The critical role of MAP-kinases and PI3K-Akt signaling pathways in inflammation and oxidative stress

Ph.D. Thesis

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Abbreviations

AP-1: activator protein-1

Akt: Protein Kinase B (PKB)

Ask1: apoptotic signal regulating kinase

BAD: Bcl-2 associated death promoter

DUSPs: dual-specifity phosphatases

EGF: endothelial growth factor

Egr-1: early growth response protein 1

ERK: extracellular signal-regulated kinase

FKHR: forkhead homolog rhabdomyosarcoma transcription factor

GSK-3β: Glycogen synthase kinase 3 beta

ICAM-1: intracellular adhesion molecule-1

IκB: inhibitors of NF-κB

IKK: inhibitor of NF-kappa B kinase

iNOS: inducible nitric oxide synthase

IRAK1: interleukin-1 receptor-associated kinase-1

IRAK4: interleukin-1 receptor-associated kinase-4

(IRF)3: interferon regulatory factor 3

JNK: c-jun N-terminal kinase

LBP: LPS binding protein

LPS: lipopolysaccharide

MAPK: mitogen activated protein kinase

MKP-1: MAPK phosphatase -1

MPT: permeability transition pore

MyD88: myeloid differentiation primary response gene 88

NAC: N-acetyl cysteine

NADPH-oxidase: nicotinamide adenine dinucleotide phosphate-oxidase

NEMO: NF-κB essential modifier

NF-kappa B: nuclear factor kappa B

P: phosphorylated

PARP: poly ADP-ribose polymerase

p38-MAPK: p38 mitogen activated protein kinase

p65: Transcription factor p65 (RelA)

p50: NF-KappaB1

PAMPs: pathogen-associated molecular patterns

PDK-1: phosphatidylinositol-dependent kinase 1

PDGF platelet-derived growth factor

PIP2: phosphatidylinositol 3,4-bisphophate

PIP3: phosphatidylinositol 3,4,5-tiphosphate

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PKG: cGMP-dependent protein kinase

PTEN: phosphatase and tension homolog deleted on chromosome 10

ROS: reactive oxygen species

Sp-1: stimulating factor-1

STAT-1: signal transducer and activator of transcription-1

TAB: TAK1-binding protein

TAK1: transforming growth factor- β-activated kinase 1

TBK1: TANK binding kinase 1

TF: transcription factor

TIR: Toll-interleukin-1 receptor

TIRAP: TIR domain-containing adaptor protein

TLRs: Toll-like receptors

TLR4: Toll-like receptor 4

TNFα: tumor necrosis factor-α

TRAF6: TNF receptor-associated factor 6

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor inducing IFN-β

1. Introduction

Inflammation and oxidative stress are main pathophysiological processes responsible for the cardiovascular morbidity and mortality. Both mechanisms activate and moderate the same intracellular signal mechanisms. Recently, investigations focus to understand signal networks because they would become potential therapeutic targets. On the other hand it may be consider to investigate the effect of new and "older" therapeutic drugs on signaling pathways.

1.1 Nuclear factor (NF)κB, mitogen activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)-Akt pathways in inflammation

Gram-negative bacterial infection is a major cause of sepsis and septic shock, a syndrome characterized by a widespread inflammatory response that triggers organ damage and ultimately organ failure. Toll-like receptors (TLRs) are key components of the innate immune system and were originally described as recognizing pathogen-associated molecular patterns (PAMPs) derived from bacteria and other microorganisms. The best characterised is TLR4, which recognises lipopolysaccharide (LPS) structural component of the outer membrane of Gram-negative bacteria. LPS induced inflammatory responses enhance the production of proinflammatory mediators, including tumor necrosis factor-α (TNFα), several interleukins (IL-1β, IL-6, IL-8), NO and reactive oxygen species (ROS) in endothelial and epithelial cells, neutrophils, macrophages, and lymphocytes. These markers are associated with gram-negative sepsis and other inflammatory diseases (Ulloa 2005). The inflammatory response was extensively studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, which are very sensitive to LPS stimulation (Wang 2002; Kim 1999; Doyle 2006). Investigation of these pathways may promote developing effective drugs for sepsis or septic shock therapy.

1.1.1 Important role of NFκB in inflammation

NFκB is a transcriptional regulator that plays a central part in responses to inflammatory signaling. NFκB family consist of five members: p65 (RelA), Rel B, c-Rel, p50 and p52. All of these family members have a highly conserved N-terminal region, called the Rel Homology Domain (RHD). The N-terminal part of the RHD contains the DNA-binding domain, the dimerization domain is located in the C-terminal region of the RHD. Close to the C-terminal end of the RHD lies the Nuclear Localization Signal (NLS), which is essential for the transport of active NFκB complexes into the nucleus. Each NF-κB/Rel protein can combine to form either homodimers or heterodimers, except of Rel B, which only forms heterodimers. Dimerization of NF-κB proteins such as RelA/RelA, p50/p50, p50/RelA, p50/c-Rel, p52/c-Rel, p65/c-Rel, p52/p52, RelB/p50 and RelB/p52 renders them differential binding specificities. These dimers can contribute to the activation of specific target genes. The most common dimer complex is the p50/RelA heterodimer, which is very important in the coordination of inflammatory and innate immune responses. Under resting state, NFκB dimers are held in the cytoplasm in an inactive form by a family of inhibitors (IκBs). Interaction between NFκB and IκB blocks ability of the former to translocate to the nucleus by masking the NLS. Several stimuli lead to phosphorylation of IκBα on two key serines (S32, S36) by IκB kinase complex (IKKα, -β, -γ). After phosphorylation, IκB is released, and undergoes ubiquitination and proteasomal degradation (Chen 1996). This process unmasks the NLS of the p50/RelA heterodimer, so it can translocate to the nucleus and become transcriptionally active by binding to specific κB sites within the promoter regions of its target genes (Vallabhapurapu 2009). IκBs (IκBα,β,ε) possess potent nuclear export signals (NES), remove NFκB proteins from the nucleus and have inhibitory effect in multiple ways. During inflammation, the activation of NFκB is switched off when the inflammatory signal terminates. IκBß and IκBε, are synthesized constitutively and reestablish NFκB inhibition, whereas synthesis of IκBα is under the control of NF-κB itself. It can enter the nucleus, remove NFκB from DNA and shuttles it back to the cytoplasm by means of a nuclear export signal. There are two primary signaling pathways that lead to activation of NFκB: the classical (canonical) one and the noncanonical (or alternative) pathways. The canonical NFκB pathway is dependent on IKKβ, which are catalytic kinases, and IKKγ (known as NEMO), which acts as a regulatory subunit. This classical pathway is related to the translocation of the p50/p65 heterodimer, which plays an important role in inflammatory response and sepsis.

The alternative pathway is associated with IKKα, which mediates the phosphorylation-dependent processing of p100 resulting in the generation of p52. P52 and RelB form heterodimer and able to enter to the nucleus.

There are several feedback mechanisms that can control the stimulatory effects of p50/p65: p50 homodimers bind to κB consensus site in the nucleus and repress transcription; p50/p65 heterodimer itself stimulates the production of IκB. In this way, NFκB limits its own activation.

A number of stimuli have been shown to activate NFκB including bacterial products, growth factors (Moynagh 2005), UV irradiation (Li N 1998) and oxidative stress. The most potent NFκB activators are the proinflammatory cytokines such as IL-1 and TNF, which can amplify NFκB activation via IL-1 and TNF receptor mediated pathways (Han SJ 2002). Considerable data indicates that NFκB activation contributes to in the pathogenesis of organ injury and lethality during sepsis. Using of anti-inflammatory agents (or NFκB inhibitors) is a double edged sword because in early sepsis its beneficial effect was confirmed but it may be harmful in the later stages, when the host's anti-inflammatory response has already been activated.

1.1.2 Effect of MAPK's and MAPK phosphatase (MKP)-1 during inflammation

The MAPKs are a heterogeneous group of enzymes responsible for phosphorylating serine and threonine amino acids in many proteins. There are currently seven families of MAPKs: extracellular regulated kinase 1/2 (ERK1/2), ERK3/4, ERK5, ERK7/8, p38 kinase, Nemo-like kinase (NLK) and c-Jun N-terminal kinase (JNK). Classical MAPKs are consisting of ERK1/2, p38, JNK and ERK5 while the atypical MAPKs include ERK3, ERK4, ERK7 and NLK. Activation of MAPKs requires dual phosphorylation of a Thr-X-Tyr motif (where X is either a Gly, Pro, or Glu) in the regulatory loop. Phosphorylation of this motif in the catalytic loop allows a conformational change and the kinase active site is revealed to allow substrate binding (Chen 2001). Ligand binding to a receptor initiates to this phosphorylation a well-conserved three-tiered kinase cascade in which a MAPK kinase kinase (MAPKKK, MAP3K, MEKK, or MKKK) activates a MAPK kinase (MAPKK, MAP2K, MEK, or MKK) which in turn activates the MAPK. The prototypic ERK1/2 pathway is mainly responsive to stimulation by growth factors, while JNK and p38 are collectively called stress activated MAPKs (SAPKs) due to their induction by physical, chemical and physiological stressors such as ultraviolet light, oxidant stress, osmotic shock, infection, and cytokines.

Excessive responses occurring in oxidative stress and in inflammation are dangerous to the host. Negative regulators modulate the strength and duration of the transduced signals and control the production of inflammatory cytokines. Inhibition of MAPK activity is effected primarily by MKPs, a family of endogenous dual-specifity phosphatases (DUSPs). The unique feature of these is their ability to dephosphorylate both tyrosine and serine/threonine residues within one substrate. To date, 11 members of the MKPs have been identified. We focused on the activation of MKP-1/DUSP1, which is an important negative-feedback regulator of the macrophage function and the TLR signaling in inflammation via inactivation of p38 and JNK (Zhao 2006).

LPS plays a crucial role in inducing the uncontrolled release of pro-inflammatory mediators, especially TNF- α, several ILs, NO and ROS from monocytes and macrophages (Veres 2004). Binding of LPS to the CD14 and TLR4/MD2 complex is critically important in the innate immune recognition of Gram-negative pathogens. Upon activation of TLR4, the adaptor protein MyD88 is recruited to the receptor, which in turn triggers a cascade of signaling events leading to the activation of transcription factor NFκB and MAPKs including ERK1/2, p38, and JNK (Zhong 2007). Experimental studies have shown that MAPK pathways are involved in activation of transcription factors such as NFκB and activator protein 1 (AP-1). These transcription factors play a significant role in expression of proinflammatory genes such as TNF- α, IL-1β, IL-6 as well as in expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). Over expression of the latter two can lead to ROS and NO production (Chiu 2008).

1.1.3 Role of poly(ADP-ribose) polymerase (PARP) activation in LPS induced inflammatory model

PARP is a nuclear DNA repair enzyme with multiple regulatory functions (Szabó 1998). PARP-1 is the most abundant isoform of the PARP enzyme family activated by single- and double-strand breaks and has critical role in the base excision repair pathway. Upon binding to damaged DNA it forms homodimers catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose, and transfer ADP-ribose from NAD^+ to specific acceptor proteins including histones and itself. PARP-1 functions as a double-edged sword. Moderate activation of PARP can be of physiological importance. PARP-1 is involved in chromatin remodeling, DNA repair, genomic stability, gene transcription by poly ADP-ribosylation (Schreiber 2006). Extensive oxidative and/or nitrosative stress triggers the overactivation of

PARP. This process rapidly depletes the intracellular NAD^+ and ATP energetic pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction, apoptotic or necrotic cell death (Uchiyama 2002). Recently, it has been reported that PARP-1 regulates signaling cascades, including JNK (Zingarelli 2004), p38 MAPK, as well as transcription factors including in stress/inflammation such as AP-1, signal transducer and activator of transcription-1 (STAT-1) and stimulating factor-1 (Sp-1) (Ha 2002). Furthermore, regulates expression of proteins implicated in inflammation at the transcriptional level such as iNOS and intercellular adhesion molecule-1 (ICAM-1), and has been shown to act as a co-activator in the NF-κB-mediated transcription (Oliver 1999).

Lorne and Virág demonstrated that LPS induces production of reactive oxygen species and activation of the nuclear enzyme poly ADP-ribose polymerase. PARP inhibitors show pronounced protection against myocardial ischemia (Zingarelli 1998; Pálfi 2005) neuronal ischemia, diabetic pancreatic damage and septic shock. Recently, inhibition of PARP was implicated in the therapy of myocardial infarction (Szabo 2005; de la Lastra 2007). Earlier studies demonstrate that the disruption of PARP genes suppresses the LPS-induced cytokine expression and NF-kappaB activation. Furthermore, potent PARP-inhibitors protected against LPS-induces tissue damage. Our previous works demonstrate that PARP inhibitors induced the phosphorylation and activation of Akt in the liver, lung and spleen of lipopolysaccharide treated mice (Veres 2003; 2004). On the other hand it was reported that PARP inhibitors protect mitochondrial membrane integrity, furthermore cellular survival via phosphatidylinositol 3-kinase and Akt pathway in oxidative stress (Tapodi 2005; Pálfi 2005). We have proved that PARP inhibition protects cells not only against NAD^+ and ATP depletion, also enhances activation of PI3-kinase-Akt pathway.

On the other hand, the inhibition of PARP-1 can reduce the oxidative stress induced JNK and p38 MAP-kinase activation (Bartha 2009; Mester 2009), moreover increase the expression of MKP-1 at both the mRNA and the protein levels (Rácz 2010). These data indicate that PARP is an important target for therapeutic intervention in the aforementioned pathophysiological conditions.

1.1.4 PI3K-Akt pathway in inflammation

The PI3Ks are membrane-anchored kinases, which catalyze the transfer of the γ-phosphate group of ATP to the D-3 position of phosphoinositides. PI3Ks can be grouped into four classes: IA, IB, II, III. Several studies have shown that LPS activation of Akt in

monocytic cells is mediated by class IA PI3K consisting of 85kD regulatory and 110 kD catalytic subunits. Activated PI3K catalyzes the phosphorylation of phosphatidylinositol 3,4-bisphophate (PIP2) to phosphatidylinositol 3,4,5-tiphosphate (PIP3). This membrane changes allow docking of phosphatidylinositol-dependent kinase 1 (PDK-1) and protein kinase B/Akt to PIP3. Akt is a serine/threonine kinase activated by dual phosphorylation of Ser^{473} and Thr^{308} by PDK1. The PI3K-Akt pathway has been shown to control a variety of cellular processes, including cell survival and proliferation. PI3K can function either as a positive or negative regulator of TLR signaling. There are several reports demonstrating a proinflammatory role for PI3K (Ozees 1999). Studies performed in murine peritoneal macrophages or the Raw 264.7 macrophage have shown that inhibition of PI3K by wortmannin enhances iNOS protein levels as well as NO production in response to LPS (Diaz-Guerra 1999; Park 1997). Several studies have found that inhibition of PI3K with wortmannin and LY294002 enhanced LPS induced activation of MAPKs, transcription factors such as AP-1, Egr-1, and NF-κB), and TNF-α and transcription factor gene expression in monocytic cells (Diaz-Guerra 1999; Guha 2002; Fukao 2003).
Deregulation of this pathway is performed via tumor suppressor phosphatase and tensin homolog (PTEN) which convert PIP3 to PIP2 resulting inhibition of the PI3K-Akt.

1.1.5 Overview of signaling pathways during inflammation

Toll-like receptors are molecular sensors for microorganisms: viruses, bacteria, fungi. TLR2 recognizes components from Gram positive bacteria, TLR3 senses viral dsRNA, TLR4 is activated by components of Gram negative bacteria, TLR9 is activated by unmethylated bacterial DNA. LPS can be bound by LPS-binding protein (LBP) and transfers to membrane-bound CD14. Binding of LPS to the CD14 and TLR4/MD2 complex results in homodimerisation and activation of TLR4. TLR signaling is dependent on the recruitment and association of adaptor molecules that contain the structurally conserved Toll/interleukin-1 receptor (TIR) domain. These adaptor molecules are necessary to recruit and activate downstream kinases and transcription factors that regulate the host inflammatory response and type I IFN production. LPS/TLR4 signaling has been divided into two pathways: a MyD88-dependent and a MyD88-independent pathway. The activation of the MyD88-dependent pathway leads to the early phase of NFκB activation and proinflammatory cytokine expression, while MyD88-independent pathway mediates the late phase of NFκB activation and the induction of IFN-inducible genes in TLR4 signaling (Kawai 2005). Upon binding of

ligand to the receptor, MyD88 recruits and activates a death domain-containing kinase IRAK4 which in turn phosphorylates IRAK1. TRAF6 is the next signaling molecule after IRAK. Phosphorylated IRAK1 dissociates from the receptor complex and binds TRAF6 which in turn interact with TAK1 and two TAK1-binding proteins (TAB1/2). TRAF6 then acts as the ubiquitin E3 ligase, and this way activates itself and its substrate TAK1, a member of the MAPKKK family (Wang 2001). TAK1 then activates downstream IKK (IκB kinase) and MAPK pathways. IKK complex (IKKα, IKKβ, IKKγ/NEMO), induces phosphorylation and subsequent degradation of IκB and translocation of the transcription factor NFκB to the nucleus, which controls the expression of proinflammatory cytokines such as IL-1, IL-6, IL-8, TNFα and a host of other factors. On the other hand, activation of the downstream MAPK pathways leads to the induction of transcription factor AP-1. The MyD88-independent pathway utilizes the adaptor protein TRAM together with TRIF leading to the activation interferon regulatory factor (IRF)3 and the subsequent induction of IRF3-dependent gene expression such as interferon-β (IFN-β). Studies using TRIF-deficient macrophages demonstrate that TRIF palys a crucial role in the activation of IRF3, and the late-phase activation of NFκB and MAPK (Yamamoto 2003). In TLR4 signaling, TRIF can also interact with TBK1. Recently it has been shown that Akt is a downstream target of TRIF/TANK-binding kinase 1 (TBK1) (Joung 2011).

1.2 Oxidative stress induced signal mechanisms

Reactive oxygen species arise from many different intracellular processes, which can be divided into two separate groups. On the one hand ROS generate as waste products during various necessary reactions and on the other hand ROS are produced intentionally, as signal transduction molecules or defense molecules during the inflammation.

The most important source of ROS is the mitochondrial electron transport chain leakage. Secondly the main source of ROS are NADPH-oxidase, xanthine oxidase, cytokine and growth factor receptors and finally metabolic processes. Phagocytic NADPH-oxidase reduces molecular oxygen, thus producing superoxide, which is the most important factor in defense against infectious pathogens. However excessive ROS production may directly induce cellular injury promoting DNA-oxidation, lipid peroxidation and protein oxidation. On the other hand ROS play a key role in redox-sensitive signaling cascades thus they can evoke both protective (adaptive) and damaging (maladaptive) processes. Under normal circumstances, the

enzymatic and non-enzymatic antioxidant defense supports the redox-homeostasis. The adequate cellular response to generation of ROS is consequently critical in order to protection against further oxidative damage and cell survival. Severity of oxidative stress determines the type of cell death. Reactive oxygen species can trigger both apoptosis and necrosis. Generally, NFκB activation promotes gene expression leading to cellular survival, but rarely NFκB activation contributes to cell death. Elevated level of ROS activates mitogen activated protein kinase (MAPKs) and inflammatory transcription factors (Shabalina 2011; Racz 2010; Zhong 2007).

1.2.1 Crucial role of MAP-kinases in oxidative stress

MAPK family plays important roles in cellular responses to a wide variety of signals induced by hormones, cytokines, growth factors and oxidative stress. One of the most important upstream regulator of MAPKs is the ASK1 which is activated under various stress conditions including oxidative stress. Many ASK1-associated proteins were identified, among which the redox protein thioredoxin was shown to constitutively interact with ASK1 and directly inhibit its kinase activity. Only the reduced form of thioredoxin interacts with ASK1 inhibiting ASK1 oligomerization and subsequent activation. ASK1 is activated when oxidants or ROS oxidize two cysteine residues in the redox center of thioredoxin, leading to formation of an intramolecular disulfide bond which results in the dissociation of thioredoxin from ASK1. ASK1 plays a pivotal role in promoting cell death under oxidative stress however, ROS activated ASK1 mediates p38 signaling leading to nonapoptotic outcomes also, such as differentiation and immune signaling (Matsuzawa 2005).

Some findings suggests that cGMP-dependent protein kinase (PKG) regulates MAPK activation also. It was demonstrated that PKG1α is a redox sensor activated by ROS. Similarly, protein kinase A (PKA) was shown to be activated by ROS through formation of intramolecular disulfide bonds and protein kinase C (PKC) activity is also regulated through redox mechanisms.

MAPK pathways are also activated by the direct inhibition of MAPK phosphatase by ROS. ROS, produced by NADPH oxidases or in mitochondria, have been shown to inhibit JNK-inactivating phosphatases through reversible oxidation of a catalytic-site resulting in sustained JNK activation.

1.2.2 PI3K-Akt pathway in oxidative stress

In *in vivo* rodent hearts and in myocardial cells *in vitro* exposed to hypoxia, activation of PI3K/Akt reduces apoptosis. Similarly 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 is associated with apoptosis in various non-myocyte cell lines (Wang 2000). Similar findings were verified by Kwon et al. in cultured rat myocardial cells. Connection between ROS and PI3K-Akt activation is clearly defined. Normally, PI3K catalyzes the synthesis of the second messenger PIP3 from PIP2. The PH domain proteins, such as PDK) and AKT serine/threonine kinases, are thus activated and mediate further downstream signaling events. The synthesis of PIP3 is negatively regulated primarily by PTEN, which dephosphorylates PIP3 back to PIP2. Through PTEN, the PI3K pathway is subject to reversible redox regulation by growth factor stimulation generated ROS. H_2O_2 effectively oxidize and inactivate PTEN through disulfide bond formation. It was also identified that endogenously produced ROS following treatment with peptide growth factors such as EGF, insulin, or PDGF causes oxidation of PTEN leading to the activation of the PI3K-Akt pathway. PTEN oxidation is reversed by peroxiredoxin II, a cytoplasmic peroxiredoxin isoform that eliminates H_2O_2, generated in response to growth factors. Additional mechanisms may include the oxidative modification of other upstream regulators of Akt, such as Src or other protein tyrosine kinases. Akt can phosphorylate a number of downstream targets leading to inactivation of glycogen synthase kinase (GSK)-3β, the pro-apoptotic Bcl-2 family member BAD (Aikawa 2000), caspase-9 (Cardone 2001), and forkhead homolog rhabdomyosarcoma transcription factor (FKHR) (Scheid 2001), as well as to activation of NFκB (Romashkova 1999), p70 ribosomal S6 kinase, and endothelial nitric oxide synthase (Dimmeler 1999; Gao 2002).

1.3 Mitochondria as a target of inflammation and oxidative stress

Mitochondria are one of the central organelles involved in cellular energy metabolism. Mitochondria produce more than 90% of the body's cellular energy in the form of ATP via oxidative phosphorylation. Mitochondria are the major sources of intracellular ROS in a resting cell and a major target of the cell death processes (Murphy 2009). Under normal

conditions, mitochondria are protected from damage by ROS via several interacting antioxidant systems, but oxidative stress can induce the damage of mtDNA leading to a cycle of ROS production. During ischemic condition, when ATP is rapidly depleted, ion pumps stop functioning, resulting in Ca^{2+} influx, 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 ROS. Mitochondrial ROS production can lead to oxidative damage to mitochondrial proteins, membranes, and DNA, interferes ATP synthesis and other essential functions. Mitochondrial oxidative damage can also increase the release of cytochrome *c* (cyt *c*) into the cytosol by mitochondrial outer membrane permeabilization, leading to apoptosis. Mitochondrial ROS production leads to the opening of mitochondrial permeability transition pore (MPT), results in a collapse of mitochondrial membrane potential and further compromises cellular energetics (Tretter 2007). The release of intermembrane proteins (apoptosis-inducing factor and endonuclease G) and their translocation to the nucleus, leads to nuclear DNA fragmentation. Together, these events trigger necrotic cell death.

There are several data where mitochondria play regulatory role in many human pathologies ranging from inborn errors of metabolism, cancer, inflammation and infections. Oxidative stress and mitochondrial dysfunction has been reported in patients (Crouser 2004) and in rat models with sepsis (Brealey 2004). Many studies have shown that direct pharmacological inhibition of the MPT pore blunts the loss of cardiac myocytes that underlies myocardial ischemia-reperfusion injury (Hausenloy 2003). The ideal drug therapy needs to be targeted to mitochondria.

1.4 Anti inflammatory and antioxidant effects of polyphenols

Red wines are rich in different types of polyphenols and anthocyanins, and they have been reported to provide greater benefit in the prevention of cardiovascular diseases. The polyphenol fraction in wine includes phenolic acids, trihydroxy stilbenes, oligomer proanthocyanidins, and flavonoids, which function as antioxidants. Polyphenols are natural aromatic compounds containing two or more phenolic hydroxyl groups. They have an important role to protect plants from microbial infection and UV irradiation (Matern & Grimming 1993), and also have positive effects on human health. Phenolic compounds have

strong antioxidant, neuroprotective effect and their anticarcinogen and cardioprotective effects are also proven (Yi 2005; Palfi 2009). Flavonoids are primarily derived from the skins, seeds, and stems of grapes while anthocyanins come predominantly from the skins.

Anthocyanins are a group of naturally occurring compounds responsible for the red-blue colour of many flowers, fruits and vegetables. They are glycosylated polyhydroxy or polymethoxi derivates of 2-phenylbenzopyrylium or flavilium salts. They are one of the major flavonoids, food ingredients from our daily diet, in the form of vegetables and red wine. Márk et al. analyzed polyphenol and anthocyanin contents of Hungarian red wine from the southernmost Hungarian wine region Villány. Mean polyphenol content was the highest in Kékfrankos (493 mg/L), mean anthocyanin content was the highest in Shiraz (2309 mg/L) (Avar 2007). Anthocyanins have also been reported to have many physiological functions such as vision improvement, anticancer, and anti-inflammatory activities. On the basis of anti-inflammatory effect the idea is raised that using of anthocyanins may be profitable in treatment of inflammatory diseases such as septic shock. Malvidin and its glycosides are primary plant pigments playing an important role to protect plants from microbial infection and UV irradiation (Matern 1993). Malvidin is responsible primarily for the color, and is included in the polyphenols of red wine together with other anthocyanidins, phenolic acids, flavonoids and trihydroxy stilbenes (Soleas 1997; Salah 1995). Recent findings indicate a potential preventive role of dietary polyphenols against chronic inflammatory diseases such as diabetes, hypertension and cardiovascular disease (Sun 2002; Yi 2005; Visioli 2011; Liu 2008). Another widely investigated nutritional polyphenol, resveratrol was found to prolong lifespan, and was suggested as a potential anti-inflammatory, anti-aging, anti-cancer and anti-cardiovascular disease agent (Kroon 2010; Udenigwe 2008; de la Lastra 2005). However, rather low bioavailability and abundance of resveratrol implies that other components may contribute substantially to the beneficial effects of red wine (Palfi 2009; Gescher 2003). A likely candidate is malvidin that exceeds resveratrol content at least 100 times in red wines (Nikfardjam 2006). Recent data describe its beneficial effects in cardiovascular disease (Quintieri 2012). On the other hand, only limited data are available about effect of malvidin on inflammatory processes and kinase signaling pathways (Chun 2008; Hou 2005; Yeh 2005). Therefore, in this study, we investigated the effect of malvidin on LPS induced processes in RAW 264.7 macrophages.

2. Aim of the study

ROS plays a key role in the pathogenesis and progression of septic shock and ischemia-reperfusion injury. To investigate direct effect of ROS and activated signaling pathways support development of new therapeutic targets. Our studies focus to identify crucial signaling pathways involved in inflammation and oxidative stress used two different models (cell culture and Langendorff perfused heart). We investigate the effect of malvidin on inflammatory response and the effect of Metoprolol, Verapamil and PARP inhibitor on ischemia-reperfusion injury.

1. We intended to determine antioxidant and anti-inflammatory effectivity of malvidin.

2. We wanted to compare antioxidant and anti-inflammatory effects of malvidin and resveratrol on the molecular basis to find out which component is most likely to account for the positive effects of moderate red wine consumption.

3. We wanted to know whether activation of PI-3K-Akt and MAPK signaling pathways are involved in the molecular action of Ca^{2+} antagonist and a β-adrenergic receptor blocker cardioprotective drugs.

4. We intended to compare the cardioprotective potential of PARP inhibition, Ca^{2+} antagonism and blocking of the β-adrenergic receptor.

3. Materials and methods

3.1 Methods in LPS induced inflammatory model

3.1.1 Chemicals

Bacterial lipopolysaccharide from *Escherichia coli* 0127:B8, trans-resveratrol and Malvidin chloride were purchased from Sigma-Aldrich Co. (Budapest, Hungary). Protease inhibitor mixture was purchased from Sigma-Aldrich Co. (Budapest, Hungary). Antibodies against phosphorylation specific extracellular signal regulated kinase ($ERK_{1/2}$) Thr^{183}–Tyr^{185}, $ERK_{1/2}$, phosphorylation specific p38 MAPK Thr^{180}–Gly–Tyr^{182}, p38-MAPK, phosphorylation specific c-Jun N-terminal kinase (JNK), JNK, phosphorylation specific Akt-1/protein kinase B-α Ser^{473}, Akt1, phosphorylation specific glycogen synthase kinase (GSK)-3β Ser^{9}, NF-κB p65 and phosphorylation specific NF-κB p65(Ser536) were purchased from Cell Signaling Technology, Kvalitex Co. (Budapest, Hungary). Antibody against N-terminal domain of actin was obtained from Sigma-Aldrich Co. (Budapest, Hungary), and MAPK phosphatase-1 (MKP-1), Histon H-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant GSK-3β, c-Jun, myelin basic protein (MBP) and myocyte enhancer factor (Mef)-2 was purchased from Abnova Gmbh (Heidelberg, Germany). JNK Inhibitor II, SB 203580, PD 98059 and Akt Inhibitor IV were from Merck Hungary Ltd. (Budapest, Hungary). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma–Aldrich Co. (Budapest, Hungary). The fluorescent mitochondrial dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) were from Molecular Probes (Leiden, Netherlands). All reagents were of the highest purity commercially available.

3.1.2 Immunoblot analysis

RAW 264.7 murine macrophage (ECACC, Salisbury, UK) and RAW-Blue™ (Cayla – InvivoGen, Toulouse, France) cells were cultured in 5% CO_2- 95% air at 37°C in Dulbecco's Modified Eagle's Medium (DMEM–endotoxin tested) with 10% fetal calf serum (FCS) and L-glutamine (Sigma-Aldrich, Budapest, Hungary). The cells were seeded at a starting density

of $2x10^6$ cells/well to a 6-well plate, cultured overnight then treated or not with 1μg/ml LPS together or without 0-100μM malvidin or resveratrol. We pre-incubated RAW 264.7 macrophages in the presence or absence of malvidin or resveratrol for 30 min before the LPS challenge. Cells were harvested in ice-cold lysis buffer containing 0,5mM sodium metavanadate, 1mM ethylenediaminetetraacetic acid (EDTA), protease inhibitor mixture and phosphate-buffered saline, pH: 7.4. Proteins were precipitated by trichloroacetic acid, washed three times with -20°C acetone, and subjected to sodium dodecylsulphate (SDS) polyacrylamide gelelectrophoresis. Proteins (30μg/lane) were separated on 12% gels and then transferred to nitrocellulose membranes. Membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at 4°C overnight at a dilution of 1:1,000. Appropriate horseradish peroxidase-conjugated secondary antibody was used for 2 h at room temperature in 1:5000 dilution (Sigma-Aldrich Co, Budapest, Hungary). Peroxidase labeling was visualized with enhanced chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL, USA). Developed films were scanned, and pixel volumes of the bands were determined using NIH Image J software. All experiments were repeated three times.

3.1.3 Cell viability assay

Cells were seeded to 96-well plates at a starting density of 2×10^4 cells/well and cultured overnight. We pre-incubated RAW 264.7 macrophages in the presence or absence of 50μM malvidin for 30 min, then exposed or not the cells to 1μg/ml LPS for 24 h. Media were replaced for fresh one without any agents containing 0.5% of the water-soluble mitochondrial dye, MTT. Incubation was continued for 3 more hours, and MTT reaction was terminated by adding 1/10 volume of 10% of SDS solution containing 0.1M HCl. The amount of water-insoluble blue formasan dye formed from MTT was proportional to the number of live cells and was determined using a 96-well plate reader (Anthos Labtech 2010; Vienna, Austria) at 550nm wavelength after dissolving the blue formasan precipitate in the acidic SDS solution. All experiments were performed in at least four parallels and repeated three times.

3.1.4 Determination of intracellular reactive oxygen species (ROS)

Intracellular ROS were determined using the oxidation-sensitive 2,4 dichlorodihydrofluorescein-diacetate (C-400, Invitrogen) fluorescent dye. Cells were seeded into 96-well plates at a starting density of $2x10^4$ cell/well, then cultured overnight. Culturing medium was replaced with a fresh one. RAW 264.7 cells were incubated or not in the presence of 1μg/ml LPS together with 0-50μM malvidin or trans-resveratrol for 22 h. Then C400 at a final concentration of 2μg/ml was added to the medium for an additional 2 h. Fluorescence was measured at 485nm excitation and 555nm emission wavelengths using Fluostar Optima (BMG Labtechnologies) fluorescent microplate reader. All experiments were performed in at least 6 parallels and repeated three times.

3.1.5 NF-κB activation assay

RAW 264.7 macrophages were transiently co-transfected with either NF-κB luciferase or control (TA-Luc) (Panomics, Santa Clara, CA, USA), and SV-β-galactosidase (pSV-β-gal) (Promega Corporation, Madison, WI, USA) plasmids by using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. 24 h after the transfection, cells were treated as indicated, and another 24 h later cell lysates were collected. Cellular proteins were assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA, Luciferase Assay System Technical Bulletin TB281). The ratio of luciferase to β-galactosidase activity served to normalize the luciferase activity to correct for any differences in transfection efficiencies.
Alternatively, RAW-Blue™ cells were treated as indicated for 24 h, then the medium was replaced by QUANTI-Blue™ detection medium (Cayla – InvivoGen, Toulouse, France) for 1h. RAW-Blue™ cells are permanently transfected with an NF-κB- and AP-1-sensitive promoter-driven alkaline phosphatase. NF-κB and AP-1 binds to the promoter upon nuclear translocation, and induces the expression of alkaline phosphatase that is detected by a dye-based assay and a plate reader.

3.1.6 Detecting mitochondrial membrane potential (Δψ)

The changes in Δψ were assayed using the Δψ dependent fluorescent dye, JC-1. RAW 264.7 cells were seeded at 1×10^6 cells/well starting density to a six-well plate containing coverslips and cultured at least overnight before the experiment. After subjecting the cells to the appropriate treatment (indicated in the figure legends), coverslips were rinsed twice in phosphate buffered saline (PBS). Coverslips were placed face down on top of a microscope slide forming a small chamber filled with PBS supplemented with 0.5% FCS and containing 5μg/ml JC1 (Molecular Probes). Cells were imaged with a Zeiss Axiovert 25 fluorescent microscope equipped with a ProgRes C12 Plus CCD camera using a 63× objective and epifluorescent illumination. For JC-1 fluorescence, cells were loaded with the dye for 15 min at 37°C, then the same microscopic field was imaged first with 546nm bandpass excitation and 590nm emission (green filter, red fluorescence), then with 450-490nm bandpass excitation and 520nm emission (blue filter, green fluorescence). Resulting images were merged. In control experiments, we did not observe considerable bleed-through between the red and green channels.

3.1.7 RNA extraction and quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) amplification

Total RNA was extracted from RAW 264.7 cells using TRIZol reagent (Sigma-Aldrich), according to the manufacturer's protocol. RNA (1μg) was reverse-transcribed with MMLV RT (RevertAid™ first-strand cDNA synthesis kit, Fermentas, Burlington, Ontario, Canada) for 1h at 42°C; final volume was 20μl. cDNA (1μl) was used for real-time PCR amplification on a Bio-Rad Mini Opticon (MJ Mini) machine. PCR was conducted over 45 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s; three-step thermal cycling preceded by an initial 95°C for 7 s using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA). PCR was performed using the following primers:
MKP-1 forward, 5'-GCATCCCTGTGGAGGACAACC-3';
MKP-1 reverse, 5'-TCCAGCATCCTTGATGGAGTCTATG-3';
β-Actin forward, 5'-GCCACCAGTTCGCCATGGAT-3';
β-Actin reverse, 5'-GCTTTGCACATGCCGGAGC-3'.

Statistical analysis of relative expression of the target gene based on comparative threshold values with efficiency correction was made with the Relative Expression Software tool (Bio-Rad CFX Manager Software), and was normalized to the housekeeping gene β-Actin. All experiments were repeated three times.

3.1.8 Preparation of nuclear protein extracts

The nuclear extracts were prepared as described previously (Tang 2007). Cells were harvested and suspended in hypotonic buffer A (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 10mM KCl, 1mM dithiothreitol (DTT), 0.1mM EDTA, and 0.5mM phenylmethylsulfonyl fluoride) for 10min on ice and vortexed for 10s. Nuclei were pelleted by centrifugation at 12000g for 20 s. The supernatants containing cytosolic proteins were collected. Nuclear pellet was suspended in buffer C (20mM HEPES, pH 7.6, 1mM EDTA, 1mM DTT, 0.5mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4M NaCl) for 30 min on ice. Nuclear protein containing supernatants were collected by centrifugation at 12000g for 20 min and stored at −70°C.

3.1.9 DNA affinity protein binding assay

After the indicated treatment, cells were harvested in buffer A, chilled on ice for 10 min and centrifuged at 12000g for 20 s. Pellets were suspended in 5 times volume of buffer C and sonicated. A 200μg aliquot of nuclear suspension was incubated with 2μg of biotinylated double-stranded oligonucleotyde corresponding to the murine consensus NF-κB binding DNA sequence (Biotin-CCTTGAAGGGATTTCCCTCC, Invitrogen) for 30 min on a 4ºC shaker-bath. Then, 30μl streptavidin coated magnetic micro particles (Sigma-Aldrich) were added, and incubation was continued for an additional 30 min. Beads were pulled down, washed 3 times with ice-cold PBS, and eluted in 25μl mercaptoethanol-free Laemmli sample buffer by a 5 min boiling. Eluted samples were subjected to immunoblot analysis. All experiments were repeated three times.

3.1.10 In vitro kinase assay

RAW 264.7 macrophages were exposed to 1μg/ml LPS for 1 h, washed in PBS and harvested in ice-cold lysis buffer containing 0,5mM sodium metavanadate, 1mM EDTA, protease inhibitor mixture and 20mM HEPES, pH: 7.4. Cell lysates were subjected to overnight immunoprecipitation at 4°C with anti-p38 MAPK, anti-JNK anti-$ERK_{1/2}$ or anti-Akt antibodies. Precipitates were collected on appropriate secondary antibody-coated magnetic micro particles (Sigma-Aldrich) for 30 min. Beads were pulled down, washed 3 times with ice-cold PBS, and incubated for 10 min at 30°C in the presence or absence of 50μM malvidin in 50 μl of buffer containing 25 mM glycerophosphate (pH 7.3), 0.5 mM dithiothreitol, 1.25 mM EGTA, 0.5 mM Na_3VO_4, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 μM okadaic acid, 0.1 mM [γ-^{32}P]ATP (250000 Bq/nmol; GE Healthcare Hungary Ltd, Budapest, Hungary) and 50 μg of recombinant Mef-2, c-Jun, MBP or GSK-3β protein. After incubation, aliquots were spotted on p81 filter paper, washed and counted for ^{32}P radioactivity. Blank values were obtained by substituting a non-immune antibody preparation for the immunoprecipitating antibodies. All experiments were repeated three times.

3.1.11 Statistical analysis

Each experiment was repeated at least three times. Values in the figures and text are expressed as mean ± S.E.M. of n observations. Statistical analysis was performed by analysis of variance followed by Student's t-test. Statistical significance was set at $p<0.05$. For determining IC_{50} values from dose-response curves, the four-parameter logistic function of GraphPad Prism software was used.

3.2 Methods in ischemia-reperfusion model

3.2.1 Chemicals

Verapamil and Metoprolol were purchased from Sigma-Aldrich Chemical Co. (Budapest, Hungary). HO-3089, a PARP inhibitor was a kind gift of Kalman Hideg (University of Pecs Medical School). All other reagents were of the highest purity commercially available.

3.2.2. Heart perfusion

Male Wistar rats weighing 300-350g were heparinized with sodium heparin (100 IU i.p.) and anesthetized with ketamine (200 mg/kg i.p.). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs. Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 Hgmm at 37°C as described before (Palfi 2005; Kovacs 2006). The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$ 25 mM $NaHCO_3$ 11 mM glucose and 0.6 mM octanoic acid without or with Verapamil (600 nM), Metoprolol (10 μM) or HO-3089 (25 μM) in combination with LY294002 (10 μM) or PD98059 (10 μM). The aforementioned compounds were administered into the perfusion medium at the beginning of a normoxic perfusion period. After a washout, non-recirculating period of 15-min, hearts were either perfused under normoxic conditions for 10 min, or were subjected to a 25-min global ischemia by closing the aortic influx and reperfused for either 15 or 45 min. The above listed compounds were administered into the perfusion medium at the beginning of normoxic perfusion. During ischemia, the hearts were submerged into the 37°C perfusion buffer. Hearts were freeze-clamped at the end of each perfusion. Myocardial energy metabolism was continuously detected by means of a ^{31}P nuclear magnetic resonance spectroscope as described before (Halmosi 2001). Functional performance of the hearts was monitored by placing a balloon catheter into the left ventricle (Deres 2005). Myocardial infarct size was determined by triphenyl tetrazolium chloride staining as described before (Palfi 2005).

3.2.3 Myocardial oxidative damage

Lipid peroxidation and mitochondrial oxidation were assessed by measuring the amount of thiobarbituric acid reactive substances (TBARS) and quantity of protein-bound aldehyde groups, respectively (Palfi 2005; Szabados 1999).

3.2.4. Immunoblot- analysis

Heart samples were prepared and Western blot analysis was performed as described before (Toth 2003). Membranes were probed overnight at 4°C with the following primary antibodies: phospho-specific anti-Akt-1 Ser^{473}, phospho-specific anti-GSK-3β Ser^{9}, phospho-specific anti-ERK1/2 Thr^{202}/Tyr^{204}, (1:1000 dilution; Cell Signaling Technology, Beverly, USA) and anti-actin (1:5000 dilution; Sigma-Aldrich Chemical Co., Budapest, Hungary). Membranes were washed six times for 5 minutes in Tris buffered saline (pH=7.5) containing 0.2% Tween prior to addition of goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000; BioRad, Budapest, Hungary). The antibody-antigen complexes were visualized by means of enhanced chemiluminescence on conventional films. After scanning, pixel densities of bands were quantified by means of NIH ImageJ program. Pixel densities of bands on the same film were normalized to that of the loading control. For combining parallel experiments to create mean ± SEM values for the same treatment group, pixel densities of bands for a given protein were further normalized to that of the ischemia-reperfusion sample. All experiments were performed at least four times.

3.2.5 Statistical analysis

All data were expressed as mean ± SEM Statistical analysis was performed by using two-factor analysis of variance with replication for correlations, and unpaired Student's *t* test for group comparisons. Differences with *p* values below 0.05 were considered as significant.

4. Results

4.1 Investigation of intracellular signaling pathways in LPS induced inflammatory model

4.1.1 Malvidin inhibited LPS-induced NF-κB activation in RAW 264.7 macrophages.

Phosphorylation of NF-κB p65 on Ser^{536} upon LPS stimulation enhances its transcriptional activity (Yang 2003). Therefore, we investigated whether malvidin affects LPS induced NF-κB p65 phosphorylation. To this end, we pre-incubated RAW 264.7 macrophages in the presence or absence of 50μM malvidin for 30 min, then exposed or not the cells to 1μg/ml LPS for 1 h, and determined steady state protein level and phosphorylation state of NF-κB by immunoblotting from whole cell homogenates. As demonstrated in Figure 1, LPS did not affect expression of NFκB, but induced phosphorylation of its p65 subunit. Malvidin effectively attenuated NF-κB phosphorylation both in the unstimulated and the LPS treated cells while did not affect expression of the protein (Fig. 1).

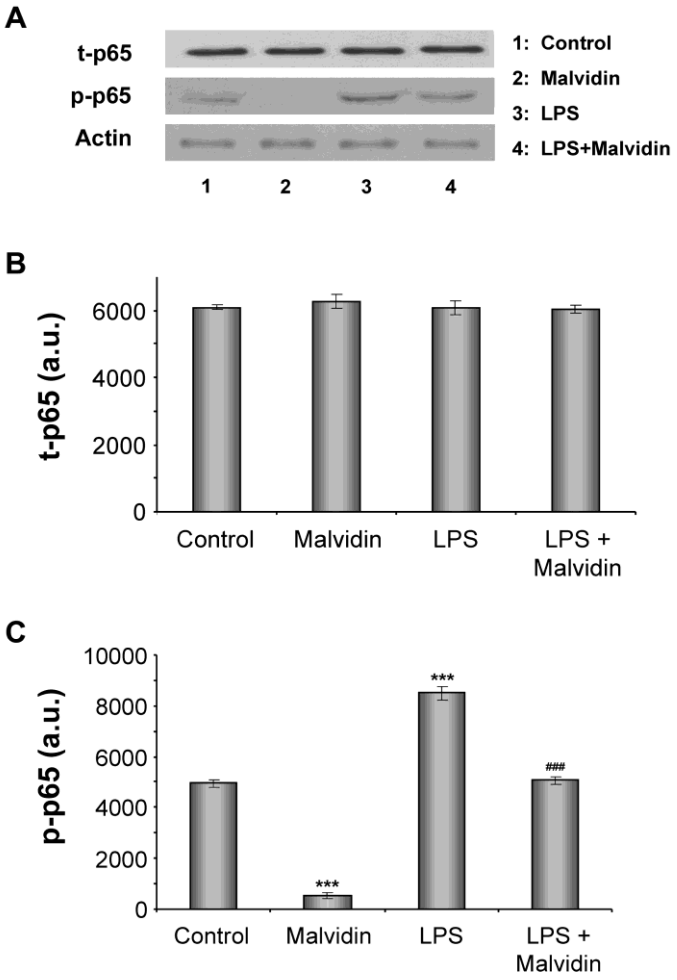


Figure 1. Effect of malvidin on LPS induced activating phosphorylation of NFκB. *Total (phosphorylated and unphosphorylated) NFκB (t- NFκB) as well as the phoshorylated form of its p65 subunit (p-NFκB) was detected by immunoblotting of whole RAW 264.7 macrophage lysates after treating the cells for 1h as indicated. Actin was used as a loading control. Representative blots (A) and densitometric evaluations (B,C) of 3 independent experiments are shown. Pixel densities were normalized to that of the actin. Values are given as means ± SEM. *** $p < 0.001$ compared to untreated control, ### $p < 0.001$ compared to LPS alone.*

Activation of NF-κB presumes its translocation to the nucleus and its binding to the DNA. To determine nuclear translocation and DNA binding of NFκB, we isolated and homogenized nuclei of RAW 264.7 macrophages subjected to the aforementioned treatment protocol, and pulled down nuclear proteins by magnetic beads using oligonucleotides of the consensus NF-κB binding sequence as bait. Proteins eluted from the beads were subjected to immunoblot analysis. Figure 2 demonstrates LPS induced nuclear translocation and DNA binding of NF-κB. Malvidin attenuated these processes in unstimulated and LPS treated cells. Furthermore, we found the same pattern of alterations in total (phosphorylated and un-phosphorylated) and phosphorylated NF-κB (Fig. 2). This indicates that most of the nuclearly translocated NF-κBs were phosphorylated.

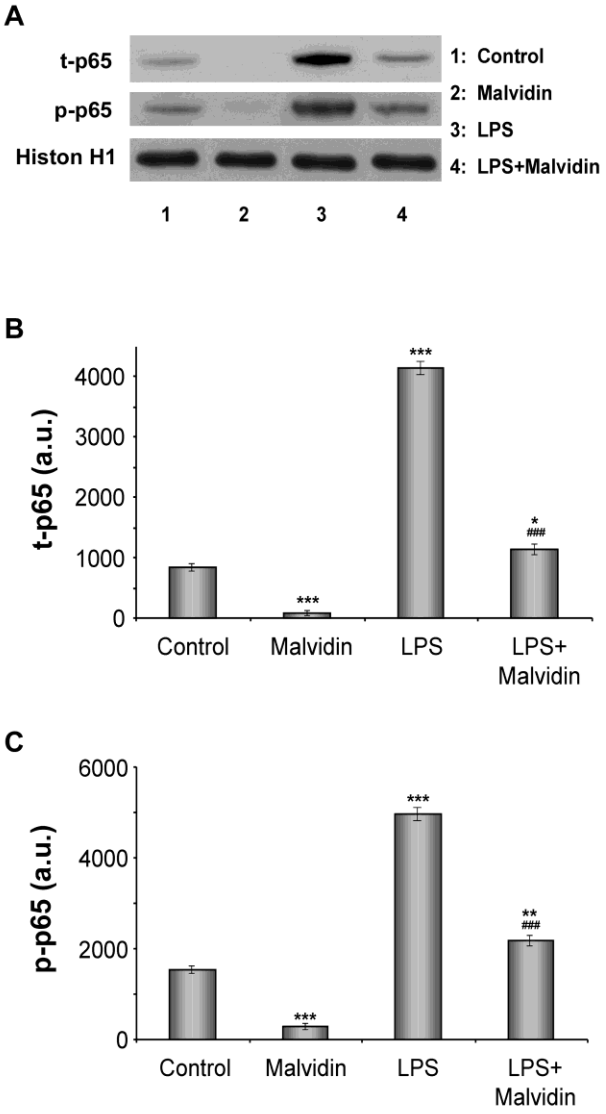


Figure 2. Effect of malvidin on LPS induced nuclear translocation and DNA binding of NFκB. *RAW 264.7 macrophages were treated for 1h as indicated, then nuclei were isolated and NFκB was extracted by using magnetic beads baited with oligonucleotides of the NFκB binding consensus sequence. Total (phosphorylated and unphosphorylated) NFκB (t-p65) as well as the phoshorylated form of its p65 subunit (p-p65) was detected by immunoblotting in the samples eluted from the beads. Histon H1 from the isolated nuclei was used as loading control. Representative blots (A) and densitometric evaluations (B,C) of three independent experiments are shown. Pixel densities were normalized to that of the histon H1. Values are given as means ± SEM. * p <0.05, ** p <0.01, *** p <0.001 compared to untreated control, ### p <0.001 compared to LPS alone. a.u.: arbitrary units.*

We confirmed the effect of malvidin on LPS induced NF-κB activation using functional luciferase reporter assay. We transiently transfected RAW 264.7 macrophages with NF-κB promoter driven luciferase construct. Due to technical reasons, we treated the cells for 24 h instead of 1 before determining luciferase activity using chemiluminescence assay. We normalized our assay by co-transfecting the cells with a β-galactosidase expressing plasmid. Similarly to our previous two experiments, we found that LPS induced the activation of NF-κB and it was attenuated by malvidin (Fig. 3A). In this assay, malvidin failed to decrease NF-κB activation in unstimulated cells (Fig. 3A).

We intended to compare NF-κB activation reducing effect of malvidin with that of trans-resveratrol. To this end, we treated RAW-Blue™ cells in the presence or absence of 0-100μM malvidin or resveratrol with 1μg/ml LPS for 24 h. RAW-Blue™ cells were permanently transfected with an NF-κB- and AP-1-sensitive promoter-driven alkaline phosphatase. Upon nuclear translocation, NF-κB and AP-1 binds to the promoter, and induces the expression of alkaline phosphatase. Alkaline phosphatase activity proportional to NF-κB activation was detected using a colour-changing substrate containing assay medium. Malvidin inhibited LPS-induced NF-κB activation with the apparent IC_{50} value of 18.1±3.2μM. Trans-resveratrol failed to inhibit NF-κB activation even at the highest concentration used (Fig. 3B).

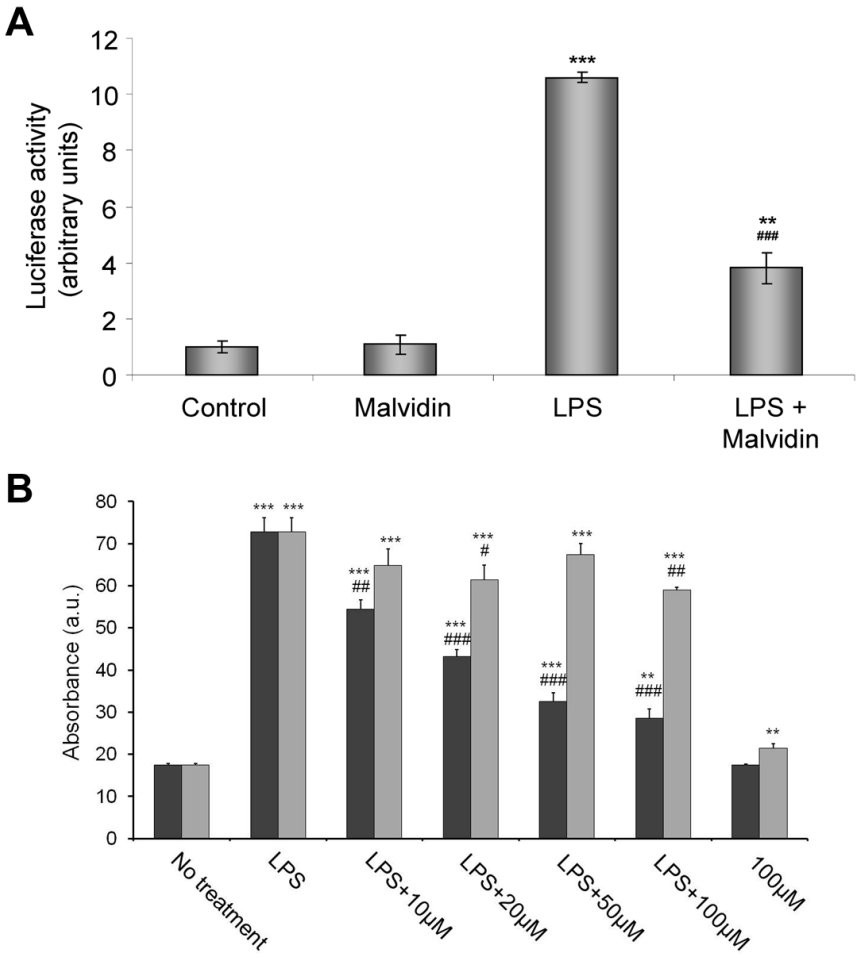


Figure 3. Effect of malvidin on LPS induced activation of NFκB in RAW 264.7 macrophages. *Cells were pretreated with 0-100μM malvidin (black bars in B) or 0-100μM trans-resveratrol (gray bars in B) for 30 min as indicated. Activation of NF-κB was assessed by a luciferase (A) or an alkaline phosphatase (B) reporter assay after 1μg/mL LPS explosion for 24 h. Values are given as means ± SEM of 4 independent experiments running in 3 parallels. ** p <0.01 , *** p <0.001 compared to untreated control, # p <0.05, ## p <0.01, ### p <0.001 compared to LPS alone. a.u.: arbitrary units.*

4.1.2 Malvidin inhibited LPS induced ROS production and PARP activation in RAW 264.7 macrophages

LPS is a well documented inducer of ROS production (Virgili 1998). Therefore, we determined the effect of malvidin on ROS production in LPS-induced RAW macrophages using an oxidation sensitive fluorescent dye, C400. The cells were pre-incubated in the presence of 0-50μM malvidin for 30 min, then exposed or not to 1μg/ml LPS for 22 h. This was followed by additional 2 h incubation after supplementing the media with C400 at a final concentration of 2μg/ml. Concentration of fluorescent C400 oxidized by the ROS was determined using fluorescence plate reader. Malvidin inhibited LPS induced ROS production in a concentration-dependent manner (Fig. 4A). We compared the antioxidant effect of malvidin with that of trans-resveratrol, and found it to be comparable (Fig. 4A). Apparent IC_{50} values for the two polyphenols were 9.0±0.8 and 6.8±0.6μM, respectively. We investigated whether decreased ROS production was due to cytotoxic effect of malvidin by using MTT assay. We found that cell viability was not affected by 50μM malvidin during the 24 h incubation period (Fig. 4B).

LPS activates PARP in RAW 264.7 macrophages as the consequence of increased intracellular ROS induced breaks of one or both strands of the DNA (Virág 2001). Therefore, we investigated the influence of malvidin on LPS induced activation of PARP. We exposed the cells to the aforementioned treatment protocol, then assessed PARP activation by immunoblot analysis of the steady state level of the enzymatic product, PAR from whole cell homogenates. We found that LPS induced PAR accumulation was attenuated by malvidin (Fig. 4C, D). Malvidin did not affect PARP activation in unstimulated cells (Fig. 4C, D).

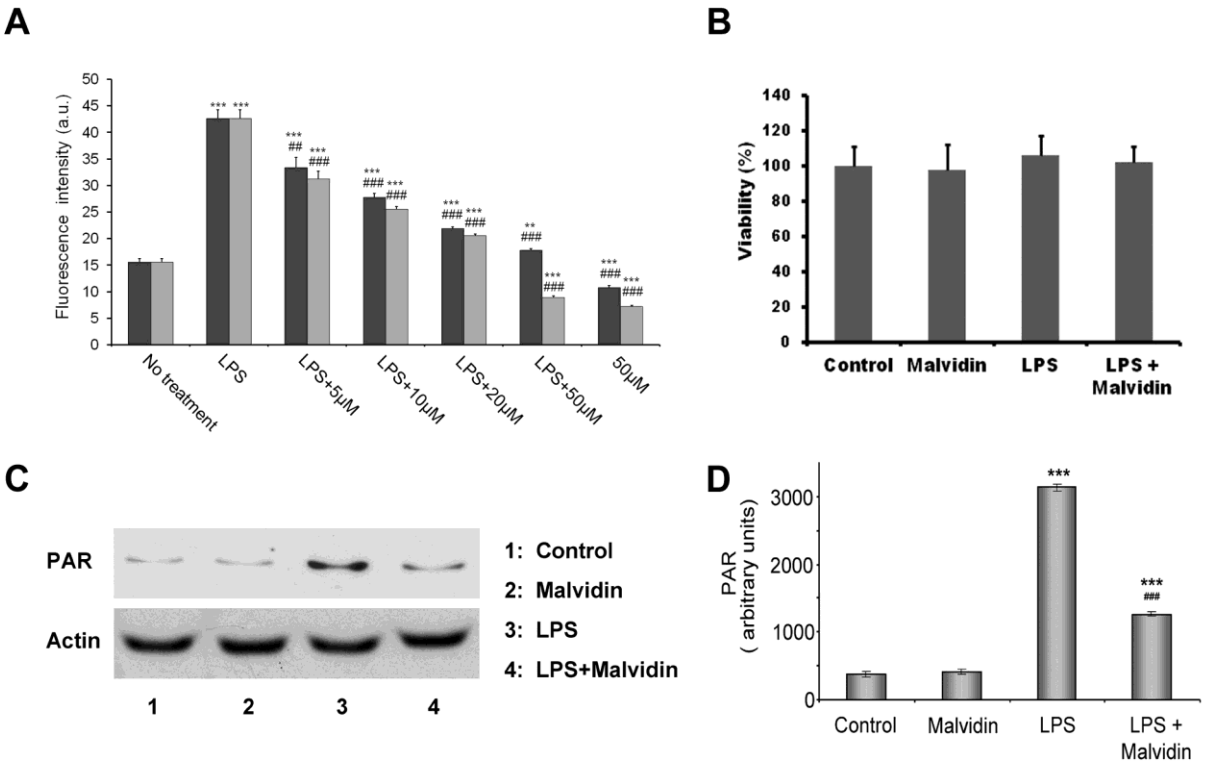


Figure 4. Effect of malvidin on LPS induced ROS production and PARP activation in RAW 264.7 macrophages. *Steady state ROS concentration in the culturing medium (A) and viability of the cells (B) was determined using the fluorescent redox dye C-400 and by the MTT method, respectively after incubating the cells for 24 h in the absence and presence of LPS together with 0-50 μM malvidin (black bars in A) or trans-resveratrol (gray bars in A) as indicated. Experiments running in 6 parallels were repeated 3 times. PARP activation was assessed by determining the steady state level of PAR using immunoblotting from whole cell lysate after treating the cells for 1h as indicated. Actin was used as loading control. Representative blots (C) and densitometric evaluations (D) of three independent experiments are shown. Pixel densities were normalized to that of the actin. Values are given as means ± SEM. ** $p < 0.01$ *** $p < 0.001$ compared to untreated control, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ compared to LPS alone. a.u.: arbitrary units.*

4.1.3 Malvidin inhibited LPS-induced MAPK activation in RAW 264.7 macrophages.

Binding of LPS to the TLR4 receptor activates multiple intracellular signaling pathways including the MAPKs (Cario 2000). Therefore, we investigated the influence of malvidin on LPS induced activation of ERK, JNK and p38-MAPK. We preincubated or not RAW 264.7 macrophages with 50μM malvidin for 30 min then treated them or not with 1μg/ml LPS for 1 h. We performed immunoblot analysis utilizing phosphorylation specific primary antibodies from whole cell homogenates. Phosphorylation and thereby activation of the studied MAPKs were increased by LPS, which was attenuated by malvidin (Fig.5). This effect of malvidin was the least effective in the case of $ERK_{1/2}$ (Fig. 5A, B), much more pronounced for p38 (Fig 5A, C) and the strongest for JNK (Fig.5 A, D). Malvidin did not exert any effect on the phosphorylation of MAPKs in unstimulated cells (Fig. 5).

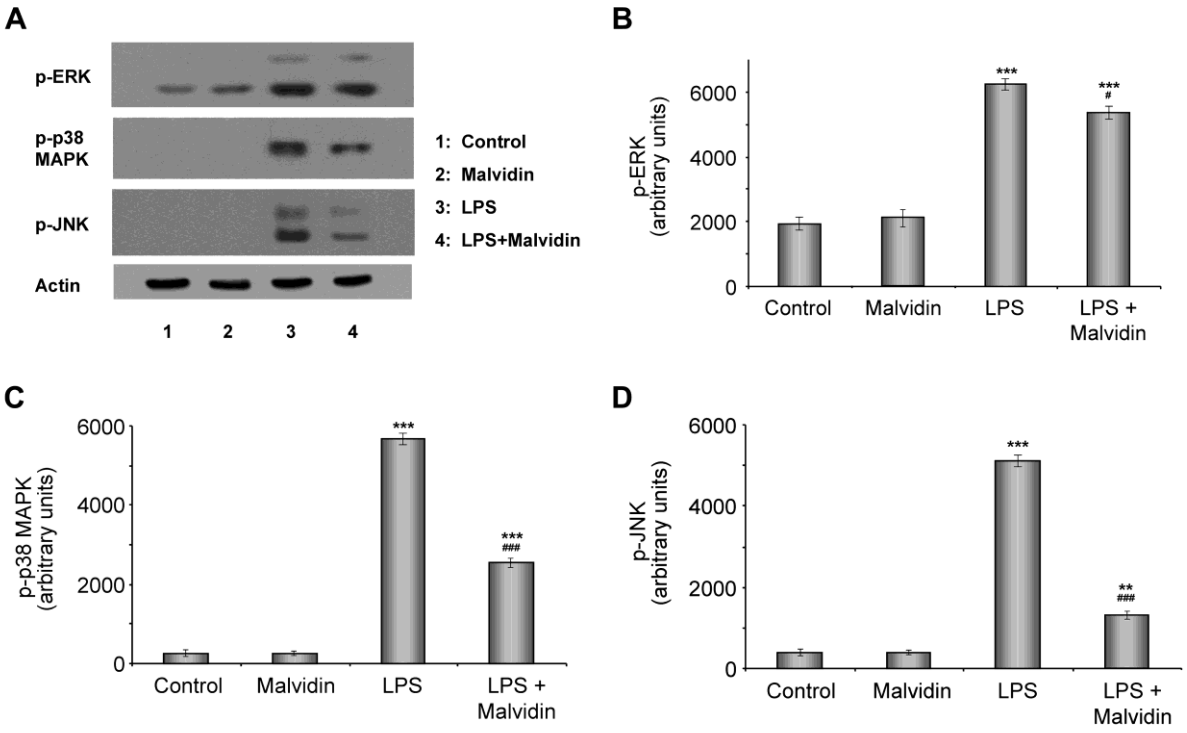


Figure 5. Effect of malvidin on LPS induced activation of ERK, p38, JNK MAPK in RAW 264.7 macrophages. *Steady state phosphorylation of ERK, p38 and JNK was detected by immunoblotting from whole cell lysate after treating the cells as indicated for 1h. Actin was used as a loading control. Representative blots (A) and densitometric evaluations (B-D) of 3 independent experiments are shown. Pixel densities were normalized to that of the actin. Values are given as means ± SEM. ** $p < 0.01$, *** $p < 0.001$ compared to untreated control, # $p < 0.05$, ### $p < 0.001$ compared to LPS alone.*

4.1.4 Malvidin enhanced MAPK phosphatase-1 (MKP-1) expression in unstimulated and LPS treated RAW 264.7 macrophages.

MKP-1 dephosphorylates thereby down-regulates the activity of all three branches of MAPKs (Wu 2007). Therefore, we determined how malvidin affects MKP-1 expression in unstimulated and LPS treated RAW 264.7 macrophages. We subjected the cells to the aforementioned treatment protocol, and performed immunoblot analysis from whole cell homogenates. After mRNA isolation and cDNA transcription, we performed Q-RT-PCR amplification assay. We found that LPS induced MKP-1 mRNA (Fig. 6C) and protein (Fig. 6A, B) expression. Malvidin increased MKP-1 expression in unstimulated cells in a much lower extent than LPS. In LPS stimulated cells, malvidin increased MKP-1 mRNA and protein much above the level that of LPS alone (Fig. 6).

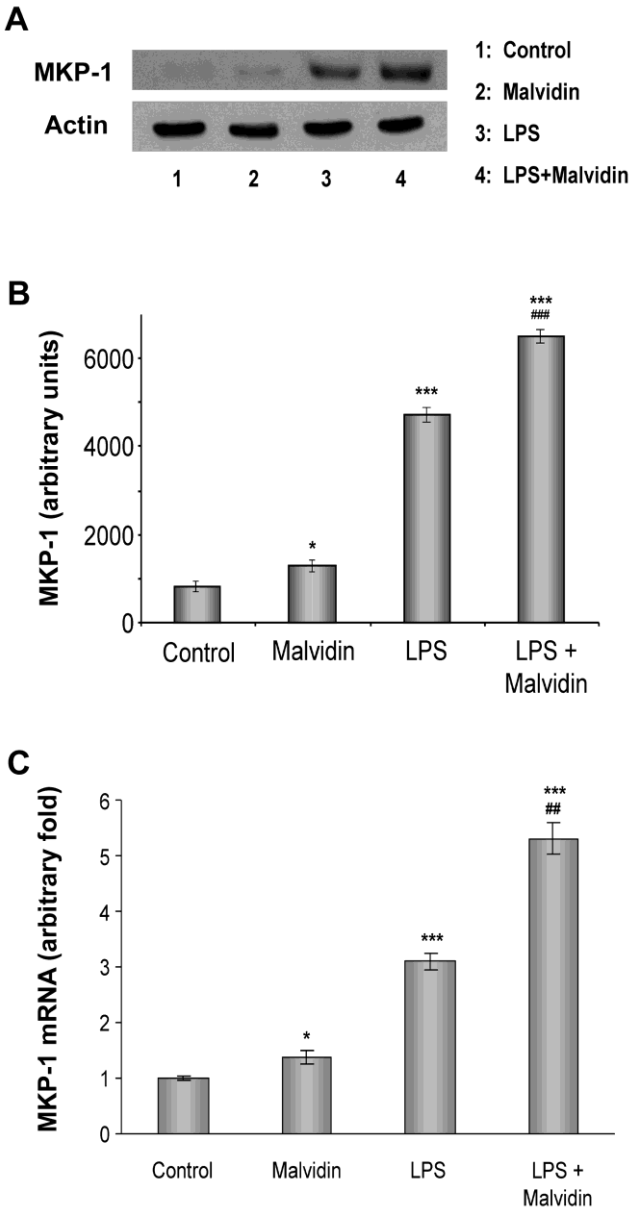


Figure 6. Effect of LPS and malvidin on MKP-1 expression in LPS treated RAW 264.7 macrophages. *Effect of LPS and malvidin on steady state MKP-1 protein level was assessed by immunoblotting from whole cell lysate after treating the cells as indicated for 1h. Actin was used as a loading control. Representative blots (A) and densitometric evaluations (B) of 3 independent experiments are shown. Pixel densities were normalized to that of the actin. MKP-1 mRNA expression (C) was determined in another aliquot of cells treated as above using Q-RT-PCR analysis. β-Actin was used as a housekeeping control gene. Specific primer sequences and PCR conditions are described in Materials and Methods. Values are given as means ± SEM. * $p < 0.05$, *** $p < 0.001$ compared to untreated control, ## $p < 0.01$ ### $p < 0.001$ compared to LPS alone.*

4.1.5 Malvidin enhanced PI-3-kinase-Akt pathway activation in unstimulated and LPS treated RAW 264.7 macrophages.

It was previously shown polyphenols modulate the phosphatidylinositol 3 (PI3)-Kinase-Akt pathway (Haller 2002). Furthermore, we previously found that activation of this cytoprotective pathway was a beneficial factor of PARP inhibition in a murine endotoxic shock model (Veres 2003). Therefore, we investigated the effect of malvidin on the phosphorylation of Akt and its down-stream target, GSK-3ß in unstimulated and LPS treated RAW 264.7 macrophages using immunoblot analysis. We followed the same experimental protocol as we did for MAPK activation studies. We found LPS increased activation of Akt as it was revealed by the phosphorylation of its Ser^{473} and GSK-3ß (Fig. 7). Malvidin increased Akt (Fig. 7A, B) and GSK-3ß (Fig. 7A, C) phosphorylation in unstimulated cells in a lower extent than LPS. In LPS stimulated cells, malvidin increased phosphorylation of both proteins much above the level that of LPS alone (Fig. 7).

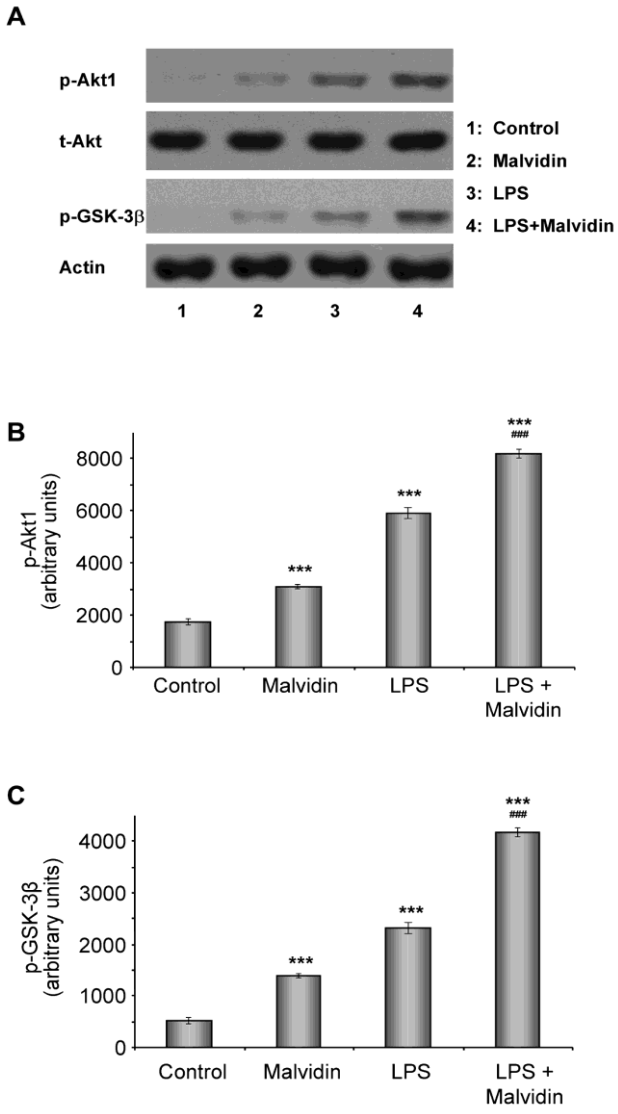


Figure 7. Effect of malvidin on LPS induced activation of Akt1 in RAW 264.7 macrophages. *Steady state level of total (phosphorylated and unphosphorylated) Akt1 (t-Akt) as well as phosphorylation of Akt1 and its down-stream target GSK-3β was detected by immunoblotting from whole cell lysate after treating the cells as indicated for 1h. Actin was used as loading control. Representative blots (A) and densitometric evaluations (B-C) of 3 independent experiments are shown. Pixel densities were normalized to actin. Values are given as means ± SEM. *** p <0.001 compared to untreated control, ### p <0.001 compared to LPS alone.*

4.1.6 Malvidin protected mitochondrial membrane potential from LPS induced depolarization in RAW264.7 macrophages.

Increased ROS and MAPK activation damages while Akt activation protects integrity of the mitochondrial membrane systems (Miyamoto 2009). To investigate the impact of LPS and malvidin on mitochondrial membrane potential, we used a cell-permeable voltage-sensitive fluorescent mitochondrial dye, JC-1, and fluorescent microscopy. After treating them according to the aforementioned protocol, we loaded the cells with JC-1 for 15 min, and acquired fluorescence images of the same area of interest in the green and red channels of the microscope. Mitochondrial membrane depolarization was indicated by the disappearance of the red component of JC-1 fluorescence while normal membrane potential was demonstrated by balanced red and green color. Figure 8 clearly demonstrates that LPS caused significant mitochondrial membrane depolarization that was attenuated by malvidin. Malvidin did not exert any effect on mitochondrial membrane integrity in unstimulated cells (Fig. 8).

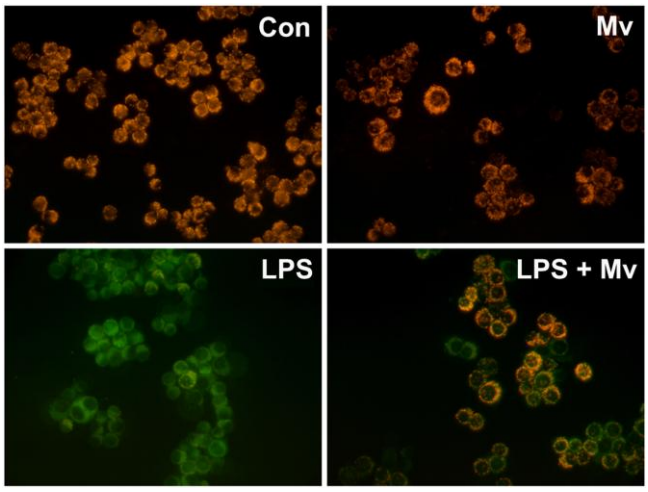


Figure 8. Effect of LPS and malvidin on mitochondrial membrane potential of RAW 264.7 macrophages. *Cells were pretreated or not with malvidin for 30 min and exposed or not to LPS for 1h. Medium was replaced to fresh one without any agents and containing 1μg/ml JC-1 membrane potential-sensitive fluorescent dye for 15 min. Green and red fluorescence images of the same field were acquired using a fluorescent microscope. Representative merged images of three independent experiments are presented. Con: control; Mv: malvidin.*

4.1.7 Malvidin, kinase inhibitors and N-acetyl cysteine (NAC) attenuate nuclear translocation and DNA binding of NF-κB in different extent.

To establish the physiological significance of malvidin's effects on signaling pathways, we compared its effect with that of various kinase inhibitors and the ROS scavenger NAC on nuclear translocation and DNA binding of NFκB. To this end, we preincubated or not RAW 264.7 macrophages with 50μM malvidin, 1μM JNK Inhibitor II, 1μM SB203580 (p38 inhibitor), 25μM PD98059 (ERK inhibitor), 5μM Akt Inhibitor IV or 3 mM NAC before 1 h exposure to 1mg/ml LPS. We isolated and homogenized nuclei of the cells subjected to the aforementioned treatment protocol, and pulled down nuclear proteins by magnetic beads using oligonucleotides of the consensus NF-κB binding sequence as bait. Proteins eluted from the beads were subjected to immunoblot analysis utilizing anti-p65 primary antibody. We found that NAC abolished LPS induced nuclear translocation and DNA binding of NF-κB. The other substances but ERK inhibitor attenuated NF-κB activation in different extent (malvidin >JNK~p38>Akt inhibitor, Fig. 9). ERK inhibition also diminished NF-κB activation, however, it did not reach the threshold of statistical significance.

Malvidin was identified as a rather potent inhibitor of cAMP phosphodiestherase (IC_{50} 23±5μM), thereby a potential indirect regulator of MAPKs (Marko 2004). We performed *in vitro* radioactive kinase assays utilizing enzymes immunoprecipitated from lysate of LPS activated RAW 264.7 macrophages and recombinant substrates to determine whether malvidin had any direct effect on the kinases studied. We found malvidin did not exert any direct effect on the MAPKs or Akt up to 50μM concentration (data not shown).

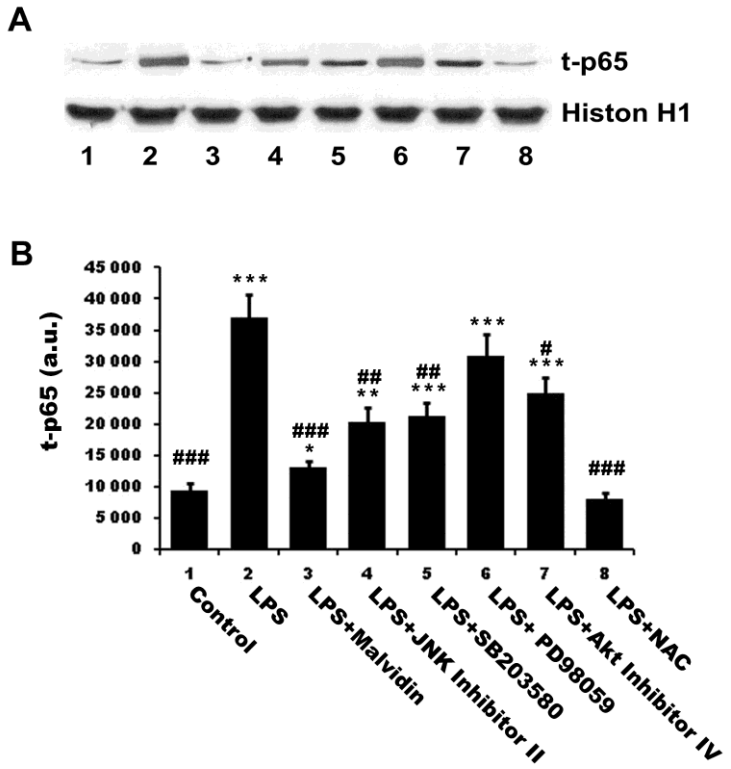


Figure 9. Effect of malvidin, kinase inhibitors and NAC on LPS induced nuclear translocation and DNA binding of NFκB. *RAW 264.7 macrophages were treated for 1h as indicated, then nuclei were isolated and NFκB was extracted using magnetic beads baited with oligonucleotides of NFκB binding consensus sequence. Total (phosphorylated and unphosphorylated) NFκB (t-p65) was detected by immunoblotting in the samples eluted from the beads. Histon H1 from the isolated nuclei was used as loading control. Representative blots (A) and densitometric evaluations (B) of 3 independent experiments are shown. Pixel densities were normalized to histon H1. Values are given as means ± SEM. * p <0.05, ** p <0.01, *** p <0.001 compared to untreated control, # p <0.05, ## p <0.01, ### p <0.001 compared to LPS alone. a.u.: arbitrary units; SB203580: p38 MAPK inhibitor; PD98059: ERK inhibitor; NAC: N-acetyl cysteine.*

4.2 Investigation of signaling pathways in ischemia-reperfusion model

4.2.1 Effect of Verapamil and Metoprolol on high-energy phosphate metabolism of Langendorff perfused hearts

Energy metabolism of Langendorff perfused hearts was monitored in the magnet of an NMR spectroscope capable of detecting real-time changes in the concentration of high-energy phosphate intermediates. Under normoxic conditions, high-energy phosphate levels were maintained, and inorganic phosphate remained close to detection level up to 90 min of perfusion. The substances studied did not affect the high-energy or inorganic phosphate levels except for Metoprolol (10 μM), which decreased ATP level by about 5% and the creatine phosphate levels by 13% (significantly different from untreated, $p<0.05$, $n=4$) by the end of 15 min normoxic equilibrating perfusion. Ischemia induced a rapid decrease in creatine phosphate and ATP levels, and a fast elevation of inorganic phosphate. Verapamil (600 nM) and Metoprolol slightly, although not significantly delayed ischemia-induced depletion of high-energy phosphates. The PARP inhibitor HO-3089 (25 μM) (Kovacs 2006) used as a positive control throughout this study could not prevent the depletion of the high-energy phosphates, however, delayed it much more effectively than Verapamil or Metoprolol did (Fig. 10A, C). Under our experimental conditions, high-energy phosphate intermediates recovered partially (to about 20% of the normoxic value) in untreated hearts. Verapamil had a slight effect on recovery of creatine phosphate levels, and Metoprolol did not affect it significantly (Fig. 10B). HO-3089 improved creatine phosphate recovery significantly better than Verapamil or Metoprolol did (43.2±4.5% vs. 35.±6.1% or 24.3±3.8%; $p<0.05$ or $p<0.001$, respectively). Similar results were observed for ATP levels (Fig. 10D). Effect of the different substances on depletion and recovery of high-energy phosphates was reflected by the inorganic phosphate levels. The substances did not affect normoxic inorganic phosphate level except for Metoprolol, which slightly, although not significantly elevated it. Verapamil and Metoprolol hardly, while the PARP inhibitor significantly delayed elevation of inorganic phosphate (Fig. 10E) resulting from the degradation of high-energy phosphates during ischemia. The PARP inhibitor also promoted faster and more complete reutilization of inorganic phosphate during reperfusion (expressed as % of the maximal value); 30.2±4.5%

for HO-3089 treated vs. 42.3±4.1% for Verapamil- and 39.7±4.2% for Metoprolol-treated hearts, (Fig. 10F, $p<0.001$ in both cases).

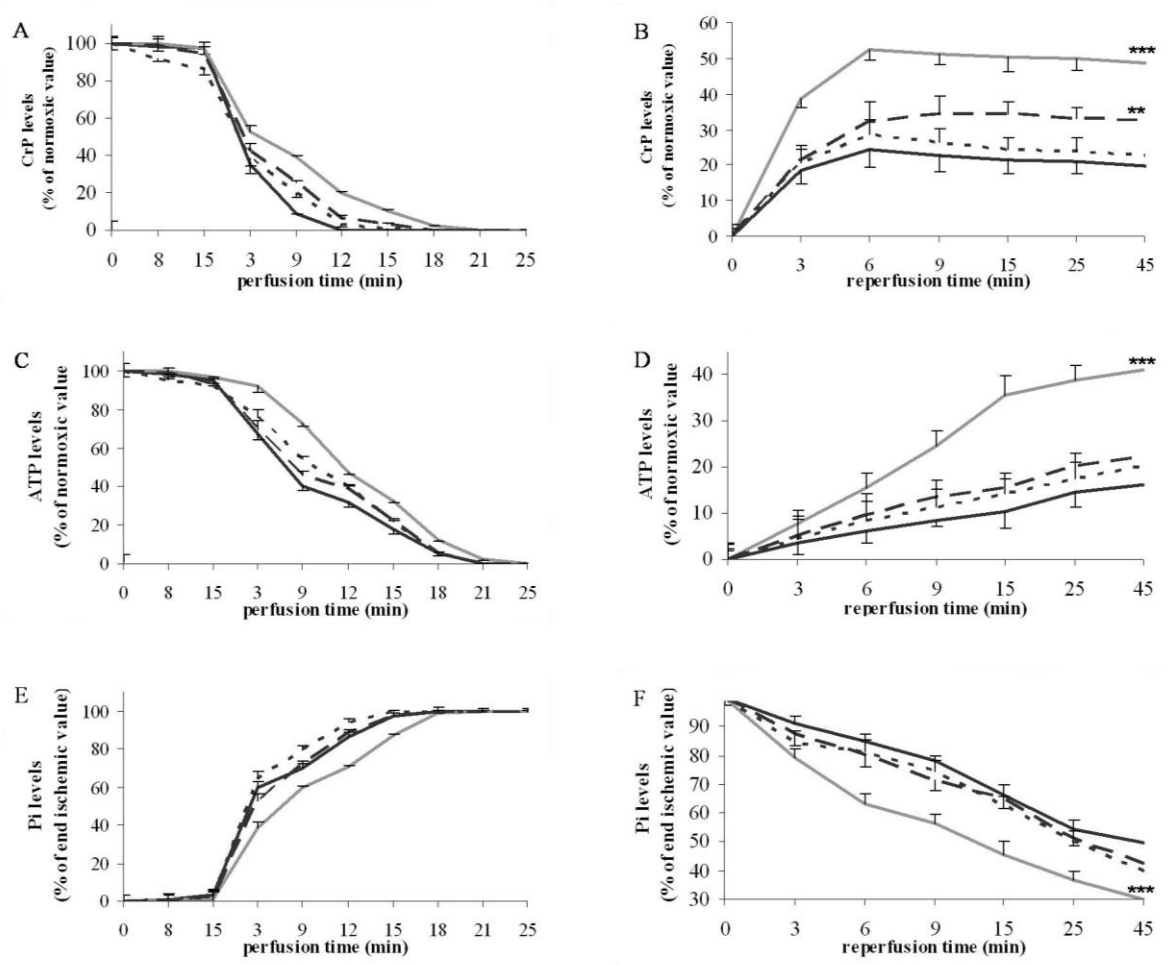


Figure 10. Effects of Verapamil, Metoprolol and HO-3089 on high-energy phosphate metabolism during ischemia-reperfusion. *Time-course of creatine phosphate (CrP; **A**,**B**), ATP (**C**,**D**) and inorganic phosphate (Pi; **E**,**F**) levels during normoxia and ischemia (**A**,**C**,**E**) as well as reperfusion (**B**,**D**,**F**) of Langendorff-perfused untreated (solid black line), HO-3089-treated (25 μM; solid gray line), Verapamil-treated (600 nM; broken line) and Metoprolol-treated (10 μM; dotted line) rat hearts. Results are expressed as mean±S.E.M. of four independent experiments. Significant difference from IR; ** $p<0.01$, *** $p<0.001$.*

4.2.2 Effect of Verapamil and Metoprolol on intracellular pH of Langendorff perfused hearts

The substances did not affect intracellular pH during the normoxic period. It markedly fell by the end of the ischemic period from 7.42±0.01 to 5.81±0.04 in untreated hearts (Fig 11A). Verapamil and Metoprolol significantly attenuated this acidification (5.97±0.07 and 6.04±0.06, respectively, $p<0.01$ for both cases); however, attenuation by HO-3089 was much more pronounced (6.18±0.05, significantly different from Verapamil and Metoprolol, $p<0.01$). Reperfusion brought about a slight recovery of the pH in untreated hearts (Fig. 11B). Verapamil and Metoprolol improved this recovery, however, the PARP inhibitor improved it significantly better (6.59±0.07 for HO-treated vs. 6.42±0.07 for Verapamil and 6.48±0.03 for Metoprolol-treated hearts; $p<0.05$ for both cases).

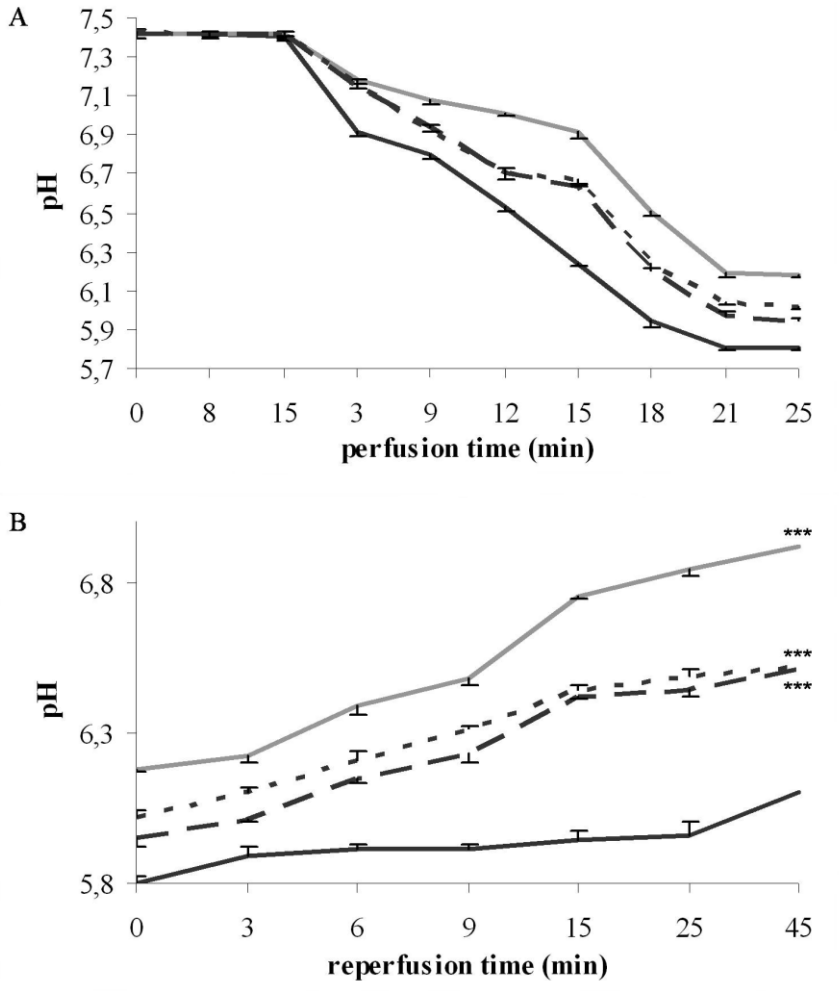


Figure 11. Effects of Verapamil, Metoprolol and HO-3089 on intracellular pH during ischemia-reperfusion. *Time-course of intracellular pH changes (**A**,**B**) during normoxia and ischemia (**A**) as well as reperfusion (**B**) of Langendorff-perfused untreated (solid black line), HO-3089-treated (25 μM; solid gray line), Verapamil-treated (600 nM; broken line) and Metoprolol-treated (10 μM; dotted line) rat hearts. Results are expressed as mean±S.E.M. of four independent experiments. Significant difference from IR; *** p<0.001.*

4.2.3 Effect of Verapamil and Metoprolol on functional recovery of postischemic hearts

To evaluate the effect of HO-3089, Verapamil and Metoprolol on the postischemic myocardial functional recovery, we inserted a balloon catheter into the left ventricle of the perfused hearts and recorded left ventricular developed pressure (LVDP), rate pressure product (RPP) and dP/dt_{max} as previously described (Toth 2003). At the end of normoxic period, LVDP was 152.2±17.5 mmHg (Fig 12A), RPP was $3.5\pm0.16\times10^4$ mmHg/min (Fig 12C) and dP/dt_{max} was 1303±187 mmHg/s (Fig 12E) for untreated hearts. During normoxic period of perfusion, Metoprolol slightly, although not significantly decreased the functional heart parameters while the other compounds did not affect them. The hearts stopped during ischemia, but restarted spontaneously during reperfusion, and the functional parameters recovered partially even in untreated hearts during the first 45-min of reperfusion. As Fig. 12 demonstrates, Verapamil and Metoprolol significantly improved the functional parameters during reperfusion, however, the PARP inhibitor had an even more pronounced effect on the recovery of all parameters ($p<0.01$) indicating that preservation of energy metabolism resulted in a proportionally better functional performance.

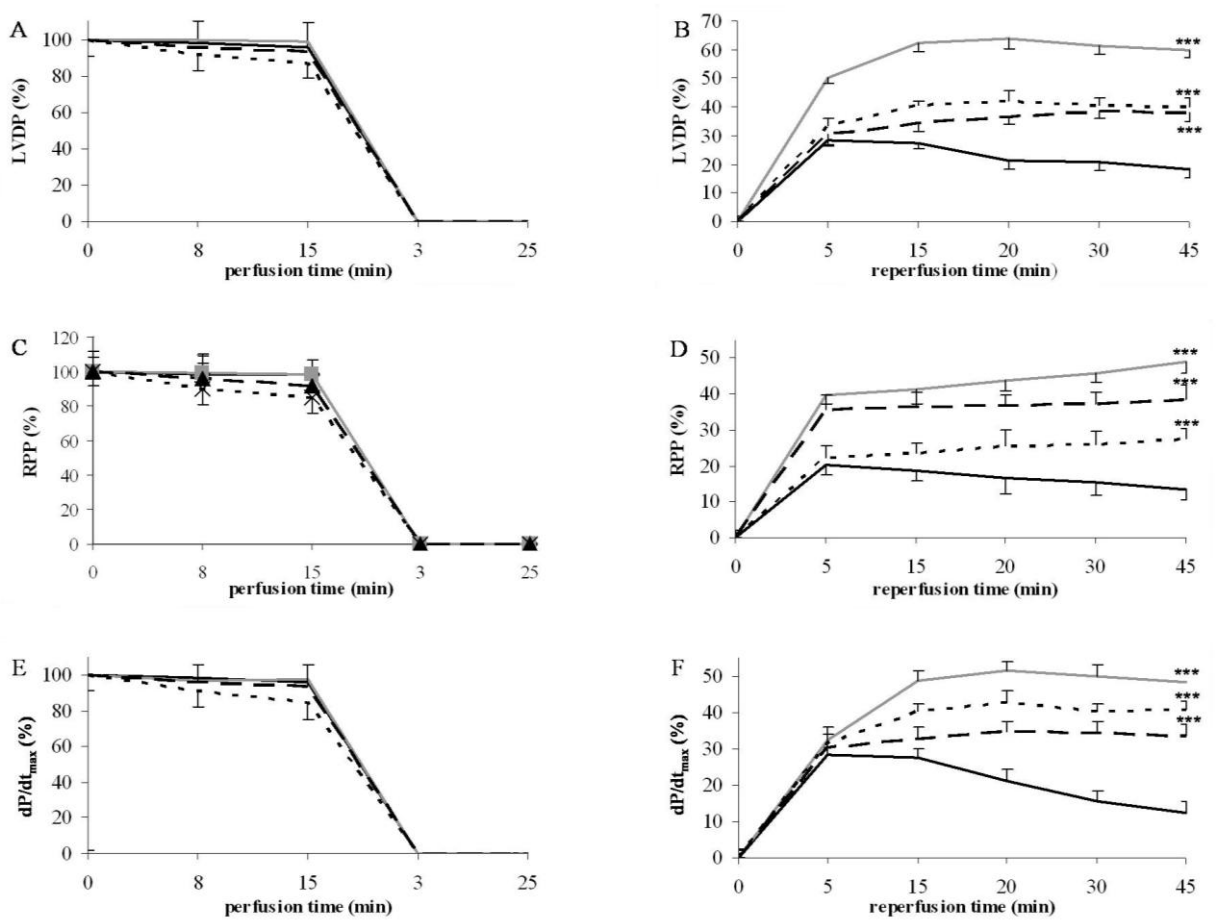


Figure 12. Effects of Verapamil, Metoprolol and HO-3089 on heart function parameters during ischemia-reperfusion. *Time-course of left ventricular developed pressure (LVDP;* ***A***,***B****), rate pressure product (RPP;* ***C***,***D****) and dP/dt$_{max}$ (****E***,***F****) during normoxia and ischemia (****A***,***C***,***E****) as well as reperfusion (****B***,***D***,***F****) of Langendorff-perfused untreated (solid black line), HO-3089-treated (25 μM; solid gray line), Verapamil-treated (600 nM; broken line) and Metoprolol-treated (10 μM; dotted line) rat hearts. Results are expressed as mean±S.E.M. of four independent experiments. Significant difference from IR; *** p<0.001.*

4.2.4 Effect of Verapamil and Metoprolol on ischemia-reperfusion induced lipid peroxidation, protein oxidation and infarct size

We assessed ischemia-reperfusion induced lipid peroxidation by measuring formation of thiobarbituric acid reactive substances from hearts that underwent ischemia-reperfusion. In untreated hearts, ischemia-reperfusion increased the amount of TBARS about three-fold compared to the normoxic hearts (Fig. 13A). Verapamil and Metoprolol significantly decreased the amount of TBARS, however, the PARP inhibitor was superior to either of them, and almost abolished the ischemia-reperfusion induced lipid peroxidation ($p<0.001$).

ROS formation during the ischemia-reperfusion cycle can also trigger protein oxidation that can be determined by measuring quantity of protein-bound aldehyde groups. Ischemia-reperfusion elevated the level of protein oxidation in untreated hearts about four-fold. Verapamil and Metoprolol attenuated this increased protein oxidation, and in this respect they almost matched the performance of the PARP inhibitor (Fig. 13B), however, the PARP inhibitor was still more effective ($p<0.05$).

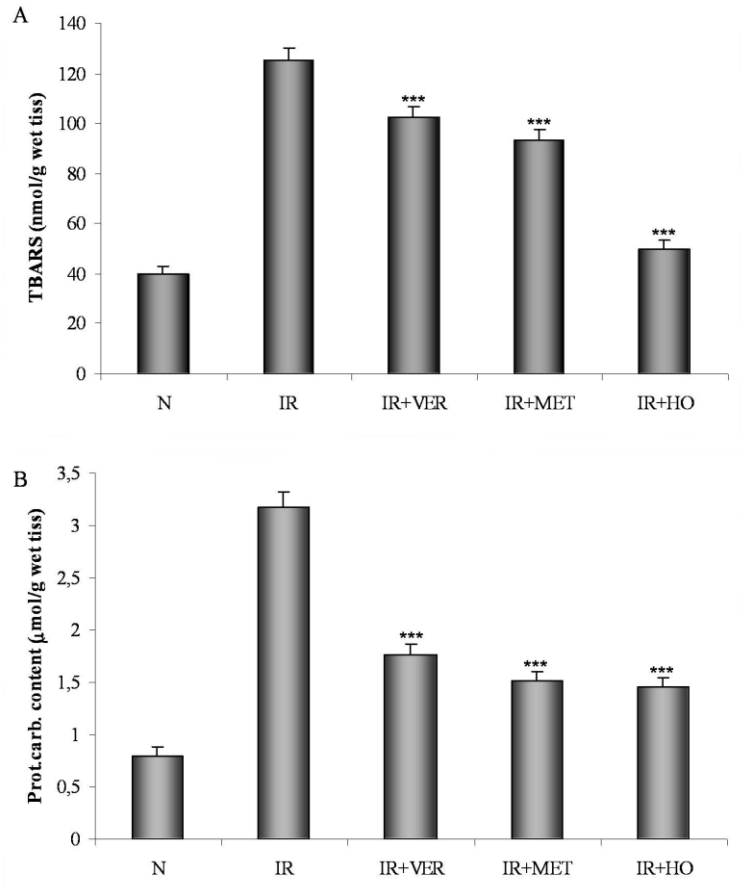


Figure 13. Effects of Verapamil, Metoprolol and HO-3089 on ischemia-reperfusion induced lipid peroxidation and protein oxidation. *Lipid peroxidation (**A**) and protein oxidation (**B**) were assessed in hearts that underwent a 25 min ischemia-45 min reperfusion cycle by measuring the amount of thiobarbituric acid reactive substances and quantity of protein-bound aldehyde groups (prot. carb. content), respectively. Results are expressed as mean±S.E.M. of four independent experiments. N: normoxia without treatment; IR: ischemia-reperfusion without treatment; IR+VER: ischemia-reperfusion in the presence of 600 nM Verapamil; IR+MET: ischemia-reperfusion in the presence of 10 µM Metoprolol; IR+HO: ischemia-reperfusion in the presence of 25 µM HO-3089. Significant difference from IR; *** p<0.001.*

Triphenyl-tetrazolium chloride staining of thick sections of the myocardium after 90 min of postischemic reperfusion revealed that both Verapamil and Metoprolol were capable of significantly diminishing infarct size (expressed as % of the total area) compared to untreated hearts (Fig. 14). In this respect, the PARP inhibitor was superior to either substance too ($p<0.001$).

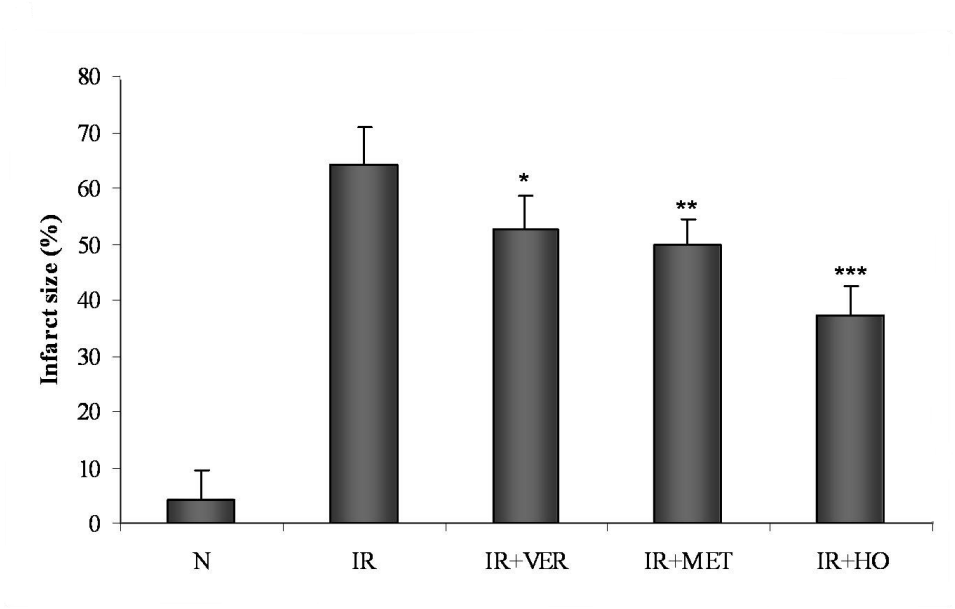


Figure 14. Effects of Verapamil, Metoprolol and HO-3089 on the size of ischemia-reperfusion induced myocardial infarction. *Myocardial infarct size was determined in hearts that underwent a 25 min ischemia-90 min reperfusion cycle by triphenyl tetrazolium chloride staining. Results are expressed as mean±S.E.M. of four independent experiments.*

*N: normoxia without treatment; IR: ischemia-reperfusion without treatment; IR+VER: ischemia-reperfusion in the presence of 600 nM Verapamil; IR+MET: ischemia-reperfusion in the presence of 10 μM Metoprolol; IR+HO: ischemia-reperfusion in the presence of 25 μM HO-3089 Significant difference from IR; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

4.2.5 Effect of Verapamil and Metoprolol on phosphorylation of ERK 1/2, Akt-1 and GSK-3β in normoxia and ischemia-reperfusion

Under normoxic conditions, phosphorylation of Akt-1 and its down-stream target GSK-3β was close to the detection limit. Verapamil and Metoprolol moderately whereas HO-3089 significantly increased activation of Akt-1 as it was revealed by increased phosphorylation of both Akt-1 and GSK-3β, and similar changes were observed for ERK1/2 (Fig. 15).

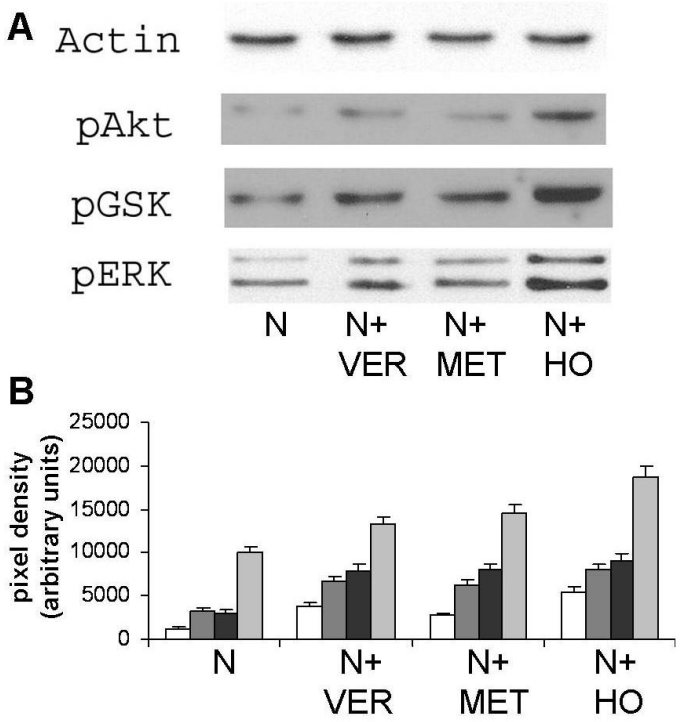
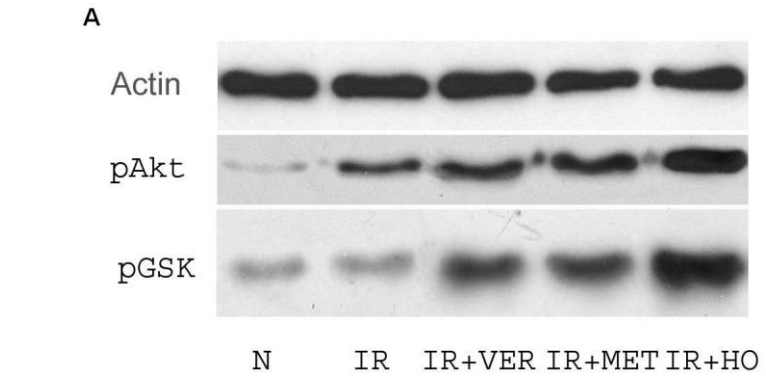
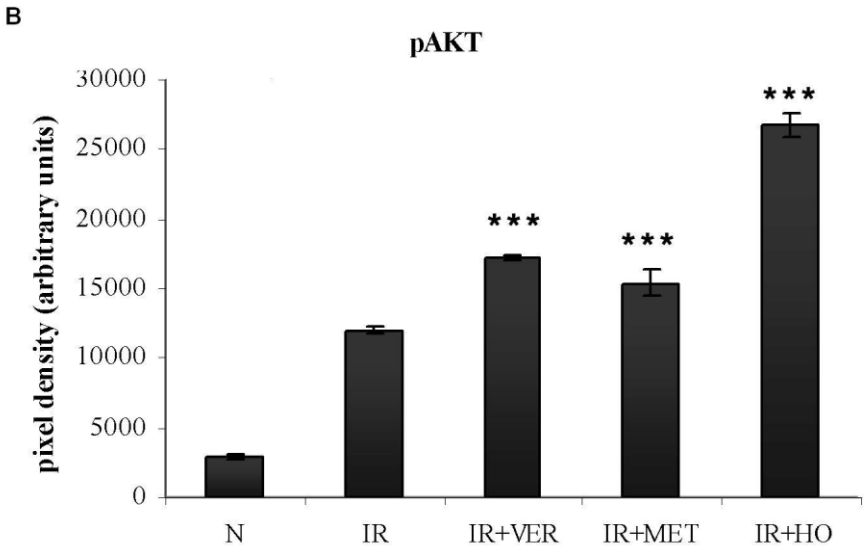


Figure 15. Effects of Verapamil, Metoprolol and HO-3089 on Akt and ERK1/2 activation in normoxic heart. *Activation of the Akt and ERK1/2 pathway was assessed by immunoblotting utilizing phosphorylation specific primary antibodies against Akt and its downstream target GSK-3β, as well as against ERK1/2 in hearts that were Langendorff-perfused for 90 min. Results are demonstrated as representative immunoblots (**A**) and mean pixel density ±S.E.M. (**B**) of four independent experiments. Actin was used as loading control. Instead of ischemia-reperfusion sample, data were normalized to N+HO sample. N: normoxia without treatment; N+VER: normoxia in the presence of 600 nM Verapamil; N+MET: normoxia in the presence of 10 μM Metoprolol; N+HO: normoxia in the presence of 25 μM HO-3089; open bars: phosphor-Akt; dark gray bars: phosphor-GSK-3β; filled bars: phosphor-ERK2; light gray bars: phosphor-ERK1.*

In agreement with previous studies (Kovacs 2006; Lee 2002), ischemia-reperfusion induced phosphorylation and so activation of Akt-1 that was further increased when Verapamil, Metoprolol or HO-3089 was present in the perfusion medium (Fig. 16A, B). This activation pattern of Akt was confirmed by according phosphorylation pattern of the down-stream target GSK-3β (Fig. 16A, C).





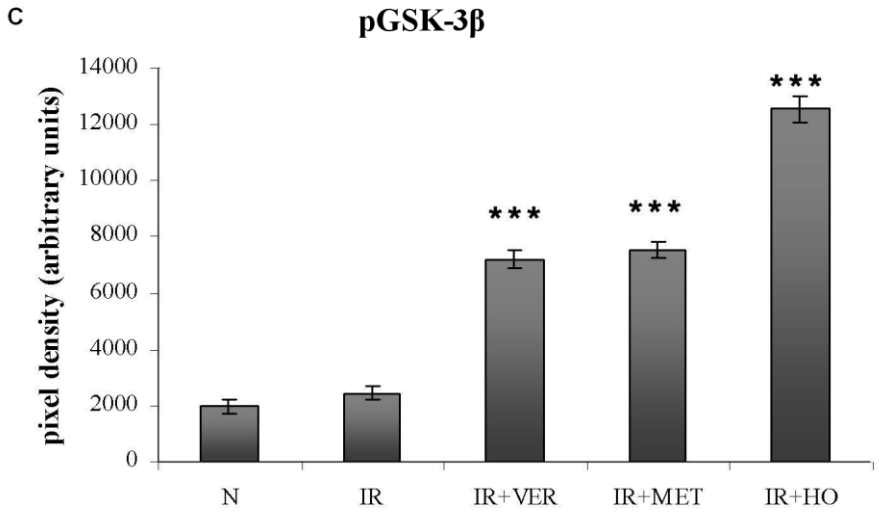
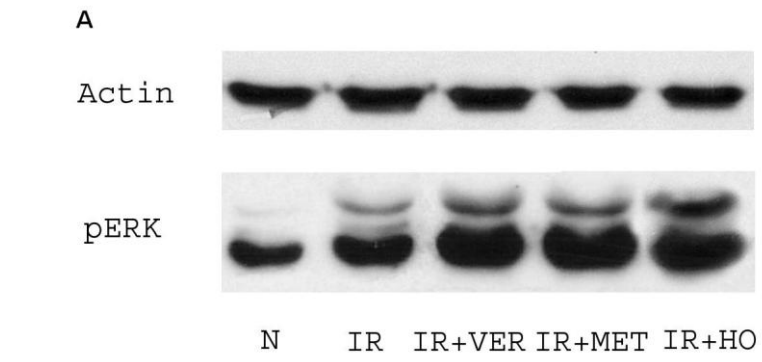
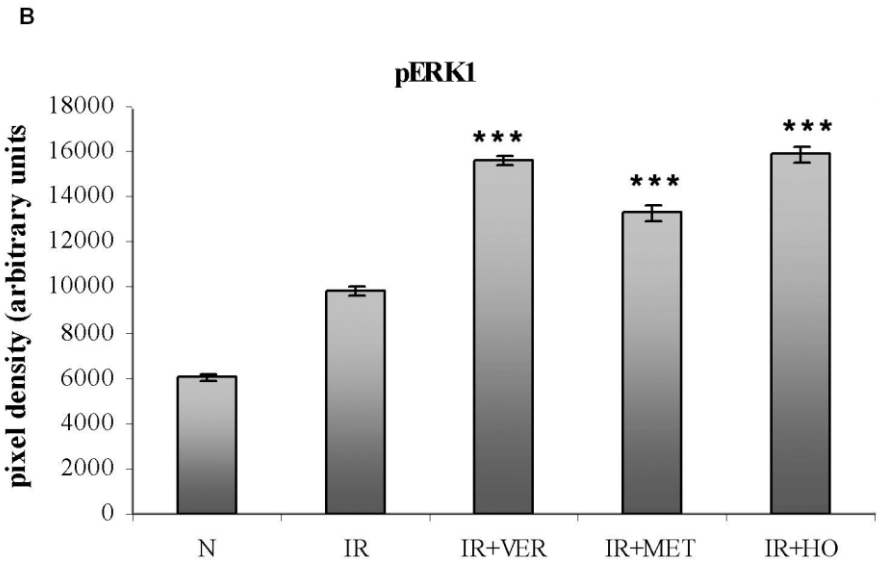


Figure 16. Effects of Verapamil, Metoprolol and HO-3089 on ischemia-reperfusion induced Akt activation. *Activation of the Akt pathway was assessed by immunoblotting utilizing phosphorylation specific primary antibodies against Akt (**A**,**B**) and its downstream target GSK-3β (**A**,**C**) in hearts that underwent a 25 min ischemia-45 min reperfusion cycle. Results are demonstrated as representative immunoblots (**A**) as well as pixel volumes of the blots (**B,C**) expressed as mean±S.E.M. of four independent experiments. Actin was used as loading control. N: normoxia without treatment; IR: ischemia-reperfusion without treatment; IR+VER: ischemia-reperfusion in the presence of 600 nM Verapamil; IR+MET: ischemia-reperfusion in the presence of 10 μM Metoprolol; IR+HO: ischemia-reperfusion in the presence of 25 μM HO-3089 Significant difference from IR; *** p<0.001.*

ERK 2 phosphorylation was close to the detection limit under normoxic conditions, was increased by ischemia-reperfusion, further increased by Verapamil and Metoprolol and even further increased by the PARP inhibitor. On the other hand, there was a considerable ERK 1 phosphorylation even under normoxic conditions that was doubled by ischemia-reperfusion and further increased by all the substances (Fig. 17).





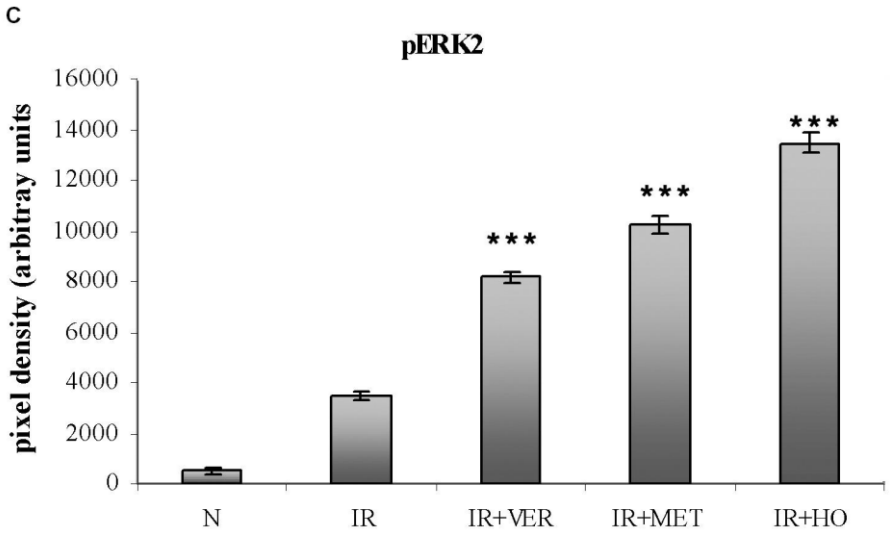


Figure 17. Effects of Verapamil, Metoprolol and HO-3089 on ischemia-reperfusion induced ERK1/2 activation. *Activation of ERK1/2 pathway was assessed by immunoblotting utilizing phosphorylation specific primary antibodies against ERK1/2 (**A,B,C**) in hearts that underwent a 25 min ischemia-45 min reperfusion cycle. Results are demonstrated as representative immunoblots (**A**) as well as pixel volumes of the blots (**B,C**) expressed as mean±S.E.M. of four independent experiments. Actin was used as loading control.*

*N: normoxia without treatment; IR: ischemia-reperfusion without treatment; IR+VER: ischemia-reperfusion in the presence of 600 nM Verapamil; IR+MET: ischemia-reperfusion in the presence of 10 μM Metoprolol; IR+HO: ischemia-reperfusion in the presence of 25 μM HO-3089 Significant difference from IR; *** $p<0.001$*

Correlation between cardioprotectivity reflected by recoveries of creatine phosphate levels and Akt, ERK1 or ERK2 activation was analyzed for the substances studied in this report and 4-hydroxyquinazoline (4OHQ), another PARP inhibitor (Kovacs 2006) by using two-factor analysis of variance with replication. We found positive correlation between recoveries of creatine phosphate levels and phosphorylation of Akt ($p<0.01$) or ERK2 ($p<0.05$). When extent of kinase activation was plotted against creatine phosphate recovery (Fig. 18), a linear correlation was observed in case of Akt for all the compounds suggesting a very strong relation between Akt activation and cardioprotection.

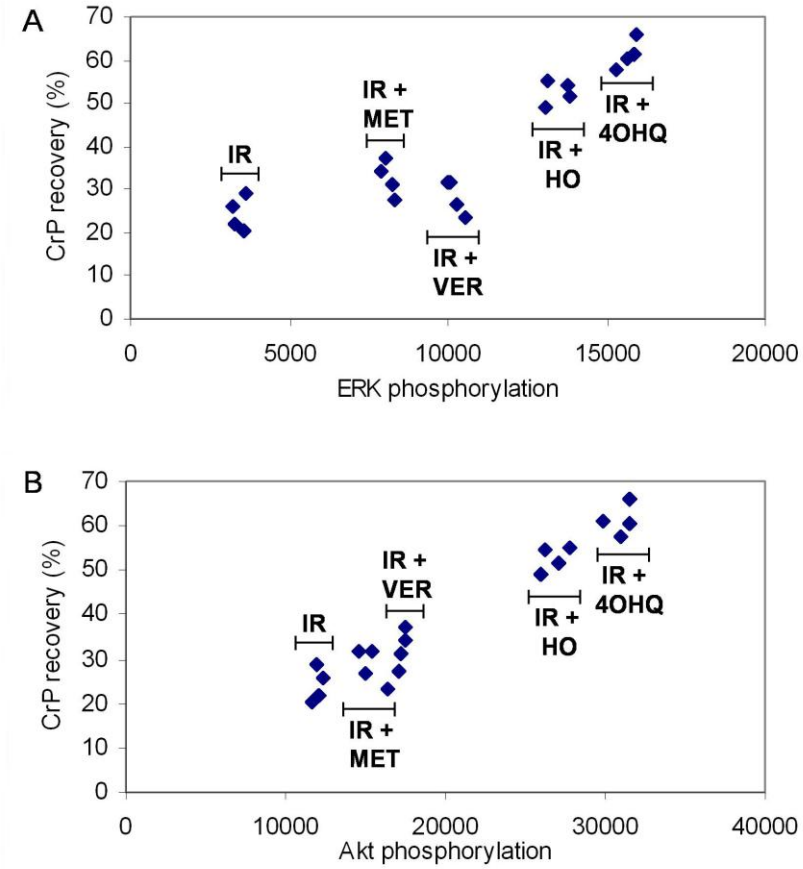


Figure 18. Correlation of cardioprotection and kinase activation during ischemia-reperfusion. *Cardioprotective effect of 10 μM Metoprolol (IR+MET), 600 nM Verapamil (IR+VER), 25 μM HO-3089 (IR+HO) and 100 μM 4-hydroxyquinazoline (IR+4OHQ, data were from [Kovacs 2006]) demonstrated by recovery of creatine phosphate in the presence of the drugs during ischemia-reperfusion (IR) of Langendorff-perfused rat hearts was plotted against activation of ERK2 (**A**) or Akt (**B**) assessed by phosphorylation specific primary antibodies and immunoblotting in the same hearts. Data were from figures 1, 7, 8 and from (Kovacs 2006) for 4OHQ. Each point represent an individual heart, and the lines with captions indicate group of hearts (n=4) of the same treatment.*

4.2.6 Effect of kinase inhibitors on the energy metabolism of Langendorff perfused hearts

In order to establish physiological significance of Akt and ERK1/2 activation in the cardioprotective effect of Verapamil, Metoprolol, we utilized an inhibitor of PI-3 kinase (upstream activator of Akt) as well as an inhibitor of MEK (upstream activator of ERK1/2) during ischemia-reperfusion of Langendorff-perfused isolated rat hearts. When applied by itself, neither LY294002 (PI3K inhibitor) nor PD98059 (MEK inhibitor) had any significant effect on recoveries of creatine phosphate (Fig. 19) and ATP (not shown) levels. However, when applied in combination with the cardioprotective substances studied, both LY294002 and PD98059 significantly diminished the recoveries of creatine phosphate (Fig. 19) and ATP (not shown) levels indicating that activation of Akt and ERK1/2 significantly contributed to the cardioprotective effect of all of the substances studied.

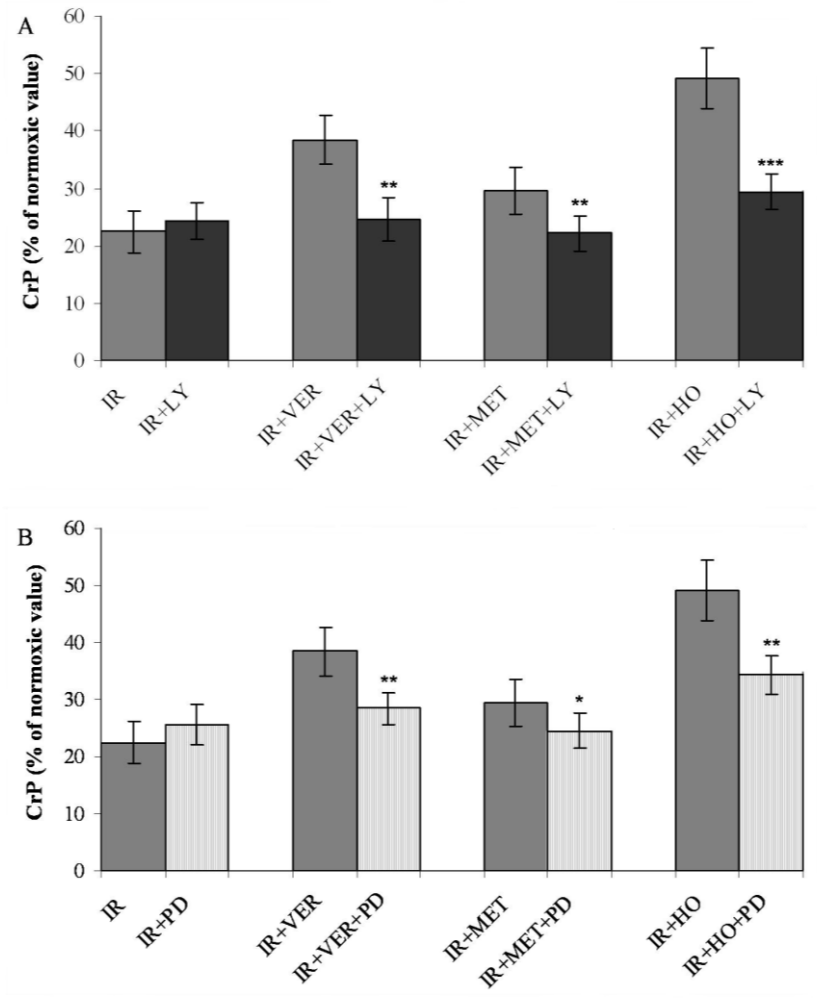


Figure 19. Effects of inhibition of Akt and ERK1/2 pathways on the cardioprotective effects of Verapamil and Metoprolol. *In Langendorff perfused hearts, recovery of creatine phosphate at the 15th minute of reperfusion following 25 min ischemia (IR) was determined by ^{31}P NMR spectroscopy, and was expressed as mean±S.E.M. of four independent experiments. The perfusion medium contained or not 10 μM LY294002 (inhibitor of the PI3K pathway, LY,* ***A****) or PD98059 (inhibitor of the MEK pathway, PD,* ***B****) in combination with 600 nM Verapamil (VER), 10 μM Metoprolol (MET), or 25 μM HO-3089 (HO). Significant difference from the hearts perfused with the same substance but in the absence of LY or PD; * p<0.05, ** p<0.01, *** p<0.001.*

5. Discussion

Oxidative stress and chronic inflammation were identified as major pathomechanisms in cardiovascular diseases. To augment and complement existing therapies, a more profound knowledge of the underlying molecular events is required. To this end, we compared cardioprotective effects of PARP inhibition with that of two major existing clinical therapies; Ca^{2+} antagonism and β-adrenergic receptor blocking. Furthermore, we investigated antioxidant and anti-inflammatory effects of malvidin to find out whether this component could account for the beneficial cardiovascular effects of moderate red wine consumption.

5.1. Antioxidant and anti-inflammatory effects of malvidin

In response to LPS, nuclear localization signal of cytosolic NF-κB becomes unmasked resulting in nuclear translocation of the transcription factor. In the nucleus, NF-κB becomes phosphorylated and acetylated, thus activated to bind to its consensus promoter DNA sequences. This binding triggers the expression of its target genes (Fig. 20) including pro-inflammatory cytokines, chemokines, adhesion proteins, COX-2 and iNOS (Doyle 2006; Baeuerle 1994; Hou 2010). These events are of pivotal importance in the development of inflammation-related chronic diseases (Purkayastha 2011). We demonstrated that malvidin attenuates activating phosphorylation, nuclear translocation and binding to consensus DNA sequence of NF-κB. These data are completely in line with the results of other groups (Wang 2002; Hou 2005; Poulose 2012). Furthermore, we found malvidin antagonise NF-κB activation at much lower concentrations than trans-resveratrol. This indicates that malvidin could account for the beneficial effects of red wine in inflammation-related chronic diseases. Furthermore, these results explain the finding of the 1999-2002 US National Health and Nutrition Examination Survey describing malvidin intake negatively correlates with serum C-reactive protein levels (Chun 2008).

Binding of LPS to TLR4 receptor triggers activation of the MAPKs (Fig. 20) via various signaling pathways such as the myeloid differentiation primary response gene (MyD)88─interleukin-1 receptor-associated kinase (IRAK)─tumor necrosis factor (TRAF)-6─transforming growth factor-β activated kinase (TAK) pathway (Wu 2007). In turn, MAPK pathways are involved in activation of the pro-inflammatory transcription factors; NF-κB and AP-1 (Wu 2007; Baeuerle 1994). In the present study, we observed malvidin attenuated LPS induced activation of all three MAPKs. However, this effect differed for the three kinases

(JNK>p38>>ERK). By using specific kinase inhibitors, we aimed to establish the significance of these results. In agreement with others (Chen 1999, Kim 2006; Lee 2004) we found JNK and p38 inhibitors significantly reduce LPS induced nuclear translocation and DNA binding of NF-κB. However, ERK inhibition was ineffective. These data indicate early inflammatory response in RAW 264.7 macrophages is mediated, at least partially, via the aforementioned pathway. On the other hand, it is likely that malvidin decreased LPS evoked MAPK activation undirectly since in *in vitro* kinase assays malvidin did not exert any effect. Most likely, it regulated MAPK activation by inhibiting other key mechanisms; ROS production.

MKP-1 is the major enzyme responsible for the dephosphorylation, thereby inactivation of all three MAPKs (Zhao 2006). It is critically involved in inflammatory signaling of macrophages, and is responsible for switching off pro-inflammatory cytokine production *in vitro* and *in vivo* (Chen 1999; Kim 2006). In agreement with others (Zhao 2006) we found increased expression of MKP-1 in the LPS stimulated macrophages both at the mRNA and protein level. However, this was accompanied by an elevated activation of the MAPKs indicating MKP-1 induction was not sufficient to suppress LPS-induced MAPK activation. Malvidin enhanced MKP-1 expression both in the unstimulated and LPS treated cells, which was accompanied by decreased activation of the MAPKs. This suggests MKP-1 expression, when augmented by malvidin, could counteract the activating mechanisms induced by TLR4 signaling (Fig. 20). However, we found significant differences among the MAPKs regarding malvidin's effectivity in reduction of their LPS induced activation. Furthermore, these differences were reflected in the anti-inflammatory effect of MAPK inhibitors. All these data indicate, the network of MAPK activation and inhibition signaling is complex, and balance of the regulating processes differs for each MAPK.

Previous studies established *in vitro* antioxidant characteristics for malvidin (Salah 1995; Sun 2009). In agreement with these results, we found that malvidin attenuates ROS production in LPS-treated RAW264.7 macrophages at an IC_{50} value comparable to that of trans-resveratrol. At the same time, this modulated a complicated network of processes produced and regulated by ROS (Fig. 20) including mitochondrial integrity and activation of MAPKs, Akt and PARP. It is feasible that LPS induced NF-κB activation in our experimental system was mediated partially via the TLR4-NADPH oxidases-ROS-PARP pathway. However, the complexity of the involved networks made it hard to distinguish between cause and consequence or identify up-stream and down-stream events. Nevertheless, it is likely that due to its antioxidant property, malvidin decreases ROS production, thereby reduces PARP and MAPK activation as well as oxidative damage to MKP-1. Reduced PARP activation leads

to decreased NF-κB and MAPK activation, increased expression of MKP-1 (Racz 2010) and activation of the PI3K─Akt pathway (Veres 2003) that together with the decreased ROS results in maintained mitochondrial integrity (Fig. 20). Importance of the antioxidant mechanism in malvidin's anti-inflammatory effect is emphasized by us and others (Lu 2012; Li 2012) reporting NAC inhibits LPS induced NF-κB activation.

Recently it has been shown that Akt is a downstream target of TRIF/TANK-binding kinase 1 (TBK1), and there is an association between endogenous TBK1 and Akt in LPS treated macrophages. TBK1 enhances phosphorylation of Akt on Ser(473), and siRNA-mediated silencing or knocking out of TKB1 compromises LPS induced Akt activation (Joung 2011). On the other hand, elevated ROS also activates the PI-3K─Akt pathway via oxidative inactivation of the phosphatase and tensin homolog (PTEN) that inactivates the pathway by dephosphorylation (Cantley 1999). Akt activation may result in mitochondrial protection by phosphorylation, thereby inactivation of Bad, and indirect NF-kappaB activation (Manukyan 2010). As we found, malvidin activated Akt both in the unstimulated and LPS-treated macrophages. Most likely, this effect of malvidin was also due to its antioxidant property. The augmented activation of Akt was most probably involved in malvidin's protective effect on LPS induced mitochondrial depolarization (Fig. 20). On the other hand, Akt was implicated in the phosphorylation thereby activation of NF-κB p65 (Sizemore 1999). Accordingly and in agreement with Zhao et al. (Zhao 2012), we found that inhibition of the PI-3K-Akt pathway attenuated NF-κB activation suggesting a partial involvement of this pathway in mediating LPS's effect. All these data suggest that Akt activating effect was unlikely to be involved in malvidin's anti-inflammatory effect.

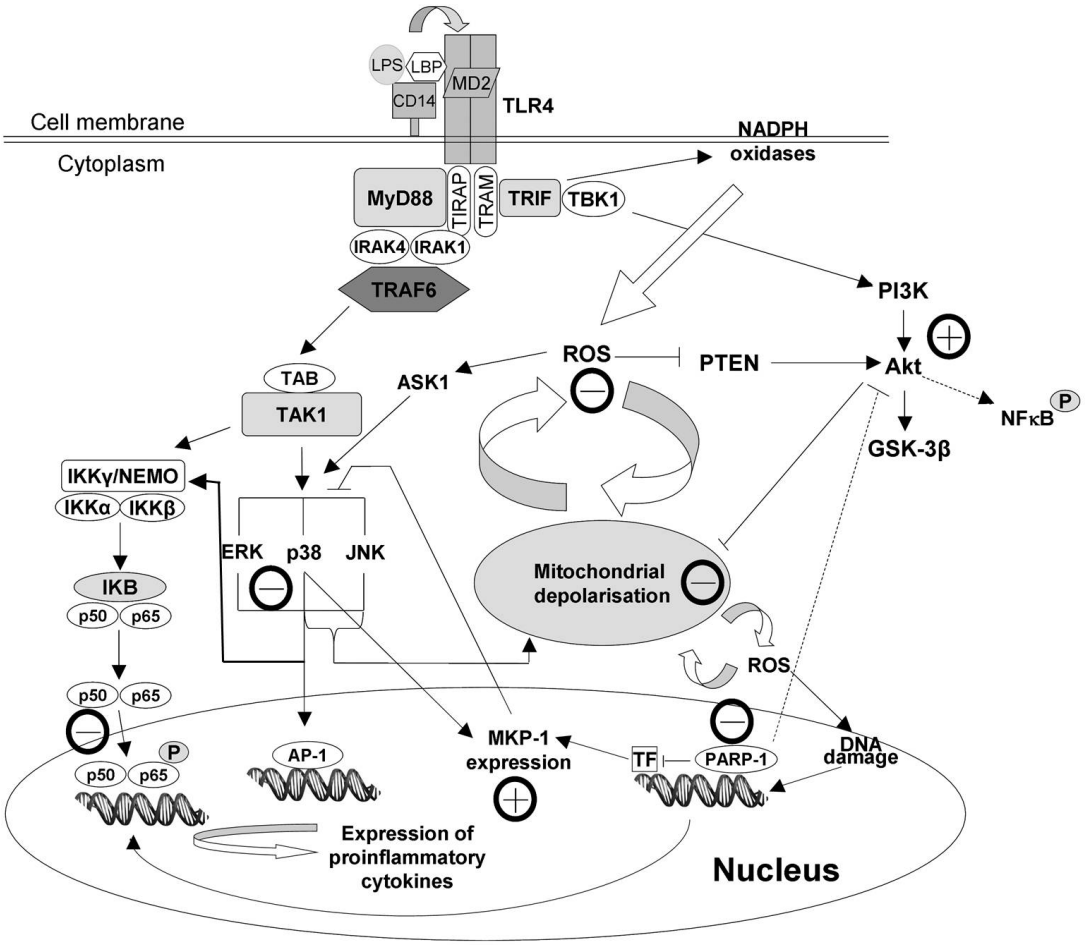


Figure 20. Effect of malvidin on LPS induced pathophysiological changes in RAW 264.7 macrophages. *Well documented effects are indicated by solid lines, whereas effects involving yet unidentified mediator(s) or events are represented by dashed line. Lines with pointed end denote activation, whereas lines with a flat end indicate inhibition. Activating or inhibitory effect of malvidin is indicated by a circled + or ─ next to the line, respectively. LPS induces activating phosphorylation, nuclear translocation and DNA binding of NFκB, induction of ROS production, PARP activation, activation of MAPKs, MKP-1 expression, activation of the phosphatidylinositol-3 kinase-Akt pathway and destabilization of the mitochondrial membrane systems. Malvidin attenuates ROS production, mitochondrial destabilization, and activation of PARP and MAPKs. It also augments Akt activation and MKP-1 expression resulting in diminished activation of NFκB.*

5.2 Comparison of cardioprotective effects of PARP inhibition, Ca^{2+} antagonism and β-adrenergic receptor blocking

In the present study, we assessed cardioprotective as well as Akt and ERK activating effects of two substances used in the clinical management of ischemic heart diseases, namely, Verapamil and Metoprolol (Dauterman 2002; Mehta 1994) in Langendorff perfused hearts during ischemia-reperfusion cycle, and compared them to that of HO-3089 (Kovacs 2006), a PARP inhibitor used as a positive control throughout the experiments.

Among other mechanisms, L-type Ca^{2+} channel antagonists were reported to protect high-energy phosphates (Buser 1989; Wolfe 1991) and cardiac functions (Farkas 1999) as well as reduction of acidosis (Markiewicz 1988), infarct size (Wolfe 1991) in different ischemia models. Cardioprotecting mechanisms of β-adrenergic receptor blockers were reported to include improvement of cardiac functions (Kalaycioglu 1999; Bradley 1984), decrease of lipid peroxidation and cardiomyocyte apoptosis (Kalaycioglu 1999; Communal 2005). Although it is very difficult to compare the results of different laboratories due to the different models and different ischemia and reperfusion protocols, our results on the different parameters of cardioprotection by Verapamil and Metoprolol were comparable with that of other laboratories.

In our experimental setup, we monitored the high-energy and inorganic phosphate levels during a 15 min equilibrating normoxic perfusion, and any significant deviation from the starting values in case of the untreated hearts resulted in the rejection of the given heart from the experiment. In the same way, we excluded those hearts treated or not, which did not regain spontaneous pulsing during the reperfusion period. Deviations from the starting values during the normoxic perfusion period were accepted as the consequence of the treatment, if it was reproducible and persisted in 45 min normoxic perfusion experiments (not shown). We used a single concentration for Verapamil and Metoprolol since they were extensively studied in the past, and their effective concentrations in Langendorff-perfusion experiments were well established. The concentration (25μM) of HO-3089 was found to protect hearts against ischemia-induced damages in previous experiments (Kovacs 2006).

According to our data, Verapamil and Metoprolol improved the recovery of creatine phosphate, ATP and pH, and the reutilization of inorganic phosphate in hearts subjected to ischemia-reperfusion significantly, although less effectively than HO-3089 did. The substances significantly attenuated oxidative myocardial damages, which were characterized by decreased lipid peroxidation and protein oxidation too. Furthermore, the favorable changes

in cardiac energetics were accompanied by improved recovery of functional cardiac parameters and reduced infarct size.

We have previously demonstrated that activation of the cytoprotective PI-3 kinase/Akt pathway significantly contributed to the cardioprotective (Kovacs 2006) and cytoprotective (Tapodi 2005) effects of PARP inhibition. Activated Akt can phosphorylate several regulatory proteins, including GSK-3β, caspase-9, BAD, or FKHR (Scheid 2001). Phosphorylation and so inactivation of pro-apoptotic BAD protein contribute to the stabilization of mitochondrial membrane systems and may prevent the release of pro-apoptotic proteins, i.e. cytochrome c or apoptosis-inducing factor (Zhou 2000). Moreover, Akt can also phosphorylate and inactivate caspase-9, which can result in the blockade of cytochrome c/Apaf-1/caspase-9/caspase-3 pathway (Crossthwaite 2002).

Although ERK 1/2 is considered to be mainly involved in growth factor-induced mitogen signaling and cellular differentiation, this kinase was reported to exhibit protective roles under circumstances of oxidative stress through the inhibition of apoptosis by downregulating c-jun N-terminal kinase (JNK) as well as caspase-3. ERK was also brought into connection with delayed cytoprotection, because its sustained activation seemed to mediate late cardiomyocyte protection after simulated ischemia-reoxygenation (Yamaguchi 2004; Goto 2002).

Because of their indicated involvement in cytoprotection, we studied effect of Verapamil and Metoprolol on Akt and ERK 1/2 phosphorylation during ischemia-reperfusion. To demonstrate activation of Akt, we determined phosphorylation of its downstream target, GSK-3β too. As we found, all the substances induced significantly elevated activation of Akt and ERK1/2 either in normoxia or after ischemia-reperfusion. We found even a strong correlation between extent of recoveries of creatine phosphate levels and Akt or ERK2 activation for the substances studied indicating a crucial role of Akt and ERK2 activation among the cardioprotective mechanisms of Verapamil and Metoprolol. An increasing number of evidence suggests that β-adrenergic receptor antagonists can induce β-arrestin-mediated trans-activation of epidermal growth factor receptor leading to activation of the down-stream target ERK (DeWire 2007), and a similar mechanism was reported for Akt activation that involved β-arrestin-1 and insulin-like growth factor 1 (Povsic 2003). Although linking of L-type calcium channel blockers to Akt and ERK activation seems more elusive, calmodulin-dependent cyclic nucleotide phospho-diesterase that was indicated as a key regulator in the cross talk between cyclic nucleotide- and Ca^{2+} -dependent as well as Ca^{2+} -independent-signaling pathways (Raju 1997; Abdel-Latif 2001; Nadif Kasri 2002) might play role.

Regardless of the mechanism, for establishing physiological significance of the activation of these kinases in the cardioprotection by the β-adrenergic receptor blocker and the Ca^{2+} antagonist, we inactivated these kinases by inhibiting their respective upstream activator kinases during the ischemia-reperfusion cycle. The finding that inhibition of these kinases significantly decreased recoveries of high-energy phosphate levels for not just the PARP inhibitor but for Verapamil and Metoprolol too clearly indicated that activation of Akt and ERK1/2 significantly contributed to the cardioprotective effects of these substances.

6. Conclusion

Ischemic heart disease and sepsis are the main causes of morbidity and mortality worldwide. Oxidative stress and inflammation play a pivotal role in both diseases. Searching for therapeutic targets we investigated the effects of naturally occurred polyphenol, malvidin and clinically used two drugs, Metoprolol and Verapamil. We investigated several signaling pathways which potentially could be therapeutic targets.

In conclusion, malvidin, the most abundant polyphenol ingredient of red wine, augments LPS-induced Akt activation and MKP-1 expression and attenuates mitochondrial destabilization, ROS production and activation of PARP as well as MAPKs resulting eventually in diminished activation of NFκB. All these data indicate that malvidin significantly contributes to the antioxidant and anti-inflammatory effects of red wine, and could, at least partially, account for the positive effects of moderate red wine consumption on inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease.

We demonstrated at the first time that activation of PI-3K-Akt and ERK1/2 pathways significantly contributed to cardioprotective effect of a Ca^{2+} antagonist and a β-adrenergic receptor blocker. Furthermore, we found a strong correlation between cardioprotective and kinase activating potencies of the substances that indicate potentiality of these kinases as drug-targets in the therapy of ischemic heart disease.

7. Achievements

1. We demonstrated that malvidin, the most abundant polyphenol ingredient of red wine, augments LPS-induced Akt activation and MKP-1 expression and attenuates mitochondrial destabilization, ROS production and activation of PARP as well as MAPKs resulting eventually in diminished activation of NFκB.

2. We found that anti-inflammatory effect of malvidin exceeded that of resveratrol. Therefore, we provided experimental evidence at the first time that malvidin rather than resveratrol could account for the positive effects of moderate red wine consumption on inflammation-mediated chronic maladies such as obesity, diabetes, hypertension and cardiovascular disease.

3. We demonstrated at the first time that activation of PI-3K-Akt and ERK1/2 pathways significantly contributed to cardioprotective effect of a Ca^{2+} antagonist and a β-adrenergic receptor blocker.

4. We found that cardioprotective effects of PARP inhibition much exceeded that of Ca^{2+} antagonism and blocking of the β-adrenergic receptor thereby confirmed the potentiality of PARP inhibitors in the management of cardiovascular diseases.

5. Furthermore, we established a strong correlation between cardioprotective and kinase activating potencies of the substances that indicate potentiality of these kinases as drug-targets in the therapy of ischemic heart disease.

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10. Publications

This work based on the following articles:

Eszter Bognár, Zsolt Sarszegi, Alíz Szabó, Balazs Debreceni, Nikoletta Kalman, Zsuzsanna Tucsek, Balazs Sumegi, Ferenc Gallyas Jr. Antioxidant and anti-inflammatory effects in RAW264.7 macrophages of Malvidin, a major red wine polyphenol. PLoS ONE 2013;8: e65355.

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Total IF: 10,422
Total citations: 13

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1. Arpad Szanto, Aliz Szabo, Zita Bognar, Balazs Radnai, Zsuzsa Tucsek, Izabella Solti, **Eszter Bognar**, Antal Tapodi, Balazs Debreceni, Ferenc Gallyas Jr., Balazs Sumegi: PARP-1 inhibition-induced activation of PI-3-kinase-Akt pathway by treatment of Taxol. **15th Euroconference on Apoptosis & 4th Training course on'Concepts and Methods in Programmed Cell Death', Portoroz, Slovenia**, October 26-31, 2007.

2. Balazs Sumegi, Anita Palfi, Krisztina Kovacs, Katalin Hanto, Peter Deres, Edina Pandur, **Eszter Bognar**, Ambrus Toth, Kalman Toth: Akt/GSK-3β, and MAP kinase cascades contribute to the protective effect of PARP inhibition. The 14th **International meeting on ADP ribosylation reactions, PARP 2005**: Bench to Bed be held in Newcastle upon Tyne-Gateshead on 5th to 7th October 2005.

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