Evaluation of Resveratrol and Tocotrienols as potential REDOX – active compounds for Cardioprotection

Dissertation for the Doctor of Philosophy Degree

Written by:
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.... So many forces and people go into the completing of my Degree of Doctor of Philosophy, but mainly standing on four pillars. I would like to dedicate my Dissertation to my Uncle, Father, Mother and my beloved Wife……

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LIST OF ABBREVIATIONS

Akt……Protein kinase B

CREB……cAMP Response-Element Binding Potein

DAG…… *sn*-1,2-Diacylglycerol

DMSO……. Dimethyl Sulfoxide

+d*P*/dt…… Rate of pressure development

-d*P*/dt……Rate of pressure decay

ERK……. Extracellular signal regulated protein kinase

eNOS……endothelial Nitric Oxide Synthase

GAPDH……Glyceraldehyde-3-phosphate dehydrogenase

HDL……. High density lipoprotein

H_2O_2…… Hydrogen peroxide

I-R…… Ischemia-reperfusion

iNOS…… inducible Nitric Oxide Synthase

IκB……Inhibitory κB

JNK…….. c-Jun N-terminal kinase

KDa ……Kilodalton

KHB……Krebs-Henseleit bicarbonate

LDL……..Low density lipoprotein

LVDP……Left Ventricular Developed Pressure

LVdp/dt……maximum first derivative of developed pressure

MAP……Mean arterial pressure

MAPK…….. Mitogen activated protein kinase

MDA…….Malondialdehyde

MEK…… Mitogen-activated protein kinase

Mn-SOD……Manganese-Superoxide dismutase

MSK…….Mitogen – and stress- activated protein kinase

NFκB……Nuclear transcription Factor κB

NO……Nitric Oxide

NOS……. Nitric Oxide Synthase

PC…….Preconditioning

PIP_2…….. Phosphatidylinositol 4,5-bisphosphate

PKA……. Protein kinase A

PKC……. Protein kinase C

PLC…….. Phospholipase C

PLD……..Phospholipase D

PI……..Phosphatidylinositol

PI-3-kinase……Phospho inositide –3- Kinase

PUFA……Polyunsaturated fatty acid

R…….. Reperfusion

ROS……..Reactive oxygen species

SOD……..Superoxide dismutase

TRF…….. Tocotrienol Rich Fraction

TUNEL…… Terminal deoxynucleotidyl transferase dUTP nick-end labeling

The heart is one of the most important organs in our body whose existence was well known from long back. The Greeks named it initially as “Kardia”, and then the Roman modified it to “Cor”. Finally it got its name “Heart” via Mediaval English – “Heorte”. Galen, the “Father of experimental physiology”, discovered that arteries carry blood & not air, by the continuous motion of pumping of the heart. Heart, is not just a pump, but it can modify the flow of the blood depending on the various performances of our body.

Since cardiovascular disease contributes in a major way to the morbidity and mortality, it is becoming a strain on the economy of many countries worldwide. Although various factors have been identified as possible causes of different cardiac diseases such as heart failure and ischemic heart disease, there is a real need to elucidate their role for the better understanding of the cardiac disease pathology and formulation of strategies for developing newer therapeutic interventions. These include myocardial shunts, valvular disease, long-standing hypertension, intracardiac or intravascular shunts, cardiac arrhythmias and hyperthyroidism, as well as other causes. Perhaps the most common cause of heart failure due to systolic and diastolic dysfunction is ischemic heart disease.

1.1. Ischemic Heart Disease:

Ischemia is a stage when there is no blood flow in a cell; as blood is the only carrier of air or oxygen, that cell is undergoing a lot of stress due to lack of oxygen. When this kind of situation arises in the heart, that disease is known as Ischemia Heart Disease. Ischemic heart disease is also known as Coronary Artery Disease, because the two coronary arteries are the major suppler of the blood in the myocardium. This disease occur due to the deposition of cholesterol plaques on the wall of these blood vessels and make those vessels narrow and ultimately getting block by those fatty deposit. This process is called Atherosclerosis, which leads ischemia to the heart muscle and this can cause damage to the heart muscle. The complete blockage of the arteries leads to myocardial infraction (MI). According to the World Health Organization, the major cause of death in the world as a whole by the year 2020 will be acute coronary occlusion (81).

Myocardial ischemia occurs when there is an imbalance between requirements and the availability of blood and oxygen in the myocardial cells. Apart from atherosclerosis, there are numerous risk factors are involved for this Myocardial Ischemia and Hypoxia. Among them smoking, diabetes mellitus, Hypercholesterolaemia, severe hypotension as in shock, aortic stenosis, Genetic and hereditary factors may also be responsible for the disease. Severe anemia may be an additional factor.

Angina pectoris is the primary symptom of ischemic heart disease, caused by transient episodes of ischemia, which is the imbalance in the myocardial oxygen supply-demand relationship and increase in myocardial oxygen demand (determined by heart rate, ventricular wall tension and ventricular contractility), by a decrease in myocardial oxygen supply (determined by coronary blood flow) (1-3).

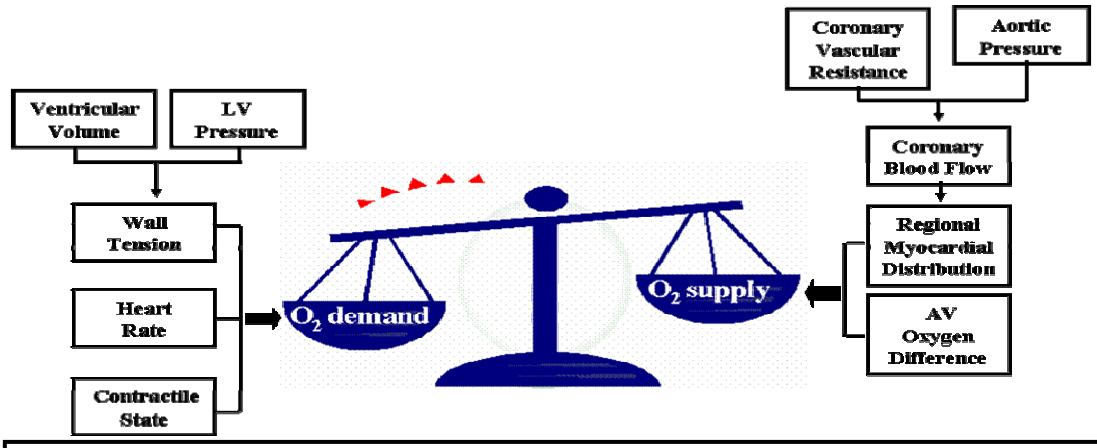


Fig. 1. Ischemic periods: an imbalance in the myocardial oxygen demand and supply relationship.

1.1.1. Reversible Injury

Hypoxia can cause the cell's aerobic respiration, i.e., oxidative phosphorylation of mitochondria that slows down the generation of ATP in the cells. Therefore, the decreased activity of ATPase is observed in the cell membrane, causing failure of the active membrane "Sodium Pump". That leads to accumulation of sodium ions intracellular and diffusion of the potassium ions out of the cell. The net gain of solute is accompanied by an iso-osmotic gain in water, producing acute cellular swelling (4).

The decrease of cellular ATP and simultaneously increase of AMP, stimulate the enzyme phosphofructokinase, which increase the rate of anaerobic glycolysis to maintain the cell energy by producing ATP from glycogen. Thus, rapidly glycogen is

decreased from the cells. This also results in reducing intracellular pH, by accumulation of lactic acid and inorganic phosphates from the hydrolysis of phosphate esters.

The next phenomenon is detachment of ribosomes from the granular endoplasmic reticulum and dissociation of polysomes into monosomes. If hypoxia continues, other alterations take place and, again, are reflections of increased membrane permeability and diminished mitochondrial function. Blebs may form at the cell surface. "Myelin figures", derived from the plasma as well as organelles membranes, are seen within the cytoplasm or extracellularly. At that point, the mitochondria appear normal, slightly swollen; the endoplasmic reticulum is dilated; the entire cell is markedly swollen.

1.1.2. Irreversible Injury

Irreversible injury is associated with vacuolization of the mitochondria, including their cristae; and finally damage in plasma membranes, and swelling of lysosomes. By reperfusion of the ischemic zone massive calcium influx happen into the cell. Amorphous, calcium-rich densities develop in the mitochondrial matrix, which cause continued loss of proteins, essential coenzymes and ribonucleic acids from the hyperpermeable membranes. The cell may also leak metabolites, which are vital for the reconstitution of ATP, thus further depleting net intracellular high-energy phosphates.

Lysosomal membranes get injured due the falling pH, followed by leakage of the cellular enzymes into the cytoplasm, activation of acid hydrolases, and enzymatic digestion of cytoplasmic and nuclear components.

After cell death, cell organelles are progressively degraded and create an empty space. The extracellular macromolecules of the cellular enzymes then enter through the interstitial space as well as through the leakage portion into the dead cell. Finally, the dead cell may become replaced by large masses, composed of phospholipids, in the form of "myelin figures". These are then either phagocytosed by other cells or degraded further into fatty acids.

Mitochondrial Dysfunction and ATP Depletion: During reperfusion, lack of oxidative phosphorylation and ATP generation can cause the inability to reverse mitochondrial dysfunction. The other phenomenon is the development of profound disturbances in membrane function.

Cell Membrane Damage: Loss of volume regulation, increased permeability to extracellular molecules such as insulin, and demonstrable plasma membrane ultra-structural defects occur in the earliest stages of this injury.

At least four potential cause have been found in the membrane damage

1. Progressive loss of membrane phospholipids.
2. Cytoskeletal abnormalities.
3. Toxic oxygen free radicals.
4. Lipid breakdown products.

Membrane injury cause the loss of membrane integrity which further cause influx of calcium from the extracellular space, where it is present in high concentrations (>10 mM), into the cells.

1.1.3. Cardiac arrhythmias

Acute myocardial infraction leads to a lethal condition called cardiac arrhythmias (5). Amongst Ventricular Tachycardia (VT) or and Ventricular Fibrillation (VF), VF is considered the major cause of premature death, called sudden cardiac death, both in North America (6) as well as in U.K (7). VF may occur within a min or hour or months after the onset of angina pectoris. VF can occur in two separate phases, first reversible injury and then the irreversible injury which leads to the infarct zone (8-10).

The mechanism for cardiac arrhythmias is not very clear yet, there is considerable controversy going on among the researchers (11, 12). Calcium overload (13), Sodium overload (14) and oxidative stress due to free radical formation (15) are the major regulator of fetal cardiac arrhythmias. Although evidence exists for a role for oxygen free radicals in the pathogenesis of reperfusion-induced arrhythmias some of them may be circumstantial, controversial or open to alternative interpretation (19). Recent studies showed the various intracellular mechanisms by the generation of free radical leads to the cardiac arrhythmias. Therefore now-a-days oxidative stress is considered as the major cause of cardiac arrhythmias (16), this has been well established by various means, like using PBN (N-tert-butyl-alpha-phenylnitrone), an organic spin trap agent designed specifically to form "stable" adducts with free radicals in electron spin resonance studies (17) or by using free radical spin trap DMPO (5,5-dimethyl-1-pyrroline-N-oxide) (18). In another study, by using superoxide dismutase (SOD), catalase, EGB 761 (Tanakan), and their combination showed significant

decrease in VF as well as VT by almost half as compared to the control values, at the same time formation of oxygen free radicals were also studied after 30 min of global ischemia followed by reperfusion in isolated rat hearts by using spin trap DMPO (20).

1.2. Cardiac Oxidative Stress

Although reperfusion of the ischemic myocardium during early stages is essential to prevent cardiac damage, reperfusion of the ischemic heart after a certain critical period has been reported to have deleterious effects due to the generation of reactive oxygen species (ROS) (21-25). Ischemia reperfusion injury may also occur after the termination of an anginal attack, there is vasospasm, platelet aggregation or collateral blood flow perfusion. Since myocardial ischemia has been shown to serve as an initial signal for the development of acute and chronic heart failure at later stages, it is believed that oxidative stress plays a significant role in different types of cardiac diseases (26-28). In fact, the involvement of ROS in ischemia-reperfusion injury has been shown directly by employing electron para-magnetic resonance spectroscopy (29, 30), spin trap [alpha]-phenyl-*N*-*tert*-butylnitrone (31) and luminal-enhanced ter-butyl-initiated chemiluminescence (32). This view is further substantiated upon observing the beneficial effects of antioxidants in hearts subjected to ischemia-reperfusion (20, 33). This oxidative stress-induced cellular damage has been estimated by measuring the levels of lipid peroxidation through different detection methods involving malondialdehyde (MDA) or thiobarbituric acid (34). Moreover, exposure of the heart or subcellular organelles to oxyradical generating systems has been reported to produce effects similar to those observed in hearts subjected to ischemia-reperfusion (35, 36).

It should be noted that small amounts of ROS are normally formed during mitochondrial respiration. On the other hand, during ischemia, the mitochondrial carriers are in a reduced state due to the degradation of the adenine nucleotide pool (37). The increase in electron leakage from the respiratory chain leads to increased formation of $O_2^{\bullet-}$ due to the interaction with molecular oxygen trapped within the inner membrane of the mitochondria. During reperfusion, there will be further leakage of electrons due to the lack of ADP resulting in increased production of $O_2^{\bullet-}$. Additionally, during ischemia, activated neutrophils secrete $O_2^{\bullet-}$ as well as oxidative and hydrolytic enzymes (38), and when plugged in the capillary bed, exacerbate the ischemic injury (39). The

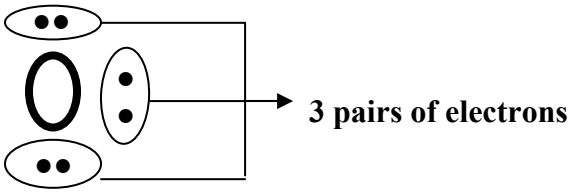
auto-oxidation of catecholamines in the ischemic myocardium also participates in the increased levels of ROS due to the formation of amino-chromes (40).

In ischemic-reperfused hearts, the increase in oxidative stress was observed to correlate well with cardiac dysfunction (16), a decrease in the antioxidant defense mechanisms (41) and an increase in lipid peroxidation (34), leading to increased membrane permeability. An increase in the levels of MDA and decreased activities of superoxide dismutase (SOD) and catalase have been reported in hearts exposed to 30 min of ischemia (32). Similar increases in the oxidative stress level were observed in the ischemic-reperfused hearts with normal levels of antioxidant activities. Regional differences were observed in the glutathione peroxidase levels, which were normal in the left ventricle of the ischemic and reperfused heart, but were increased in the right ventricle (32). Hearts treated with SOD plus catalase showed a decrease in infarct size (42), an improvement in cardiac function (20) and sarcoplasmic reticular regulatory function associated with Ca^{2+}/calmodulin protein kinase (43). The depressed SL Ca^{2+}-pump, Na^{+}–Ca^{2+} exchange and Na^{+}–K^{+} ATPase activities in the ischemia-reperfused hearts were also prevented with a combination of SOD plus catalase (44).

Overexpression of Mn-SOD in transgenic mice demonstrated a decrease in the ischemia-reperfusion injury as reflected by improved cardiac performance and decreased lactate dehydrogenase release (45). A similar protection was also reported in conscious rabbits genetically treated with adenovirus-mediated SOD transfer (46). Hearse et al (47) suggested a link between reperfusion ventricular fibrillation and the generation of free radicals. SR dysfunction due to excessive amounts of free radicals generated during reperfusion leading to Ca^{2+}-overload has been suggested to be a possible mechanism for the arrhythmias (47). Depression in the L-type Ca^{2+}-channel density, Na^{+}–Ca^{2+} exchange and Ca^{2+}-pump ATPase activities have also been reported in ischemic heart disease (48, 49). Other abnormalities include defects in the SL superficial stores of Ca^{2+}, depressed SL Na^{+}–K^{+} ATPase (50), and decreased myofilament responsiveness to Ca^{2+} due to thiol group oxidation (51) and inhibition of the myofibrillar creatine kinase activity (52). These results are consistent with the view that oxidative stress may result in the occurrence of subsequent arrrhythmias, myocardial cell damage and cardiac dysfunction due to ischemia-reperfusion injury (16).

1.3. ROS (Reactive Oxygen Species)

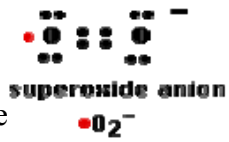
From the atomic structure of oxygen atoms we know that the outermost shell (L-shell) of o-atoms contains six (6) electrons that mean three (3) pair of electrons.

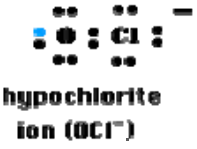


Formation of ROS:

Reactive Oxygen Species are formed in normal cellular process in minute amount by several different mechanisms:

1. Interaction of ionizing radiation with biological molecules
2. Unavoidable byproduct of cellular respiration
3. Synthesized by dedicated enzymes in phagocytic cells like neutrophils and macrophages
    - NADPH oxidase (Both phagocytes)
    - Myeloperoxide (neutrophils only)
4. Electrons transport chains
5. Cytochrom P-450 Reaction
6. UV radiation Reaction





Reactive Oxygen Species are:

1. Molecules like hydrogen peroxide.
2. Ions like the hypochlorite ion.
3. Radicals like the hydroxyl radical.
4. Superoxide anion.

By losing electron from the O-atoms paired electronic configuration genarates unpaired electronic configuration. Whenever there is an unpaired electron in the outermost shell that is known as radical and that atom becomes reactive. That's why it is called ROS or "free radicals."

1.4. Redox Signaling

ROS are produced endogenously as a consequence of normal cell functions or derived from external sources. They pose a threat to cells living in an aerobic environment because they can result in DNA, protein and lipid damage. As mentioned previously, ROS also play an important role in the pathophysiology of many diseases, including ischemic heart disease. All cells contain a number of antioxidant defense mechanisms to minimize fluctuations in ROS, however, when ROS generation exceeds a cell's antioxidant capacity, the result is a condition known as oxidative stress. A host's survival then depends upon the ability of its cells and tissues to either adapt to or resist this stress. A number of stress response mechanisms have evolved to protect cells from oxidative insult and these mechanisms are rapidly activated. Some of these pathways are preferentially linked to enhanced survival, while others are associated with cell death. In ischemic heart disease, ROS, along with abnormal lipid metabolism and calcium homeostasis, gives rise to the "death signal" resulting in apoptotic cell death that leads to an infracted heart. The mammalian heart is also protected against ischemic injury by several lines of defense. The first line of defense consists of intracellular antioxidants such as superoxide dismutase, catalase and other protective enzyme systems. Recently, it has become apparent that the heart produces oxidative stress-inducible proteins in an attempt to counteract the invading ROS and that these proteins can also function as a defense system. I-R injury is likewise associated with the induction of a number of both pro- and anti- apoptotic genes and transcription factors (53).

The finding of the production of ROS during the agonist-induced activation of NF-κB provided the first concrete evidence for the role of ROS as a second messenger. NF-κB regulates the inducible expression of a number of genes involved both in cell survival and death. For example NF-κB has been found to regulate the anti-apoptotic gene, Bcl-2, and the pro-apoptotic factors, bax and p53, in the ischemic/reperfused myocardium (53).

At the cellular level oxidative stress gives rise to a variety of responses, which range from proliferation to growth arrest to cell death. The final outcome varies depending on the cell type, as well as the dose of the ROS. The final observed effect is a balance between a varieties of intracellular stress signaling pathways that have been activated in response to oxidative stress. These pathways modulate transcription factors

and enzymes which then induce changes in gene expression (Fig. 2). Some of these pathways are linked directly to enhanced survival while others are known to produce cell death. A variety of other pathways produce an effect depending on the circumstances.

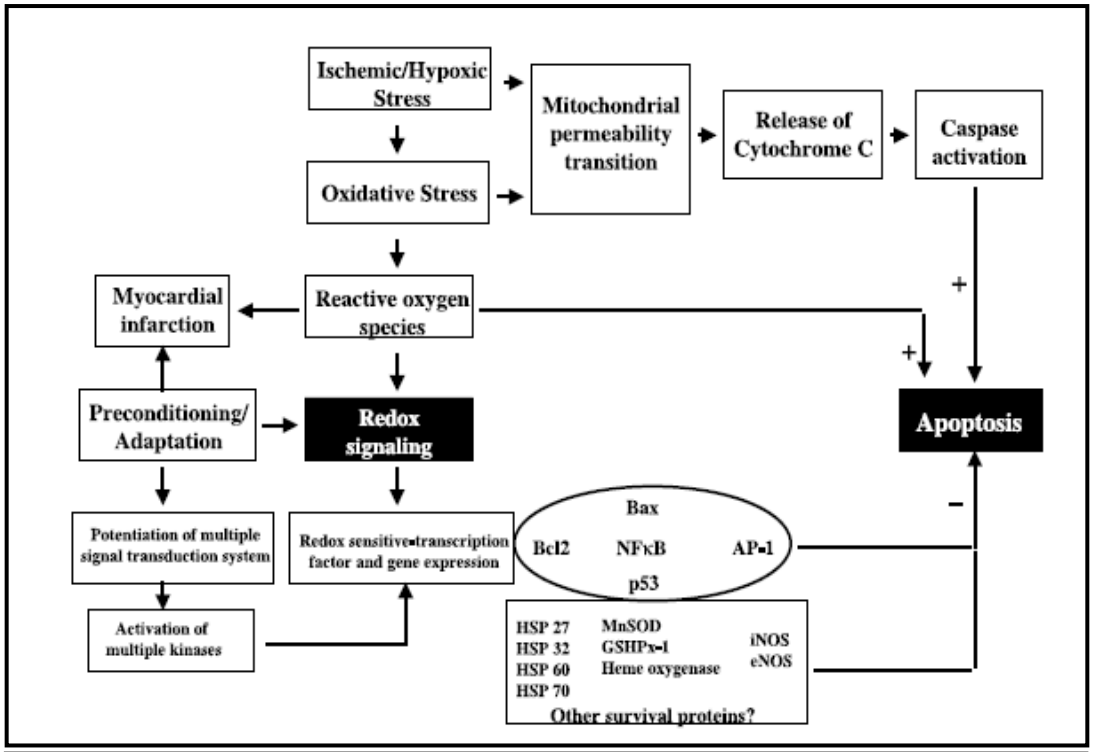


Fig. 2. REDOX regulated survival signal

1.4.3. Death and survival machinery

Bcl-2 is an anti-death gene that functions as an intracellular antioxidant. Recent analysis of the Bcl-2 gene family reveals an intricate network that regulates apoptosis. Within this Bcl-2 gene family, some members suppress apoptosis while others can induce apoptosis (54). Among the proteins coded by the genes of this family, Bcl-2 and Bcl-x_L act as cell death repressors (55), while Bax and an alternatively sliced Bcl-x product, Bcl-xs, promote cell death (56). When in excess of Bcl-2, Bax counteracts the repressive action of Bcl-2 on apoptosis. Likewise, excess Bcl-xs antagonizes the function of BCL-x_L. Therefore, a critical balance between the Bcl-2, Bax, and Bcl-$x_{L/S}$ molecules may determine the fate of cells in response to cytotoxic agents, or environmental or oxidative stress. It has also been reported that p53 activates the transcription of the Bax gene via p53-response elements while downregulating the expression of Bcl-2 (57).

Overexpression of Bcl-2 can block apoptosis initiated by several stimuli. For instance, the activation of Bcl-2 was associated with the inhibition of apoptosis (58, 59). By inducing Bcl-2 expression and reducing Bax expression, preconditioning converts the death signal triggered by ischemia/reperfusion (which decreases Bcl-2 and increases Bax) into survival signal.

1.4.2. PKC signaling

PKC is a family of at least 11 phospholipid-dependent serine-threonine kinases. There is enough evidence that a variety of PKC isoforms also act as major modulators of the myocyte death machinery, having both pro- and anti-apoptotic effects. A great majority of these have been identified in the mammalian heart (60). PKC are susceptible to redox regulation and various antioxidants can inhibit PKC-dependent cellular responses. Both pro-survival and apoptotic functions for PKC during oxidative stress have been described (60). The 11 enzymes of the PKC family are often broken down into three distinct subgroups: classical PKCs (α, βI, βII, γ), which are activated by calcium and diacylglycerol (DAG); novel PKCs (δ, ε, η, θ), which are activated by DAG but not calcium; and finally the atypical PKCs (λ/ι, ζ), which are insensitive to both DAG and calcium, but are activated by distinct lipids (60). The classical pathway for PKC activation is by G-protein coupled receptors, which through hydrolysis of phospholipids elevate intracellular levels of DAG and calcium (61). However, PKC activation is not limited to seven transmembrane receptors, as growth factor receptors, nitric oxide, and ROS can also activate select PKC isoforms in the myocardium (60). PKC activation is associated with translocation to different subcellular compartments, mediated by interaction of the kinases with proteins termed as receptors for activated C kinase or RACKs (62). The discovery that each PKC isoform binds to its own specific RACK has proved extremely useful as it has allowed the generation of small peptides that either facilitate or abrogate this interaction, and therefore the activation of individual PKC isoforms is possible.

Select PKC isoforms have been shown to regulate cardiac hypertrophy, calcium handling, and contractile protein function. In this regard, most attention has focused upon two PKC isoforms, specifically PKC δ and PKC ε, which although exhibiting very similar structures, have disparate effects on myocyte survival and death. Studies in non-cardiac cell lines have demonstrated a pro-death action for PKC δ (63), and the same

seems to be true in cardiac myocytes. In one study (64), it has been demonstrated that adenoviral infection with constitutively active PKC δ, but not PKC ε, directly induced apoptosis in rat ventricular myocytes. Consistent with this observation, inhibition of PKC δ translocation attenuated hyperglycemia-induced DNA laddering and TUNEL (Terminal deoxy nucleotidyl transferase Biotin-dUTP Nick End Labeling) staining (60). Therefore activation of PKC δ appears to contribute to ischemic injury in cardiac myocytes. For example, using small peptides that specifically regulate PKC δ, it has been demonstrated that activation of PKC δ exacerbates ischemia-reperfusion-induced injury in rat myocytes (60), whereas inhibition of PKC δ translocation blocks ischemia-induced apoptosis in isolated rat heart (60). Conversely, PKC ε appears to exert an anti-apoptotic effect in myocytes. Inhibition of PKC ε translocation attenuated both hypoxia and phorbol-induced protection against simulated ischemia in neonatal myocytes (65). Likewise, activation of PKC ε was associated with the ability of ischemic preconditioning to block ischemia-induced apoptosis (60). Therefore the majority of the *in vitro* data implicate PKC δ as being pro-apoptotic and PKCε as being anti-apoptotic in myocytes, especially in the context of ischemia-reperfusion injury.

The hypothesis that PKC δ is pro-apoptotic while PKC ε is anti-apoptotic is also supported by a number of *in vivo* reports. Intra-coronary administration of a PKC δ translocation inhibitor peptide was able to significantly attenuated ischemia-reperfusion-induced infarction and caspase-3 cleavage in the pig (66). Transgenic expression of the same PKC δ inhibitory peptide also blocked ischemia-reperfusion-induced injury in the mouse, whereas overexpression of a PKC δ activator peptide augmented ischemic injury (67, 68). In contrast, transgenic expression of a PKC ε activator peptide was able to blunt apoptosis and therefore heart failure in $G\alpha_q$-overexpressing transgenic mice (69), while expression of a PKC ε inhibitory peptide had the opposite effect. Moreover, transgene-mediated activation of PKC ε protected the myocardium against ischemia-reperfusion injury (67, 69).

The potential mechanisms by which two such structurally similar kinases could have opposing effects on the apoptotic process are still unclear. One possibility is that signal specificity lies in the signaling events downstream of each PKC isoform. For example, in myocytes, adenoviral PKC δ was found to selectively activate the JNK and p38 MAPK pathways, whereas PKC ε activated the ERK pathway (60). Given the proposed pro-apoptotic actions of JNK/p38 versus the anti-apoptotic actions of ERK, such differential signaling may account for the different survival/death effects of each

PKC isoform. Another potential protective mechanism was suggested by the observation that PKCε-dependent cardioprotection was associated with activation of the anti-apoptotic kinase Akt (70). Differences may also exist as to how each kinase might affect members of the pro-death and pro-survival pathways that directly impact the mitochondria. Both PKC δ and PKC ε have been localized to the mitochondria in the heart (60). Indeed, translocation of PKC δ to the mitochondrion has been shown to be essential for apoptosis in a variety of cell lines and is associated with mitochondrial permeability transition and activation of the mitochondrial death pathway (60). In contrast, PKC ε can prevent mitochondrial-mediated cell death (71) through a mechanism that appears to involve association with and inhibition of mitochondrial pore opening, and is associated with protection against ischemic injury in mice expressing active PKC ε (72). Whether each PKC isoform can directly affect the pore itself, potentially through direct phosphorylation, or whether it is through secondary effectors such as JNK or ERK remains to be tested. The ability of PKC isoforms to affect mitochondrial-dependent apoptosis may also be related to modulation of Bcl-2-family proteins. PKC ε has been reported to bind to and inhibit the pro-apoptotic protein Bax (60), and PKC ε transgenesis is associated with phosphorylation of Bad (73). On the other hand, ischemia-reperfusion-induced dephosphorylation, and presumably activation of Bad was prevented by PKC δ inhibition (60).

In summary, according to recent investigations, specific mechanisms have been identified whereby PKC δ and PKC ε might antithetically regulate cardiac myocyte apoptosis.

1.4.3. Thioredoxin regulation survival machinery

From the biochemical point of view of redox signaling, it is clear that the oxidizing conditions are maintained by the stabilizing disulfides in the extracellular surface, while the intracellular environment is maintained in the reduced state with the help of free sulfhydryl groups. The principle disulfide reductase responsible for maintaining intracellular milieu in the reduced state is a low molecular weight redox-active protein with two cysteine residues in its active sites, thioredoxin (74). Thioredoxin appears to play a crucial role in the redox regulation of the ROS signaling during and/or following ischemia/reperfusion. Thioredoxin is ubiquitously present in mammalian cells including hearts. They possess dithiol/disulfide active site and can

serve as electron donors for enzymes including thioredoxin peroxidases and ribonucleotide reductases (75). There are numerous studies, which showed that the thioredoxin is induced by the oxidative stress (75). There are two major thioredoxins: Trx1, a cytosolic and nuclear form, and thioredoxin 2 (Trx2), a mitochondrial form. Trx1 is the major thioredoxin-redox protein, which is responsible for most of the biological signals for thioredoxin including the supply of reducing equivalents to thioredoxin peroxidases and ribonucleotide reductase, and regulation of transcription factor activity (75).

There are many beneficial effects of thioredoxin which have been shown by various researchers, like thioredoxin may be an important component of the cellular defense against cardiac injury (76) or oxidized thioredoxin was found to be released into plasma of the patients undergoing cardiopulmonary bypass surgery (77) or endurance training by swimming accompanied by a reduction of ischemia/reperfusion-induced oxidative stress with a concomitant increase in thioredoxin reductase resulted in a protection against myocardial ischemia/reperfusion injury (78) or human thioredoxin attenuated hypoxia-reoxygenation injury of murine endothelial cells in a thiol-free condition suggesting thioredoxin protection of myocardial injury through a novel redox-signaling pathway (79). Thioredoxin, thus, plays a crucial role in thiol-redox control of cell function through transcription regulation of target genes including NFκB, which control numerous gene expressions. Several recent studies have demonstrated an increase in DNA binding of NFκB and AP-1 in the ischemic/reperfused myocardium (75). A recent study demonstrated a reduction of thioredoxin-1 (Trx1) protein in the ischemic reperfused myocardium. When the same heart was adapted to ischemic stress by preconditioning with repeated cyclic episodes of small duration of ischemia and reperfusion, there was an increased induction of Trx1 expression. Inhibition of Trx1 expression resulted in reduced postischemic ventricular recovery and increased myocardial infarct size in the preconditioned heart (80).

Therefore in summary we can say that the thioredoxin may be one of the factors which precondition the heart by redox signaling mechanism.

1.6. Ischemic Preconditioning

Interestingly it has been found that patients with one or few episode of angina pectoris earlier could make the heart more tolerant from this deadly condition. After more than 15 years of extensive research work, researcher finally can explain this fact. The underline cause of this fact they termed as “Ischemic Preconditioning”. Ischemic preconditioning is the protective adaptive phenomenon produced by short periods of ischemic stress resulting in a marked, albeit temporary, resistance of the myocardium to a subsequent more prolonged period of that same insult (82). It was first described by Murry and colleagues in 1986 showing that preconditioning the dog heart with four brief periods of ischemia, each 5 min in duration, caused the heart to tolerate a subsequent 40-min ischemic insult with less fraction of the infraction than that realized in nonpreconditioned hearts (83). Since this report, many other researchers have demonstrated the profound protective effect that can be achieved with either a single short cycle of ischemia and reperfusion or a number of such cycles preceding a prolonged ischemic episode (84).

The basis for the protection of ischemic preconditioning of the myocardium is that short periods of ischemic stress do not cause irreversible cell injury but paradoxically to an adaptive mechanism within the heart that leads to resistance to a subsequent ischemic insult. Two different phases of protection following the preconditioning stimulus are proposed. The first phase occurs within minutes, and is extremely profound but transient and the delayed phase (also known as the second window of protection), takes hours to become apparent and can last for days or even longer periods. The mechanism of preconditioning is not completely understood. The most possible mechanisms for this protection involve the release of endogenous substances from the ischemic myocardium (for example adenosine, bradykinin, nitric oxide, prostacyclin, norepinephrine, and opioids) with the possible involvement of G_i protein, ATP-sensitive K^+ channels (K_{ATP}), and protein kinase C (PKC).

There are growing evidences to imply that the duration and number of cycles of ischemia and reperfusion necessary to confer protection may vary between species and the end point analyzed. Preconditioning protection includes reduced tissue necrosis, attenuated life-threatening arrhythmias and recovery of contractile dysfunction.

1.5.1. Characteristics of Preconditioning

Murry reported in 1986 that short preconditioning periods of ischemia reduced the severity of myocyte necrosis related to a subsequent prolonged occlusion and reperfusion of the same coronary artery (83). This remarkable decrease in infarct size by coronary artery occlusions has also been demonstrated in vivo even in species with a low collateral coronary blood flow such as rabbits and rats (84). In addition, it is proved by Schott and colleagues that ischemic preconditioning reduces infarct size in swine myocardium (85).

There is no concrete evidence where preconditioning decreases stunning effect. But in one or two days stunned myocardium recovers fully. Stunning effect is the loss of contractility just immediately after ischemia. Ovize et al. showed that after 15 min or less time of ischemia the heart stuns but there is no infraction (86), in most of the species. Landymore et al. (87) noted that in sheep myocardium preconditioning effect attenuates stunning effect. Finally Sekili et al. (88) as well as Goto et al. (89) revealed that adenosine release is one of the major factors, which plays an important role during preconditioning, which decrease the stunning effect. More recent study (90) also showed adenosine A_1 and A_{2a} receptors take important role in preconditioning of myocardial stunning.

The incidence of ventricular arrhythmias in preconditioning group was reduced significantly compared with non-preconditioning group. This antiarrhythmic protection is pronounced, but transient and it might be even more important feature of ischemic tolerance. This anti arrhythmic protective effect with preconditioning could be due to the reduced intracellular calcium release and accumulation with decreased inositol (1,4,5) triphosphate release (91). Other possible mechanisms may be involved, such as slow energy metabolism, opening of K^+ channels, and reduced free radical production by preserving myocardial ATP (92) and more recent study showed during Antiarrhythmic effect of ischemic preconditioning, bradykinin and sarcolemmal play an important role (93).

Preconditioning can lead to improved recovery of cardiac contractile function following reperfusion of the ischemic myocardium, except that the ischemia has caused very marked functional deterioration. Preconditioning greatly accelerates the time of onset of contracture and also increases the degree of contracture (94). After reperfusion, the developed pressure is significantly greater in preconditioned hearts. There is

evidence for improved contractile function as the rate of contraction (+dp/dt) is significantly higher in preconditioned rat hearts, the same as the rate of relaxation (-dp/dt) (95). There are several studies with isolated rat heart (96) where postischemic cardiac function commonly used as an end point to show the cardioprotective effect of the ischemic preconditioning. The functional protection achieved by preconditioning appears to be in part due to a reduction in myocardial stunning and a reduction in cell death.

1.5.2. Possible Mechanisms

There are now several proposed mechanisms for the initiation of preconditioning induced protection: adenosine receptor and α_1-receptor stimulation, involvement of G protein and K^+ channel, release of endothelium derived mediators, low metabolism and protein kinase C stimulation. However, evidence in every species or against every index of injury cannot imply the same mechanisms.

One of the possible mechanisms of preconditioning is around the rate of metabolism during the prolonged ischemic period. The preconditioning protocol itself caused a reduction in ATP depletion and secondly the preconditioning reduced the accumulation of glucose-1-phosphate, glucose-6-phosphate and lactate, suggesting reduced rates of glycogen breakdown and anaerobic glycolysis. In addition, the severity of intracellular acidosis during the sustained ischemic period was significantly less in preconditioned hearts and lactate release in reperfusion in preconditioned hearts was about half of that of control (97). Therefore, it can be concluded that preconditioning may be due to reduced harmful glycolytic intermediates: the reduced glycolytic rate may be responsible for the reduced acidosis during ischemia in the preconditioned hearts and that both preservation of ATP and limitation of the cellular accumulation of catabolites may be responsible for delaying ischemic cell death.

Most important observation is that only exogenous (not endogenous) source of NO initiates preconditioning in both rabbits (98) and in endothelial NO synthase (eNOS) knock out mouse models (99). In addition, Schultz et al. (100) have described the opioids-induced preconditioning via the activation of the δ_1-opioid receptors and a $G_{i/o}$ protein-mediated mechanism. Apart from these pathways, several studies have shown the activation of the p44 isoform of extracelluar signal-regulated kinase subsequent to PKC-δ activation, tyrosine kinases and mitochondrial ATP-sensitive K^+

(K_{ATP}) channels activation in the development of opioid-induced cardioprotection (101, 102). Prostacyclin, which is capable of reducing the severity of ischemia-induced arrhythmias, has also been considered as another possible agent in preconditioning of the heart (103). Since bradykinin infusion in isolated rabbit and rat hearts mimicked preconditioning and administration of bradykinin B_2 receptor blocker abolished the protection effects of I/P on infarction and cardiac function, these observations indicate the involvement of bradykinin receptor activation during IP mediated cardiac protection (104). Bradykinin B_2 receptor activation stimulates phospholipase C mediated release of inositol 1,4,5-trisphosphate and diacylglycerol, which activate PKC leading to cardioprotection in isolated I/R hearts. In addition, opening of mitochondrial K_{ATP} channels have been shown to be associated with bradykinin receptor activation mediated reduction of necrotic cell death in IP hearts (105).

Activation of adenosine receptors, mainly A_1, and A_3, has found to be cardioprotective by virtue of their abilities to precondition the heart (106). Cardiac adenosine A_1 receptors are coupled to adenylate cyclase via inhibitory G protein (G_i) leading to reduced cAMP formation and attenuated phosphorylation through protein kinase A. The adenosine A_1 activated G_i protein is also negatively linked to the Ca^{2+} channel membrane protein and positively linked to K^+ channels.

Two sub-types of α_1–adrenoceptors are found, viz. α_{1A} and α_{1B} which have been demonstrated to be associated with the cardioprotective effect of IP (107). α_1-Adrenergic receptor stimulation has been shown to cause hydrolysis of phosphoinositides resulting in an increased production of $InsP_3$ and diacylglycerol, which activates PKC (108), which leads to cardioprotection through p38 mitogen activated protein kinase (MAPK) signaling. Loubani et al. (109) have shown the involvement of mitochondrial K_{ATP} channels in α_1-adrenergic receptor mediated cardioprotection in human myocardium.

The proposed mechanism of opening K_{ATP} channels in IP was first suggested by Auchampach and colleagues (110) demonstrating that opening these channels with administration of K_{ATP} channel openers mimics preconditioning by reducing infarct size and blocking these channels prevent the effect of both preconditioning and adenosine on reduction of myocardial infarct size and attenuation of reperfusion-induced arrhythmias.

There are several other proposed mechanisms for the preconditioning effect, like activation of different anti-apoptotic isoform of PKC or avtivation of tyrosine kinase or

activation of MAPK or induction of free radical production which then by redox signaling protects the myocardium.

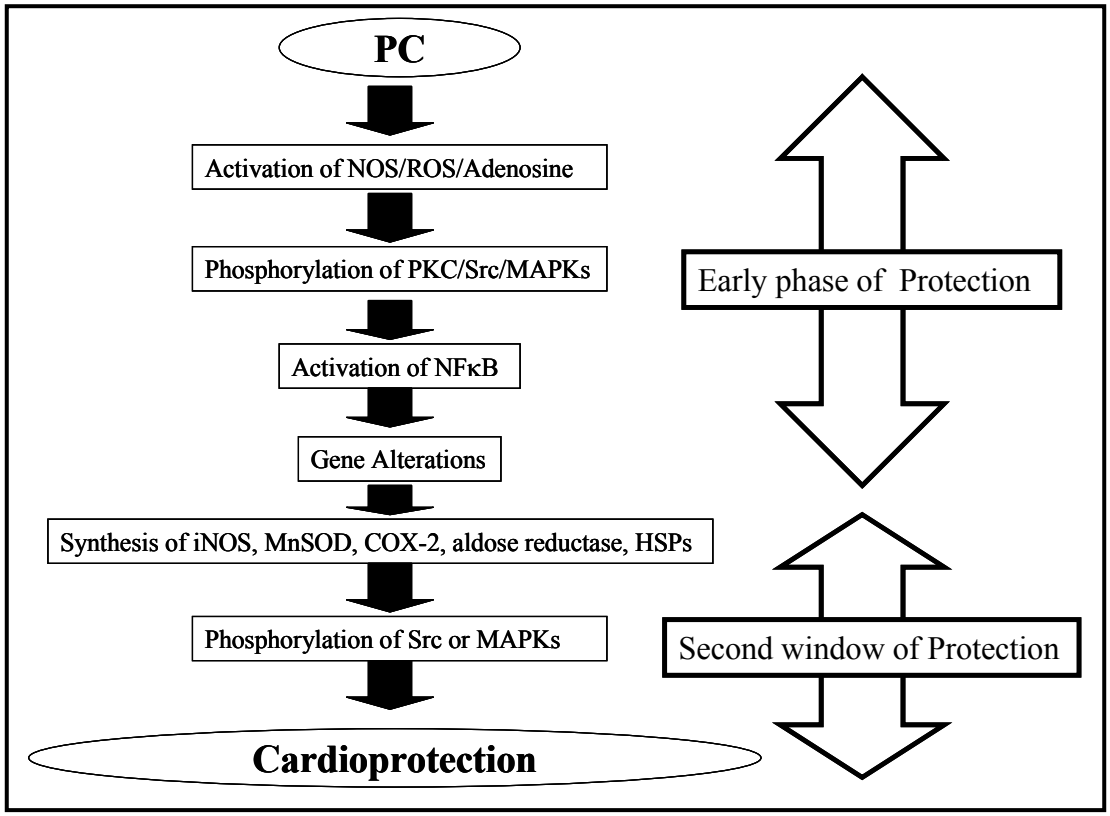


Fig. 3: Schemetic diagram of Ischemic preconditioning, which leads to cardioprotection

1.6. Antioxidants

Antioxidants are the molecules that scavenge the free radicals from the cells, by donating one electron. It also may work as terminator of the free radical genarator by reducing the energy of the free radical or by interrupting an oxidizing chain reaction to minimise the damage cause by the free radicals.

There are several enzymes which normally released from the various tissues, including superoxide dismutase (SOD), catalase and glutathione peroxidase, that neutralise the effect of free radicals. Apart from the natural antioxidant produced from the tissues there are some minerals like Mg, Zn, Se and Cu which act as an antioxidant by inducing the secretion of these enzymes that act as antioxidants. Mg, Zn and Cu acts as a building block (helps to form the enzymes) nutrients for SOD secretion and Se for glutathione peroxidase. In addition to enzymes, many vitamins and some other minerals act as potent antioxidants, such as various polyphenolic compounds like vitamine E,

vitamine C, uric acid, beta-carotene, lutein, lycopene, vitamin B_2, co enzyme Q_{10} and cysteine (an amino acid). Herbs such as bilberry, turmeric, gape seed, pine bark extracts, palm oil, rice bran oil, green tea and ginkgo bilova can also provide powerful antioxidant protection. Another example is NAC (N-acytilecystine), a cell permeable potent antioxidant.

Many centuries ago, Hippocrates the father of modern medicine said "let food be your medicine". Today's medicine is beginning to realize the importance of this statement. Natural sources of antioxidants are getting importance by the researchers, as these are almost free from the side effects, which are very common in case of drugs. Recently, natural polyphenols are getting considerable amount of attention by the various research groups. Polyphenolic antioxidants, chemically, are cyclic benzene compounds including one or more hydroxyl groups connected directly to the ring structure. Polyphenols are categorized into two groups: flavonoids and nonflavonoids. Among the several natural polyphenols, resveratrol, present mainly in red wine and grapes, tocotrienols, present in palm oil and rice bran oil, and green tea extract are considering for the clinical trials for their exceptional pharmacological preconditioning property.

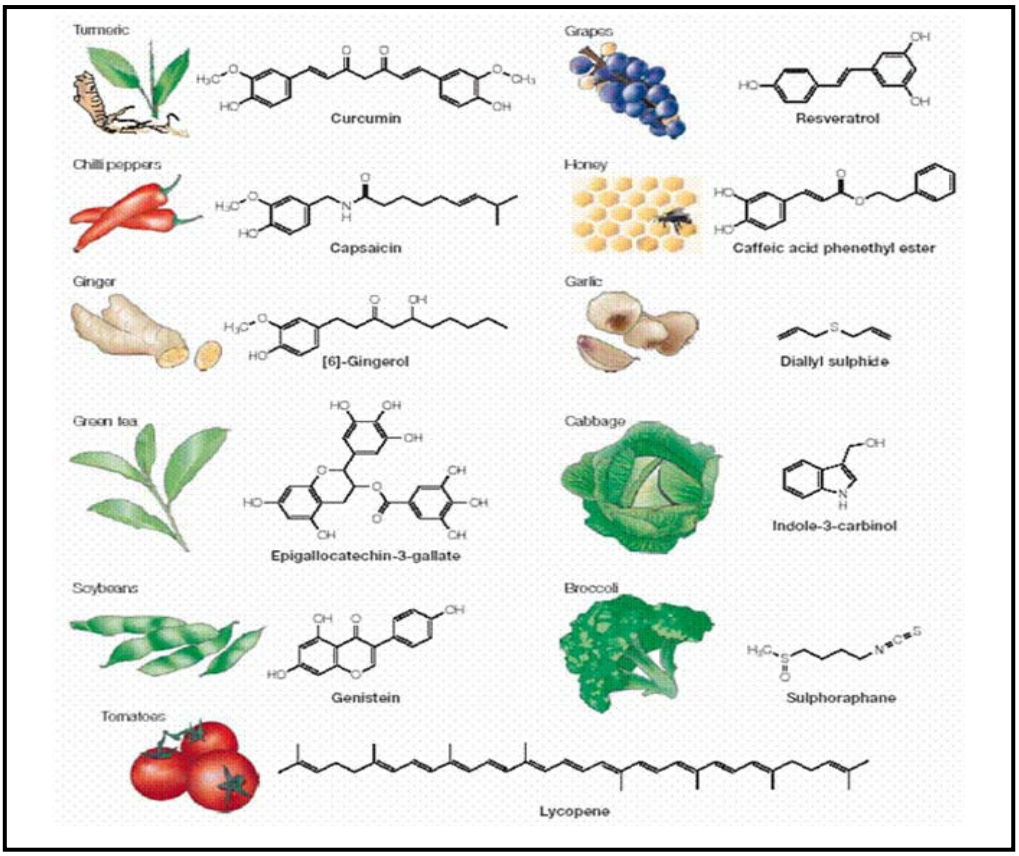


Fig. 4: Examples of some natural polyphenols

1.7. Resveratrol

The current popular propositions about the benefits of "moderate wine drinking" dates back through history and was first proposed by the Father of Medicine, Hippocrates of Kos in Greece. Epidemiological and experimental studies have revealed that mild-to-moderate drinking of wine, particularly red wine, attenuates the cardiovascular, cerebrovascular, and peripheral vascular risk. However, the experimental basis for such health benefits is not fully understood. The cardiovascular benefits of red wine became the hub of research activity after the observation of the 'French paradox' by Renaud and associates who, in 1992, found that there was a low mortality rate from ischemic heart disease among French people despite their high consumption of saturated fats and the prevalence of other risk factors such as smoking. This was attributed to so-called 'Mediterranean diet', which includes a large intake of wine. The cardioprotective effect of wine has been attributed to both components of wine; the alcoholic portion and more importantly, alcohol-free portion containing antioxidants. Wines are manufactured from grapes, which also contain a large variety of antioxidants including resveratrol, catechin, epicatechin and proanthocyanidins. Resveratrol (trans-3, 4', 5-trihydroxystilbene) is mainly found in the grape seeds and grape skin.

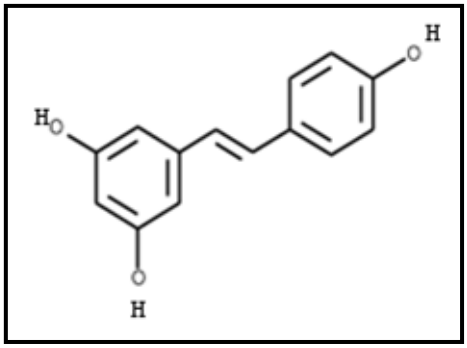


Fig 5: Chemical structure of resveratrol

Resveratrol has been shown to decrease the vulnerability of LDL to endure lipid peroxidation in vivo by several different mechanisms. The first mechanism is where it acts as a free radical scavenger by assuming the role of a reducing agent of hydrogen atom-donating molecules. Another mechanism is by diminishing the capacity of metal to generate free radicals through using phenolics to chelate transition metal ions. Lastly, promotion of LDL associated lipid peroxides and the hydrolysis of arterial cells can be done by protecting or increasing serum paraoxonase activity (111). It can also possibly ease LDL oxidation by reducing macrophage oxidative stress through inhibition of

cellular oxygenases such as 15-lipoxygenase, cytochrome p450, NADPH oxidase, and myeloperoxidase, or even by triggering cellular antioxidants such as the glutathione system. In addition to these resveratrol binds with LDL molecules and are efficiently absorbed, thus protecting LDL from peroxidation. Studies have shown that resveratrol significantly reduces the number of smooth muscle cells (SMCs) in the thickened intima of rabbits with atherosclerosis (111). It is known that resveratrol is able to cause DNA strand breakage in the presence of copper ions (111); Cu (I) is reduced by a complex with Cu (II) that is formed by resveratrol bound to cellular DNA thus causing the redox cycling of copper (111). Eventually, this results in the production of pro-oxidants acting as DNA cleaving agents.

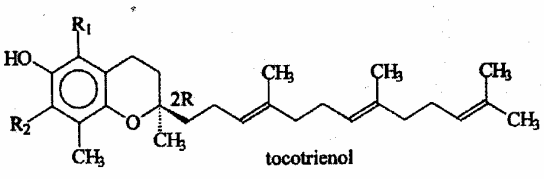
Recently, a number of studies have demonstrated that resveratrol given prior to ischemic arrest could protect the heart from ischemia and/or reperfusion injury (59, 112). The role of nitric oxide (NO) is found to be one of the important mechanisms of pharmacological preconditioning by resveratrol (59). A recent study (113) has suggested that the coordinated up-regulation of iNOS–VEGF–KDR–eNOS, is one of the resveratrol preconditioning mechanisms. Another study (59) has indicated that adenosine receptors have an important function in the resveratrol preconditioning. It has been suggested that adenosine A_1 and A_3 receptors, but not A_{2a} or A_{2b} receptors, play a critical role in the pharmacological preconditioning by resveratrol. Resveratrol likely activates both adenosine A_1 and A_3 receptors which phosphorylate PI-3 Kinase which then phosphorylates protein kinase B (Akt) and thus preconditions the heart by producing NO as well as by the activation of antioxidant transcription factor Bcl-2. In another study, HO-1 has been reported to play an important role in resveratrol preconditioning (114). Apart from this, the same study showed an important cardioprotective effect of resveratrol. Kaga et. al. (114) showed that resveratrol may protect the heart from ischemia-reperfusion injury by angiogenesis; this is due the VEGF up-regulating property of resveratrol (113). Another possible way of cardioprotective effect of resveratrol is due to its up-regulating property of Trx-1 (114). Up-regulation of Trx-1 can protects the heart by various ways, by generating survival signal to reduce the apoptotic cell death or by angiogenesis may be by activating VEGF up-regulation. Resveratrol attenuates various soluble intercellular cytokines like ICAM, VCAM and e-Selectin through improvement in the endothelium function which reduces the infarct size (115). Apart from the REDOX signaling mechanism, resveratrol also protects the heart as a potent antioxidant by scavenging free radicals and inhibiting lipid

peroxidation both in vitro and in vivo (116). Thus, resveratrol inhibits apoptotic cell death as well as release and/or generation of inflammatory mediators. Some of the mechanisms for the cardioprotective effects of resveratrol are depicted.

1.8. Tocotrienols

Vitamin E was discovered in 1922 at Berkeley University in California. It was first found to play a vital role in the fertility of rats. Drs. Evans and Bishop found that rats fed a semi purified diet grew well but in all female pregnancies, pups would die in the womb. When the rats' diet was expanded to include lettuce and wheat germ, healthy pups were born. They called the factor that was lacking in the semi purified diet 'Factor X'. The missing factor was found to be a fat soluble alcohol later named vitamin E. For over a decade the exact composition of vitamin E, referred to as the 'antisterility' vitamin, remained unknown and as a result, research into its potential health benefits was stalled.

The first component identified was α-tocopherol. It was named as such from the Greek *tokos (offspring)* and *pheros (to bear)*. It is the most abundant form of vitamin E found in blood and body tissue. Vitamin E actually refers to a family of eight antioxidants; four tocopherol isomers (α-, β-, γ-, and δ-) and four tocotrienol isomers (α-, β-, γ-, and δ-). Tocotrienols and tocopherols differ from each other in their aliphatic tail. Tocopherols have a saturated side chain where as tocotrienols have unsaturated side chains. The isomers of tocotrienol and tocopherols differ in the methyl substitution on their chromayl ring. In the tocotrienols, α-tocotrienol has 3 methyl groups, β-tocotrienol has 2 methyl groups, γ-tocotrienol has 2 methyl groups, and δ-tocotrienol has one methyl group.



R_1	R_2	
CH_3	CH_3	**α**
CH_3	H	**β**
H	CH_3	**γ**
H	H	**δ**

Fig. 6: Chemical structure of tocotrienol

Vitamin E tocotrienols are abundant in cereal grains including soy beans, oats, rice, bran as well as in palm oil. Recent studies have shown many health benefits of

tocotrienols including anticancer and tumor-suppressive activities as well as inhibition of lipid peroxidation in biological membranes. Dietary tocotrienols are derived from plant sources, mainly palm oil from palm fruits. The tocotrienol-rich fraction (TRF) from palm oil is composed primarily of 26% α-tocopherol, 26% α-tocotrienol, 36% γ-tocotrienol, and 12% δ-tocotrienol (117). Many studies have demonstrated the anticancer properties of tocotrienols. In a study (118), TRF was shown to inhibit proliferation of estrogen receptor-negative MDA-MB-435 human breast cancer cells with 50% inhibitory concentrations (IC_{50}) of 180, 90, 30, and 90 μg/mL, respectively, whereas α-tocopherol had no effect at concentrations up to 500 μg/mL. In further studies, 1:1 combinations of γ- or δ-tocotrienols with tamoxifen were found to inhibit the proliferation of estrogen receptor-positive MCF-7 cells as measured by [^{3}H] thymidine incorporation. Tocotrienols also suppressed the growth of murine b16 melanomas in vitro and in vivo (120). TRF has excellent free radical scavenging capacity (117). Numerous studies (117, 119) show that it is a potent inhibitor of lipid peroxidation and protein peroxidation in rat microsomes and mitochondria. At low concentrations of 5 μM, TRF, mainly γ-tocotrienol and to a lesser extent α- and δ-tocotrienols, significantly inhibited oxidative damage to both lipids and proteins in rat brain mitochondria. Studies on effects of γ-tocotrienols on endothelial nitric oxide synthase (NOS) activity in spontaneously hypertensive rats have reported that upon treatment with antioxidant γ -tocotrienol increased the NO activity and concomitantly reduced the blood pressure and enhanced total antioxidant status in plasma and blood vessels (119). In general, TRF has significantly higher antioxidant ability as compared to tocopherols. This can be explained by the structural difference between the saturated side chain of tocopherols and the unsaturated side chain of tocotrienols. The molecular mobility of polyenoic lipids in the membrane bi-layer (composed mainly of unsaturated fatty acid) is much higher than that of saturated lipids hence tocotrienols are more mobile and less restricted in their interaction with lipid radicals in membranes than tocopherols. This is further supported by the higher effectiveness of tocotrienols in processes that may involve oxidative stress such as in red blood cells where tocotrienols have more potency against oxidative haemolysis than α-tocopherols (117) as well as the better antitumor activity of tocotrienols as compared to α-tocopherols (117). TRF is known to reduce the production of total serum cholesterol. Studies (120) show that TRF reduces concentrations of plasma cholesterol and apolipoprotein B, thromboxane B2 and platelet factor 4 indicating its ability to protect against endothelial dysfunction and

platelet aggregation. In neuronal cells, tocotrienols inhibited glutamate-induced $pp60^{c\text{-}Src}$ kinase activation of HT4 neuronal cells (121). Tocotrienols are implicated in a wide range of neurological diseases, including epilepsy, cerebral ischemia, Huntington's disease and Parkinson's disease by blocking cystine uptake via amino acid transporter Xc^-, resulting in a significant depletion of cellular glutathione (GSH).

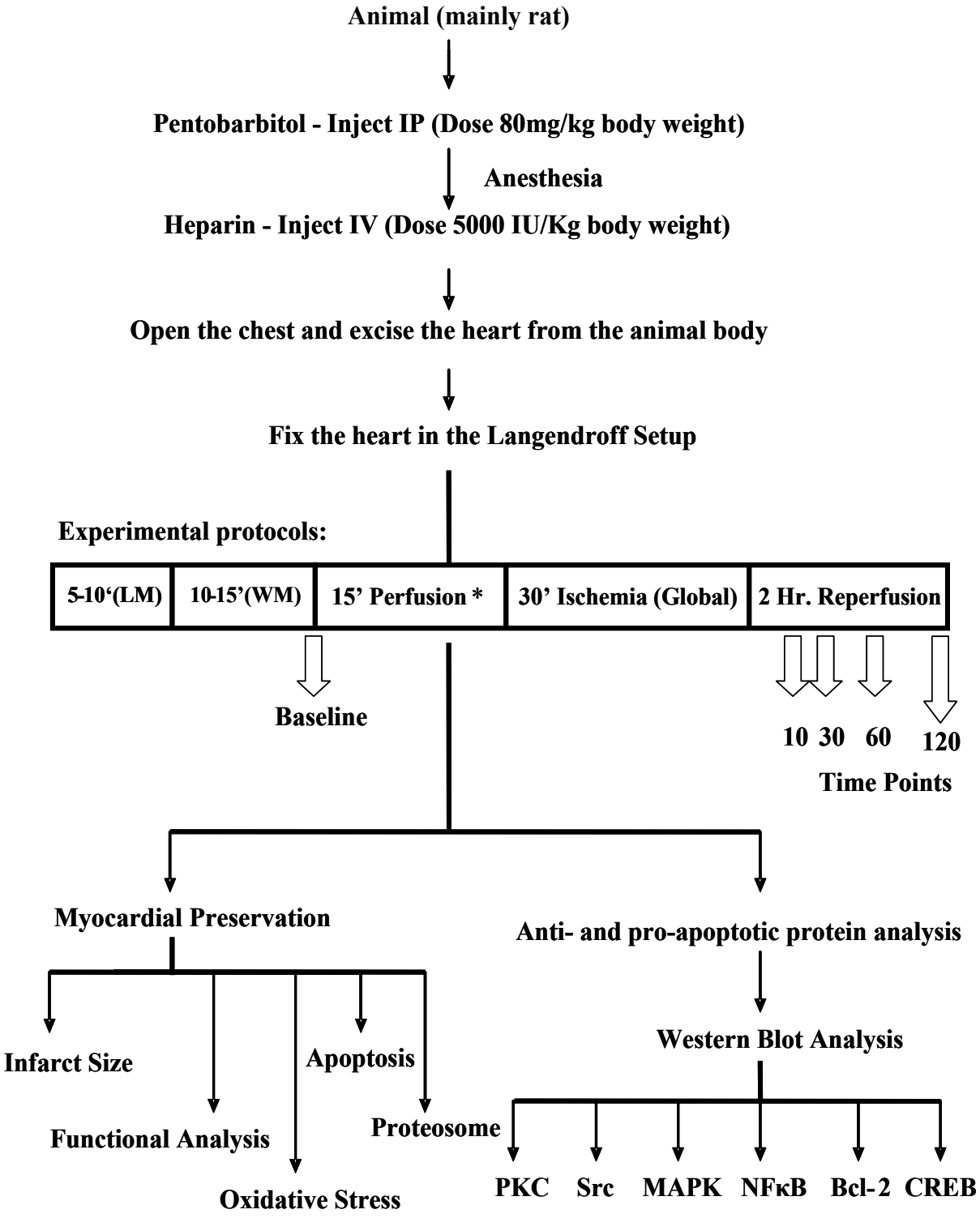
Tocotrienols offer protection against atherosclerosis by preventing LDL oxidation. There are several mechanisms for LDL lowering property of tocotrienols. One of the mechanisms is via the lecithin-cholesteryl ester transferase, which functions to convert free cholesterol to cholesterol ester with in HDL particle, increase HDL. HDL cholesterol is protective against CHD. Other possible mechanisms are, tocotrienols help to convert LDL cholesterol, present in the arteries, to VLDL then this VLDL turns into IDL then finally HDL. Lastly tocotrienols activate the HDL cholesterols and they destroy LDL by phagocytosis; and transform HDL within the HDL particle only (122). Recently, it has been found that it may be desmethyl or didesmethyl, two very recently discovered isoforms; of tocotrienols might be involved to down-regulate 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) (123). This HMG CoA is the key element to generate LDL. Various clinical trials have showed that by down-regulating HMG CoA, 60% LDL reduction can be possible.

Aims of this study

Based on the reports from other laboratories and previous work done by our own research group, the overall objective of this study was to describe that with the therapeutic dose resveratrol and tocotrienols can reduce the ischemia/reperfusion injury, and serve as a cardioprotective agent. In this approach we utilized several strategies such as the investigation of free radicals scavenging properties of resveratrol and tocotrienols in order to protect cardiomyocytes from the oxidative stress, determination of optimal dose and time-points for the cardioprotective effect of resveratrol and tocotrienols, investigation of some pro-apoptotic protein degradation, in addition to this investigation of the pharmacological preconditioning effect of resveratrol and tocotrienols and the underlying intracellular signaling pathways; with special emphasis on the redox signaling mechanisms.

The specific aims were:

1. To further evaluate resveratrol and tocotrienols as cardioprotective compounds by first determining the therapeutic dose of these compounds for and then carefully examining their role in preconditioning by following analysis :
    - Cardiac function analysis
    - Infarct Size determination
    - Apoptosis
    - Neucrosis
2. To examine the survival pathways of intracellular signaling by resveratrol and tocotrienols by studying potential molecular targets of death vs. survival signaling pathways including PI3Kinase, Akt, MAPKinase, p38 MAPKinase, cSrc, cJUN, cJNK, PKC and their downstream Transcription Factor gene including NFκB, Bcl-2, Bad, CREB etc.
3. By using specific inhibitors of each protein target, to evaluate the survival pathway and reconfirming the cardioprotective mechanism of resveratrol and tocotrienols.
4. To determine potential redox regulation of resveratrol and tocotrienols by examining their in-vivo antioxidant effects using either ESR or MDA formation.


Animal (mainly rat)
Pentobarbitol - Inject IP (Dose 80mg/kg body weight)
Anesthesia
Heparin - Inject IV (Dose 5000 IU/Kg body weight)
Open the chest and excise the heart from the animal body
Fix the heart in the Langendroff Setup
Experimental protocols:
5-10'(LM)
10-15'(WM)
15' Perfusion *
30' Ischemia (Global)
2 Hr. Reperfusion
Baseline
10 30 60 120
Time Points
Myocardial Preservation
Anti- and pro-apoptotic protein analysis
Infarct Size
Functional Analysis
Oxidative Stress
Apoptosis
Proteosome
Western Blot Analysis
PKC Src MAPK NFκB Bcl-2 CREB

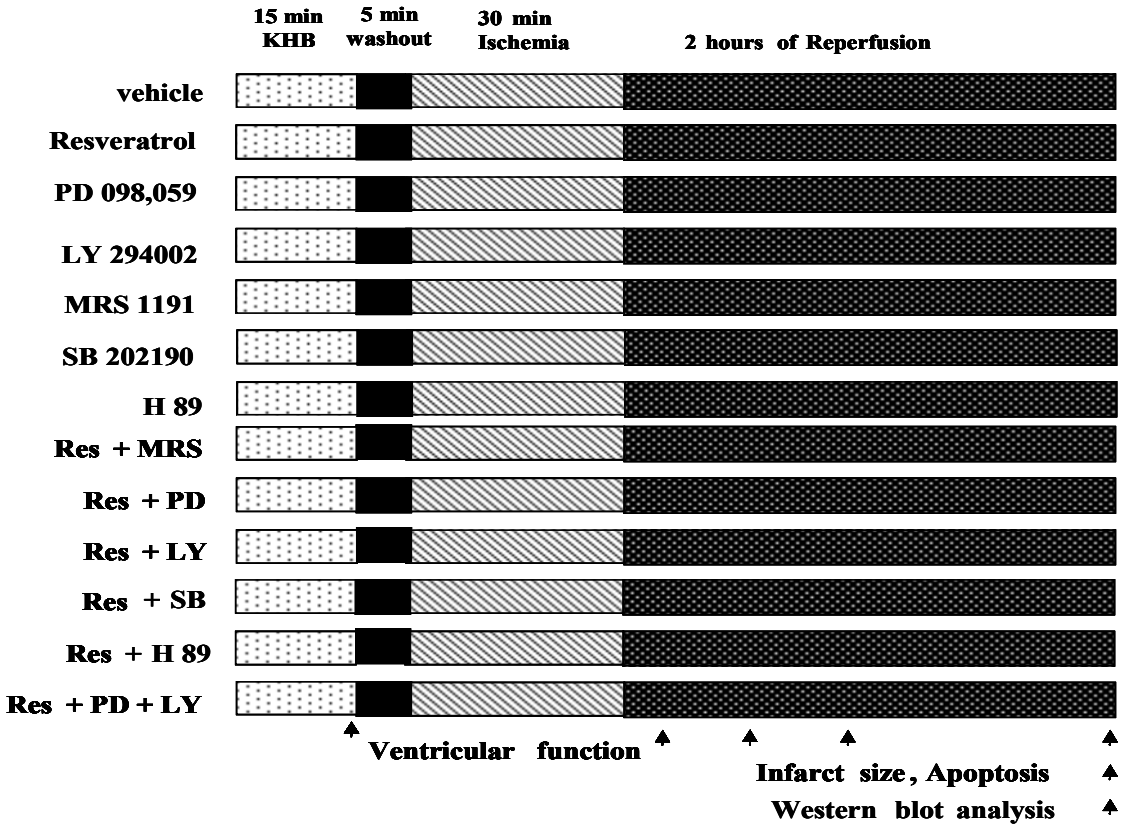
4.1. Materials

Resveratrol (trans-3, 4', 5-trihydroxystilbene), a natural phytoalexin, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). A highly specific blocker of the adenosine A_3 receptor MRS 1191 (3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6–phenyl-1,4-(±)–dihydropyridine-3,5-dicar-boxylate) and PD 098,059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), a MEK inhibitor and p38MAPK blocker, SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole) were also purchased from the same company. LY 294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), a PI-3-kinase inhibitor and MSK-1 blocker, H-89 (N-[2-(p-Bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) were purchased from Calbiochem Corp. (San Diego, CA, USA). All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise mentioned. Tocotrienol Rich Factor (TRF) was supplied by the Malaysia Palm Oil Board. The drugs were dissolved in DMSO, and the aliquots were kept at 4°C. Control experiments used the vehicle (0.01% DMSO) only.

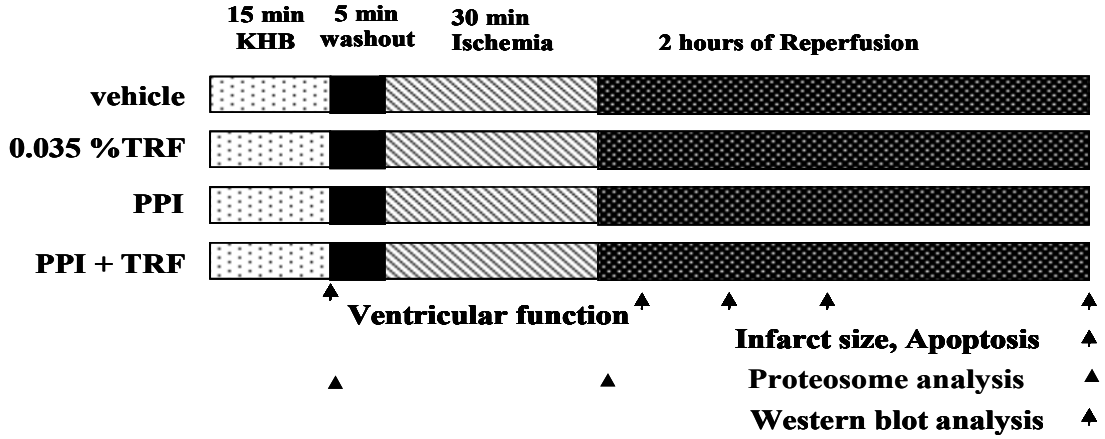
4.5. Animals

All animals used in this study received humane care in compliance with the principles of the laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Publication Number NIH 85-23, revised 1985). Sprague Dawley male rats weighing between 250-300 gm were fed *ad libitum* regular rat chow with free access to water until the start of the experimental procedure. The rats were randomly assigned to one of the following groups (Figure 6): pre-perfused the isolated hearts for 15 min with KHB i) containing 0.1% DMSO as a control group; ii) KHB containing 10 μM resveratrol; iii) 10 μM resveratrol + 1 μM MRS 1191; iv) 10 μM resveratrol + 3 μM LY 294002; v) 10 μM resveratrol + 20 μM PD098059 vi) 10 μM resveratrol + 20 μM 098059 + 3 μM LY 294002; vii) 10 μM resveratrol + 10 μM SB 202190; or viii) 10 μM resveratrol + 1 μM H-89. All hearts were then subjected to 30 min ischemia followed by 2 h reperfusion. Control experiments were performed with vehicle (DMSO) only, MRS 1191 only, LY 294002 only or PD 098059 only or SB 202190 only or H 89 only.

For TRF study the groups are: i) control (vehicle); ii) 0.035 % TRF; iii) 5 μM PPI or iv) 0.035 % TRF + 5 μM PPI. TRF was dissolved in ethanol.



Experimental Protocol (Resveratrol)



Experimental Protocol (TRF)

Fig. 6: Experimental protocol. Isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of vehicle only or with various combinations of drugs. The arrows represent the time points at which various parameters were measured. First level of five arrows represents the five different points where the ventricular functions were recorded. Second level arrow represents the point where infarct size and apoptosis were measured, and the third level arrow shows the point where the tissue was taken for the Western blot analysis or proteosome analysis.

4.5. Isolated working heart preparation

Rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), (Abbott Laboratories, North Chicago, IL, USA) and anticoagulant with heparin sodium (500 IU/kg., i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ, USA). After ensuring sufficient depth of anesthesia thoracotomy was performed, hearts were perfused in the retrograde Langendorff mode at 37 °C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5 min washout period. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mM: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2, and glucose 10). The Langendorff preparation was switched to the working mode following the washout period as previously described.

At the end of 10 min, after the attainment of steady state cardiac function, baseline functional parameters were recorded. The circuit was then switched back to the retrograde mode and hearts were perfused either KHB with vehicle or adenosine A_3 receptor antagonist or the other inhibitors (Control), Resveratrol at a concentration of 10 μM or a combination of resveratrol and adenosine A_3 receptors antagonist or the inhibitors for a duration of 15 min. In case of TRF study, after the steady state of the heart in the apparatus the heart were perfused with vehicle (ethanol) or PPI (control), TRF at a concentration 0.035% or a combination of TRF and PPI. This was followed by a 5-min washout with KHB buffer, and then the hearts were subjected to global ischemia for 30 min and then 2 h of reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for post ischemic stabilization and there after, in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 10-, 30-, 60- and 120- min reperfusion.

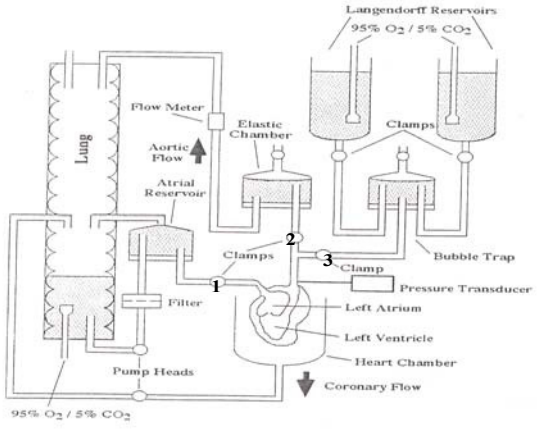


Fig. 7: Experimental working heart preparation:

The Langendorff perfusion is converted to working heart mode by opening clamps 1 and 2, and closing 3.

4.4. Cardiac function assessment

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH, USA) connected to a side arm of the aortic cannula, the signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA, USA). Heart Rate (HR), Left Ventricular develops pressure (LVDP) (defined as the difference of the maximum systolic and diastolic aortic pressures), and the first derivative of developed pressure (dP/dT) were all derived or calculated from the continuously obtained pressure signal. Aortic flow (AF) was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL, USA) and coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart.

4.5. Infarct size estimation

At the end of reperfusion, a 10 % (w/v) solution of triphenyl tetrazolium in phosphate buffer was infused into aortic cannula for about 10 min at 37 °C. The hearts were excised and stored at –70 °C. Sections (0.8 mm) of frozen heart were fixed in 2% Para formaldehyde, placed between two cover slips and digitally imaged using a Microtek ScanMaker 600z. To quantitative the areas of interest in pixels, a NIH image 5.1 (a public-domain software package) were used. The infarct size was quantified and expressed in pixels.

4.6. TUNEL Assay for assessment of Apoptotic Cell Death

Immunohistochemical detection of apoptotic cells was carried out using TUNEL assay by using APOP TAG® kit (Oncor, Gaaithesburg, MD). After 2 hr of reperfusion, the heart tissues were immediately put in 10% formalin for 24-48 hrs then the tissues were washed with water and put it in 70 % ethanol for dehydration and finally fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic moulds and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed and tissues became embedded in paraffin on the plastic moulds. Prior to analyzing tissues for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, 70%). Then tissues were

incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

4.7. Western blot Analysis

Left ventricles from the hearts were homogenized in a buffer containing 25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA and 1 mM PMSF. High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 50 μg total membrane proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto 0.45-μm polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with phospho-Akt, Akt, phosphor-ERK 1/2, ERK 1/2, phospho-p38MAPK, p38MAPK, phosphor-MAPKAPK2, MAPKAPK2, phosphor-MSK1, MSK1, CREB, and p-CREB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for overnight at 4°C. Primary antibodies were diluted in TBS-T according to the manufacturer's instructions. Horseradish peroxidase-labeled anti-mouse or anti-rabbit (according to the manufacturer's instructions) IgG (Bio-Rad, CA, USA) was also diluted according to the manufacturer's instructions in TBS-T and used as a secondary antibody. Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). The linearity of the Western blot procedure used for the quantification in subsequent blotting experiments 50 μg membrane protein was used because it is in the linear range. The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

4.8. Proteasome activity

Proteasome activity was determined in cell lysate. Frozen cardiac tissue was homogenized in HEPES buffer (137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 0.01% digitonin) without protease inhibitors at 4°C and then centrifuged at 10,000 *g* to obtain the soluble fraction. Cell supernatant (50

μg of protein) was incubated in 50 mmol/l Tris·HCl buffer, pH 7.8, containing 20 mM KCl, 0.5 mM $MgCl_2$, and 1 mM DTT for 1 h with 75 μM succinyl-LLVY-methylcoumarin (Biomol Research Laboratory, Plymouth Meeting, PA). Hydrolysis was stopped by addition of ice-cold ethanol and dilution with 0.125 mol/l sodium borate, pH 9.0, and fluorescence products were monitored at 380-nm excitation and 440-nm emission. The reaction was carried out in the absence and presence of the proteasome inhibitor lactacystin (5 μM; Biomol Research Laboratory) to differentiate between nonproteasome- and proteasome-mediated peptide hydrolysis and with or without 0.0625–0.125 mmol/l ATP (with or without lactacystin) to differentiate between 20S and 26S proteasome, respectively. Results are expressed as percentage of control, because storage of tissue samples, even at –80°C, can result in interassay variation. Care was taken to avoid freeze thawing of tissue samples more than once and to match experimental samples with preischemic controls that had been stored for similar amounts of time.

4.9. Statistical analysis

The values for myocardial functional parameters, total and infarct volumes and infarct sizes and cardiomyocyte apoptosis are all expressed as the mean ± standard error of mean (SEM). Analysis of variance test followed by Bonferoni's correction was first carried out to test for any differences between the mean values of all groups. If differences between established, the values of the treated groups were compared with those of the control group by a modified t-test. The results were considered significant if $p<0.05$.

5.1. Effects of Resveratrol on Myocardial Function

There were no differences in baseline function amongst all the thirteen groups. In general, there were no significant differences between resveratrol vs. control, PD98059, SB202190, LY 294002, MRS 1191 and H-89 as well as vs. Resveratrol + PD98059, Resveratrol + SB202190, Resveratrol + LY 294002, Resveratrol + MRS 1191 or Resveratrol + H-89 vs. Resveratrol on heart rate and coronary flow (Table 1). As was expected, upon reperfusion, the absolute values of all functional parameters were decreased in all the groups as compared with the respective baseline values. Resveratrol group displayed significant recovery of post ischemic myocardial function. Aortic flow (Table 1) was markedly higher in the resveratrol group from R-30 onwards at the all rest three points; R-30 (66.1 ± 3.62 ml/min vs. 36.02 ± 12.7 ml/min), R-60 (43.5 ± 5.34 ml/min vs. 19.24 ± 6.48 ml/min) and R-120 (14.9 ± 2.36 ml/min vs. 4.28 ± 1.43 ml/min). The cardio protective effects of resveratrol were evidenced by significant differences in the LVDP from R-30 onwards at the all rest three points (Table 1)**,** the difference was especially apparent at R-30 (121.7 $\pm$ 2.64 mm Hg vs. 103.57 $\pm$ 7.1 mm Hg), R-60 (110.27 ± 1.2 mmHg vs. 88.02 ± 9.57 mmHg) and at R-120 (87.8 ± 1.74 mm Hg vs. 52.5 ± 4.56 mm Hg). For LVdp/dt, resveratrol mediated increased recovery was apparent at R-60 (2843 $\pm$ 79.48 mmHg/sec vs. 1880.5 $\pm$ 403.3 mm Hg/sec) and at R-120 (1391.8±104.7 mm Hg/sec vs. 899.83±86.75 mmHg/sec). With the use of PD098059, SB202190 and LY 294002, resveratrol partially lost it cardio-protective effect, but with the combination of PD + LY, MRS 1191 and H-89, resveratrol significantly lost its cardio protective effects, which were evidenced by significant differences in the post ischemic period of LVDP from R-30 onwards at all of the three time points. PD 098,059 or SB 202190 or LY 294002 did not decrease the LVDP at R-30 or at R-60 level but with H-89 and Res + PD + LY the decrease was prominent both at R-30 (105.5 $\pm$ 5.78 mm Hg and 103 $\pm$ 2.8 mm Hg, respectively, vs. 121.7 ± 2.64 mm Hg) and R-60 (83.9 $\pm$ 4.75 mm Hg and 84 $\pm$ 2.3 mm Hg, respectively, vs. 110.27 ± 1.2 mm Hg) (Table 1) and with MRS 1191 the decrease is significant at R-60 onwards, at R-60 (97.9 $\pm$ 4.2 mm Hg vs. 110.27 ± 1.2 mm Hg). At R-120 the decrease is significant with all the inhibitors, PD 098059, SB 202190, LY 294002, MRS 1191, the combination of PD and LY and H-89, [R-120 (61.35 $\pm$ 4.05 mmHg, 68.62 $\pm$ 3.59 mmHg, 70 $\pm$ 12 mm Hg, 75.7 $\pm$ 3,7 mm Hg, 59 $\pm$ 4.7 mm Hg and 60.63 $\pm$ 6.27 mmHg, respectively, vs. 87.8 $\pm$ 1.74 mmHg)]. The same effect of PD 098,059, SB 202190, LY

294002, MRS 1191, PD + LY and H-89 on resveratrol preconditioning also reflects from the significant decrease of LVdp/dt at R-60 (2114.7 ± 119.29 mmHg/sec, 1760.5 ± 158.44 mmHg/sec 1,908.67 ± 249.55 mmHg/sec, 2,077±286.4 mmHg/sec, 1716 ± 92 mmHg/sec and 1633.17 ± 225.59 mmHg/sec, respectively, vs. 2843 ± 79.48 mmHg/sec) and at R-120 (877.7 ± 187.27 mmHg/sec, 866.7 ± 96.61 mmHg/sec, 932.33 ± 207.45 mmHg/sec, 980.16 ± 62.5 mmHg/sec, 707 ± 71 mmHg/sec and 862.3 ± 126.32 mm Hg/sec, respectively, vs. 1425.2 ± 109.72 mmHg/sec). But with PD + LY and H-89 the significant decrease also observed at R-30 (2623 ± 27 mmHg/sec and 2566.5 ± 143.17 mmHg/sec, respectively, vs. 3012.7 ± 64.02 mmHg/sec) apart from the other time points (Table 1). This was also confirmed from the Aortic flow value; which is markedly lower at R-30 onwards at all the rest two time points with all the inhibitors (Table 1).

Table 1

Effects of Resveratrol and the inhibitors of MEK (1/2), p38 MAPK, PI-3- Kinase, Adenosine A_3 receptors and MSK 1 on ventricular functions

	Group	Baseline	10 min R	30 min R	60 min R	120 min R
Heart Rate (beats/min)	**Control**	345 ± 31	316 ± 26	365 ± 17	344 ± 35	414 ± 24
	Resveratrol	352 ± 15	368 ± 20	382 ± 21	394 ± 16	411 ± 13
	PD 098,059	383 ± 17	371 ± 17	392 ± 6.5	405 ± 5.7	439 ± 5.9
	SB 202190	364 ± 20	346 ± 18	388 ± 22	399 ± 16	416 ± 14
	LY 294002	380 ± 21	358 ± 19	388 ± 23	411 ± 24	441 ± 38
	MRS 1191	390 ± 20	372 ± 20	388 ± 18	408 ± 8.7	422 ± 6.2
	H 89	374 ± 26	355 ± 12	372 ± 20	402 ± 14	428 ± 8
	Res + PD	415 ± 26	372 ± 20	386 ± 14	406 ± 9.8	426 ± 10.2
	Res + SB	383 ± 22	378 ± 28	396 ± 12	412 ± 7	442 ± 12
	Res + LY	377 ± 28	395 ± 5.7	391 ± 18	420 ± 3.4	458 ± 8.2
	Res + MRS	364 ± 23	368 ± 17	384 ± 13	413 ± 8.8	433 ± 7.7
	Res + PD + LY	385 ± 14	355 ± 12	393 ± 6.9	416 ± 4.1	438 ± 3.4
	Res + H 89	368 ± 24	370 ± 30	390 ± 16	422 ± 9,8	440 ± 4.6
LVDP (mm Hg)	**Control**	127 ± 3.2	107 ± 5.4	104 ± 7.1	88 ± 9.6	53 ± 4.6
	Resveratrol	126 ± 3	116 ± 2.4	122 ± 2.6 *	110 ± 1.2 *	88 ± 1.7 *
	PD 098,059	127 ± 3.2	109 ± 4	111 ± 4.4	92 ± 3	57 ± 5.1
	SB 202190	125.7 ± 3.4	104.3 ± 3.4	101.9 ± 3.5	84.3 ± 5.6	56.6 ± 5.8
	LY 294002	131 ± 1.6	123 ± 6.7	127 ± 7.1	100 ± 8.9	52 ± 6.4
	MRS 1191	119 ± 4.2	106 ± 3.7	97 ± 3.1	80 ± 4	40 ± 2.7
	H 89	128.2 ± 4.8	108.6 ± 4.6	108.5 ± 4.85	89.3 ± 5.1	60.3 ± 4.9
	Res + PD	129 ± 2.5	116 ± 4	116 ± 3.4	107 ± 3.4	61 ± 4 #
	Res + SB	127 ± 2.2	110.5 ± 2.5	111.4 ± 4.6	98.2 ± 6	68.6 ± 3.6 #
	Res + LY	127 ± 5.3	109 ± 2.1	109 ± 5.4	92 ± 9.9	70 ± 12
	Res + MRS	123 ± 2.8	102 ± 3.7	109 ± 3.1	98 ± 4.2 #	76 ± 3.7 #
	Res + PD + LY	128 ± 1.2	102 ± 4	103 ± 2.8 #	84 ± 2.3 #	59 ± 4.7 #
	Res + H 89	127.9 ± 2.9	108.6 ± 5.6	105.5 ± 5.8 #	83.9 ± 4.8 #	60 ± 6.3 #

Cont……

Table 1 (Cont...)

	Group	Baseline	10 min R	30 min R	60 min R	120 min R
LVdp/dt (mm Hg/sec)	**Control**	3319 ± 115	2412 ± 250	2472 ± 235	1881 ± 403	900 ± 87
	Resveratrol	3324 ± 95	2720 ± 131	3013 ± 64 *	2843 ± 79 *	1425 ± 110 *
	PD 098,059	3365 ± 82	2470 ± 68	2600 ± 159	1853 ± 100	843 ± 78
	SB 202190	3253 ± 116	2544 ± 130	2527 ± 177	1717 ± 217	946 ± 213
	LY 294002	3044 ± 115	2536 ± 76	2266 ± 78	1705 ± 129	996 ± 98
	MRS 1191	2985 ± 152	2493 ± 230	2371 ± 151	1572 ± 231	898 ± 78
	H 89	3289 ± 177	2668 ± 154	2613 ± 167	1822 ± 245	885 ± 97
	Res + PD	3578 ± 115	2998 ± 127	2902 ± 113	2115 ± 119 #	878 ± 187 #
	Res + SB	3270 ± 67	2552 ± 45	2885 ± 113	1761 ± 158 #	867 ± 97 #
	Res + LY	3116 ± 21	2261 ± 85	2347 ± 136	1908 ± 250	932 ± 207
	Res + MRS	2932 ± 212	2164 ± 270	2326 ± 238	2077 ± 286 #	980 ± 63 #
	Res + PD + LY	3218 ± 52	2524 ± 86	2623 ± 27 #	1716 ± 92 #	707 ± 71 #
	Res + H 89	3251 ± 62	2430 ± 193	2567 ± 143 #	1633 ± 226 #	862 ± 126 #
Aortic Flow (ml/min)	**Control**	72 ± 5.2	43 ± 12.9	36 ± 12.7	19 ± 6.5	4.3 ± 1.4
	Resveratrol	72 ± 3.5	61 ± 4.3	66 ± 3.6 *	44 ± 5.3 *	14.9 ± 2.4 *
	PD 098,059	71 ± 3.4	46 ± 3.8	41 ± 4.8	20 ± 3	3.65 ± 1
	SB 202190	64 ± 5	35 ± 7.8	32 ± 5	12.5 ± 2.7	2.1 ± 0.5
	LY 294002	71 ± 3.5	40 ± 2.6	27 ± 2.7	8.7 ± 1.2	6.6 ± 1
	MRS 1191	60 ± 3.2	44 ± 3.8	32 ± 4.6	14 ± 2.2	2.8 ± 1
	H 89	73 ± 5.2	45 ± 6.5	43.7 ± 5.5	21 ± 6.6	3.3 ± 1.37
	Res + PD	79 ± 2.2	49 ± 8	44 ± 7.4 #	19 ± 5.7 #	3 ± 1 #
	Res + SB	75 ± 4	41 ± 8.8	42 ± 6.7 #	20 ± 3.6 #	3.5 ± 1.1 #
	Res + LY	75 ± 4.4	62 ± 4.3	58 ± 7.4	34 ± 11.6	11 ± 7.5
	Res + MRS	63 ± 3.9	36 ± 5.3	39 ± 5 #	29 ± 5.7 #	7 ± 1 #
	Res + PD + LY	64 ± 7	35 ± 9.8	31 ± 6.9 #	10 ± 3.4 #	1.9 ± 1 #
	Res + H 89	73 ± 3.2	45 ± 6.7	44 ± 5 #	17 ± 6.9 #	2.5 ± 0.9 #
Coronary Flow (ml/min)	**Control**	24 ± 1.4	22 ± 1	23 ± 1.9	23 ± 1.3	22 ± 1.2
	Resveratrol	24 ± 1.3	23 ± 1.5	26 ± 1.1	25 ± 1.6	23 ± 1.5
	PD 098,059	21 ± 1.4	20 ± 1.2	21 ± 1	21 ± 1.5	20 ± 1.8
	SB 202190	20.4 ± 1.5	19.5 ± 0.9	20.1 ± 1.1	20.3 ± 1.1	20.7 ± 0.9
	LY 294002	24 ± 1.2	22 ± 1.1	24 ± 1.1	25 ± 1.5	25 ± 1.5
	MRS 1191	28 ± 1	25 ± 1.2	23 ± 1	22 ± 1	20 ± 1
	H 89	21 ± 1.5	20 ± 0.6	22 ± 1	21 ± 0.6	20 ± 1
	Res + PD	20 ± 1.5	23 ± 2	22 ± 1.8	22 ± 1.1	21 ± 1
	Res + SB	25 ± 0.6	21 ± 1	22 ± 1	20 ± 1	20 ± 1.2
	Res + LY	22 ± 1.3	21 ± 1.5	21 ± 0.2	23 ± 2.3	23 ± 2.3
	Res + MRS	28 ± 1	28 ± 1	28 ± 1.4	27 ± 1.5	25 ± 1.5
	Res + PD + LY	21 ± 1	19 ± 1.1	20 ± 1	20 ± 1.4	19 ± 1.9
	Res + H 89	24 ± 1.3	21 ± 1.6	23 ± 1.7	22 ± 1.4	20 ± 1.6

LVDP: Left ventricular developed pressure; LVdp/dt: Maximum first derivatives of developed pressure; R: Reperfusion.

Results are expressed as mean ± SEM of 6 animals as group * $p<0.05$ Res vs. Control and # $p<0.05$ (Res + PD) or (Res + SB) or (Res + LY) or (Res + MRS) or (Res + PD + LY) or (Res + H 89) vs. Reseratrol.

5.2. Effects of TRF on ventricular function.

There were no differences in baseline function among the six groups. In general, there were no significant differences between TRF and control in heart rate and coronary flow (Table 2). As expected, on reperfusion, the absolute values of all functional parameters were decreased in all groups compared with the respective baseline values. TRF displayed significant recovery of postischemic myocardial function. The cardioprotective effects of TRF were demonstrated by significant differences in the left ventricular dP/dt from 30 min of reperfusion onward, the difference is especially apparent at 60 and 120 of reperfusion and also in left ventricular developed pressure at 120 min of reperfusion. AF was markedly higher in the TRF group from 30 min of perfusion onward. This is also confirmed from the AF value, which is markedly lower throughout the reperfusion period (Table 2). Similar to TRF, PPI also improved postischemic ventricular recovery.

Table 2

Effects of palm tocotrienol and PP 1 on postischemic ventricular function

	Group	Baseline	10 min	30 min	60 min	120 min
Heart Rate (beats/min)	Control	345.32 ± 42.55	332.27 ± 24.22	339.57 ± 15.4	344.43 ± 34.57	414.28 ± 24.38
	TRF	359.62 ± 29.77	347.92 ± 33.16	340.67 ± 30.86	350.15 ± 31.74	391.47 ± 34.5
	PPI	322.67 ± 29.48	373.4 ± 11.45	362.37 ± 8.98	387.23 + 3.08	428.23 ± 4.53
	TRF + PPI	339.37 ± 14.48	319.08 ± 19.72	358.1 ± 23.56	383.3 ± 17.9	401.7 ± 18.84
LVDP (mm Hg)	Control	126.76 ± 3.16	107.43 ± 5.40	103.56 ± 7.10	88.01 ± 9.57	42.5 ± 7.62
	TRF	126.27 ± 3.55	106.56 ± 3.75	103.76 ± 3.56	99.13 ± 1.67	80.83 ± 3.57 *
	PPI	118.1 ± 2.06	105.16 ± 2.33	108.6 ± 2.74	94.23 ± 3.85	55.53 ± 1.79
	TRF + PPI	125.45 ± 2.97	113.03 ± 1.7	109.32 ± 3.68	94.78 ± 3.78	65.93 ± 4.32 #
LVdp/dt (mmHg/sec)	Control	3068.83 ± 132	2630.66 ± 121.9	2365 ± 34.73	968.3 ± 97.22	481 ± 84.54
	TRF	2990.17 ± 189.9	2273.67 ± 183.6	2128.6 ± 203	1928.6 ± 164.8 *	1404.3 ± 148.7 *
	PPI	3189.67 ± 137.4	2479.3 ± 399.61	2563.3 ± 357.64	1446.67 ± 240.77	594.3 ± 75.24
	TRF + PPI	2916 ± 74.45	2333 ± 110.68	2259.67 ± 191.78	1790.5 ± 90.7	810.67 ± 137.22 #
Aotic flow (mL/min)	Control	58.57 ± 4.5	39.68 ± 6.21	35.31 ± 8.41	9.19 ± 2.39	2.5 ± 0.41
	TRF	65.17 ± 7.7	35.76 ± 6.64	32.93 ± 8.68	21.72 ± 4.72 *	7.44 ± 1.76 *
	PPI	66 ± 2.17	38.4 ± 3.64	42.6 ± 2.1	14.8 ± 2.86	3.63 ± 0.61
	TRF + PPI	65.02 ± 3.86	46.85 ± 4.97	39.45 ± 4.51	17.58 ± 3.47	2.81 ± 1.02 #
Coronaryflow (mL/min)	Control	26.6 ± 0.7	21.5 ± 0.36	20.2 ± 0.77	18.65 ± 0.78	16.8 ± 1.03
	TRF	24 ± 2.66	23.3 ± 2.28	23.1 ± 2.37	21.9 ± 2.52	18 ± 1.65
	PPI	23 ± 0.53	22.8 ± 0.6	23.4 ± 1.93	20.8 ± 1.78	18.2 ± 1.44
	TRF + PPI	24.8 ± 1.85	25.3 ± 2.25	25.3 ± 2.34	24.8 ± 2.32	20.8 ± 1.85

LVDP : Left ventricular developed pressure; LVdp/dt : Maximum first derivatives of developed pressure.

Results are expressed as mean + SEM of 6 animals as group * $p < 0.05$ TRF vs. Control and # p< (TRF + PPI) vs. TRF

5.3. Effects of Resveratrol on Myocardial Infarct size

Infarct size (percent of infarct vs. total area at risk) was noticeably reduced in resveratrol group as compared to the control (18.17 ± 2.08% vs. 34.7 ± 2.74%) (Fig. 8). This infarct zone was increased significantly when resveratrol were used along with PD 98,059, SB 202190, LY 294002 and MRS 1191 (29.8 ± 1.98 %, 30.4 ± 2.44 %, 24.9 ± 2.3 % and 26.33 ± 2.45 %, respectively, vs. 18.17 ± 2.08%). When resveratrol was used along with the combination of (PD + LY) and H-89 the infarct zone was further increased compared to the other inhibitors (30.05 ± 4.9 % and 33.6 ± 2.62 %) as shown in the Figure 8.

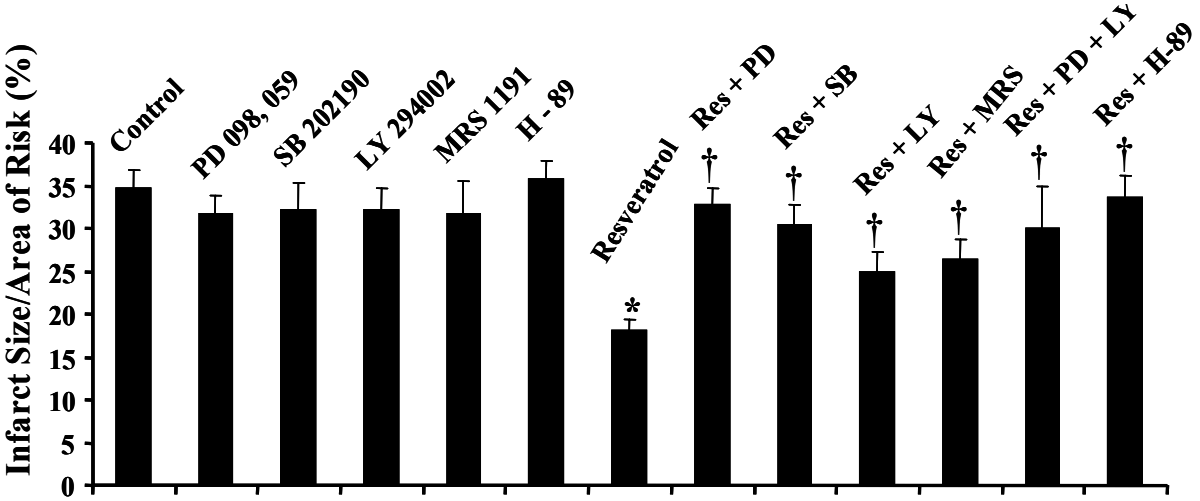


Fig. 8: Effect of resveratrol anf different drugs on myocardial infarct size. The isolated hearts from control (n = 6) or resveratrol preperfused rats in the absence or presence of either these drugs (n = 6) were subjected to 30-min global ischemia followed by 2 h of reperfusion in working mode. Infarct size was measured by triphenyl tetrazolium dye method. Results are expressed as means ± S.E.M. *, p < 0.05 versus control; †, p< 0.05 versus resveratrol.

5.4. Effects of TRF on myocardial infarct size

Infarct size (percentage of infarct vs. total area at risk) was significantly higher in the hearts subjected to 30 min of ischemia and 2 h of reperfusion than in the hearts that were not subjected to the ischemia-reperfusion protocol (almost at the baseline level, data not shown). The values were noticeably reduced in TRF and PPI groups compared with the group subjected to ischemia-reperfusion: 25.1 ± 2.45 and 25.6 ± 2.33%, respectively, vs. 33.43 ± 2.44% (Fig. 9). The infarct size was further reduced when a combination of TRF and PPI was used (20.5 ± 2.2% vs. 33.43 ± 2.44%; Fig. 9).

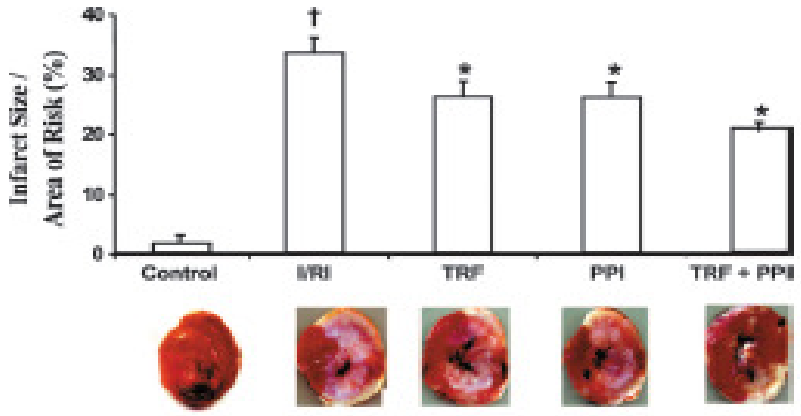


Fig. 9: Effect of TRF on myocardial infarct size, measured by TTC staining methods.

5. 5. Effects of Resveratrol on Cardiomyocyte Apoptosis

The percent of apoptotic cardiomyocytes was significantly reduced in resveratrol group as compared to the control (3.7±1.2% vs. 22.7±1.5%). This apoptotic cell death was increased significantly when resveratrol was used along with MRS 1191. Thus, the apoptosis was significantly higher in the resveratrol + MRS1191 group as compared to the resveratrol group (20.9 ± 1.7% vs. 3.7 ± 1.2%); as shown in the Figure 10. Inhibition of PI-3-kinase with LY 294002 or MEK with PD 098059 or p38 MAPK with SB 202190 also increased the number of apoptotic cardiomyocytes to (17.4 ± 1.2%, 18.9 ±1.4 % and 11.8 ± 1.6%, respectively) compare to resveratrol group. The cardiomyocyte apoptosis was further increased when the combination of PD 098059 and LY 294002 and H-89 were used with resveratrol (24.3 $\pm$ 1.1 % and 23.6 $\pm$ 2.1 %).

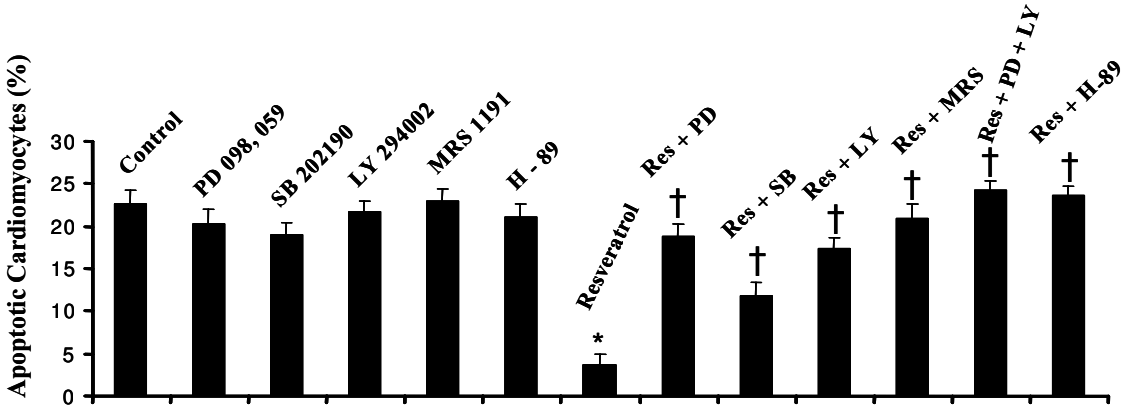


Fig. 10: Effect of resveratrol and various drugs on cardiomyocyte apoptosis, measured by TUNNEL assay methods. Results are expressed as means ± S.E.M. *, $p < 0.05$ versus control; †, $p<0.05$ versus resveratrol

5. 6. Effects of TRF on cardiomyocyte apoptosis

Ischemia-reperfusion caused the cells to undergo apoptosis, as expected. The percentage of apoptotic cardiomyocytes was significantly reduced in the TRF and PPI groups compared with the control group: 5.7 ± 1.3 and 6.6 ± 2.2%, respectively, vs. 22.0 ± 1.7% (Fig. 11). As observed for the infarct size, the apoptotic cardiomyocytes were also further reduced when a combination of TRF and PPI was used: 4.8 ± 0.8% vs. 22.0 ± 1.7% (Fig. 11).

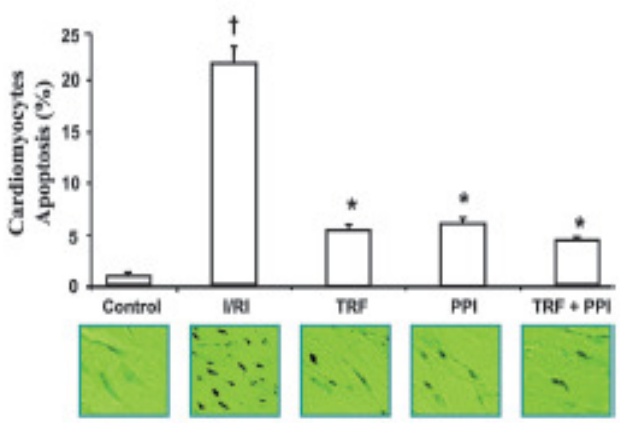


Fig. 11: Effect of TRF on cardiomyocyte apoptosis, measured by TUNNEL assay methods in conjunction with antibody against α-myosin heavy chain. Results are expressed as means ± S.E.M. *, $p < 0.05$ versus control.

5. 7. Effects of TRF on ventricular arrhythmias

The total incidence of VF (sustained and nonsustained) was significantly reduced with TRF and PPI from its control value of 90% to 30% and 40%, respectively (Fig. 12), indicating antiarrhythmic effects of tocotrienol and PPI.

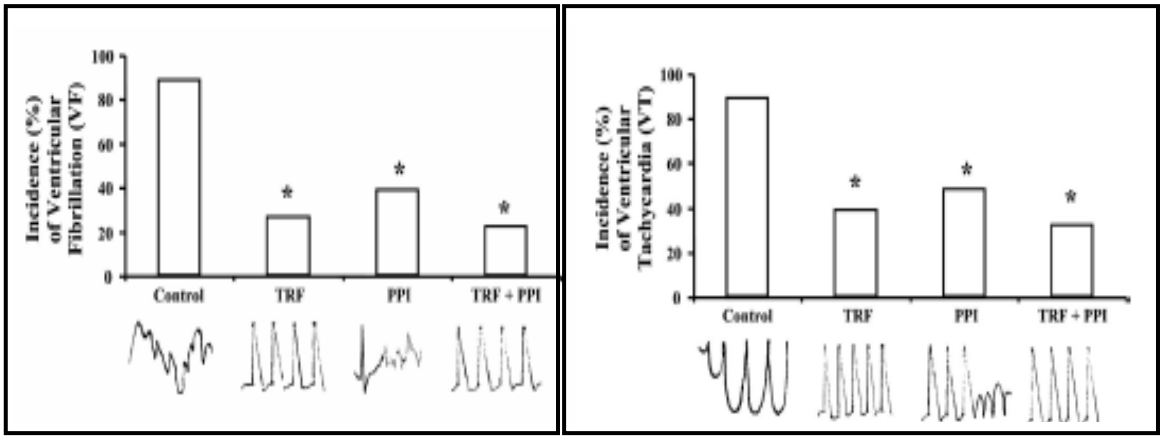


Fig. 12: Effects of TRF in the presence or absence of PPI on ventricular fibrillation (VF) and ventricular tachycardia (VT). Isolated hearts from control rats ($n = 10$) and TRF-preperfused rats in the absence or presences of PPI ($n = 10$) were subjected to 30 min of global ischemia. VF and VT were determined by contractibility of the heart and with the help of cardiac functions. *$P < 0.05$ vs. control.

5. 8. Effects of Resveratrol on the phosphorylation of Akt, p38MAPK, MAPKAP kinase2, MSK-1 and CREB

Resveratrol significantly enhanced the phosphorylation of Akt and CREB. As shown in Figure 13, phosphorylation of Akt was increased by 10-12 fold and CREB by 6-7 folds. The resveratrol-mediated induction of Akt and its subsequent phosphorylation was reduced significantly by MRS 1191 and LY 294002, but not with PD 098059. In contrast, any one of the three blockers inhibited the phosphorylation of CREB. As shown in Figure 13 B, LY 294002 and PD 098059 partially, but MRS 1191 and LY294002 plus PD 098059 almost completely abolished resveratrol mediated CREB phosphorylation.

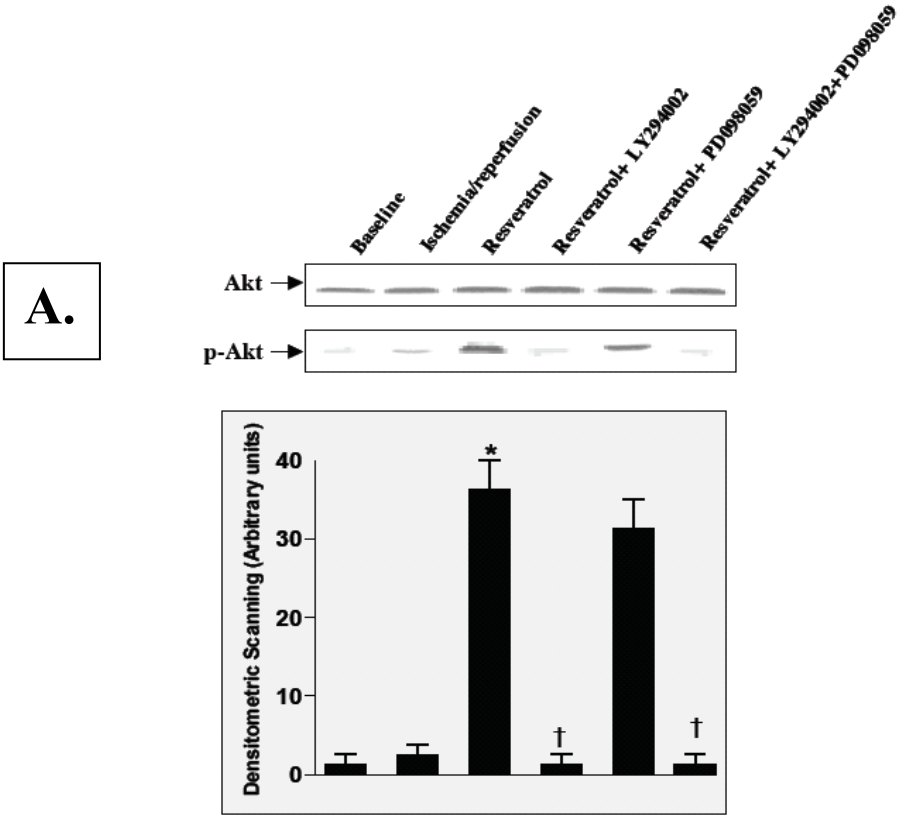
Resveratrol significantly enhanced the phosphorylation of MAP kinases. As shown in Figure 14, phosphorylation of ERK1/2, p38 MAP kinase, and MAPKAP kinase 2 was increased significantly as compared to control. Resveratrol-mediated increased phosphorylation of ERK1/2 (Figure 14, top) was reduced by PD98059, but not with SB202190, increased phosphorylation of p38MAP kinase (Figure 14, middle) and MAPKAP kinase was reduced by SB202190, but not with PD98059 (Figure 14, middle and bottom).

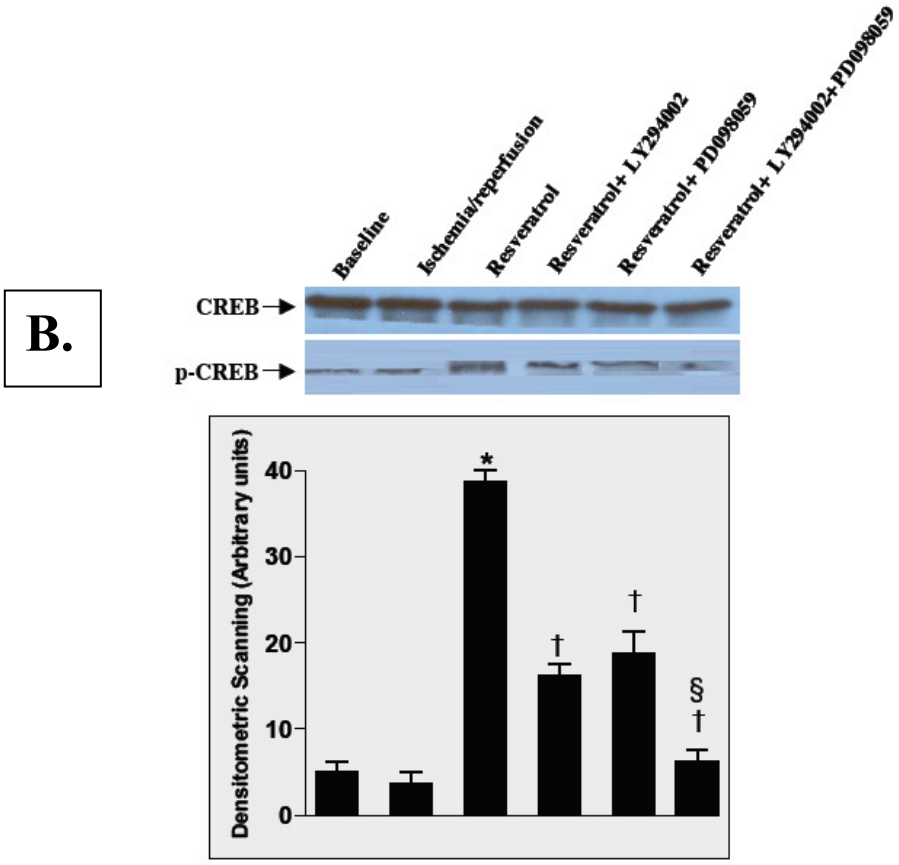
The phosphorylation pattern of MSK-1 and CREB is shown in Figure 6. Resveratrol increased the phosphorylation of both MSK-1 (Figure 15, top) and CREB (Figure 15, bottom). Increased phosphorylation of MSK-1 and CREB was reduced significantly by either PD98059 or SB202190. Resveratrol-mediated increased phosphorylation of MSK-1 and CREB was almost abolished by H-89.

Fig. 13: A. Western blot analysis of Akt and phosphorylated Akt (p-Akt) proteins. The results of p-Akt are shown in bar graphs as means ± S.E.M. of six experiments per group. Representative Western blots are shown at the top of the bar graphs. The density of the Akt blots did not change, which also served as control.

B. Western blot analysis of CREB and p-CREB proteins. The results of p-CREB are shown in bar graphs as means ± S.E.M. of six experiments per group. Representative Western blots are shown at the top of the bar graphs. The density of the CREB blots did not change, which also served as control.

*, $p < 0.05$ versus baseline or ischemia/reperfusion., †, $p < 0.05$ versus resveratrol alone;§, $p < 0.05$ versus resveratrol + LY294002 or resveratrol + PD098059.


A.
Baseline
Ischemia/reperfusion
Resveratrol
Resveratrol+ LY294002
Resveratrol+ PD098059
Resveratrol+ LY294002+PD098059
Akt
p-Akt
Densitometric Scanning (Arbitrary units)
0
10
20
30
40
*
†
†


B.
Baseline
Ischemia/reperfusion
Resveratrol
Resveratrol+ LY294002
Resveratrol+ PD098059
Resveratrol+ LY294002+PD098059
CREB
p-CREB
Densitometric Scanning (Arbitrary units)
0
10
20
30
40
*
†
†
†
§
†

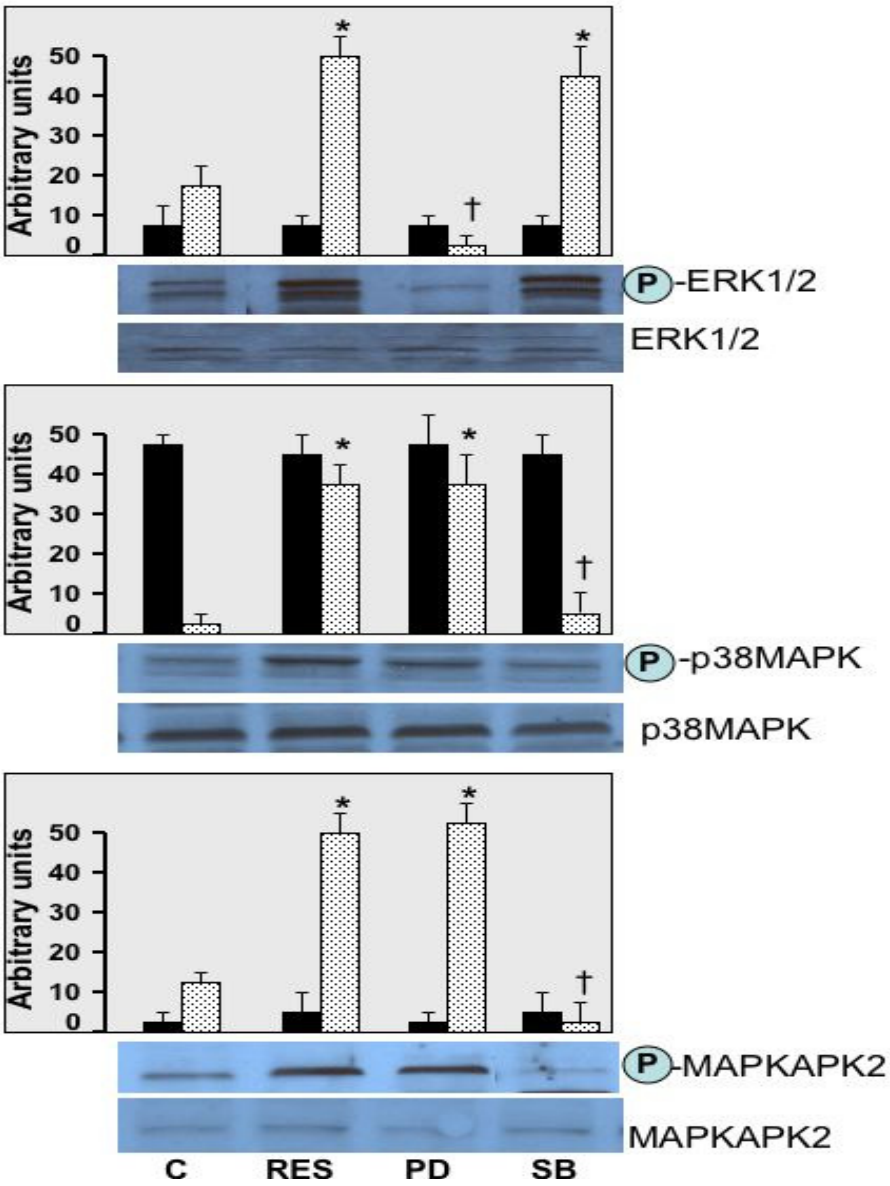


Fig. 14: Effects of resveratrol and various inhibitors used to block the effects of resveratrol preconditioning on the phosphorylation of ERK1/2 (top), p38MAPK (middle) and MAPKAP kinase2 (bottom). The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of resveratrol without or with the inhibitors. The hearts was made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. At the end of the experiments the hearts were frozen at liquid nitrogen temperature for subsequent determination of the protein phosphorylation by Western blot analysis. The phosphorylated proteins are shown on the top of non-phosphorylated proteins, which also served as the controls. The average of four experiments (Means±SEM) is shown as bar graphs on the top of the representative Western blots.

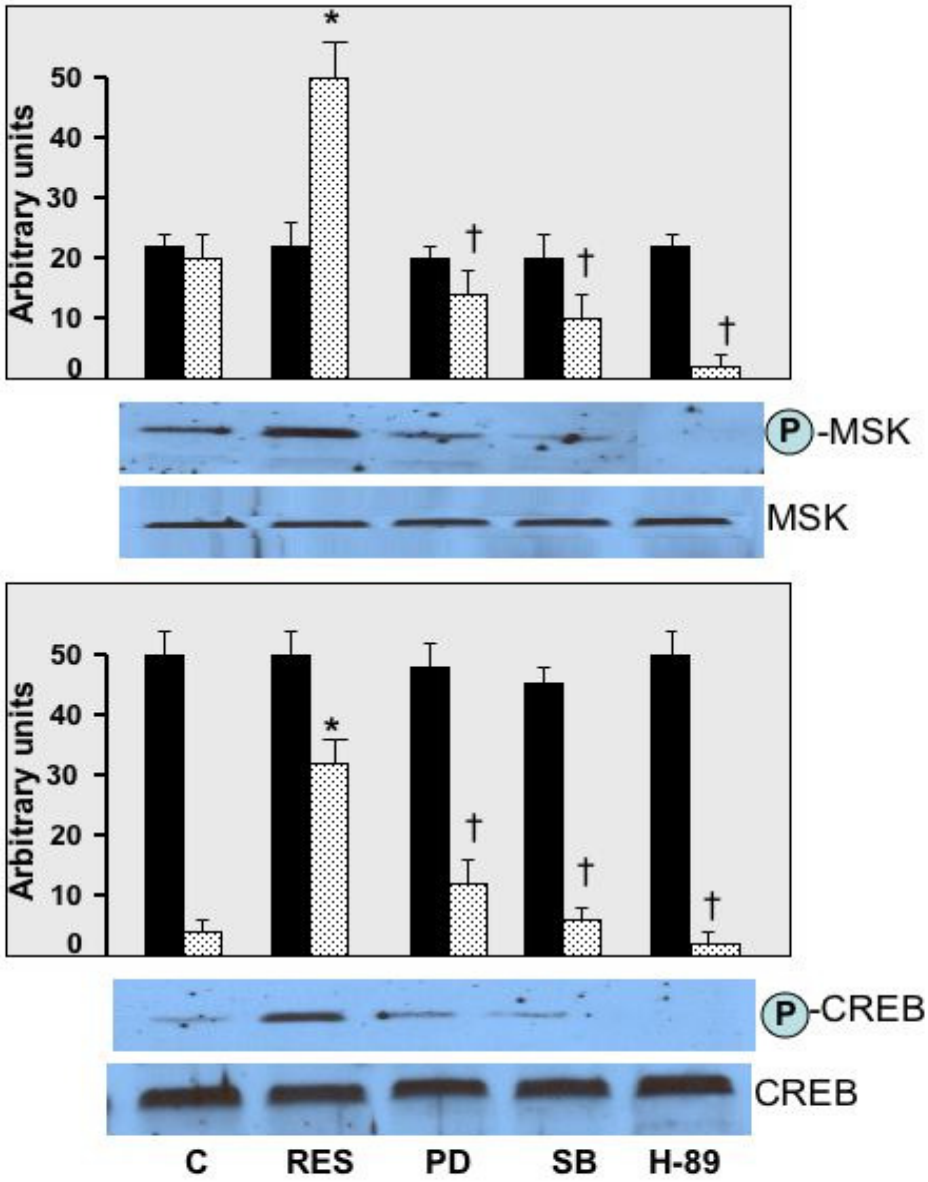


Fig. 15: Effects of resveratrol and various inhibitors used to block the effects of resveratrol preconditioning on the phosphorylation of MSK-1 (top) and CREB (bottom). The isolated rat hearts were perfused for 15 min with KHB buffer in the absence or presence of resveratrol without or with the inhibitors. The hearts were made globally ischemic for 30 min followed by 2 h of reperfusion in the working mode. At the end of the experiments the hearts were frozen at liquid nitrogen temperature for subsequent determination of the protein phosphorylation by Western blot analysis. The phosphorylated proteins are shown on the top of non-phosphorylated proteins, which also served as the controls. The average of four experiments (Means±SEM) is shown as bar graphs on the top of the representative Western blots.

5. 9. Effects of TRF on c-Src expression and phosphorylation

Because previous studies indicated negative regulation of c-Src with vitamin E, we determined whether TRF could reduce the ischemia-reperfusion-induced upregulation of c-Src. The results show that ischemia-reperfusion significantly increased the induction and phosphorylation of c-Src activities (Fig. 16). TRF minimally, but significantly, affected the ischemia-reperfusion-mediated increase in c-Src induction. TRF-mediated reduction of c-Src phosphorylation was highly significant. PPI completely abolished c-Src expression (data not shown).

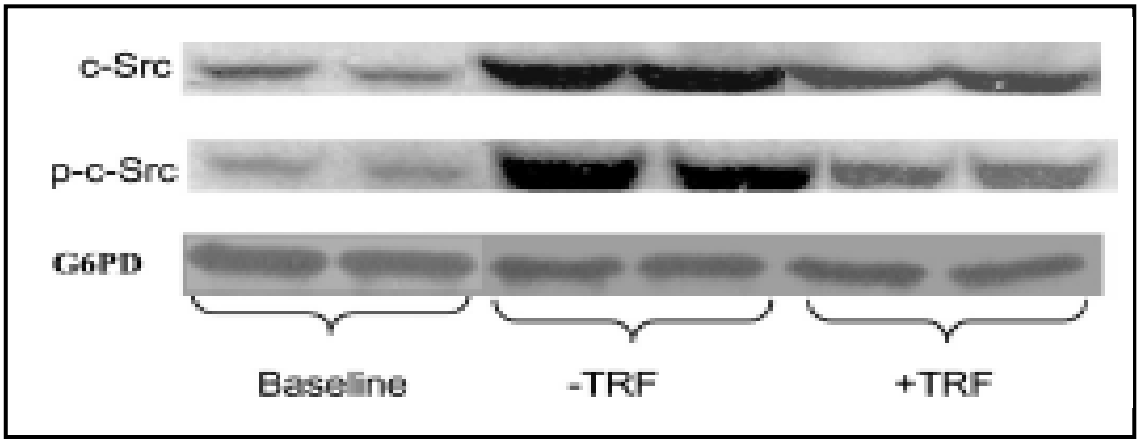


Fig. 16: Western blot analysis of phosphorylated c-Src (p-c-Src) and c-Src protein. Results are from 2 different hearts for each group. PPI completely blocked expression of c-Src; hence, it is not shown. G6PD, glucose-6-phosphate dehydrogenase.

5. 10. Effects of TRF on ischemic and postischemic proteasome activities

As illustrated in Fig. 17, 20S and 26S proteasome activities were significantly ($P < 0.05$) depressed by 45 and 46%, respectively, after ischemia. Preischemic treatment of hearts with TRF not only prevented this decrease, but it actually appeared to activate both proteasomes to levels significantly ($P < 0.05$) higher than the ischemic values. After 120 min of reperfusion, 20S proteasome activity recovered to a level not different from baseline, and TRF had no effect. However, 26S proteasome activity was still significantly ($P < 0.05$) depressed by 58%, which was prevented in the TRF-treated hearts (Fig. 17). Treatment of hearts with PPI had no protective effects on postischemic proteasome activities (data not shown).

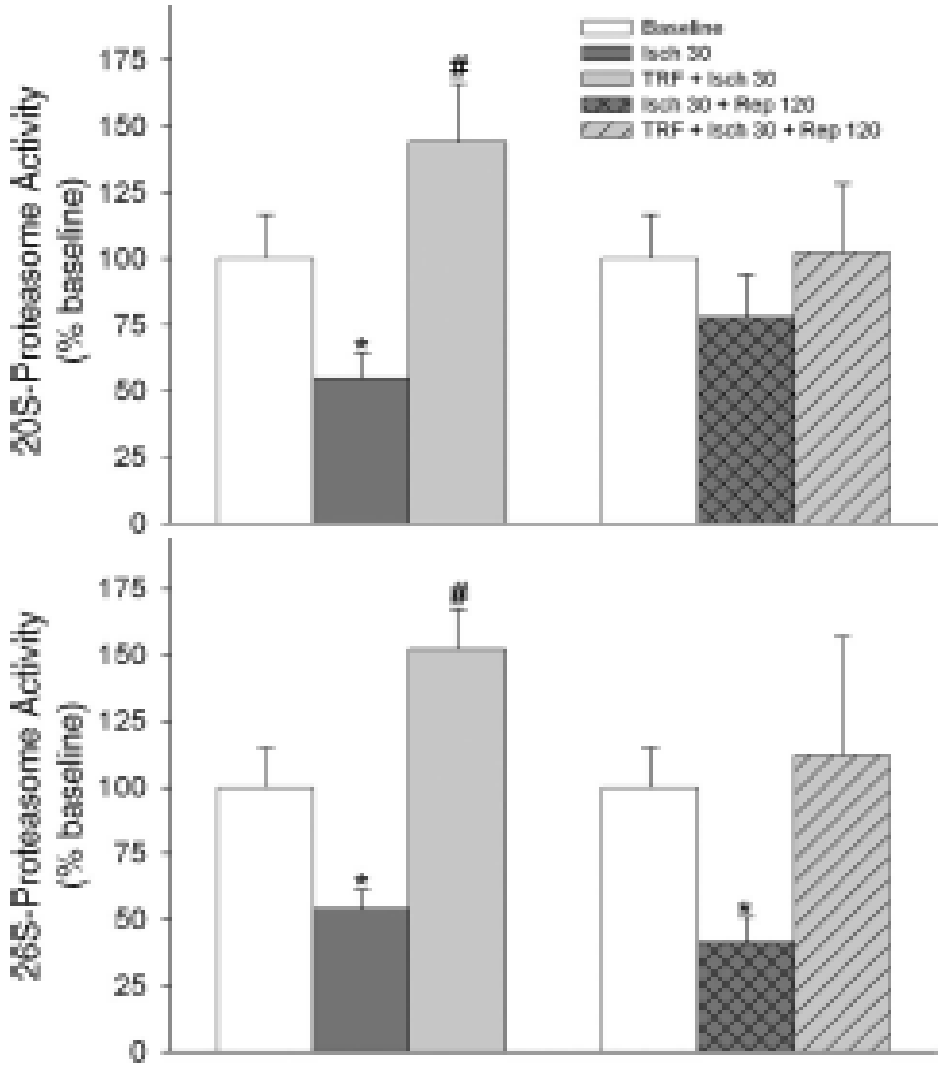


Fig. 17: TRF prevents ischemia-associated decreases in proteasome activities. Hearts treated with TRF and subjected to ischemia and reperfusion were harvested, and 20S and 26S proteasome activities were determined. Values are means ± SE of 5–11 hearts, expressed as percentage of baseline (1,261 ± 210.6 and 612 ± 95 fluorescence units·h^{-1}·mg $protein^{-1}$ for 20S and 26S proteosome, respectively). Isch 30, 30 min of ischemia; Rep 120, 120 min of reperfusion. **P* < 0.05 vs. baseline. $^{\#}P < 0.05$ vs. 30 min of ischemia.

The results of my studies demonstrated that resveratrol phosphorylated both Akt and CREB that were blocked by MRS 1191, which also abolished cardioprotective abilities of resveratrol. LY 294002 completely inhibited Akt phosphorylation, but partially blocked the phosphorylation of CREB. Inhibition of PI-3- kinase also partially blocked resveratrol's ability to precondition the heart. PD 098059 partially blocked the phosphorylation of CREB and resveratrol-mediated cardioprotection. Pre-perfusing the hearts with LY294002 and PD 098059 together completely abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A_3 receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and –independent pathways leading to cardioprotection. The most important finding of my study is that resveratrol increased the phosphorylation of what by ERK1/2 and p38 MAPK, which in turn phosphorylated MSK-1 leading to the activation of CREB, suggesting that phosphorylation of MSK-1 and subsequent activation of CREB, occurred via both p38MAPK ERK1/2. Resveratrol also increased the phosphorylation of MAPKAP kinase 2, the downstream target of p38MAP kinase. In agreement with these results, cardioprotective abilities of resveratrol were partially abolished either with an ERK1/2 inhibitor, PD 098,059 or with a p38 inhibitor, SB202190 and almost completely with a MSK-1 blocker, H-89.

Resveratrol is abundant in grapes and nuts (59). The cardioprotective effects of red wine have been attributed to the resveratrol present in red wine (124) through diverse mechanisms including its ability to inhibit low density lipoprotein (LDL) (111), to block platelet aggregation (125) and induce NO production (124). A recent study demonstrated that resveratrol reduced myocardial ischemia reperfusion injury by inducing the expression of iNOS (115). Reseveratrol was ineffective in the hearts of the knockout mice devoid of any copies of iNOS (126).

A number of studies including our own have indicated the ability of resveratrol to pharmacologically precondition the heart (59, 124,127). Ischemic preconditioning (PC) refers to the paradoxical mechanism by which cyclic episodes of brief reversible ischemia, each followed by another brief periods of reperfusion render the heart tolerant to subsequent ischemic reperfusion injury (128-130). PC is a complex phenomenon, which occurs through multiple interrelated cascades of events. A variety of neurohumoral factors are released during the onset of PC that include among many intracellular mediators, adenosine (131-136). Both adenosine A_1 and A_3

receptors have been implicated in PC-mediated cardioprotection (131, 134, 137). The same adenosine has recently been implicated in resveratrol preconditioning (138). In addition, nitric oxide (NO), which is a powerful mediator of PC (131, 137), is also involved in resveratrol PC (59, 138). Resveratrol activates both iNOS and eNOS, which presumably contributed towards the ability of resveratrol to provide cardioprotection. In a recent study, resveratrol failed to precondition mouse hearts devoid of any copies of iNOS gene suggesting a crucial role of iNOS in resveratrol PC (126). A study from our laboratory have indicated the role of that both A_1 and A_3 receptors are involved in resveratrol preconditioning, and both use PI-3-kinase-Akt signaling pathway (59). The resveratrol-mediated increased Akt phosphorylation occurred at serine 478 site. The increased Akt phosphorylation was blocked by MRS and CPT, suggesting the involvement of both A_1 and A_3 receptor in Akt signaling. Interestingly, LY 294002 abolished the cardioprotective effects of resveratrol indicating PI-3-kinase as the upstream signaling molecule for resveratrol PC.

Akt is a critical regulator of PI-3-kinase-mediated cell survival (139). A large number of studies have demonstrated in various cell types that constitutive activation of Akt is sufficient to block cell death induced by a variety of apoptotic stimuli (140). Akt is activated by PC as a result of activation of PI-3-kinase leading to the activation of PKC and endothelial NO synthase (eNOS) (141). Several recent studies showed phosphorylation of Akt as a result of adenosine A_3 receptor activation. For example, A_3 adenosine receptor activation triggered phosphorylation of PKB/Akt and protected rat basophilic leukemia 2H3 mast cells from apoptosis (142). Low concentrations of ethanol activate cell survival promoting PI-3-kinase/Akt pathway in endothelial cells by an adenosine receptor-dependent mechanism (143). In another study, adenosine receptor was found to regulate insulin-induced activation of PI-3-kinase/PKB in rat adipocytes (144).

Protein kinase B or Akt has been recognized as a survival factor by its ability to inhibit apoptosis (145). Akt, a member of serine/threonine kinase family, is a major target of PI-3-kinase, which enhances the level of lipid second messenger, PI-3,4,5-triphosphate upon stimulation leading to its binding to PH domain of Akt (146). This results in the translocation of Akt from cytosol to plasma membrane, where it becomes activated by phosphorylation on Thr^{308} and Ser^{473}. Finally, Akt is detached from the membrane, and goes to both cytosol and nucleus, where it regulates gene expression by the stimulation of transcription factors including NFkB and CREB (147). Several down

stream targets of Akt have been recognized to be apoptosis regulatory molecules including Bcl-2-family member BAD (148), and cAMP response element-binding protein (CREB) (149). We recently showed that resveratrol could induce the expression of Bcl-2, which was inhibited by A_1 and A_3 receptor antagonism. Additionally, the downstream target molecules of Bcl_2, BAD and CREB were phosphorylated with resveratrol. CSC and MRS 1191 significantly inhibited the phosphorylation of BAD, indicating that resveratrol-mediated Akt-Bad survival signal was regulated by both A_1 and A_3 adenosine receptors PI-3-kinase and Akt signaling pathways were also found to play a critical role in the prevention of apoptotic cell death by adenosine A_3 receptor activation (59).

CREB, a major nuclear transcription factor that transduces cAMP activation of gene transcription, is another regulatory downstream target molecule of Akt (149). CREB has been recognized as an important nuclear factor for cell survival. Overexpresson of a dominant negative CREB transgene induced apoptosis in T cells (150). A recent study showed that CREB contributed to cell survival in response to growth factor stimulation (151). Our results showed simultaneous induction of CREB and Bcl-2 in response to resveratrol treatment. The promoter region of Bcl-2 contains a cAMP-response element (CRE) site and the transcription factor CREB has been recognized as a positive regulator of Bcl_2 expression. Like NFκB, CREB is also a target for several signaling pathways mediated by a variety of stimulation. For example, IGF-1-stimulated CREB phosphorylation was decreased by Wortmannin, an inhibitor of PI-3-kinase, suggesting a role of Akt in CREB activation.

An alternative survival pathway via CREB that may bypass PI-3-kinase-Akt signaling has recently been described (152). In this report, CREB phosphorylation was found to occur through the activation of the MAP kinase pathway via activation of p90rsk. In another study, relaxin activated CREB through a Akt-independent signaling pathway (153), In this case, CREB may be phosphorylated via MEK/MAPK/p90rsk/CREB or cAMP-PKA signaling pathway, or both (154). In another related study, dopamine induced PI-3-kinase independent activation of Akt in striatal neurons indicating a new route to CREB phosphorylation (155).

Resveratrol has been known to modulate MAP kinase signaling. Among the three MAP kinases, ERK1/2 is involved in cell proliferation, while p38MAPK and JNK are activated in response to environmental stress. In undifferentiated cells, a small amount (1 μM) of resveratrol can induce phosphorylation of ERK1/2 (156). In retinoic

acid differentiated cells, the same amount of resveratrol induced an increase in ERK1/2 phosphorylation. Another study showed increased phosphorylation of ERK1/2, JNKs and p38MAPK in the mouse epidermal cells, which subsequently enhanced serine-15 phosphorylation of p53 (157). Dominant negative mutant of ERK2 or p38MAPK depressed phosphorylation of p53 at serine-15. In this study, overexpression of dominant-negative mutant of JNK1 had no effect on this phosphorylation. In papillary and follicular thyroid carcinoma cell lines, relatively higher amount of resveratrol (1-10 mM) induced activation and nuclear translocation of ERK1/2 (158). Interestingly, at higher concentration (even at 50-100 μM) resveratrol appears to inhibit phosphorylation of MAPK. At 37 mM concentration, resveratrol depressed MAPK activity and reduced phosphorylation of ERK1/2, JNK1 and p38 MAPK at active sites (159). Another related study showed that resveratrol activated JNKs at the same dose that inhibited tumor promoter-induced cell transformation (160). Thus, it appears that resveratrol can cause activation of MAPK in some cells, while it inhibits MAPK in others. Moreover, activation/inhibition seems to be concentration-dependent; in general, it is stimulatory at lower concentration and inhibitory at higher concentration. In the present study, resveratrol at 10 μM concentrations enhanced the phosphorylation of p38MAPK and ERK1/2. In concert, inhibition of p38MAPK with SB 202190 or ERK1/2 with PD098059 partially abolished the effect of preconditioning. MSK-1, a mitogen- and stress-activated protein kinase-1, is situated downstream of ERK1/2 and p38MAPK. MSK1 that belongs to AGC family of kinases and is related in structure to ribosomal p70 S6 subfamily can be activated by both ERK1/2 and p38MAPK. MSK-1 as well as MSK-2 can be directly activated both in vitro and in vivo by p42/44 ERK and p38MAPK in cultured cells (161). In another study, MSK-1 and MSK-2 activities were increased 400-500% and 200-300%, respectively, in exercised muscle along with an increase in MAPKAP kinase 2 (162). In a related study, ERK1/2 phosphorylation increased 7.8-fold and p38 MAPK phosphorylation increased 4.4-fold after the exercise (Yu et al, 2001). The activity of MAPKAP kinase 2, the downstream target of p38 MAPK, increased 3.1-fold while MSK-1, downstream of both ERK1/2 and p38MAPK increased 2.4-fold at the same time. In the present study, resveratrol-mediated increase in MSK-1 appears to be the result of the activation of both p38MAPK and ERK1/2, because inhibition of either p38MAPK or ERK1/2 resulted in partial downregulation of MSK-1.

MSK-1 is required for cyclic AMP response element (CRE)-binding protein (CREB) and the closely related activating transcription factor (ATF1) activation after mutagenic or stress stimuli. Upon phosphorylation, they recruit the co-activator CREB binding protein thereby effecting phosphorylation. Recently, resveratrol was found to phosphorylate CREB via adenosine A1 and A3 receptors through the activation of Akt survival pathway (59). Another related study demonstrated activation of CREB by resveratrol though Akt- dependent as well as Akt-independent pathways (163). Several distinct pathways can induce CREB, which is an important nuclear factor for cell survival. For example, growth factors and stress can induce CREB phosphorylation through the activation of downstream targets of MAP kinase signaling pathways including classical ERK pathway and stress activated p38MAPK pathway (149). Recent studies determined that MSKs are the major growth factor-regulated CREB kinase (164). In the present study, resveratrol-mediated CREB activation appears to occur through the phosphorylation of MSK-1 since the inhibition of MSK-1 abolished the phosphorylation of CREB. Previous studies demonstrated the involvement of CREB in transmitting resveratrol-mediated survival signal through the activation of Bcl-2 (59). Thus, it appears that resveratrol activates CREB through the phosphorylation of MSK1.

MAPKAP kinase 2 is the downstream target for p38MAPK. A large number of reports exist in the literature indicating that MAPKAP kinase 2 plays a crucial role in preconditioning (58). Preconditioning potentiates the phosphorylation of p38MAPK leading to the phosphorylation of MAPKAP kinase 2, which in turn upregulates heat shock protein 27 (HSP 27) (165). In this study, resveratrol could phosphorylate MAPKAP kinase 2 via the activation of p38MAP kinase as the MAPKAP kinase 2 phosphorylation was partially blocked with SB 202190.

In another segment of my study, I looked at the cardioprotective effect of TRF derived from palm oil. In that study, there are several salient features. *1*) Palm tocotrienol was found to provide cardioprotection, as evidenced by reduction of the ischemia-reperfusion-mediated increase in ventricular dysfunction, ventricular arrhythmias, and myocardial infarct size. *2*) Palm tocotrienol reduced ischemia-reperfusion-induced activation of c-Src activities. *3*) Tocotrienol stabilized proteasomes by preventing the ischemia-reperfusion-mediated reduction of 26S and 20S proteasomes. The results of the study thus showed, for the first time, that beneficial effects of tocotrienol are due to its ability to reduce c-Src activation, which is linked

with the stabilization of proteasomes. Tocotrienols have extremely short half-lives; after oral ingestion, they are not recognized by α-tocotrienol transport protein, which also accounts for their low bioavailability. For this reason, TRF was used in an acute experiment to determine its immediate effects on the ischemic-reperfused myocardium. The results indicate that tocotrienol readily blocks the ischemia-reperfusion-mediated increase in Src kinase activation and proteasome inactivation, thereby providing cardioprotection.

The Src kinases belong to the family of nonreceptor tyrosine kinases, which mediate a wide variety of intracellular signaling, including those mediating DNA synthesis and proliferation. Activation of Src kinase is associated with many degenerative diseases, including cardiovascular diseases, oncogenesis, and neurodegenerative diseases (166). In mammalian tumors expressing the *neu* protooncogene, c-Src tyrosine kinase activity is elevated (167). Markedly elevated levels of c-Src kinase activity were detected in human skin tumors (168). Myocardial ischemia-reperfusion caused an induction of c-Src protein expression (169); inhibition of c-Src with PPI reduces the extent of cellular injury.

Signaling pathways of Src kinase involve its activation through the activated cell surface receptors. For example, binding of ligand to platelet-derived growth factor receptors causes ligand to be associated with and to activate the Src family kinases (170), which trigger a cascade of events leading to entry into the S phase and subsequent DNA replication (171). The Src kinases are also involved in progression of the G_2-to-M transition of the cell cycle (172). In addition, Src kinases can also transduce signals in response to cell-cell or cell-matrix adhesion (173).

The present studies confirm previous studies (174, 175) that presented evidence of inactivation of 20S and 26S proteasomes during myocardial ischemia-reperfusion. When proteasome is inhibited, cell cycle regulatory proteins and proapoptotic factors, normally inactivated by this complex, can accumulate. Several studies have shown that many activated protein kinases, such as protein kinase C (176) and several members of the Src family of kinases (177-179), undergo suicide regulation, whereby the activated form is rapidly ubiquitinated and, thereby, becomes a target for the 26S proteasome. For example, Blk, an Src family member, is recognized by the E3 ubiquitin-protein ligase E6AP, which promotes its ubiquitination and subsequent degradation by the 26S proteasome (179). Other studies (180, 181) indicate that Src itself is degraded in a ubiquitin-dependent manner and that the active form is specifically targeted for

degradation, thus indicating a negative regulatory function for the proteasome. It is conceivable that decreased proteasome activity in postischemic hearts accounts for part of the observed increased Src and phosphorylated Src, which normally signals ubiquitination (181). This interpretation is strongly supported by the observation that preserving proteasome activity and, in particular, 26S proteasome activity, mitigates this increase. On the other hand, PPI has no protective effects on postischemic proteasome activities but, rather, directly inhibits c-Src, thus exerting an overall cytoprotective effect that is not mediated through the proteasome. In combination, these results support the conclusion that c-Src activation has a large role in postischemic cardiac injury and dysfunction.

The notion that preserving proteasome function in the postischemic heart can be protective may appear to be at odds with two studies (182, 183) that suggest protective effects on the ischemic myocardium of the proteasome inhibitor PS-519 (Millennium Pharmaceuticals). These studies (182, 183) used the inhibitor to limit the inflammatory response by decreasing leukocyte adhesion to endothelial cells. One of these studies (182) demonstrated positive effects in the leukocyte-supplemented crystalloid-perfused heart preparation but failed to observe any effect of the inhibitor, positive or negative, in the absence of the leukocytes. Although both of these studies (182, 183) determined peripheral leukocyte 20S proteasome activity, neither measured myocardial 20S nor 26S proteasome activity, nor did they measure levels of ubiquitin-conjugated proteins, and it is not clear whether the beneficial effect was related to myocardial proteasomes. The ability of proteasome inhibitors to decrease the inflammatory response has been well documented (184) and, besides effects on leukocyte adhesion, has been attributed to decreased ubiquitin-mediated degradation of NF-κB nuclear translocation (185). Whether a proteasome inhibitor has a beneficial (anti-inflammatory) or negative (proapoptotic) effect is notoriously dose related (186) and will be somewhat dependent on degrees of proteasome activity in the different tissues (i.e., leukocyte vs. heart). When little or no proteasome inhibition is present, such as after brief ischemia, a decrease in leukocyte-mediated inflammation may be beneficial. However, in the presence of decreased proteasome activity, an inhibitor that adds to ischemia-mediated proteasome inhibition may tilt the cell toward death, but an agent, such as TRF, which protects the proteasome, may be beneficial, as shown in this study.

The mechanism by which TRF preserves proteasome activity is not completely understood from these studies. It is tempting to speculate that TRF acts as an

antioxidant, preventing oxidative inactivation during ischemia. The 20S and 26S proteasomes have been shown to be vulnerable to oxidative inactivation, with 26S proteasome significantly more vulnerable (181). Indeed, Bulteau et al. (174) showed that subunits of the 20S proteasome are oxidatively modified during myocardial ischemia. However, a recent study (187) suggests that certain antioxidants isolated from cruciferous vegetables are capable of upregulating expression of several subunits of the proteasome, an effect observed 24 h after treatment. In light of the rather short treatment and posttreatment intervals used in the present experiments, it is unlikely that proteasome upregulation could account for the increased proteasome activity. Nonetheless, proteasome activity was increased to levels greater than baseline, indicating activation. This suggests mechanisms that include other than simple antioxidation, possibly redox effects, as has been suggested for the 20S proteasome (188).

We have initiated a study aimed at identifying the sub-cellular mechanism of action of resveratrol and tocotrienols, derived from palm oil, in the pathogenesis of ischemic heart disease and furthermore to reveal the redox signaling pathways for the cardioprotection of these two natural polyphenols. In order to accomplish these goals, we have utilized several strategies in order to investigate the cardioprotective role of resveratrol and tocotrienols derived from palm oil.

Our major findings were the following:

- Resveratrol preconditioning is mediated by adenosine A_3 receptors that trigger CREB phosphorylation via both PI3-kinase-Akt and via MEK-CREB pathways. Resveratrol-mediated phosphorylation of Akt and CREB was blocked by MRS-1191, which also abolished cardioprotective abilities of resveratrol, indicating a crucial role of adenosine A_3 receptor for resveratrol preconditioning. That LY294002 completely inhibited Akt phosphorylation but partially blocked the phosphorylation of CREB, resulting in partial inhibition of resveratrol's ability to precondition the heart, suggests that PI3-kinase-Akt-CREB signaling pathway is at least partially responsible for the cardioprotection achieved by resveratrol. Partial blockage of CREB phosphorylation and resveratrol-mediated cardioprotection by PD098059 indicates negative role of PI3-kinase/Akt signaling in CREB activation. This receives further support from the finding that LY294002 and PD098059 together abolished the phosphorylation of CREB simultaneously inhibiting resveratrol-mediated cardioprotection. The results indicate that resveratrol preconditions the hearts through adenosine A_3 receptor signaling that triggers the phosphorylation of CREB through both Akt-dependent and -independent pathways, leading to cardioprotection.
- The results of the present study showed for the first time that resveratrol triggers a preconditioning-like survival signaling by activating MAP kinase signaling pathway. Thus, resveratrol activates both ERK1/2 and p38MAPK both of which contributes towards the phosphorylation of MSK-1. There appears to be two downstream targets for p38MAPK, MSK-1 and MAPKAP kinase 2. MSK-1 in turn activates CREB, which was previously shown to transmit survival signal by activating Bcl 2.
- Ischemia-reperfusion caused ventricular dysfunction, electrical rhythm disturbances, and increased myocardial infarct size. PPI or TRF could reverse the ischemia-reperfusion-mediated cardiac dysfunction. Ischemia-reperfusion also upregulated c-Src expression and phosphorylation. Although TRF only minimally

affected c-Src expression, it significantly inhibited the phosphorylation of c-Src. Ischemia-reperfusion reduced 20S and 26S proteasome activities, an effect prevented by TRF pretreatment. PPI exerted a cardioprotective effect that is not mediated by the proteasome but, rather, through direct inhibition of c-Src. The results of this study support a role for c-Src in postischemic cardiac injury and dysfunction and demonstrate direct cardioprotective effects of TRF. The cardioprotective properties of TRF appear to be due to inhibition of c-Src activation and proteasome stabilization.

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List of Publications

[1] Das S, Powell SR, Wang P, Divald A, Nesaretnam K, Tosaki A, Cordis GA, Maulik N, Das DK. Cardioprotection with palm tocotrienol: antioxidant activity of tocotrienol is linked with its ability to stabilize proteasomes. Am. J. Physiol. Heart Circ. Physiol. 2005 Jul; 289(1):H361-H367.

[2] Das S, Tosaki A, Bagchi D, Maulik N, Das DK. Resveratrol-mediated activation of cAMP response element-binding protein through adenosine A3 receptor by Akt-dependent and -independent pathways. J. Pharmacol. Exp. Ther. 2005 Aug; 314(2):762-769.

[3] Das S, Tosaki A, Bagchi D, Maulik N, Das DK. POTENTIATION OF A SURVIVAL SIGNAL IN THE ISCHEMIC HEART BY RESVERATROL THROUGH p38MAPK-MSK-1-CREB SIGNALING. J. Pharmacol. Exp. Ther. (in press).