

Protection against ischemia/reperfusion:
role of the farnesol and red palm oil

Summary of PhD Thesis

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INTRODUCTION

Acute myocardial infarction and ischemia/reperfusion injury

Ischemic heart diseases including acute myocardial infarction are currently the leading causes of morbidity and mortality in the world. Myocardial ischemia can be described as an imbalance between oxygen and nutrient supply and demand of myocardial tissue. The definitive therapy for myocardial infarction is still the rapid reclamation of blood flow. Early reperfusion at least partly saves ischemic myocardium; however, reperfusion exacerbates further complications such as diminished cardiac contractile function (stunning) and arrhythmias. Therefore, the prevention of ischemic heart disease is very important to avoid the enormous clinical, economic, and social costs of cardiovascular disease. The process involves damage caused by ischemia and reperfusion is called ischemia/reperfusion injury. The development of effective cardioprotective agents and pretreatments against ischemia/reperfusion injury is of great importance and remains a foremost experimental goal of heart research.

Hypercholesterolemia

Incidence of hypercholesterolemia is constantly growing worldwide. Hypercholesterolemia is a well-known risk factor for atherosclerosis and therefore, coronary heart diseases, including myocardial infarction. Hypercholesterolemia usually results of nutritional factors combined with decreased physical activity, and underlying polygenic predispositions. It is known, that hypercholesterolemia increases the incidence of cardiovascular diseases through atherosclerosis; however, several studies showed that hypercholesterolemia exerts direct myocardial effects independent of the development of atherosclerosis both in humans and animal models. We used the hearts of male Wistar rats to study the direct cardiac effects of dietary cholesterol as rats show moderate increase in serum cholesterol level due to a high-cholesterol diet without substantial development of atherosclerosis. Hypercholesterolemia independently from the vascular effects negatively influences myocardial performance: impairs systolic as well as diastolic contractile function, aggravates the deleterious effects of ischemia/reperfusion injury, and interferes with pre- and postconditioning (endogenous cardioprotective mechanisms). Furthermore, it has been shown that myocardial function impaired in cholesterol fed rats and this impaired function is in connection with increased peroxynitrite level caused by cholesterol. Cholesterol is the bulk end-product of the mevalonate pathway. HMG-CoA reductase enzyme is the rate limiting step of mevalonate pathway. The end product cholesterol represses the transcription of the HMG-CoA reductase gene. The most commonly used cholesterol lowering drugs, statins, are competitive inhibitors of HMG-CoA reductase enzyme. In humans a number of large clinical trials have shown that chronic administration of statins have potent cholesterol-lowering effects and reduce cardiovascular morbidity and mortality. On the other hand, it is well known that chronic treatment with statins may exhibit a number of extrahepatic adverse

effects, such as myopathy and rhabdomyolysis. Therefore, it is reasonable to develop new, safer therapeutic tool to influence mevalonate pathway.

Mevalonate pathway intermediates

Farnesol is a 15-carbon sesquiterpenoid molecule. Bentinger et al. (1998) has shown in rats that farnesol could be phosphorylated in vivo to form farnesyl-pyrophosphate which is a key branching point in the mevalonate pathway and therefore, farnesol is able to modulate the mevalonate pathway. We have previously shown that administration of farnesol restored the protective effect of preconditioning in cholesterol fed rats; however, it is not known if farnesol itself is able to protect the heart against ischemia/reperfusion injury. Farnesol is naturally occurring mainly in aromatic fruits and is known as a powerful antioxidant. Farnesyl-pyrophosphate can be further metabolized to the mevalonate pathway end-products i.e. cholesterol, coenzyme Q, and dolichol. Moreover, farnesyl-pyrophosphate is a precursor for the prenylation (farnesylation and/or geranylgeranylation) of several intracellular proteins. Protein prenylation is a common post-translational modification of several intracellular proteins and prerequisite for their physiological function (e.g.: γ subunit of heterotrimeric G-proteins, nuclear lamins, Ras, Rho, Rac and Rab. Although several G-proteins are involved in the ischemia/reperfusion injury, the role of their prenylation has not been investigated. Farnesyl pyrophosphate is also a precursor for the synthesis of different forms of coenzyme Q. Coenzyme Q plays a major role in the mitochondrial electron-transport chain and serves as an endogenous antioxidant. Coenzyme Q is protective against myocardial ischemia/reperfusion-injury in animal studies and it is a registered drug for adjuvant therapy of heart failure worldwide. Dolichol, another derivative of farnesyl pyrophosphate, plays a role in the synthesis of oligosaccharide part of glycoproteins; however, its role in ischemia/reperfusion is not known. Farnesol also has been shown to exert an antioxidant effect in-vivo in rat model. This finding may support the potential cardioprotective effect of farnesol, since reactive oxygen and nitrogen species play a pivotal role in ischemia/reperfusion injury.

Red palm oil

Red palm oil (RPO) is natural oil obtained from oil palm fruit (*Elaeis guineensis*). RPO is an antioxidant rich oil which contains approximately 50% saturated and 50% unsaturated fatty acids. Carotenoids and vitamin E (75% of which is tocotrienol) are the most abundant antioxidants in this oil. Both of these antioxidants are contained at a level of at least 500 ppm in RPO. The cocktail of antioxidants within RPO is believed to have synergistic effects. The oil offers cardioprotection, by activation of several different protective pathways which work synergistically together. Dietary RPO supplementation has previously been shown to offer protection against ischemia/reperfusion injury in the isolated perfused heart. Esterhuysen and co-workers (2005) showed that dietary RPO

supplementation could improve post ischemic functional recovery in rats fed with standard rat chow or rat chow supplied with RPO.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of zinc dependent endopeptidases that recognized for their ability to remodel the extracellular matrix. MMPs involved in remodeling the extracellular matrix during various physiological functions such as embryonic development, angiogenesis, morphogenesis, wound healing, tissue repair and play a role in several pathological conditions such as cancer, cardiovascular disease, arthritis, inflammation, diabetes, etc. There are evidences that in pathological condition MMPs are able to proteolytically cleave intracellular proteins e.g.: troponin I. MMPs are synthesized in a latent form (zymogen or pro-MMP), and are activated by proteolytic removal of autoinhibitory propeptide domain or by conformational changes induced by denaturing agents or oxidative stress molecules such as peroxynitrite. Recently it was found that MMP2 plays a role in ischemia/reperfusion damage in the heart as ischemia induced activation and release of MMP-2 is a major effector of acute mechanical dysfunction after ischemia/reperfusion in rat hearts. Increased MMP2 activity has also been associated with hypercholesterolemic diets. This may be due to the increased peroxynitrite production within the myocardium. The RPO has antioxidant properties and therefore, may inhibit the peroxynitrite induced activation of MMP2.

In a recent study it was shown that red palm oil reduced infarct size in a model of ischemia/reperfusion injury. However, this model investigated only hearts of healthy animals. The intention of the current study was therefore, to use a high cholesterol feeding model to ensure that the model is more clinically relevant and to establish confirmation that red palm oil protection are indeed applicable in unhealthy diets. In all red palm oil fed studies up to date, results have indicated post-ischemic involvement of certain cellular biochemical pathways. However, none of these studies provided any evidence of pre-ischemic protection by red palm oil. Mechanism of RPO induced protection is not known; however we hypothesized that RPO may attenuate peroxynitrite induced MMP activation.

Aims

The aims of this study were: 1) to investigate the cardioprotective effect of farnesol and 2) to determine whether the antioxidant effect of farnesol or the increased coenzyme Q, protein prenylation and dolichol formation may protect myocardium against ischemia/reperfusion injury. Furthermore, our aims were 3) to investigate the effects of dietary RPO supplementation on myocardial infarct size in the hearts of rats on a cholesterol enriched diet and 4) to determine whether MMP2 activity was altered by RPO supplementation, both pre- and post-ischemically.

MATERIALS AND METHODS

Experimental design and isolated heart perfusion protocol of study 1

Here we assessed the effect of subchronic farnesol treatment on ischemic tolerance and biochemical changes of the heart. Male Wistar rats were randomly assigned to the following groups: oral administration of vehicle (2.5% methylcellulose) or 0.2, 1, 5, and 50 mg/kg/day farnesol (SAFC Supply Solution, St Louis, MO), respectively for 12 days. On day 13, the effect of farnesol on cardiac ischemic tolerance and biochemical changes was tested. Therefore, rat hearts were isolated and were subjected either to (i) 30 min coronary occlusion followed by 120 min reperfusion to measure infarct size in all groups or to (ii) 10 min of perfusion before tissue sampling for biochemical assays. Cardiac levels of mevalonate pathway end-products (prenylated proteins, cholesterol, coenzyme Q9, Q10, and dolichol), and 3-nitrotyrosine (marker of oxidative/nitrosative stress) were measured from the tissue samples in separate experiments, respectively.

Experimental design and isolated heart perfusion protocol of study 2

Male Wistar rats were divided into four groups. Rats in these groups were placed on the following diets, respectively:

Group 1: Standard rat chow diet for 9 weeks (Norm)

Group 2: 2% cholesterol-enriched diet for 9 weeks (Chol)

Group 3: Standard rat chow (SRC) supplemented with 200 µl RPO (Norm+RPO) per day for the last 5 weeks of the 9 week period

Group 4: 2% cholesterol-enriched diet for nine weeks supplemented with 200 µl RPO per day for the last 5 weeks of the 9 week period (Chol+RPO)

After the feeding period, hearts were isolated, mounted on a Langendorff perfusion apparatus. After mounting, hearts were subjected to 10 minutes of stabilization, followed by 30 minutes of normothermic global ischemia and 120 minutes of reperfusion. At the end of the perfusion protocol infarct size was measured.

Infarct size determination

To assess ischemic tolerance of the heart, infarct size was measured as follows. In case of coronary occlusion (study 1) at the end of the perfusion protocol, the coronary artery was reoccluded and 5 ml of 0.1% Evans blue dye was injected into the aorta to delineate the area at risk zone and after that the hearts were frozen. In case of global ischemia (study 2) the hearts were frozen without Evans blue dye. Frozen hearts were then sliced, and incubated for 10 min at 37°C in 1% triphenyltetrazolium chloride to delineate infarcted tissue. Slices were then fixed in formalin solution 10 min at 37°C and then placed in phosphate buffer (pH 7.4). Heart slices were then scanned into a computer and analyzed using infarct size planimetry software (Infarctsize™ 2.4 Pharmahungary, Szeged, Hungary). Infarct size was expressed as a percentage of the area at risk. In case of coronary

occlusion (study 1) the area at risk was calculated as a percentage of the total ventricular area. In case of global ischemia (study 2) the area at risk was the total ventricular area.

Measurement of myocardial protein geranylgeranylation and farnesylation

The nucleophilic cleavage of the allylic thiol bond by 2-naphthol provides for quantitative determination of the cysteine-bound prenyl groups of prenylated proteins. The level of cysteine-bound farnesyl and geranylgeranyl groups were measured by HPLC method as previously described. Briefly, 200 mg tissue samples were delipidated and mixed with 2.5 ml reagent mixture. Then dioxane was removed and dimethylformamide was added to samples and they were heated. Samples were extracted in hexane and were measured by HPLC.

Measurement of myocardial cholesterol

Ten mg ventricular homogenates were used for tissue cholesterol measurement (Cholesterol/Cholesterol Ester Quantification kit; BioVision, Mountain View, CA).

Measurement of myocardial coenzyme Q

The level of cardiac coenzyme Q9 and Q10 were measured from four hundred mg ventricular homogenate by a HPLC following lipid extraction.

Measurement of myocardial dolichol

For the measurement of cardiac dolichol level minced cardiac tissues (400 mg) were mixed with pyrogallol in methanol and KOH. After that hydrolysis was performed in a at 100°C and the mixture was extracted three times with diethyl ether:petroleum ether (1:1). The pooled extracts were dried under nitrogen. The samples were resolved with isopropanol:acetonitrile and dolichol was measured by HPLC.

Measurement of nitrotyrosine

Cardiac free 3-nitrotyrosine content, as markers for peroxynitrite-induced oxidative/nitrosative stress, was measured from 50 mg ventricular tissue homogenate by ELISA (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Measurement of protein concentration

Protein concentrations were measured by the BCA Protein Assay kit (Thermo, Rockford, IL) according to the instructions.

Neonatal cardiomyocyte culture experiments

To study direct cardiocytoprotective effect of farnesol we investigated whether farnesol protects cardiomyocytes subjected to simulated ischemia/reperfusion. One day old neonatal cardiomyocyte cultures were treated with farnesol (0.0032 – 250 μ M) for 2 days. After farnesol treatment, cardiomyocyte cultures were subjected 4 h simulated ischemia and 2 h simulated reperfusion. To assess cell viability cardiomyocytes were incubated with 1 μ M calcein acetoxymethyl ester (calcein-AM, Sigma, St Louis, MO) for 30 min. Fluorescence intensity was measured with a fluorescence plate

reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) at 490-nm excitation and 520-nm emission filters.

MMP2 zymography

Coronary effluent collected for 10 minutes before ischemia and the first 10 minutes of reperfusion was concentrated by Amicon ultra filtration tubes (Millipore, Billerica, MA). Gelatinolytic activities of MMPs contained by concentrated coronary flow were examined by zymography. Polyacrylamide gels were copolymerized with gelatin, and sample proteins were separated by electrophoresis. Following electrophoresis, gels were washed and incubated. Then gels were stained and digitally scanned, and band intensities were quantified.

Serum cholesterol and triglyceride measurement

Serum cholesterol and triglyceride were measured using a test kit supplied by Diagnosticum Zrt. (Budapest, Hungary) as described previously.

RESULTS

Study 1

To assess the cardioprotective effect of different oral doses of farnesol, infarct size was measured after 30 min regional ischemia and 120 min of reperfusion. Infarct size was significantly decreased by 1 mg/kg/day farnesol. However, 0.2, 5, and 50 mg/kg/day farnesol treatment did not significantly decrease infarct size. Therefore, the infarct size reducing effect of farnesol showed a U-shaped dose-response relationship.

In separate sets of experiments, to assess the effect of cardioprotective (1 mg/kg/day) dose of farnesol on mevalonate pathway end-products, we measured cardiac protein prenylation, cholesterol, coenzyme Q, and dolichol. We found that oral farnesol treatment significantly increased cardiac protein-bound geranylgeranyl level. However, farnesol did not influence cardiac protein-bound farnesyl level, cardiac cholesterol, coenzyme Q9 and Q10, and dolichol levels significantly.

To assess the antioxidant effect of the cardioprotective dose of farnesol in the heart, in separate experiments, we measured cardiac 3-nitrotyrosine level as marker of cardiac peroxynitrite which is a major player in cardiac oxidative and nitrosative stress. We found that the cardioprotective dose of farnesol (1 mg/kg/day) did not affect cardiac 3-nitrotyrosine level. Therefore, to further assess if farnesol at a higher dose may show an antioxidant effect, in separate experiment, we measured 3-nitrotyrosine in the 50 mg/kg/day farnesol-treated group. This dose of farnesol (50 mg/kg/day) significantly decreased cardiac 3-nitrotyrosine level; however, it did not show any cardioprotective effect.

To assess if farnesol has a direct cardiocytoprotective effect, we measured cell viability of cardiomyocytes subjected to simulated ischemia/reperfusion. Farnesol (0.0032 – 50 μ M) protected

cardiomyocytes in a bell-shaped concentration-response manner similarly to that observed in the isolated heart experiments. However, 250 μ M farnesol showed a cytotoxic effect.

Study 2

The body and heart weight of the RPO supplemented rats were significantly decreased when compared to the Norm group and also to the Chol+RPO group after the feeding period.

There were no significant differences in the coronary effluent of any of the groups after the supplementation period. Coronary effluent of all groups except the RPO group was significantly decreased after ischemia, when compared to their baseline values. There were no significant differences in the heart rates before or after ischemia.

Cholesterol-enriched diet alone increased myocardial infarct size compared to normal diet. RPO supplementation significantly reduced infarct size in Norm+RPO and also in Chol+RPO groups. Infarct size in Chol+RPO group was comparable to the group fed with normal diet.

Before ischemia activity of the 75kDa isoform of MMP2 was significantly lower in the Chol+RPO group when compared to the Chol group. After ischemia MMP2 (72kDa isoform) activity of the RPO supplemented group was significantly increased when compared to rats with normal diet. There were no significant differences in the serum total cholesterol and triglyceride level among groups.

DISCUSSION

Study 1

This is the first demonstration that oral farnesol treatment reduces infarct size in the rat heart following ischemia/reperfusion. We also found that farnesol shows a direct cytoprotective effect in cardiomyocytes subjected to simulated ischemia/reperfusion. Furthermore, we found that the *in vivo* cardioprotective dose of farnesol (1 mg/kg/day for 12 days) increased the geranylgeranylation of cardiac proteins, however, it did not affect other mevalonate pathway-derived end-products (cardiac cholesterol, coenzyme Q, dolichol) and cardiac peroxynitrite marker 3-nitrotyrosine level significantly. This shows that the cardioprotective effect of farnesol is independent of its antioxidant effect but may involve changes in protein geranylgeranylation.

It is known that mammalian cells can utilize farnesol for protein prenylation and for synthesis of other mevalonate pathway derivatives (cholesterol, coenzyme Q, and dolichol). Bentinger et al. (1998) has shown in rats that farnesol could be phosphorylated *in vivo* to form farnesyl-pyrophosphate, which could re-enter biosynthetic reactions. Our present results support this finding since administration of exogenous farnesol increased total myocardial protein geranylgeranylation and reduced infarct size.

Prenylation (protein farnesylation and geranylgeranylation) is a lipid post-translational modification of proteins involving the irreversible covalent attachment of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid to conserved cysteine residues at or near the C-terminus of numerous cellular proteins. Prenyl groups act as hydrophobic membrane anchors and play key role in the maturation of proteins, regulation of protein targeting, function of proteins, and controlling apoptosis. Large number of proteins is known to be prenylated, such as e.g. γ subunit of heterotrimeric G-proteins, nuclear lamins, and some members of the Ras superfamily of small GTPases e.g.: Ras, Rho, Rac, Rab subfamily. Some of these proteins have been shown to be involved in cardioprotection. Brar et al. (2004) also showed that cardioprotective effect of urocortin-II against ischemia/reperfusion injury in rat heart was completely abolished by the Ras inhibitor manumycin A. It was also shown that infarct size was increased in cardiac specific RhoA knockout mice hearts and was significantly decreased in cardiac-specific RhoA transgenic mice. It was also reported that activation of c-Jun N-terminal kinase through the Rac1/cdc42-TAK-1 pathway promotes survival of cardiac myocytes after hypoxia-reoxygenation. Members of the Rab subfamily are geranylgeranylated while the other small GTPases either farnesylated or geranylgeranylated. In our present study, we measured total protein farnesylation and geranylgeranylation and found that farnesol treatment significantly increased total myocardial protein geranylgeranylation but not farnesylation. However, the reason for the discrepancy between farnesol-induced protein geranylgeranylation and farnesylation remained unknown and it was not investigated in our present

study. Protein prenylation is catalysed by prenyl transferase enzymes, which can be classified into two main functional classes: (i) the CAAX prenyl transferases including farnesyl transferase and geranylgeranyl transferase type 1, and (ii) the Rab geranylgeranyl transferase (geranylgeranyl transferase type 2). CAAX prenyltransferase activities are highly selective for their isoprenoid diphosphate substrates: farnesyl transferase for farnesyl-pyrophosphate and geranylgeranyl transferase type 1 for geranylgeranyl-pyrophosphate. However, farnesyl transferase can also bind geranylgeranyl-pyrophosphate with low affinity, yet the enzyme is unable to transfer the geranylgeranyl group to substrate proteins, indicating that geranylgeranyl-pyrophosphate is an inhibitor of farnesyl transferase. This might explain our findings regarding the difference between farnesol-induced protein geranylgeranylation and farnesylation.

Farnesyl-pyrophosphate can be utilized for synthesis of cholesterol, coenzyme Q, and dolichol. In our present study, we have observed that cardiac tissue cholesterol content expressed in ng/mg protein was approximately 10-fold higher than the level of geranylgeranyl group of protein; however, farnesol treatment did not modify cardiac cholesterol production.

In our present study, we have found that the cardiac tissue content of coenzyme Q9 was comparable to that of protein-bound geranylgeranyl group. Cardiac level of coenzyme Q10 content was approximately 10-fold less than coenzyme Q9 or protein-bound geranylgeranyl group. Our result is in concordance with the findings of Matejíková et al. (2008) who reported that the rat heart contains approximately 10-fold more coenzyme Q9 than coenzyme Q10. Coenzyme Q is a well-known antioxidant and cardioprotective molecule. In our present study, farnesol treatment failed to alter coenzyme Q levels, which suggest that farnesol-induced cardioprotection is independent of changes in coenzyme Q levels. In our experiment the dolichol content of cardiac tissue did not change, but it was 10-fold lower than protein-bound geranylgeranyl content. The role of dolichol in ischemia/reperfusion is not known in the literature. However, it was shown that dolichol kinase deficiency causes congenital dilated cardiomyopathy in patients.

Farnesol has been shown to exert antioxidant effect *in vivo*. However, in our present study the cardioprotective dose of farnesol (1 mg/kg/day) failed to decrease peroxynitrite formation, a major player in oxidative/nitrosative stress. However, 50 mg/kg/day farnesol, which did not show any cardioprotective effect, significantly decreased 3-nitrotyrosine a marker of peroxynitrite, demonstrating an antioxidant effect of farnesol. These results show that the cardioprotective effect of farnesol is independent from its antioxidant effect.

We have found here that oral farnesol reduced infarct size in a U shaped dose-dependent manner showing the maximum efficacy at 1 mg/kg/day dose. Moreover, here we also demonstrated that farnesol exerts a similar concentration-dependent direct cytoprotective effect in cardiomyocytes *in vitro*. These results show that the cardioprotective effect of farnesol is not dependent on any

systemic effect; however, it might be based on a direct cardiocytoprotective action. The reason for the inefficiency of higher farnesol doses (5 and 50 mg/kg/day in in-vivo experiments and 2, 10, 50, 250 μ M in neonatal rat cardiomyocyte experiments) has not been revealed in this study. However, one may speculate that the potential pro-apoptotic effect of farnesol may interfere with its cardioprotective effect at higher doses. Indeed, Chagas et al. (2009) found in a partial hepatectomy model that farnesol (250 mg/kg for 2 consecutive weeks) induced apoptosis in rat hepatocytes. Joo et al. (2007) reported that 250 μ M farnesol induced apoptosis in human lung carcinoma cell line. Indeed, we have found here that 250 μ M farnesol radically decreased the viability of cardiomyocytes. The present study has clearly shown the cardioprotective effect of farnesol and revealed several aspects of its mechanism; however its cellular mechanism has not been fully explored. An obvious limitation of the present study is that total protein farnesylation and geranylgeranylation were measured and individual protein prenylation was not examined. Further studies are necessary to identify the specific geranylgeranylated proteins, which may play a role in the cardioprotective effect of farnesol.

Study 2

Our results show that dietary RPO supplementation reversed the negative effects of cholesterol supplementation in the ischemia/reperfusion rat heart model. Furthermore, dietary RPO supplementation reduced myocardial infarct size in cholesterol supplemented rats. Previous studies have shown that dietary RPO supplementation improved functional recovery of cholesterol fed rats after ischemia. In the present study cholesterol supplementation was carried out for a longer period (nine weeks versus six weeks in previous studies). This indicates that RPO could effectively protect hearts against ischemia/reperfusion injury, despite a longer duration of cholesterol feeding. Osipov and co-workers (2009) found that hypercholesterolemic pigs had increased left ventricular function throughout the ischemia/reperfusion period when compared to normal pigs. This was, however, associated with an increased infarct size and increased apoptotic markers. Our results together with previous studies showed that dietary RPO can attenuate the harmful effects of cholesterol supplementation in the ischemia/reperfusion model. Our total serum cholesterol results were not increased in the cholesterol fed rats. This was expected, as a previous study employed a similar model of cholesterol feeding in rats, without achieving significant changes in serum cholesterol. The effects of the cholesterol feeding on the cardiovascular system are however clearly discussed in the study of Giricz and coworkers (2003) through the depletion of nitric oxide, and our current study by increased myocardial infarct size.

Coronary flow in the Norm group was reduced by 70%, in the Chol group by 64%, in the Chol+RPO group by 57% and in the Norm+RPO group by 33% after ischemia. This indicates that RPO attenuated

the fall in coronary flow after ischemia. This may suggest that RPO supplementation improves vascular function during reperfusion.

MMP2 activity was measured before ischemia in the cholesterol supplemented groups and compared to a Norm control group and a RPO supplemented group, as it may be expected that the cholesterol supplemented groups may have increased oxidative stress after supplementation. As increased oxidative stress leads to activation of MMP2 through redox modification of its regulatory subunit, this would be associated with changes in MMP2 activity before ischemia which would be expected to be absent in normal rats. Our results demonstrate for the first time that dietary RPO supplementation may alter myocardial oxidative stress before ischemia in cholesterol fed rats, as MMP2 activity was reduced before ischemia. The reduction in MMP2 activity before ischemia in rats supplemented with both cholesterol and RPO suggests that RPO was able to reduce oxidative stress in these rats. This would most probably be achieved through quenching of ROS, which is generated in greater proportions in cholesterol supplemented rats. Increased generation of ROS and oxidative stress would normally be associated with activation of MMP2. As increased activity of MMP2 may lead to either cardiac remodeling, or tissue damage, this reduction in MMP2 activity may play a role in RPO mediated protection against ischemia/reperfusion injury. However, MMP2 activity of the Norm+RPO was increased during reperfusion, when compared to normal rats without RPO supplementation. This would normally be associated with increased myocardial susceptibility to ischemia/reperfusion injury. RPO was able to reduce myocardial infarct size in cholesterol fed rats, despite increased activity of MMP2 in reperfusion found in normal rats. This suggests MMP2 activity may only play a protective role in cholesterol fed rats, and that other protective pathways are responsible for RPO mediated protection in normal rats.

The aim of this study was to investigate whether MMP2 activity was involved in RPO mediated protection of cholesterol fed rat hearts against ischemia/reperfusion. Our results suggest that MMP2 activity may play a role in RPO mediated protection of the hearts of the cholesterol fed rats, but not the hearts of SRC fed rats. This suggests that more pathways of protection may play a role in this protection. Kruger and co-workers (2007) found that RPO supplementation of cholesterol fed rats led to decreased phosphorylation of pro-apoptotic molecules, p38 and JNK. This coincided with increased phosphorylation of the pro-survival kinase ERK early in reperfusion, which leads to reduced apoptosis. Apoptosis has been shown to play a role in the detrimental effects of hypercholesterolemia in the heart. Inhibition of apoptosis may therefore, explain the protective effects of RPO in this model.

Acute myocardial infarction is currently the leading causes of morbidity and mortality in the world. Myocardial infarction has several risk factors and the development of these risk factors takes

decades when they finally lead to the cardiovascular event. Rapid reclamation of blood flow to the ischemic zone can decrease the mortality of myocardial infarction but it is still high. Therefore, the prevention of myocardial infarction is foremost goal. Here we presented oral pretreatments with two widely used natural food additives which decrease ischemia/reperfusion injury. It may improve the compliance of patients (with risk factors) and increase the effectiveness of prevention of ischemic heart diseases that these materials are natural and orally usable. However, further investigations are needed to understand the mechanism of protective effect of farnesol and RPO. Further understanding of the biochemical mechanism of the protective effect of farnesol and RPO may help to develop new and more effective prevention of acute myocardial infarction.

New Observations

Study 1

- 1) We demonstrated that oral farnesol treatment reduces ischemia/reperfusion injury.
- 2) We showed that the cardioprotective effect of farnesol likely involves increased protein geranylgeranylation and seems to be independent of the other end-products of mevalonate pathway and the antioxidant effect of farnesol.

Study 2

- 1) We showed that dietary RPO supplementation attenuated increased susceptibility of cholesterol fed rat hearts to ischemia/reperfusion injury as evidenced by reduced infarct size.
- 2) Myocardial MMP2 activity was reduced in cholesterol and RPO supplemented rat hearts before ischemia, but not after ischemia associated with decreased infarct size.

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LIST OF PUBLICATIONS**List of full papers directly related to the subject of thesis**

Cardioprotection by Farnesol: Role of the Mevalonate Pathway. **Szűcs G**, Murlasits Z, Török S, Kocsis GF, Pálóczi J, Görbe A, Csont T, Csonka C, Ferdinandy P. Cardiovasc Drugs Ther. 2013 May 15. [Epub ahead of print]

IF.: 2.673

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IF.: 2.170

Cumulative impact factor of papers directly related to the thesis: 4.843

List of full papers indirectly related to the subject of thesis

MicroRNA-25-dependent up-regulation of NADPH oxidase 4 (NOX4) mediates hypercholesterolemia-induced oxidative/nitrative stress and subsequent dysfunction in the heart. Varga ZV, Kupai K, Szűcs G, Gáspár R, Pálóczi J, Faragó N, Zvara A, Puskás LG, Rázga Z, Tiszlavicz L, Bencsik P, Görbe A, Csonka C, Ferdinandy P, Csont T. J Mol Cell Cardiol. 2013 [Epub ahead of print]

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Cumulative impact factor of papers indirectly related to the thesis: 11.596