion that inactivation of GSK-3β by phosphorylation at Ser9 is a common mechanism of protection of cardiomyocytes against necrosis in many cardioprotective interventions. Although the role of GSK-3β in apoptosis of cardiomyocytes has not been fully clarified, evidence to date supports its significant contribution to apoptosis induced by ischemia/reperfusion, hypoxia/re-oxygenation, β-adrenoceptor activation and pressure overload. Apoptosis by these insults was suppressed by overexpression of the adrenomedullin gene or kallikrein gene or treatment with statins, all of which induced phosphorylation of GSK-3β. Furthermore, this protection from apoptosis was inhibited by dominant-negative Akt or active GSK-3β mutant and mimicked by pharmacological inhibitors of GSK-3. These findings indicate that GSK-3β activity determines the fate of cardiomyocytes exposed to apoptosis inducers. However, the intracellular localization of phospho- GSK-3β responsible for the anti-apoptotic function and the mechanism downstream of GSK-3β phosphorylation in cardiomyocytes remain unclear.It is likely that translocation of phosphorylated GSK-3β to the mitochondria is important for inhibition of oxidant stress-induced apoptosis of cardiomyocytes. Results suggest that phosphorylation of GSK-3β pre-existing in the mitochondria by Akt affords protection from oxidant stress-induced apoptosis, possibly by suppressing Bax translocation in cardiomyocytes. Phosphorylation of GSK-3β in the mitochondria is possibly achieved by translocation of Akt to the mitochondria.

Taken together it may be useful to find out some efficient strategies to directly or indirectly inhibit GSK-3β for protection of cardiomyocytes.

2.1.3 Endoplasmatic reticulum stress response and unfolded protein response

The endoplasmic reticulum (ER) is a multifunctional intracellular organelle supporting many processes required by virtually every mammalian cell, including cardiomyocytes. It performs diverse functions, including protein synthesis, translocation across the membrane, integration into the membrane, folding, posttranslational modification including N-linked glycosylation, and synthesis of phospholipids and steroids on the cytoplasmic side of the ER membrane, and regulation of Ca(2+) homeostasis. Perturbation of ER-associated functions results in ER stress via the activation of complex cytoplasmic and nuclear signaling pathways, collectively termed the unfolded protein response (UPR) (also known as misfolded protein response), leading to upregulation of expression of ER resident chaperones, inhibition of protein synthesis and activation of protein degradation. The UPR has been associated with numerous human pathologies, and it may play an important role in the pathophysiology of the heart. ER stress responses, ER Ca^{2+} buffering, and protein and lipid turnover impact many cardiac functions, including energy metabolism, cardiogenesis, ischemic/reperfusion, cardiomyopathies, and heart failure. Connection between oxidative stress and endoplasmatic reticulum stress response is not understood exactly. In some animal models data suggested that ER stress occurs likely downstream of oxidative stress en route to cardiac dysfunction.

2.1.4 ROS and mitochondria

Mitochondria are one of the most vulnerable organelles to cope with oxidative stress caused by several damages such as ischemia-reperfusion injury or toxic effects. Since mitochondria are involved not only with bioenergetics but also with oxidative damage through ROS generation and cell signaling leading to apoptosis.

During ischemia, when ATP is progressively depleted, ion pumps cannot function resulting in a rise in calcium Ca(2+), which further accelerates ATP depletion. The rise in Ca(2+) during ischemia and reperfusion leads to mitochondrial Ca(2+) accumulation, particularly during reperfusion when oxygen is reintroduced. Reintroduction of oxygen allows generation of ATP; however, damage to the electron transport chain results in increased mitochondrial generation of reactive oxygen species (ROS). Mitochondrial Ca(2+) overload and increased ROS can result in opening of the mitochondrial permeability transition pore, which further compromises cellular energetics (Tretter 2007). The resultant low ATP and altered ion

homeostasis result in rupture of the plasma membrane and cell death. Mitochondria have long been proposed as central players in cell death, since the mitochondria are central to the synthesis of both ATP and ROS and since mitochondrial and cytosolic Ca(2+) overload are key components of cell death. Many cardioprotective mechanisms converge on the mitochondria to reduce cell death. Reducing Ca(2+) overload and reducing ROS have both been reported to reduce ischemic injury. Preconditioning activates a number of signaling pathways that reduce Ca(2+) overload and reduce activation of the mitochondrial permeability transition pore. The mitochondrial targets of cardioprotective signals are discussed in detail.

The endothelium is vital to the proper functioning in the heart, in particular due to its production of nitric oxide (NO) which regulates vascular tone. Damage to the endothelium contributes to the development of atherosclerosis, and hence to possible myocardial infarction and subsequent heart failure. Like most cells, endothelial cells contain mitochondria, despite their having relatively little dependence on oxidative phosphorylation for ATP production. However, endothelial mitochondria are centrally involved in maintaining the fine regulatory balance between mitochondrial calcium concentration, reactive oxygen species (ROS) production, and NO. This raises the question of whether damage to endothelial mitochondria would have repercussions in terms of the development of heart disease. In fact, increasingly nuanced techniques enabling restricted transgenic expression of antioxidant proteins in mice has demonstrated that mitochondrial ROS do contribute to endothelial damage. New pharmaceutical approaches designed to target protective molecules such as ROS scavengers to the mitochondria promise to be effective in preventing heart diseases. As well as protecting cardiomyocytes, these drugs may have the added benefit of preventing damage to the endothelial mitochondria. However, much remains to be done in understanding the contribution that mitochondria make to endothelial function.

2.2 Oxidative stress and endothelial dysfunction

2.2.1 Connection between ROS, nitric oxide and ADMA

Asymmetrical dimethylarginine (ADMA) is a naturally occurring L-arginine analogue derived from the proteolysis of proteins containing methylated arginine residues. It is synthesized when arginine residues in the nuclear proteins are methylated through the action of the protein arginine methyltransferases (PRMTs), which are largely distributed throughout the human body, through a posttranslational change that adds one or two methyl groups to the nitrogens of the guanidine incorporated into the proteins. There are two types of PRMTs, with several isoforms: type 1 catalyzes the formation of ADMA, and type 2 catalyzes the formation of the symmetric dimethylarginine (SDMA); but both enzymes can transfer the methyl radical, producing the NG-monomethyl-L-arginine (L-NMMA). Of these, only the asymmetrically methylated species (ADMA and L-NMMA) are inhibitors of NOS; SDMA is not. ADMA inhibits the three isoforms of NOS, and is equipotent with L-NMMA. It can also uncouple the enzyme, generate superoxides, and it interfaces with other targets in the cell. The administration of ADMA in rats causes an increase in the renal vascular resistance and blood pressure, confirming its biological action *in vivo*. Its levels are much higher intracellularly than extracellularly, sufficient in some cases to inhibit NOS, as demonstrated with cultivated endothelial cells. However, an independent additional action modality was demonstrated *in vivo*, in which the chronic infusion induced vascular injuries in eNOS knockout mice. By now, numerous studies suggest that an elevated plasma level of ADMA is associated with endothelial dysfunction and is a risk factor for several human diseases, such as hyperhomocysteinanemia, hypertension, coronary artery disease, peripheral arterial occlusive disease, pulmonary hypertension, and preeclampsia.

Oxidative stress has been shown to increase the activity of arginine-methylating and ADMA-degrading enzymes, leading to increased ADMA concentrations; moreover, high ADMA levels further contribute to the vascular oxidative stress burden in a positive feedback fashion (Sydow and Munzel 2003). It has previously demonstrated that ADMA concentration-dependently increased oxidative stress measured as superoxide production by cultured human endothelial cells. ADMA also activated the oxidant-responsive transcription factor NF—kappa B, resulting in enhanced monocyte chemoattractant protein-1 (MCP-1) and endothelial adhesiveness for monocytes. MCP-1 and interleukin-8 (IL-8) are major chemokines for

leukocyte trafficking and were found to be highly expressed in human atherosclerotic lesions. Recently it has been reported that cellular senescence, the limited ability of primary human cells to divide when cultured in vitro, is accelerated by increased oxidative stress, and it is reduced by NO donor treatment.

ADMA is known to upregulate several components of microvascular RAS, leading to increased production of angiotensin II, which then activates NADPH oxidase and increases ROS production (Veresh 2008). ADMA improves the p38 mitogen-activated protein kinase activity in human coronary artery endothelial cells, which may provide a link between ADMA and RAS, because ACE protein expression has been shown to be regulated by p38 mitogen-activated protein kinase.

ADMA regulates endothelial permeability and endothelial barrier function. Several possible mechanisms have been proposed to explain the effects of ADMA on endothelial barrier function. A previous study indicated that ADMA increased pulmonary endothelial permeability both in vitro and in vivo, and that this effect was mediated by nitric oxide, acting via protein kinase G and independent of ROS formation. Others have demonstrated that ADMA compromises the integrity of the glomerular filtration barrier by altering the bioavailability of nitric oxide and superoxide, and that nitric oxide (NO)- independent activation of soluble guanylyl cyclase preserves the integrity of this barrier under conditions of NO depletion (Sharma 2009). ADMA markedly downregulated connexin43 expression and damaged gap junction function in human umbilical vein endothelial cells by increasing the production of intracellular ROS and inducing phosphorylation of p38-MAPK (Jia 2009).

2.2.2 ROS and arterial stiffnes

$O_2^{\bullet-}$ is generally the first ROS generated by vascular cells. All vascular cells can generate $O_2^{\bullet-}$ (Pagano et al. 1995), with different cell types containing various $O_2^{\bullet-}$-generating systems. Xanthine oxidase, NADH oxidase and nitric oxide synthase (NOS) are present in endthrlial cells, while NADPH oxidase is present in vascular smooth muscle cells, endothelial cells and adventitial fibroblasts. Additionally, vascular cytochrome P450 can also generate $O_2^{\bullet-}$ and is expressed in virtually all vascular cell types. The role of NADPH oxidase as a key ROS-generating enzyme has been widely reported. Vascular oxidative stress has been reported to mediate vascular remodelling. It has been reported that H_2O_2 generated from NADPH oxidase-derived $O_2^{\bullet-}$ played a critical role in Ang II-induced hypertrophy of cultured VSMCs by activating growth-related signalling pathways and growth factors. Earlier studies

suggested a paracrine effect of ROS on medial hypertrophy. Subsequently, it has been reported that Ang II-induced NADPH oxidase-derived ROS in the adventitia and intima of the aorta was concomitant with medial hypertrophy. Overexpression of human catalase in VSMCs decreased Ang II-induced hypertrophy in vivo and adventitia-specific expression of catalase decreased NADPH oxidase activity in the adventitia, and reduced both medial ROS levels and medial hypertrophy (Liu et al. 2004). These data strongly support the paracrine effect of adventitial NADPH oxidase-derived ROS, primarily H_2O_2, on vascular hypertrophy. Adventitial fibroblasts are involved in arterial repair and serve as one of the major sources of vascular ROS production (Pagano et al. 1997; Wang et al. 1998, 1999; Ardanaz and Pagano 2006). Inhibition of adventitial ROS production has been demonstrated to decrease neointimal proliferation, hyperplasia and VSMC hypertrophy (Liu et al. 2004). These results would strongly suggest that adventitial-derived ROS, generated by NADPH oxidase, are a critical mediator of vascular wall remodelling and repair after injury.

Either vascular remodelling or increased redistribution of collagen and elastin in vascular wall play an important role in pathophysiological processes. Excessive deposition of extracellular matrix proteins, especially collagen, due to pathological conditions such as systemic hypertension occurs throughout the vessel, with initial build-up taking place in the adventitial region of arterioles. Increased production of collagen has been demonstrated in adventitial fibroblasts due to stimulation with several mitogens, including Ang II (An et al. 2006) and endothelin-1 (An et al. 2007). The increased synthesis of procollagen, the precursor of collagen, has been observed in cardiac fibroblasts by several other substances and forces, including mechanical load (Carver et al. 1991), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-1). Transforming growth factor β (TGF-β) also serves to upregulate procollagen production in both cardiac fibroblasts and interstitial fibroblasts of the pulmonary vascular system. Additionally, stimulation with TGF-β, PDGF, or bFGF leads to a decrease in collagen degradation.

Connection between ROS and collagen synthesis is well defined. Ang II-induced procollagen-α1(I) expression was inhibited by $O_2^{\bullet -}$ scavengers and NADPH oxidase inhibitors Moreover, Ang II-induced procollagen-α1(I) levels were decreased in cells overexpressing SOD1. These findings have important implications for disease states associated with remodelling of the vasculature. Moreover, fibrillar collagen accumulation could conceivably contribute to vascular stiffness and changes in vascular compliance. Endothelin-1 is a potent

peptide growth factor and is well known to consent to many pathological conditions, such as hypertension and atherosclerosis. Although ET-1 is synthesized primarily by endothelial cells under physiological conditions, it can be synthetized by vascular adventitial fibroblasts. Hence, Ang II-induced oxidative stress regulated ET-1 release, which in turn mediated collagen synthesis in adventitial fibroblasts. These findings reveal the mechanism that contributes to ET-1 release and collagen deposition in the overall remodelling of the arterial wall in various pathological conditions.

The arterial stiffness closely related to functional changes of smooth muscle of arteries and deposition of collagen and elastin in large artery wall also. Vasoactive mediators can alter vessel diameter by up to 30% independent of changes of blood pressure and peripheral resistance. This fact proves the significant dynamic contribution of vascular smooth muscle to large artery stiffness.

2.2.3 Clinical assessment of arterial stiffness

Clinical assessment of arterial stiffness may be determined by analysis of pulse wave velocity and augmentation index. These parameters are derived from analysis of central and peripheral pressure waveform. Central arterial pressure waveforms essentially vary from peripheral pulse wave because changes in vessel stiffening throughout the vascular tree cause location-dependent changes in the pressure waveform. In addition, central arterial waveforms will be influenced by the reflective wave phenomenon as described by Nichols and O'Rourke (2005). The systolic waveform leaves the aortic root and passes to the periphery, where smaller arterioles provide multiple points of reflection. A resulting 'reflective' wave is generated and returns to central arteries (O'Rourke and Kelly 1993). In healthy individuals with healthy compliant arteries, the reflective wave will go back to the central vasculature in diastole and augment diastolic coronary arterial blood flow. The speed of the advancing wave is termed pulse wave velocity (PWV) (Lehmann et al 1998). With advancing age, a combination of increased reflective capacity at peripheral sites and faster PWV within stiffened vessels causes premature augmentation of the systolic waveform, forming a 'late systolic peak' on waveform analysis (Figure 4). This explains the differences between the brachial and aortic pressure waveforms, which may be as high as 20 mmHg (Pauca et al 1992). The central pressure waveform is important in view of determining left ventricular workload, which is relatively independent of the brachial pressure.

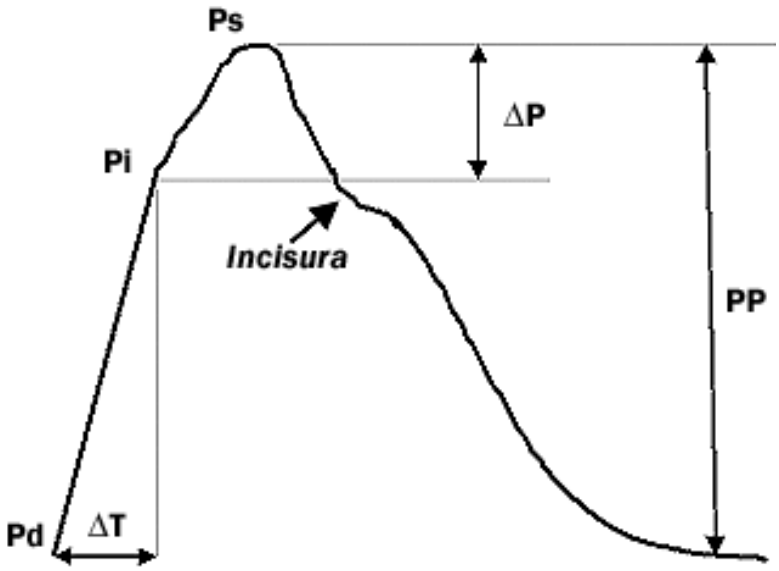


Figure 4. Waveform of central arterial pressure incisura represents the reflective wave. Augmentation index is calculated by Aix= ΔP/PPX100

A technique involves the use of an applanation tonometer able to noninvasively record the peripheral pulse pressure wave and generate a corresponding central arterial waveform to record the radial pulse wave. Applanation tonometry causes partial flattening of the arterial wall and equilibration of intra-arterial circumferential pressure. The accuracy of arterial tonometry in recording peripheral waveforms has been described by previous investigators who evaluated waveforms derived from noninvasive tonometry and direct arterial puncture (Cohn et al 1995). The central arterial waveform can subsequently be derived from the peripheral waveform using a validated generalized transfer factor (Takazawa et al 1998). This is then expressed in terms of an Augmentation Index (AIx) which can be used to assess vascular stiffness. Pulse wave analysis has more recently been used as a noninvasive tool to assess endothelial function (Wilkinson, Hall, et al 2002).

The analysis of PWV uses a similar system that calculates pulse wave propagation velocity between two sites, commonly the carotid and femoral pulses, or carotid and radial (Oliver and Webb 2003), although brachial-ankle PWV has been assessed by some (Katayama et al 2004). Pulse wave velocity is inversely proportional to vessel stiffness and distensibility. Waveform data are recorded from two sites using noninvasive tonometry and stored electronically. Following documentation of the distance between the two recording sites, determination of the pulse transit time allows calculation of PWV. In order to assess pulse transit time a correlation point is identified within the pressure waveform, which may be the foot of the pressure wave (using SphygmoCor system) or the point of maximal upstroke

(using Complior system) (Millasseau et al 2005). Elevation of PWV leads to augmentation of the ascending aortic systolic waveform as previously outlined, resulting in higher left ventricular afterload and amplification of pulse pressure (Nichols 2005). Pulse wave analysis and PWV are both noninvasive simple techniques that can be used to assess vascular stiffness in research and clinical settings. Both techniques are influenced by factors that may confound data. For example, elevation in pulse rate will lower the AIx as a result of a reduction in reflective wave amplitude, and does not represent a change in vascular stiffness (Wilkinson, Mohammed, et al 2002). Consequently, a correction factor has been suggested to standardize for variation in heart rate (Wilkinson et al 2000). In addition, an inverse relationship between AIx, PWV, and height has been described, which may result from shorter reflective wave propagation time in individuals with short stature (McGrath et al 2001). Using PWA and PWV, vascular stiffness has been assessed and identified as an independent risk marker for cardiovascular mortality (Laurent et al 2001; Meaume et al 2001) and cerebrovascular events (Laurent and Boutouyrie 2005), and has a prognostic value equivalent to currently available biomarkers.

2.3 Myocardium- and endothel- protection with antioxidants

Several studies have reported beneficial effects of a therapy with antioxidant agents, including trace elements and other antioxidants, against the endothelial dysfunction, ischemia-reperfusion injury in heart or in cases of cardiotoxic agent induced myocardial damage. Antioxidants act through one of three mechanisms to prevent oxidant-induced cell damages. They can reduce the generation of ROS, scavenge ROS, or interfere with ROS-induced alterations. Modulating mitochondrial activity is an important possibility to control ROS production. Hence, using different drugs to reduce fatty acid oxidation and of trace elements such as zinc and selenium as antioxidants, and physical exercise to induce mitochondrial adaptation, contribute to the prevention of ROS-related cardiac and endothelial dysfunction. The paradigm that inhibiting the overproduction of superoxides and peroxides would prevent cardiac diseases has been difficult to verify using conventional antioxidants like vitamin E or catalytic antioxidants such as SOD/CAT mimetics. Although the beneficial effects of several antioxidant agents were varified in animal models, the favourable effects failed in some large multicenter randomised human trials. So this fact inspires to discover newer antioxidant agents treating oxidative stress effectively.

3. Aim of the sudy

The aim of our study was double: (i) to investigate the protective effect of new antioxidant and PARP-inhibitor nicotinic amidoxime derivate BGP-15 to imatinib-induced cardiotoxicity in animal model and (ii) to assess the beneficial effect of physical exercise on endothel function considering oxidative stress. In detail,

1. to identify the mechanism by which imatinib induces cardiotoxicity and to determine how BGP-15 can modulate these processes to perform its cytoprotective effects
2. to investigate how imatinib can compromise energy metabolism in Langendorff perfused rat heart, and can whether BGP-15 counteract these effects
3. to identify the possible role of JNK and p38 MAP kinase activation in the imatinib- induced cardiotoxicity, and suggests that BGP-15 by suppressing these MAP kinases and by the activation of PI-3-kinase Akt pathways can ameliorate imatinib-induced cardiotoxicity.
4. to investigate the effect of single bout maximal physical exercise on oxidative stress, asymmetric dimethylarginine, a competitive inhibitor of eNOS.
5. to evaluate the effect of physical exercise on arterial stiffnes in well-trained swimmers and runners.

4. Experimental study

BGP-15, a PARP-inhibitor, prevents imatinib-induced cardiotoxicity by activating Akt-GSK-3beta pathway and suppressing JNK and p38 MAP kinase activation

4.1 Introduction

Imatinib-mesylate (Gleevec), a potent specific inhibitor of the Bcr-Abl tyrosine kinase, has been used successfully for the treatment of advanced–phase chronic myeloid leukemia (CML) (Deninger et al 2005, Cohen et al 2002, Czechowskaet al 2005) Bcr-Abl is a constitutively active tyrosine kinase in leukemic cells and activates several signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K/Akt). BCR-ABL also activates pro-survival pathways in leukemic cells including Jak-STAT and Grb2-ERK signaling pathways (Van Etten 2004), which can lead to increased spontaneous and DNA damage-induced proliferation frequency.

Although the detection of imatinib related cardiotoxicity has been based largely on physical examination and medical history, the laboratory based evidence is still missing. A comprehensive study was published by Kerkela et al (2006), showing that imatinib treated patients developed chronic heart failure, which could be modelled on C57BL6 mice as well after prolonged administration of imatinib. The underlying mechanisms of cardiotoxicity involved the activation of endoplasmic reticulum (ER) stress response, the collapse of the mitochondrial membrane potential, the release of cythochrome c into the cytosol, a reduction in cellular ATP content and cell death (Kerkela et al 2006). Further evidence of congestive heart failure development has been reported by another clinical study, which investigated the toxicity of imatinib in sixteen CML and GIST patients (Park 2006), and strengthened the presumption of cardiotoxicity (Orphanos 2009).

In vitro experiments revealed that at least two different pathways, one involving caspase activation and another one is PARP-1 enzyme mediated pathway, coexisted in imatinib induced apoptosis (Moehring 2005). The treatment of BaF3BA cells with a broad caspase inhibitor alone was not sufficient to completely block imatinib induced apoptosis, however, co-administration with PARP-inhibitor PJ34 resulted in an increased cytoprotection (Moehring 2005). Consequences of pathophysiological PARP-1 enzyme activation in cardiomyocytes have been well established (Szabó 2005, Pacher 2007). Over-activation of

PARP-1 enzyme can induce rapid cellular NAD^+ and ATP pool depletion leading to mitochondrial dysfunction and can suppress the activity of the PI-3-kinase-Akt pathway resulting in necrotic or apoptotic cell death. Mitochondrial dysfunction in turn can further impair energy metabolism and increase mitochondrial ROS production manifesting in lipid peroxidation, protein oxidation and DNA damage. Earlier studies demonstrated the beneficial effect of BGP-15 on oxidative stress (Halmosi 2001, Szabados 2002). BGP-15 is a nicotinic amidoxime derivate (Fig.5.) which was originally developed against insulin resistance. BGP-15 is a potent insulin sensitizer (Literáti-Nagy B 2009). Data generated by our group showed that multitarget agent BGP-15 successfully inhibited the activation of PARP-1 enzyme and protected the mitochondria from oxidative damage under condition of ischemia-reperfusion on a Langendorff rat heart model (Halmosi 2001, Szabados 2002).

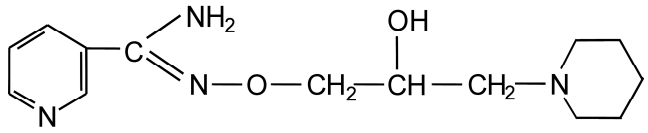


Fig.5. Chemical structure of BGP-15 (O-(2-hydroxy-3-piperidinepropyl)-pyridine-carbonic acid-amidoxime dihydrochloride)

The aim of our study was to identify the mechanisms by which imatinib induces cardiotoxicity in a Langendorff perfused rat heart model and to determine how PARP inhibitor and antioxidant agent BGP-15 can modulate these processes. We investigated whether imatinib mesylate administration could lead to oxidative stress and alterations in cardiac energy metabolism and the ability of BGP-15 to counteract these effects. We studied the possible role of JNK and p38 MAP kinase activation in imatinib induced cardiotoxicity, and the potential beneficial effect of BGP-15 on these processes.

4.2 Materials and methods

4.2.1 Chemicals

4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl] amino]-phenyl] benzamide methanesulfonate (imatinib mesylate, Gleevec) was donated by the Department of Internal Medicine, University of Pecs. BGP-15 was synthesised by N-Gene Research and Development, Ltd., Hungary. Antibodies against phospho-specific extracellular

signal regulated kinase ($ERK_{1/2}$) Thr^{183}–Tyr^{185}, phospho-specific p38 mitogen activated protein kinase (p38-MAPK) Thr^{180}–Gly–Tyr^{182}, phospho-specific c-Jun N-terminal kinase (JNK), phospho-specific Akt-1/protein kinase B-α Ser^{473} and phospho-specific glycogen synthase kinase (GSK)-3β Ser^{9} were purchased from Cell Signalling Technology, Kvalitex Co., Budapest, Hungary. Antibody against N-terminal domain of actin was obtained from Sigma-Aldrich Co, Budapest, Hungary. Anti-PARP was obtained from Alexis Biotechnology, London, U.K. All other highly purified reagents were commercially available.

4.2.2 Experimental animals

Male Wistar rats weighting 300g to 350g were used for this study. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.2.3 Heart perfusion

Rats were anticoagulated with sodium heparin (100 U, i.p.) and were anesthetized using ketamine (200 mg/kg, i.p.). Hearts were immediately removed and arrested in ice-cold perfusion buffer. Each heart was then canulated through the aorta and perfused at 37°C by the Langendorff method at a constant perfusion pressure equivalent to 70 Hgmm. Retrograde aortic perfusion was maintained with a modified phosphate free- Krebs–Henseleit (KH) buffer, containing 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.25 mM $CaCl_2$, 1.8 mM octanoic acid and 11 mM glucose. The KH buffer was filtered through a 0.22 µM Millipore filter to remove any particulate contaminants and was saturated with a mixture of 95% O_2 and 5% CO_2 resulting in a pH of 7.4. After a non-recirculating washout period of 10 min, hearts underwent 60 minute perfusion. Imatinib and/or BGP-15 were administered into the perfusion medium at the beginning of baseline perfusion. Heart perfusion models were distributed into 4 groups with 10 perfused heart model in each group: 1. normoxic control group, 2. normoxic perfusion with BGP-15, 3. normoxic perfusion with imatinib and 4. normoxic perfusion with BGP-15 and imatinib. Hearts were freeze-clamped at the end of each perfusion.

4.2.4 NMR spectroscopy and data analysis

Cardiac energy metabolism was monitored *in situ* during the perfusion by ^{31}P NMR spectroscopy through quantification of creatine phosphate (δ= 0.0 ppm). NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument (Varian Inc., Palo Alto, CA, USA). ^{31}P measurements (161.90 MHz) of the perfused hearts were run at 37°C in a Z•SPEC 20 mm broadband probe (Nalorac Co., Martinez, CA, USA), applying WALTZ proton decoupling (γB_2 = 2 kHz) during the acquisition only. Field homogeneity was adjusted following the ^{1}H signal ($w_{1/2}$ = 10–15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each FID. 45° flip angle pulses were employed after a 1.25 s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16 K. Under the above mentioned circumstances the relative concentrations of the species can be taken proportional to the peak areas. Peak areas were determined by deconvolution of simulated spectra fitted to experimental spectra obtained after referencing (δ=0 for creatine phosphate) and baseline correction using Vnmr 6.1C software (Varian Inc., Palo Alto, CA, USA). Amounts of individual metabolite levels in each spectrum were expressed as their ratio to the first creatine phosphate (PCr) amount.

4.2.5 Lipid peroxidation

Determination of thiobarbituric acid reactive-substances (TBARs) in the heart was performed according to a modified method of Serbinova et al (1989). Malondialdehyde, formed by the breakdown of polyunsaturated fatty acids, served as an indicator of the extent of lipid peroxidation. Malondialdehyde reacted with thiobarbituric acid to give a red species absorbing at 535 nm. Amount of 50 mg frozen cardiac tissue was homogenized in 6.5% trichloroacetic acid (TCA) and in a reagent containing 15% TCA, 0.375% thiobarbituric acid (TBA) and 0.25% HCl. Homogenates were then heated for 15 min in a boiling water bath, cooled, centrifuged, and the absorbance of the supernatant was compared at 535 nm with a blank sample that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nmol/g wet tissue (Serbinova et al 1989).

4.2.6 Protein oxidation

Fifty milligrams of freeze-clamped perfused heart tissue were homogenized with 2 ml 10% trichloroacetic acid (TCA) and was divided into two equal parts. After 10 min centrifugation at 3000 g, 2 ml 2N hydrochloric acid containing 0.2% dinitrophenyl hydrazine was given to the pellets. The pellets were then vortexed, treated with 50% TCA and the protein content was collected by centrifugation. The protein carbonyl content was determined by means of the 2,4-dinitrophenyl-hydrazine-method (Butterfield 1997).

4.2.7 Western blot analysis

Fifty mg of heart samples were homogenised in ice-cold Tris buffer (50 mM, 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 (2 h with 3% non-fat milk in Tris-buffered saline) membranes were probed overnight at 4^0C with antibodies (1:1000dilution) recognising the following antigens: GAPDH, pAkt, pGSK-3β, p$ERK_{1/2}$, pp38, pJNK and anti-PARP. Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) before addition of anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3000 dilution; Bio-Rad, Budapest, Hungary). Membranes were washed six times for 5 min in TBST. The antibody-antigen complexes were visualized by means of enhanced chemiluminescence on conventional films. Quantification of band intensities (E_{540}) of the blots was performed by a DU-62 spectrophotometer equipped with a densitometry attachment (Beckman Coulter Inc., Fullerton, CA) and ImageJ (public domain) software. Data representing three independent experiments are expressed as percentage of the untreated control (mean ± S.E.M.).

4.2.8 Statistical analysis

Statistical analysis was performed using ANOVA and all data were expressed as mean ± SEM. Two-way repeated measures ANOVA was used to evaluate the statistical significance of differences among groups for levels of PCI. Bonferroni post hoc analysis was used for

specific comparisons, when significant differences were detected for the treatment-by-time interactions, Differences were considered statistically significant at the level of $p<0.05$.

4.3 Results

4.3.1 Effect of BGP-15 on myocardial energy metabolism of Langendorff perfused rat hearts in the presence of imatinib

Under our experimental conditions creatine phosphate levels of untreated Langendorff perfused rat hearts showed a slight decrease during the 60-minute perfusion. Cardiac PCr levels were decreased by 18% compared to untreated hearts when 20 mg/l imatinib was added to the perfusate. Co-administration of imatinib and BGP-15 revealed that BGP-15 in a concentration of 200 mg/l was able to prevent the decrease of imatinib induced PCr levels. Administration of BGP-15 alone increased cardiac PCr levels over the normoxic values (Fig.6.). The change of ATP concentration followed a similar pattern to the change in creatine phosphate level in our investigated time period. Imatinib treated hearts showed significant decrease in ATP levels whereas co-administration with BGP-15 successfully prevented this effect. ATP levels in the presence of BGP-15 alone were not significantly different from untreated, control cases (Fig.7.). Inorganic phosphate showed moderate increase during the perfusion reaching 15-20% of the normoxic PCr level after 30 min (data not shown).

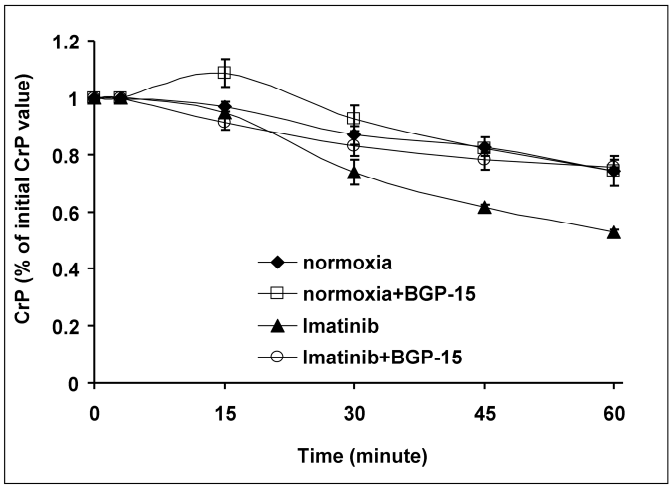


Fig.6. Effect of BGP-15 and/or Imatinib on cardiac creatine phosphate levels in Langendorff perfused rat hearts. Creatine phosphate amounts, determined by in situ ^{31}P NMR spectroscopy, are expressed as their ratio to initial creatine phosphate amount. Values are given as means ± SEM. ($p<0.01$)

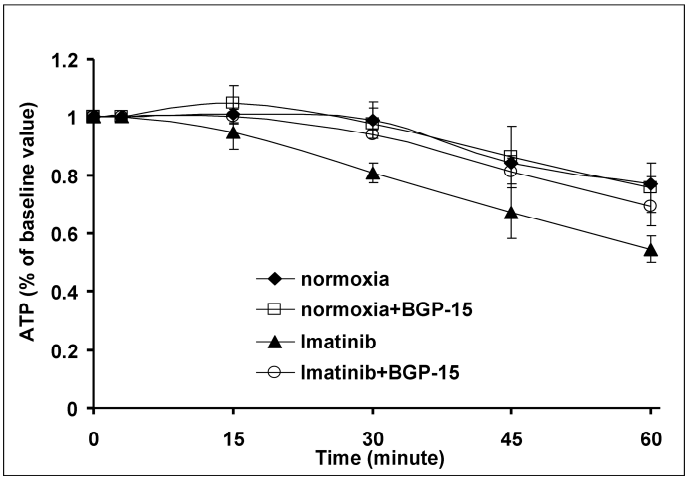


Fig.7. Effect of BGP-15 and/or Imatinib on cardiac ATP levels in Langendorff perfused rat hearts. ATP amounts, determined by in situ ^{31}P NMR spectroscopy, is expressed as their ratio to initial ATP amount. Values are given as means ± SEM. ($p<0.01$)

4.3.2 Effect of BGP-15 on myocardial oxidative damages induced by imatinib

Determination of lipid peroxidation after one hour normoxic perfusion showed that administration of imatinib significantly ($p<0.01$) increased thiobarbituric reactive substance (TBARS) formation compared to untreated hearts. On the other hand, when both imatinib and BGP-15 were present in the perfusate, the formation of TBARS was significantly diminished. BGP-15 alone did not alter TBARS formation (Fig.8.). In the case of protein oxidation we observed that presence of imatinib markedly elevated the level of protein-bound aldehyde groups as compared to untreated hearts. This phenomenon was significantly inhibited ($p<0.01$) by the co administration of imatinib and BGP-15. However, BGP-15 alone had no significant influence on the level of protein oxidation (Fig. 9).

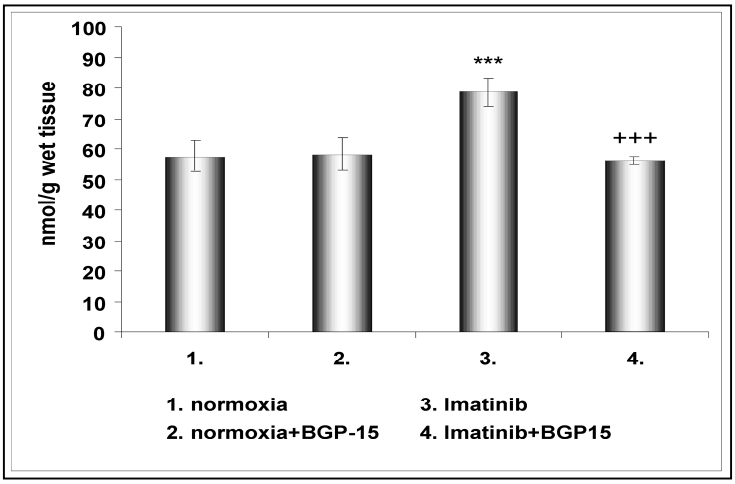


*Fig.8. Effect of BGP-15 on imatinib induced lipid peroxidation in Langendorff perfused rat hearts. The figure demonstrates the quantity of thiobarbituric reactive substances in various animal groups. Values are given as means ± SEM. (*** p<0.001 compared to normoxic levels, +++ p<0.001 compared to imatinib treated levels).*

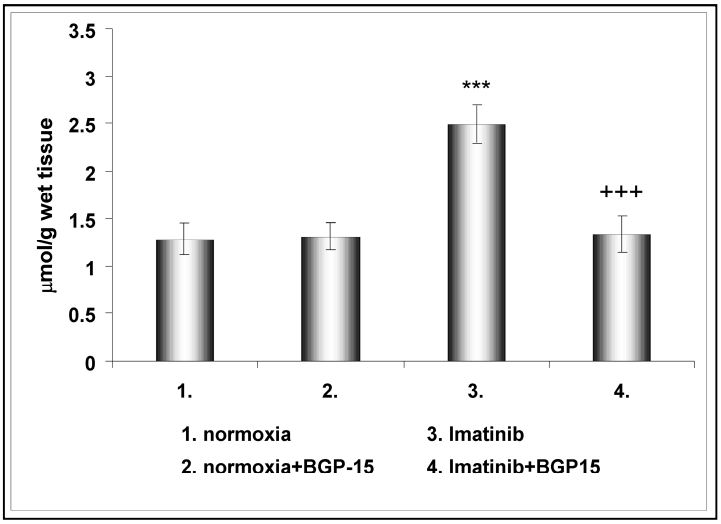


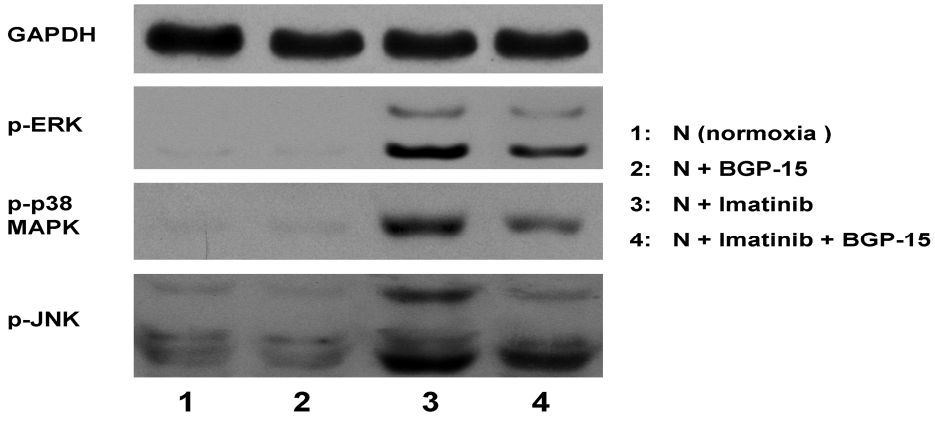
*Fig.9. Effect of BGP-15 on imatinib induced protein oxidation in Langendorff perfused rat hearts. The figure demonstrates the protein carbonyl content in various groups. Values are given as means ± SEM. (*** p<0.001 compared to normoxic levels, +++ p<0.001 compared to imatinib treated levels).*

4.3.3 Effects of imatinib and BGP-15 in signaling pathways

Akt, GSK-3ß, ERK, JNK and p38-MAPK phosphorylation were examined by Western blot in the following samples: without treatment (normoxia) (1), treatment with BGP-15 (2), imatinib (3) and with imatinib+BGP-15 (4). Imatinib induced the activation of MAP kinases (ERK1/2, p38 and JNK). JNK and p38 MAP kinase contribute to cell death (Baines 2005),

and inflammatory reactions (Gil 2007) therefore could play a significant role in the imatinib induced cardiotoxicity. BGP-15 in the presence of imatinib suppressed the activation of JNK and p38 MAP kinase, and this effect of BGP-15 could be significant in its protective role (Fig.10).

A.



B.

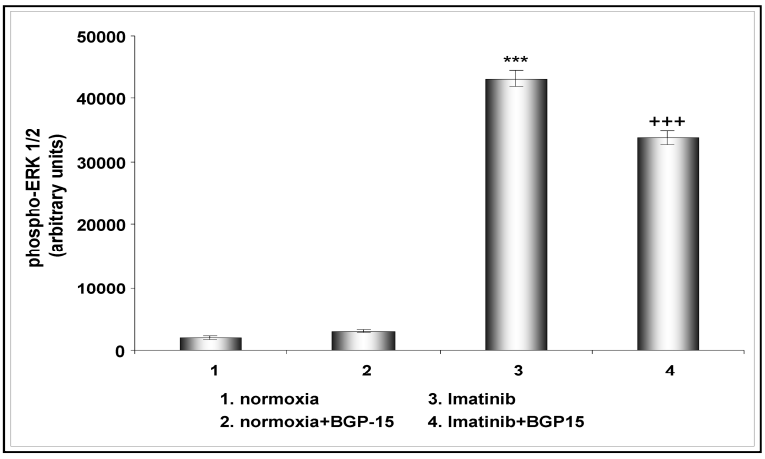
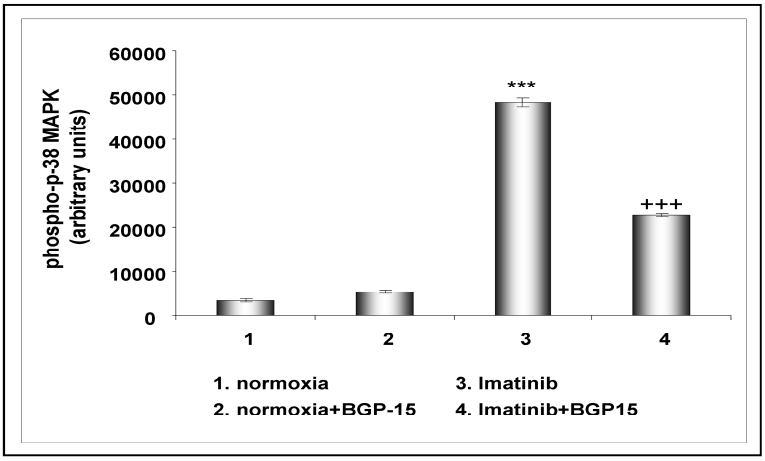


Fig.10. Phosphorylation of ERK, JNK and p38-MAPK in Langendorff perfused (60 min) rat hearts under normal conditions (normoxia), after treatment with BGP-15, with imatinib and with BGP-15+imatinib.
***A**: Representative Western blot analysis of ERK, JNK, p38-MAPK phosphorylation and densitometric evaluations are shown.**B**: p-ERK*

C.



D.

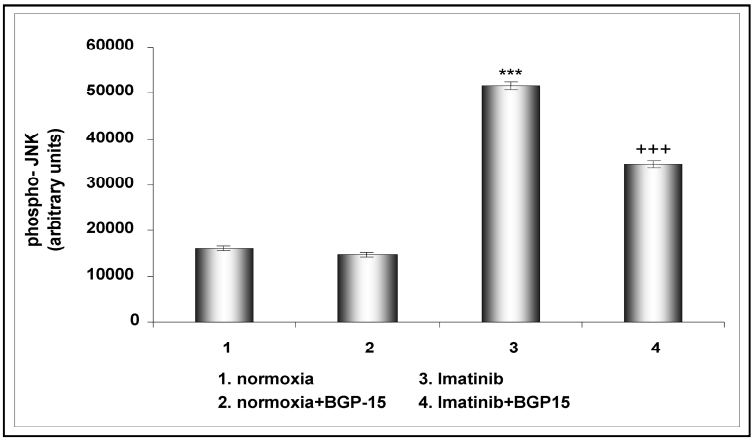
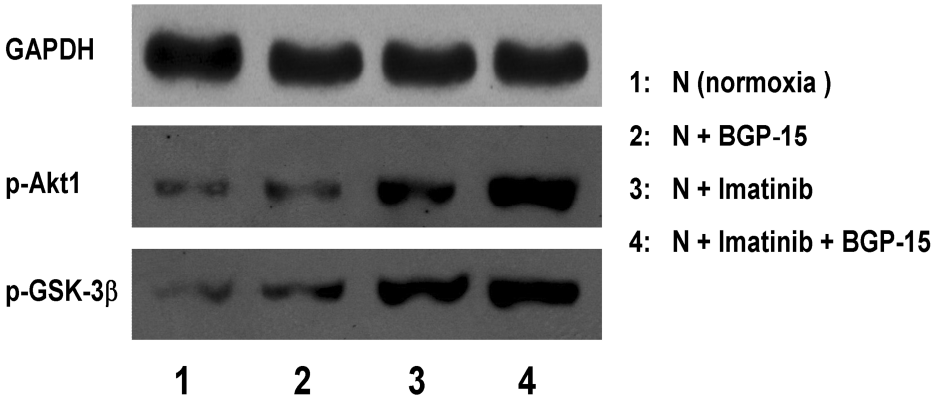


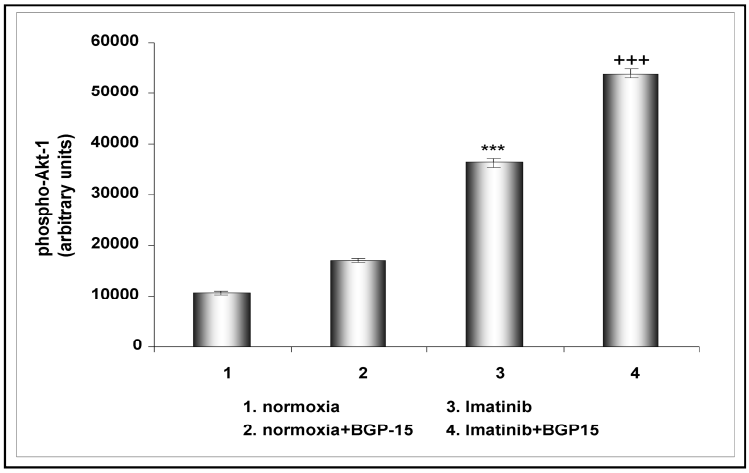
Fig.10 C: densitometric evaluations are shown of p38-MAPK and ***D:*** *densitometric evaluations are shown of p-JNK: Values are given as means ± SEM. (*** $p<0.001$ *compared to normoxic levels, +++* $p<0.001$ *compared to imatinib treated levels).*

Imatinib induced the phosphorylation of Akt and GSK-3ß, but in the presence of imatinib BGP-15 further induced the phosphorylation of these kinases (Fig.11). Phosphorylation and activation of Akt in the myocardium may play a protective role under stress conditions by maintaining mitochondrial membrane integrity (Tapodi 2005) and by phosphorylation and inactivation of GSK-3ß (Liang 2003).

A.



B.



C.

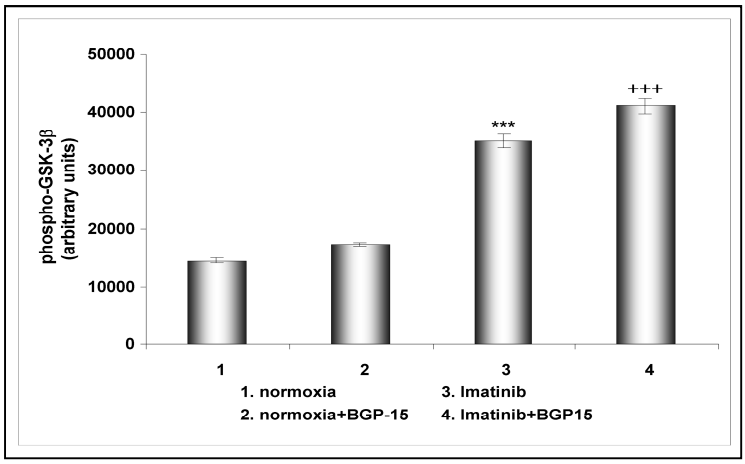
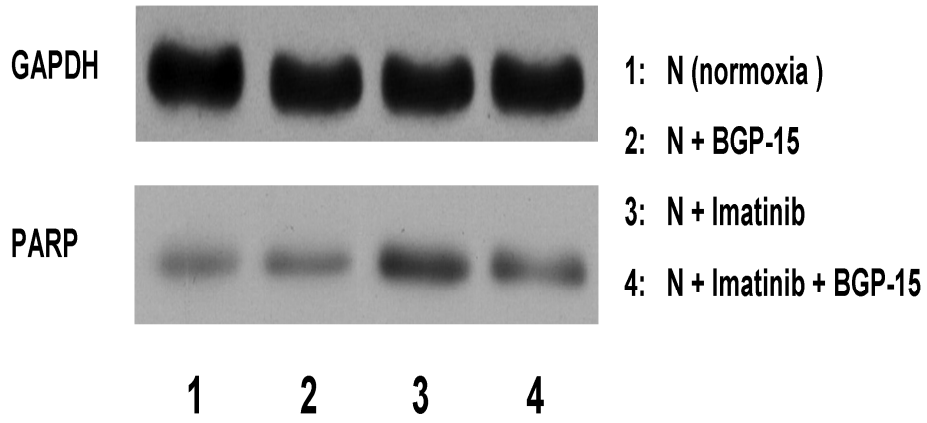


Fig.11. Phosphorylation of Akt1 and GSK-3β in Langendorff perfused (60 min) rat hearts under normal conditions (normoxia) and after treatment with BGP-15, with imatinib and with BGP-15+imatinib.
A**: Representative Western blot analysis of p-Akt1, and p-GSK-3β phosphorylation amd densitometric evaluation is shown.**B**: p-Akt1 and **C**: p-Gsk3beta. Values are given as means ± SEM. (p<0.001 compared to normoxic levels, +++ p<0.001 compared to imatinib treated levels).*

4.3.4 Effects of imatinib and BGP-15 on PARPactivation

One hour perfusion with imatinib resulted roboust PARP-1 activation. BGP-15 administered alone had no effect of PARP activation, but when co-administered with imatinib successfully reduced the effect of imatinib on PARP-1 activation (Fig.12.).

A.



B.

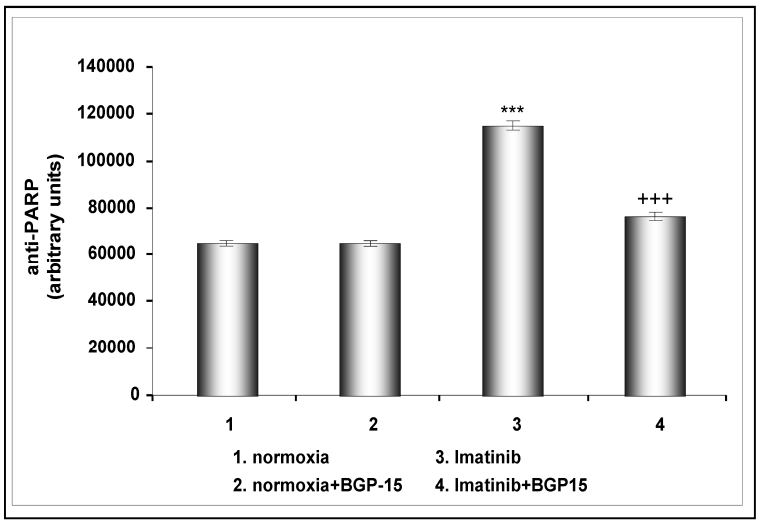


Fig.12. *Effect of BGP-15 on imatinib-induced PARP-1 activation under normal conditions (normoxia) and after treatment with BGP-15, with imatinib and with BGP-15+imatinib. Representative Western blot analysis of PARP(A.) and densitometric evaluation (B.)) are shown. Values are given as means ± SEM (*** p<0.001 compared to normoxic levels, +++ p<0.001 compared to imatinib treated levels). (n=3 in each group)*

4.4 Discussion

To date our study is a pioneering work to elaborate the effect of BGP-15 on imatinib induced energy run-down, oxidative damages and signaling mechanisms on an isolated Langendorff perfused rat heart model. BGP-15 is a nicotinic amidoxime derivate originally developed against insulin resistance. Studies investigating the effects of BGP-15 revealed that this multitarget agent is a potent cardioprotectant under conditions of ischemia-reperfusion by inhibiting over activation of PARP-1 enzyme and stabilizing the mitochondria, thus preventing ROS generation along the mitochondrial respiratory chain (Halmosi et al 2001, Toth et al 2010, Kovacs et al 2004). Furthermore hepatoprotective (Nagy et al 2010), nephroprotective (Rácz et al 2002) and neuroprotective (Bárdos et al 2003) effects of BGP-15 were also published. Recently BGP-15 has been introduced as a potent insulin sensitizer (Literáti-Nagy2003, 2010).

Earlier studies performed on myocardium cell cultures confirmed that imatinib treatment might lead to the collapse of mitochondrial membrane potential and the loss of membrane integrity (Kerkela et al 2006). The significant decrease of mitochondrial membrane potential plays a pivotal role in the development of cardiotoxicity (Van Etten 2004). Earlier data clearly defined the structural damage of mitochondria detected by electron microscopy both in human myocardial histological samples and animal models after imatinib treatment. The connection between the deterioration of mitochondrial function and ATP depletion is also well demonstrated. A well-known cytostatic agent, Herceptin causes 35% decrease in myocardial ATP level, contrary to imatinib, which generates 65% decline in ATP concentration. The reduction of ATP level may significantly run down many cellular processes. Earlier studies show that both necrotic and apoptotic cell death coexist in cultured cardiomyocytes. This fact may introduce apoptosis as an ATP-dependent process.

Our study is a pioneering work in that it has elaborated energy run-down in isolated, perfused Langendorff rat heart-model. Using ^{31}P NMR spectroscopy, we managed to show significant ATP and creatine-phosphate depletion in perfused rat heart *in situ*. The detection of energy-depletion after long-time imatinib administration has been well established by earlier reports. By perfusing the heart imatinib-containing solution we have shed light on a new aspect of a relatively quick energy run-down. Accordingly, it is worth to note that not only long-term administration but also its “acute phase” effect of imatinib can deteriorate cellular reactions in general. Energy depletion may compromise the cardiac function and can lead to a

compensatory remodelling and heart failure (Pálfi et al 2006). ATP-depletion may also compromise ion transports, mainly increase the cytoplasmic free Ca^{2+} level. The increased level of calcium in cytoplasm causes an increase in the level of calcium in mitochondria, which can lead to mitochondrial permeability pore formation and enhanced mitochondrial ROS production, which then causes enhanced oxidative damages (Murphy 2006) Mitochondrial damages can lead to the activation of apoptotic pathways, which can result in the loss of cardiomyocytes in the damaged heart regions (Subramanian et al 2010). The increase in ROS production and the imbalance of ion concentrations can lead to the rupture of plasma membrane and necrotic cell death. BGP-15 protects the mitochondrial membrane system against oxidative damages (Nagy 2010). This effect of BGP-15 can be crucial in the heart, because mitochondrial energy production is predominant (Balaban 2009), and the protection of the mitochondrial membrane system as well as the membrane potential is prerequisite of active mitochondrial ATP production. Therefore stress conditions were induced by different mechanisms, in all these cases BGP-15 decreased oxidative damages. These data suggested that BGP-15 had significant mitochondrial protecting effects (Halmosi et al 2001, Szabados 2002, Nagy 2010), regarding to protection against mitochondria related apoptotic pathway (Hori 2009) Our data show that the imatinib induced significant oxidative damage in the heart (Fig. 4 and 5), could be reversed by BGP-15, and this observed effect is in good agreement with the mechanism found in ischemic-reperfusion models (Halmosi et al 2001, Szabados 2002, Nagy 2010).

Kerkala et al. showed in cultured cells that imatinib treatment induced the activation of the endoplasmic reticulum (ER) stress response, the collapse of the mitochondrial membrane potential, the release of cytochrome c into the cytosol, a reduction in cellular ATP content and cell death. In our perfused heart system we found oxidative damages, ATP depletion and the effect of imatinib treatment on signaling pathways, which can lead to cell death (Fig.6-11). The activation of JNK and p38 MAP kinase as a consequence of imatinib treatment can play significant role in the mitochondrial depolarization and the activation of mitochondrial related apoptotic pathway (Duplain 2006). The role of p38 and Akt activation in the cardiotoxic effect is controversial, because these signaling pathways were not dysregulated in other studies (e.g. Kerkela et al). This discrepancy should be lain on the different model system. Furthermore the dose of imatinib was higher than the regular plasma concentration in humans, therefore the direct toxic effect of imatinib must be considered. However Akt activation could be explained theoretically because excessive ROS generation inactivates intracellular PTEN

which normally inhibits PI3-kinase-Akt pathway. Therefore inhibition of PTEN could activate Akt pathway. In our model p38 pathway was activated also. Earlier studies clearly defined that imatinib treatment induces ER stress response which enhances JNK and p38 activation via IRE1-ASK1 pathway. During the imatinib induced oxidative stress oxidation of the Ask1 inhibitor thioredoxins lead to the activation of Ask1 kinase, which is an upstream kinase of JNK and p38 MAP kinases (Matsuzawa 2008). A consequence, the modulation of these kinases can provide a mechanism by which we could protect the heart during imatinib treatment. Here, we showed that by combining BGP-15 with imatinib we could prevent the imatinib induced activation of JNK and p38 MAP kinase in the heart and prevent the oxidative stress and as well as ATP depletion (Fig.7. 9-11). The protective mechanism of BGP-15 is likely mediated by mitochondrial protection, because our previous data indicated that BGP-15 did not interfere with the ER stress response (Nagy 2010), while our other data indicated a mitochondrial protective effect of BGP-15. Furthermore, we found that BGP-15 activated PI-3-kinase Akt pathway, which is a well-known cytoprotective pathway. Akt activation protects cells by preventing the collapse of mitochondrial membrane system in oxidative stress (Tapodi et al 2005) which is the further evidence of BGP-15-mediated mitochondria protecting effect. In this paper we used the well-known cytostatic agent imatinib mesylate to induce cardiotoxicy in perfused Langendorff rat hearts, and tried to identify mechanism of cardiotoxicity *in situ*. Our data showed for the first time that imatinib induced oxidative stress compromised energy metabolism and the activation of potentially cell death inducing kinases (JNK and p38 MAP kinases). These data raised the possibility that the modulation of these pathways could prevent the toxic cardiac effect of imatinib mesylate.

Imatinib treatment leads to a rapid increase in poly(ADP-ribosyl)ation (PAR), preceding the loss of mitochondrial membrane integrity and DNA fragmentation. It is also important to note that the inhibition of PAR in imatinib-treated cells partially prevented cell death to an extent comparable to that observed after caspase inhibition (Moehring 2005). Previous data showed that BGP-15 protected cells in oxidative stress by protecting the mitochondrial membrane system (Halmosi 2001, Nagy 2010)], which suppressed the activation of nuclear poly-ADP-ribosylation. Our data support the earlier findings that imatinib treatment induces PARP activation. Furthermore, we demonstrated that BGP-15 was protected against PARP activation and the toxic cardiac effect of imatinib *in situ*.

The mechanism by which BGP-15 prevented the imatinib-induced cardiotoxicity is likely regarded to its mitochondrial protective role, because BGP-15 counteracted the oxidative

stress induced effect of imatinib and prevented the imatinib mesylate induced activation of JNK and p38 MAP kinase. Furthermore, BGP-15 attenuated PARP-1 activation and induced the activation of PI-3-kinase – Akt pathway, which can also contribute to the mitochondrial protection (Fig.13).

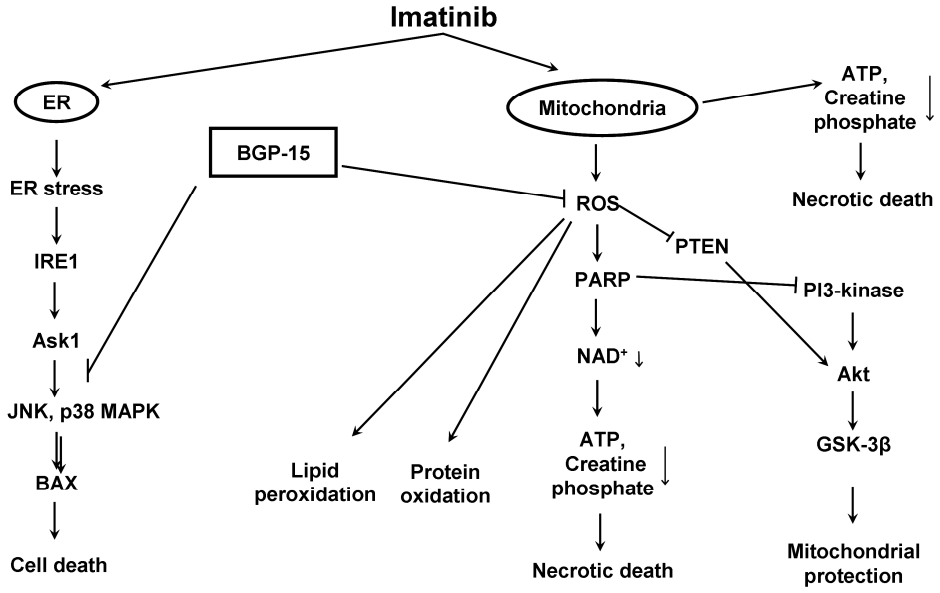


Fig.13. Molecular mechanisms of imatinib mesylate-induced cell death and possible regulatory points of BGP-15. Imatinib induces ER stress leading to activation IRE1-Ask1 pathway and further activation of JNK and p38 MAPK. Releasing of BAX and imatinib itself induce mitochondrial membrane depolarisation leading to ATP depletion, cytochrome c (Cyt c) release, and features of necrotic and apoptotic cell death. During mitochondrial damage excessive ROS generation enhances PARP-1 activation leading to NAD^+ and ATP depletion, which eventually cause necrosis. Furthermore ROS-mediated PTEN inactivation could activate PI3-Akt pathway. Inhibition of PARP-1 activity suppresses both processes leading to attenuation of NAD^+ depletion, mitochondrial protection and cell survival.

5. Clinical investigation

Effect of acute physical exercise on vascular regulation, oxidative stress, ADMA and arterial stiffness

5.1 Introduction

Among the cardiovascular risk factors, arterial stiffness and endiothelial dysfunction are believed to be the most important. Regular endurance exercise is associated with lower cardiovascular risk in middle-aged and older patients (Blair et al 1989, Manson et al 2002, Mora et al 2007). The mechanism of this beneficial effect is not understood exactly. Earlier studies demonstrate various impact of exercise to arterial stiffness, oxidative stress and endothelial dysfunction.

5.1.1 Relationship between exercise training, oxidative stress and asymmetric dimethylarginine

Oxidative stress, defined as the imbalance in production of ROS over antioxidant defense is the most important step to endothelial dysfunction. Superoxide reacts with NO synthesised by endothelial NO synthase generating oxidant peroxynitrite, which induces NO inactivation, decreases NO bioavaibility and uncouples NO sysnthase. Previous studies suggest that oxidative stress involves enhanced NADPH oxidase activity leading to ROS over generation and eNOS inactivation. On the other hand eNOS activity is deeply influenced by ADMA such as competitive inhibitor.

Asymetrical dimetylarginine (ADMA) is a metylated arginine analogue derived from proteolysis of proteins containing metylated arginine. Elevated plasma level of ADMA is associated with endothelial dysfunction. Several previous studies suggest that elevated plasma level of ADMA is a risk factor for numerous human diseases such as hypertension, diabetes mellitus, peripheral arterial disease, preeclampsisa, coronary artery disease, renal diseases.
Production of ADMA is correlated with age and the plasma level is dependent from activity of catabolic enzyme, dimetyarginin dimetilaminohydrolase. ADMA is directly involved in the pathogenesis of atherosclerosis by influencing of oxidative stress and bioavailability of nitrogen monoxide. Furthermore previous studies suggest the connection between ADMA and

rennin-angiotensin system. ADMA can increase superoxide production modulated by stimulation of angiotensin-II – NADP(H) oxidase pathway. (Veress et al 2008).

The impact of physical exercise to endothelial function and oxidative stress was widely investigated in the two last decades. Some previous studies suggest that long term periodic physical training has a beneficial effect on elevated plasma level of ADMA. These studies demonstrate that endurance training reduces circulating ADMA level in patients with coronary artery disease (Richter 2005), type I diabetes mellitus and metabolic syndrome. (Mittermayer 2005, Gomes 2008) It proves oneself to be non-sustained when patients discard the regular physical exercise

Some studies proved that physical fitness was negatively correlated with oxidative stress markers such as lipid peroxidation, DNA oxidation and protein oxidation in postmenopausal women. Moreover higher values of antioxidant enzymes (catalase and glutathione peroxidase) were found in the fit group and the positive relationship between glutathione proxidase and fitness variables (VO2max, MET). This fact supports the earlier finding that regular exercise increases the antioxidant defense in reaction to sudden rise of ROS generation during a single bout of exercise. The acute physical exercise is presumed to generate free radicals by three ways: 1. during the exercise increasing of catecholamine level and its metabolism produce free radicals, 2. production of lactic acid promote conversion of superoxide into strongly damaging hydroxyl, 3. inflammatory response to muscle damage.

On the one hand antioxidant defense depends on dietary intake of antioxidant vitamins and endogenous production of antioxidant compounds and on the other hand repetitive exercise bouts can upregulate antioxidant defense. (Dékány 2006) For example in postmenopausal women the fittness capacity negatively correlates with oxidative stress level and positively with antioxidant defense (catalase and glutathion). The fittness capacity closely has bearing on blood pressure also. Higher VO2max associates with lower protein and lipid peroxidation levels (MDA, 8OhdG). (Pialoux 2009)

5.1.2 Relationship between exercise training and arterial stiffness

Arterial stiffness is an important factor for cardiovascular performance and an independent predictor of cardiovascular risk. Moreover increased aortic augmentation index is associated with coronary artery disease. (Agabiti-Rosei 2007) Correlation between central pressures and cardiovascular risk is well known not only in patients with coronary disease but also in manifestly healthy subjects. Additionally the late systolic augmentation of the central

pressure waveform is associated with an increase in left ventricular mass index independent of age and mean blood pressure (Agabiti-Rosei 2009). Some studies investigated the effect of exercise training on arterial stiffness in healthy young athlets compared to recreationally active individuals, and in patients with coronary artery disease (CAD). Augmentation index was lower in competitive endurance athletes who have higher fitness levels and who exercise at a higher intensity, for a longer duration, and more frequently as compared with recreationally active individuals. These differences may be due to functional changes that occur as a result of training. (Edwards 2005)

Similarly in patients with CAD augmentation index was lower significantly after 12 weeks of exercise. These results suggest that endurance exercise training improves systemic arterial stiffness in individuals with CAD (Edwards 2004). But in another study, healthy youngs were investigated after 6 day endurance daily exercise, surprisingly no significant changes were observed at 75 bpm in AI(x), vascular conductanc, heart rate variability or VO(2peak). However pulse wave velocity was significantly decreased. These data indicate that changes in arterial stiffness are independent of changes in heart rate variability or vascular conductance. Relatively pure data are available regarding acute effect of single bout exercise on arterial stiffness and on endothelial function.

Marathon runners were investigated before and after marathon race. A significant fall in wave reflections was observed after marathon race, whereas aortic stiffness was not altered. Moreover, marathon runners have increased aortic stiffness and pressures, whereas wave reflections indexes do not differ compared to controls (Vlachopoulos et al 2010). The marathon running is an extreme burden for athlets. So much remains to be understood about the impact of common physical exercise on arterial stiffness and oxidative stress.

The aim of our study was to investigate the effect of single bout maximal physical exercise on oxidative stress, asymmetric dimetihylarginine and arterial stiffness in well-trained swimmers and runners.

5.2 Patients and methods

5.2.1 Subjects

21 young men well-trained athletes (mean age 22.4 y ±3.4 years) were enrolled into the study after signing an informed consent. Their basic clinical characteristics are summarized in Table 1. All of the participants had normal resting electrocardiogram and echocardiogram and they did not use any medication at the time of the study.

5.2.2 Experimental design

5.2.2.1 Standard spiroergometry

Symptom-limited exercise was performed by tredmail spiroergometry (CASE 6.5, respiratory function was analysed by LF-8 system) using Bruce protocol, which increases the workload gradually every 3 min. Vital signs, blood pressure, 12-lead electrocardiogram were detected regularly. Oxygen uptake and other respiratory parameters (minute ventilation, carbon dioxide output) were measured at rest and during the exercise. Maximal oxygen uptake, maximal workload (MET) and anaerob treshold (AT) were determined during the exercis test.

5.2.2.2 Measurements of arterial stiffness

The novelty of the Arteriograph (TensioMed Kft., Budapest, Hungary,) device is that a simple upper arm cuff is used as a sensor, but in a very special condition: the cuff is pressurized at least 35 mmHg over of the actual systolic pressure (S_{35}). By creating this stop-flow condition a small diaphragm will develop in the brachial artery at the level of the upper edge of the over-pressurized cuff. The central pressure changes as early (direct) systolic wave (P_1), late (reflected) systolic wave (P_2) and diastolic wave(s) (P_3) will reach this point and cause a beat on the membrane like a drumstick. Since the upper arm tissues are practically uncompressible, the energy propagates and reaches the skin/over-pressurized cuff edge, where it causes a very small volume/pressure change in the cuff. These very small suprasystolic pressure changes are recorded by a high-fidelity pressure sensor in the device. In this situation the conduit arteries (subclavian, axillary, brachial) act like a cannula to transfer the central

pressure changes to the edge-position sensor (similar to the central pressure measurement during cardiac catheterization). It is worth mentioning, that in this set-up (stop-flow, occluded artery) the local influence of the characteristics of the brachial wall artery are practically eliminated, and the received curves are pure pressure waves.

The Arteriograph first measures the actual systolic and diastolic blood pressure and heart rate oscillometrically, and then the device decompresses the cuff. In a few seconds the device starts to inflate the cuff again, first to the actually measured diastolic pressure, then to the suprasystolic (actually measured systolic +35 mmHg) pressure and records the signals for 8 (optionally up to 10) seconds at both cuff pressure levels. All of the signals received by the tonometer are transmitted wireless to a notebook or desktop PC. The data analysis is performed by the software designed for this purpose. The software of the device determines the augmentation index by using the formula:

$$\text{Aix (\%)} = (P_2\text{-}P_1)/PP \times 100$$

where P_1 is the first (direct), P_2 is the late (reflected) systolic wave's amplitude and PP is the pulse pressure.

To determine PWVao the Arteriograph uses the physiological behaviour of the wave reflection, namely that the ejected direct (first systolic) pulse wave is reflected back mostly from the aortic bifurcation. The device measures the time interval between the peaks of the direct (first) and reflected (late) systolic wave (return time – RT), which is equal to the time that the pulse wave needs to travel down to the bifurcation and back. Both for the invasive and non-invasive PWVao calculation the sternal notch–upper edge of the pubic bone (jugulum-symphysis = "Jug-Sy") distance was used. Care was taken to avoid the overestimation of the distance by putting the belt on the curvature due to large mammas or abdomen. Instead, parallel distance was measured between these anatomical points. The PWVao was calculated by using the formula:

$$PWV_{ao}\ (m/s) = \frac{\text{Jug-Sy (meter)}}{\text{RT/2 (second)}}$$

The calculation of the central SBP in the Arteriograph was based on the relationship between the brachial and central SBP on the basis of the late systolic wave amplitude. The blood pressure measurement algorithm in the device has been validated. (Horváth IG 2010)

Augmentation index and pulse wave velocity were measured before and after exercise stress test at the end of recovery phase. The measurements were performed in a supine position and were accepted if the quality indicator of the recordings was within the acceptable range (i.e., the SD of the beat-to-beat measured PWVao values were less than 1.1 m/s).

5.2.2.3 Blood sampling

Measurement of ADMA, SDMA and arginine

Venous blood samples were obtained before, immediately after and 4 hours after the stress test, respectively. Samples were centrifuged and serum was removed and was frozen in -20˚C. Serum ADMA, SDMA, arginine levels were measured.

Solid phase extraction (SPE) of the analytes was performed according to the method of Nonaka et al. 250 µL serum sample was mixed with 700 µL pH = 9.00 borate buffer and L-homoarginine hydrochloride (Sigma, HArg) was used as internal standard (50 µL of 1000 µmol/L solution). The resulting mixture was passed through OASIS® MCX 3cc SPE cartridges at 750 mbar in a 12-column manifold (J. T. Baker). Washing was done respectively by borate buffer, water and methanol (Sigma). The analytes were eluted with a mixture of concentrated aqueous ammonia (Reanal), water and methanol with a volume ratio of 10/40/50. The solvent was evaporated beginning under nitrogen atmosphere and finished in vacuum at 60 C. The dry residue was dissolved in 200 µL ultrafiltered deionized water (Millipore, Milli-Q) and derivatized according to Molnar-Perl et al. The samples (200 µL) were mixed with 63 µL reagent solution containing OPA (*ortho*-phthaldialdehyde from Fluka) and MPA (3-mercaptopropionic acid from Aldrich) and incubated at RT for 10 min then cooled down to 5 °C. For HPLC analysis derivatized samples of 10 µL were injected into a Waters 2695 Separations Module equipped with thermostable autosampler (5 °C) and column module (35 °C). Separation was achieved with a Waters Symmetry SB C18 (4.6 x 150 mm, 3.5 µm) column and detected by a Waters 2745 Fluorescent detector (Waters Milford, MA, USA). Gradient elution at a flow rate of 1 mL/min was applied during the analysis with two mobile phases: **A** (20 mM $(NH_4)_2CO_3$ in water, pH = 7.50 ± 0.05) and **B** (acetonitrile). The gradient program was as follows: 0-16 min: 91% **A** and 9% **B**, 16-17 min: linear change to 70 % **A** and 30 % **B** and hold this for 5 minutes, 22-23 min: linear change to 91 % **A**, 9% **B** and hold this for 12 minutes. The last two phases were to wash and regenerate the column for the

next sample. Arginine and homoarginine were detected at λ_{ex} = 337 nm, λ_{em} = 520 nm, and λ_{em} = 454 nm was used for asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). With the described method baseline separation was achieved.

Measurement of lipid peroxidation

Quantitative determinations of peroxides were performed by Oxystat ELISA method. The LDL-oxidation processes the general characteristics of lipid peroxidation reactions. In most instance three conseutive time phase can clearly be detected.

1. lag phase – radical capture by antioxidants, duration 1/2-1 hours
2. propagation phase – lipid peroxidation, duration 1-2 hours
3. decomposition phase- decomposition of lipid hydroperoxides, duration 15 hours

In our study the Biomedica Oxystat assay was used wich measures the total peroxide concentration formed in the propagation phase of the LDL oxidation process. Blood samples were collected 2 hours later at the end of exercise stress test considering duration of propagation phase. Using the Oxystat assay the normal range exists between 0-400 μmol/l.

5.2.3 Statistical analysis

Independent sample t tests were used to compare oxidative stress marker Oxystat, setum ADMA level, augmentation index and pulse wave velocity before and after physical exercise test. Pearson correlation coefficients between Aux, PWV and oxidative stress markers were estimated. Statistical analysis was performed with SPSS (version 15.0, SPSS Inc.)

5.3 Results

All measured data of well trained athletes in our cohort are presented in descriptive statistics table. (Table 1.) Paired data show mean and SEM before and after exercise test High level of Vo2max, MET and anaerob treshold levels demonstrate that athletes were very well-trained. Serum arginine levels (70,77 ±27,002 versus 74,96±22,97 μmol/l), serum ADMA (0,79±0.15 versus 0,73±0,16 μmol/l) and SDMA levels (0,58±0,12 versus 0,57±0,10 μmol/l) were not significantly different before and after exercise stress test. (Fig.14,15,16.) The oxidative stress response was evaluated by analyzing blood oxidized LDL level before and 2 hours later after exercise. Oxystat data show surprisingly no significant changes before and afetr exercise (260,76±40,50 versus 267,08±68,02 μmol/l) though normally physical exercise increases oxidative stress level. (Fig.17.)

Table 1. Characteristics of the athletes who underwent single- bout exercise stress test

(Data are presented as mean ± SEM)

	N	Mean ± SEM
Age (year)	21	22.4 ± 3.4 years
RR syst.(before)	21	127.74 ± 3.39 mmHg
RRdiast (before)	21	82.5 ± 2.2 mmHg
RR syst. (after)	21	128.47 ± 2.74 mmHg
RR diast (after)	21	76.4 ± 3.4 mmHg
RR syst max	21	180.6 ± 4.2 mmHg
RR diast max.	21	82.8 ± 2.1 mmHg
MET	21	19.65 ± 0.25
AT	21	28.52 ± 2.16
MAX.WORK LOAD	21	52.16 ± 1.26 ml/kg/min
ATREF	21	60.31 ± 2.56 %

Abbreviations:
RR syst before: systolic blood pressure measured before exercise stress test
RR syst. after: systolic blood pressure measured after exercise stress test
RR diast before: diastolic blood pressure measured before exercise stress test
RR diast. after: diastolic blood pressure measured after exercise stress test
RR syst and diast max. systolic and diastolic blood pressure measured at maximal work load
MET: metabolic unit, calculated maximal work load
AT: anaerob treshold measured during exercise stress test
MAX WORK LOAD: measured maximal oxygen uptake at maximal work load during exercise test
ATREF: measured anaerob treshold level /age-related predicted value

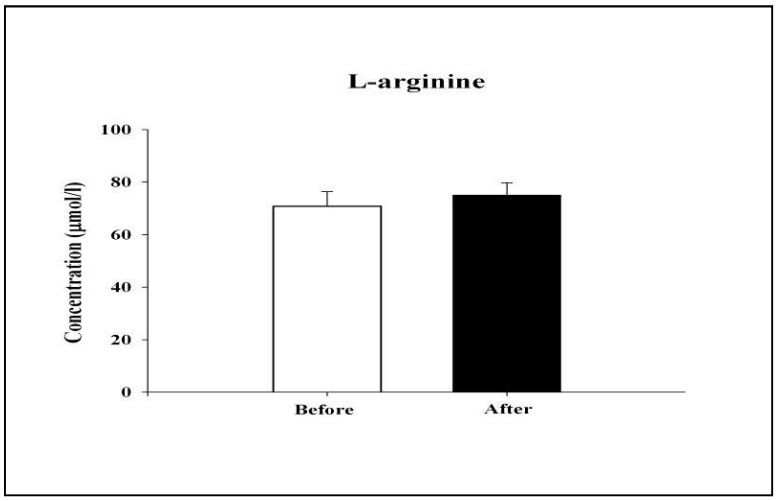


Fig. 14.Mean and SD of serum arginin (70,77 ±27,00 versus 74,96±22,97 µmol/l) There was no significant difference before and after exercise p<0.49

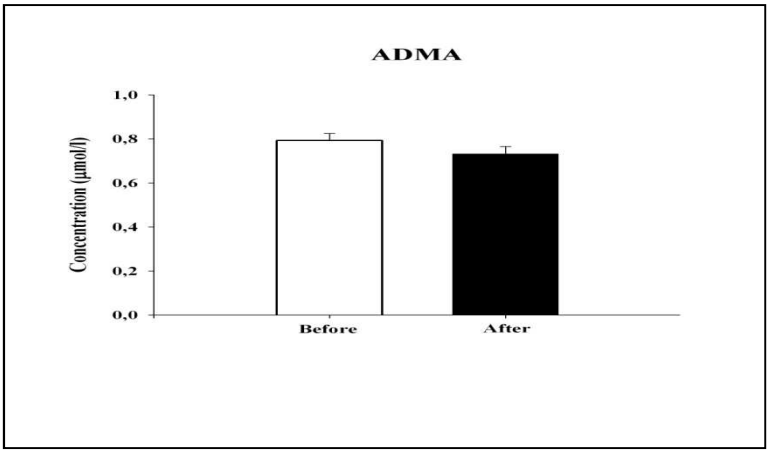


Fig. 15.Mean and SD of serum ADMA 0,79±0.15 versus 0,731±0,16 µmol/l. There was no significant difference before and after exercise p<0.13.

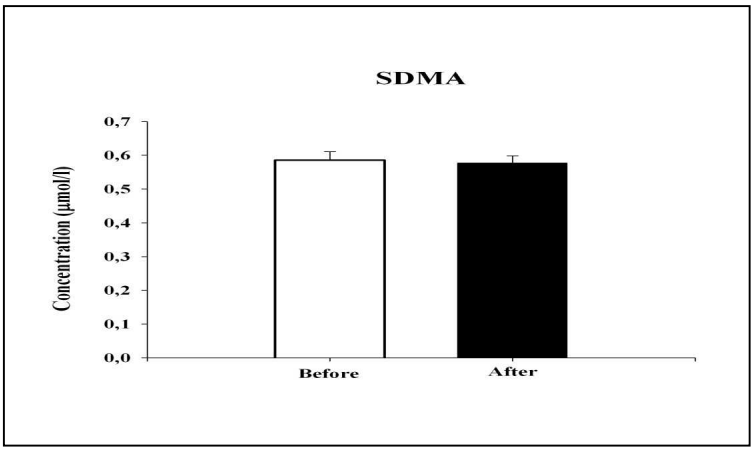


Fig.16. SDMA levels 0,58±0,12 versus 0,57±0,10 µmol/l were not statistically significantly different before and after exercise stress test. p<0.57

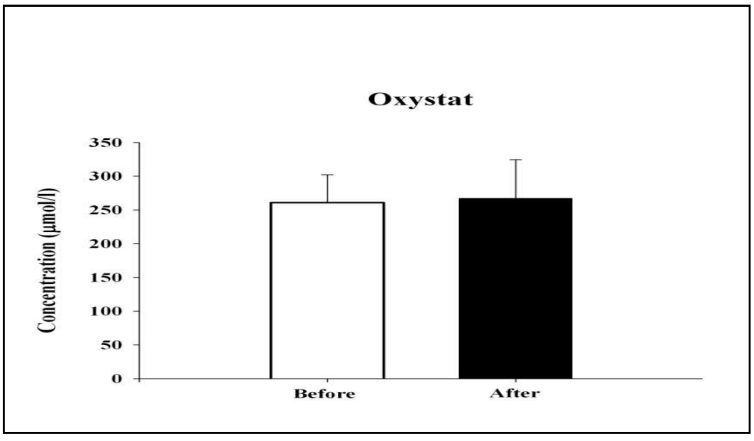


Fig.17. Mean and SD of serum oxidized LDL (260,76±40,50 versus 267,08±68,02 µmol/l) There was no significant difference before and after exercise. p<0.43

AIX is a complex composite measurement, derived from many dynamic variables, such as PWV, ventricular contractility and the resistance of the small arteries.Both pre-test and pos-test augmentation index values were in normal range. Augmentation index significantly dropped after exercise stress test compared topre-test levels. (-50,92±16,43 versus -70,13±8,589 p<0.001) (Fig.18.) PWV values were not significantly differed before and after stress test. (6,49±1,001 versus 6,27±1,037 p<0.48) and existed in normal range. (Fig.19.)

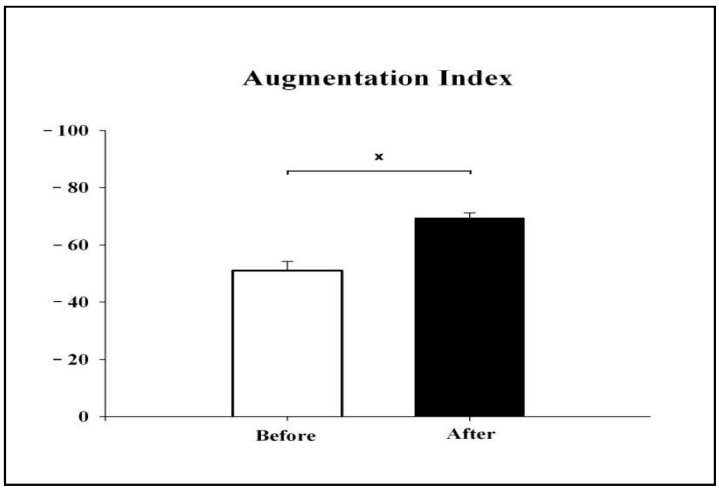


Fig. 18. Augmentation index significantly decreased after exercise compared to pre-test augmentation index -50,9211±16,43041 versus -70,131 6±8,58966 p<0.001

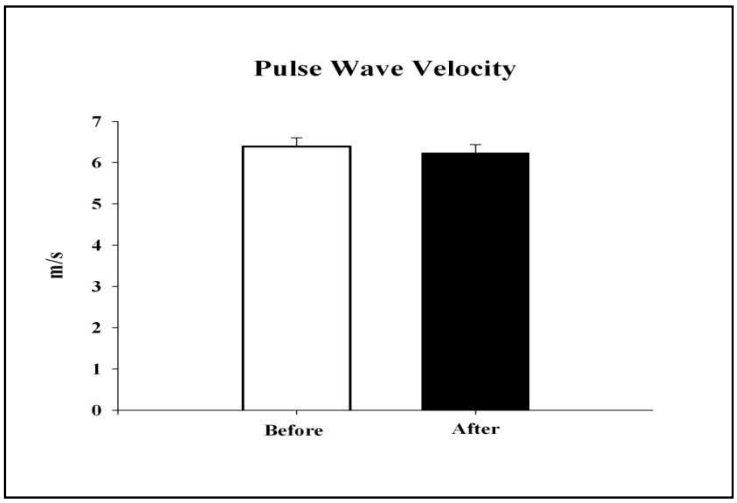


Fig. 19. Mean values of pulse wave velocity before and after exercise. There is no significant effect of exercise on PWV

5.4 Discussion

Several studies have reported that exercise training can protect against myocardial infarction in animal model and has been associated with improved survival following an ischaemic event in humans. The background of protective mechanisms is not fully understood. On the one hand the endurance exercise can reduce risk factors related to cardiovascular disease, including obesity, high blood pressure, and high lipid level and insulin resistance. On the other hand the exercise training has an independent beneficial effect related to the reduction of these risk factors. Moreover earlier studies demonstrate the infarct sparing effects of exercise both during long-term (10 weeks) and short-term (1–5 days) training regimens.

Although the physiological and cardioprotective effects of exercise training have previously been documented, the signalling mechanisms that mediate these effects have not been fully understood. However, it is known that exercise training increases a number of classical signalling molecules. For instance, exercise can increase components of the endogenous antioxidant defences (i.e. superoxide dismutase and catalase), increase the expression of heat shock proteins, activate ATP-sensitive potassium (KATP) channels, increase the expression and activity of endothelial nitric oxide synthase (eNOS) resulting in increased NO levels. (Calvert 2011) NO plays a central role in both blood delivery and the capacity of cells to extract several substances. First, the release of NO from endothelial cells in response to shear stress induces vasodilatation of arteries in both the skeletal muscle and the heart to increase blood flow. Second, NO has been reported to alter carbohydrate metabolism in skeletal muscle through an enhancement of glucose uptake and inhibition of glycolysis. Furthermore to its effects on matching blood supply to metabolic demands during exercise, NO is also responsible for some of the atheroprotective effects of exercise through its ability to inhibit inflammatory cells and platelets from adhering to the vascular surface. Not only eNOS and NO play a key role in these processes but also asymmetric dimethylarginine, such as a competitive inhibitor of eNOS can alter the exercise induced beneficial changes. Exposure of human endothelial cells to mild or moderate shear stress causes overexpression of protein methylase-1 (PRMT-1) and the release of ADMA via activation of the NF-κB pathway. Shear stress at higher magnitude, however, enhances the degradation of ADMA via stimulation of dimethylarginine dimethylaminohydrolase (DDAH) activity and reduces ADMA level to baseline (*Osanai 2003*). Interestingly, the activity of the enzymes involved in the formation and degradation of ADMA such as PRMT-1 and DDAH

are regulated in a redox-sensitive fashion. Thus, oxidative stress enhances the activity of PRMT-1 and inhibits the activity of DDAH leading to increased ADMA concentration. Under those conditions, ADMA could inhibit eNOS activity and/or even uncouple the enzyme, which would further increase oxidative stress (Scalera 2006). In contrast, reduced oxidative stress enhances the activity of DDAH (Scalera 2004), causing decreased ADMA levels.

Earlier studies have demonstrated that regular endurance exercise can reduce the serum level of ADMA. Hovewer the minimum duration of exercise training needed to achieve a decrease in the levels of ADMA and provide cardioprotection is not clearly understood. In addition some question remains unanswered. How high can exercise training increase levels of antioxidant defense and decrease the level of ADMA and how long does it take to reach a new steady-state level? Will the new steady-state levels of be maintained over time? Additionally, how can the different types of exercise training, alter the antioxidant defense and ADMA levels?

Our findings suggest that asymmetric dimethylarginine, symmetric dimethyarginine and L-arginine are not modulated by acute maximal exercise stress in healthy sportsmen. The maximal physical exercise rapidly elevates shear stress; which theoretically should induce an increasing ADMA level. In opposite, acute phase changes cannot be detected after single bout exercise it seems that repetitive endurance exercise evolves protective effect. Moreover the endurance exercise training can reduce ADMA level after 8-12 weeks training programme and the lower level remains sustained if the patient did not give up the training. Between two decisive points the time-course is not defined.

Under physiological conditions, small amounts of reactive oxygen species (ROS) produced as a consequence of electron transfer reactions in mitochondria, peroxisomes, and cytosol and during the exercise the amount of ROS rapidly elevates. If the cellular antioxidant capacity is sufficient antioxidants can capture reactive oxygen species. Two most important antioxidant enzymes are SOD and catalase. Previous findings suggest that short durations of exercise training are not sufficient to increase SOD expression but catalase activity was increased in response to 8 days of exercise training and by 9 days of rest, catalase activity had returned to baseline levels. Interestingly, the protective effect of SOD and catalase against myocardial infarction is remained unclear (Lennon 2004). It seems that the duration and intensity of the endurance training determines which endogenous antioxidants will be over expressed. Furthermore an increase in endogenous antioxidants may be sufficient to provide cardioprotection in response to exercise, but is not necessary. The acute physical exercise is

presumed to generate free radicals which can enhance lipid peroxidation. Our findings demonstrate that the level of lipid peroxidation was not significantly different before and after maximal physical exercise stress test. The endurance exercise induces elevated antioxidant defense, which can cope with ROS generation related to acute physical stress.

Arterial stiffness parameters are commonly used for this purpose - to identify structural and functional changes in the arteries on the development of atherosclerotic disease. The independent predictive value of aortic stiffness has been demonstrated for fatal stroke, all-cause and cardiovascular mortalities, fatal and non-fatal coronary events in hypertensive, diabetic, end-stage renal disease, in elderly patients and in the general population. Among stiffness parameters, the aortic pulse wave velocity (PWV), and the wave reflection, measured as augmentation index (AIx) were determined. Moreover, carotid-femoral pulse wave velocity (PWV), a direct measure of aortic stiffness, has become increasingly important for total cardiovascular (CV) risk estimation. PWV is accepted as the most simple and reproducible method to assess arterial stiffness.

Aix is defined as the difference between the second and first systolic peaks of the pressure wave, expressed as a percentage of the pulse pressure. Therefore AIX is a complex composite measurement, derived from many dynamic variables, such as PWV, ventricular contractility and the resistance of the small arteries.

Our findings support earlier establishment that pulse wave velocity is not modulated by physical exercise. This fact is explained by good elasticity of aorta in young healthy sportsmen. Recent study demonstrates that not only endurance exercise but a single bout exercise can reduce augmentation index. The rapid decreasing of Aix after exercise test may indicate physiological exercise-related vascular response namely dropping of peripheral vascular resistance. This non-invasive method may be eligible to evaluate physiological and pathological vascular response to physical exercise. It has been shown that AIX is an independent predictor of mortality in patients with end-stage renal disease and the extent of angiographic coronary artery disease (London 2001, Weber 2004). Moreover it may be a predictor of endothelial dysfunction. Further investigations are required to appoint the sensitivity and specificity of this method.

6. Conclusion

Oxidative stress is one of the most important pathophysiological processes leading to myocardial cell damage, endothelial dysfunction and endothelial cell damage. Aim of our experimental investigation was to identify underlying mechanism of oxidative stress and myocardial cell damage in typical cytotoxic agent –induced perfused heart model. Our data demonstrate cytotoxic agent-induced myocardial cell damage exist on the basis of energy-deplettion, enhanced lipid peroxidation and protein oxidation. Among signalling mechanisms, the prodeath effects of activation of JNK-p38-MAPK pathway was confirmed.

Furthermore oxidative stress can damage not only myocardial cells but also endothelial cells. In our clinical study we investigated the role of ADMA and lipid peroxidation in context of physical exercise – induced oxidative stress. In both experimental and clinical studies we evaluated the protective effect of using a new exogenous antioxidant agent (BGP-15) and the beneficial effect of elevation of endogenous antioxidant defense by endurance exercise. Effectiveness of both methods were confirmed. Furthermore we realised the usefullness of measuring of arterial stiffness on clinical evaluation of endothel function.

7. Novel findings

1. Our findings demonstrated that BGP-15 can protect against imatinib induced energy depletion.

2. Imatinib induced lipid peroxidation and protein oxidation were decreased by using BGP-15.

3. An early step of imatinib induced ER stress response is the Akt activation. Over-expression of these protective signaling pathways is significantly higher after treatment with BGP-15 +imatinib.

4. Our data showed for the first time that imatinib induces oxidative stress, compromised energy metabolism and the activation of potentially cell death inducing kinases (JNK and p38 MAP kinases). BGP-15 could prevent the activation of these prodeath signalling mechanisms.

5. Our findings demonstrate that asymmetric dimethylarginine, symmetric dimethyarginine and L-arginine is not modulated by acute maximal exercise stress in well-trained healthy patients

6. Endurance exercise induces elevated antioxidant defense, which can cope with ROS generation related to acute physical stress and it protects against lipid-peroxidation

7. Measuring of PWV and Aix is eligible to evaluate psysiological and pathological vascular response to physical exercise. Rapid decreasing of Aix after exercise test may indicate the physiological exercise-related vascular response namely dropping of peripheral vascular resistance.

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9. Publications

9.1 Publications related to thesis

9.1.1 Full papers

***Sarszegi Z**, Bognar E, Gaszner B, Konyi A, Gallyas F Jr, Sumegi B, Berente Z.* BGP-15, a PARP inhibitor prevents imatinib induced cardiotoxicity by activating Akt-GSK-3beta pathway and suppressing JNK and p38 MAP kinase activation.
Mol Cell Biochem 2012.Febr.14. Equib ahead of print IF:2.168

***Sarszegi Z**, Ajtay Z, Lenkey Z, Nemeth A, Cziraki A, Czeh A, Lustyik G, Biri B, Kék Si, Szabados S.* Impact of acute physical exercise on vascular regulation, oxidative stress, ADMA and arterial stiffness
Med Sci Monit submitted, under review

*Gaszner B, Lenke Zsy, Illyés M, **Sarszegi Z**, Horváth I, Magyari B, Molnar F, Cziraki A.* Comparison of aortic and carotid arterial stiffness parameters in patients with verified coronary artery disease
Clin Cardiol. 2012 35(1):26-31.IF:1.807

*Csordás A, Pankotai E, Snipes JA, Cselenyák A, **Sárszegi Z**, Cziráki A, Gaszner B, Papp L, Benko R, Kiss L, Kovács E, Kollai M, Szabó C, Busija DW, Lacza Z.*
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A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death.
Free Radic Biol Med 2006;41(5):835-48. IF:5.440

*Cziráki A, **Sárszegi Zs**, Gaszner B, Illyés M, Papp L*: A vaszkuláris endothel és a pulmonális epitel noninvazív vizsgálata iszkémiás szívbetegekben., Cardiol Hung 35:A50, 2005

9.1.2 Abstracts

***Sárszegi Zs**, Lenkey ZS, Németh Á, Kéki S, Lustyik Gy, Cziráki A, Szabados S:* A maximális fizikai terhelés hatása a vaszkuláris regulációra, oxidatív stresszre, ADMA szintre és az artériás stiffnessre Card Hung 2011 május Suppl F72.

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10. Acknowledgement

I owe my most sincere gratitude to my tutor Balázs Sümegi and Attila Cziráki for introducing me to the basic science of molecular cardiology, and to the tools to investigate its properties.
I would also especially like to thank Professor Erzsébet Rőth former support, expert guidance and mentorship.
I thank the friendship and scientific support to Balázs Gaszner and Attila Kónyi
And last but not least my warmest thanks to my wife and my children for their supports and patience during long years