The role of the endogenous antioxidant enzyme, glutathione S-transferase /GST/ on cultured cardiomyocytes under oxidative stress conditions

Summary of Ph.D. Thesis

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1. INTRODUCTION

Myocardial ischemia-reperfusion injury is crucially involved in the pathogenesis of cardiovascular diseases. It has been well investigated that oxidative stress following ischaemic-reperfusion injury is a major apoptotic stimulus in many cardiac diseases. Under normal circumstances the endogenous antioxidant systems neutralise the harmful effects of free radicals. Pathophysiological conditions (such as hypoxia, ischemia, early reperfusion, etc) the amount of nascent oxygen free radicals and reactive oxygen intermediers (ROI) beyond the capacity of endogenous antioxidants and the oxidative stress developes.

Although recently surgical interventions more frequent in myocardial diseases, but to avoid the ischemic-reperfusion injury is not solved. The role of oxygen free radicals in reperfusion injury is well known, accordingly to decrease of these harmful agents is very important. Catalase, superoxide dismutase (SOD), glutathione peroxidase and repaire enzymes are in the first line of antioxidant protection, but recently among other antioxidant enzymes researches are focus on *glutathione S-transferase (GST)*.

Myocyte loss during ischemic-reperfusion injury involves both apoptotic and necrotic cell death. Therefore, it is reasonable to think that the balance of cell survival and death is critical during the pathological evolution of postischemic cardiac dysfunction. The intricate relationship between signal transduction and this balance of survival and death in oxidative stress and ischemic-reperfusion injury make the investigations necessary to be focused on MAP kinases.

1.1. GLUTATHIONE AND GLUTATHIONE S-TRANSFERASE

Glutathione (GSH) is the predominant low-molecular-weight thiol (0.5–10 mmol/L) in cells. Most of the cellular GSH (85–90%) is present in the cytosol, with the remainder in many organelles (including the mitochondria, nuclear matrix, and peroxisomes).

Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species).

GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells. It is important to note that shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways (including protein kinase B, calcineurin, nuclear factor κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase), thereby reducing cell proliferation and increasing apoptosis.

A major factor that affects glutathione homeostasis is its utilization by conjugation, primarily via glutathione S-transferase (GST).

Glutathione S-transferases (GSTs), are members of a multigene family of isoenzymes ubiquitously expressed in most living organisms. It was subsequently shown that these enzymes catalyze the conjugation of glutathione (GSH) to a variety of electrophilic compounds, thus establishing the now widely accepted role of GSTs as cell housekeepers involved in the detoxification of endogenous as well as exogenous substances.

The GSTs encompass three major families of proteins: (1) cytosolic, (2) mitochondrial, and (3) microsomal (also referred to as membrane-associated proteins in eicosanoid and glutathione /MAPEG/), of which the cytosolic GSTs constitute the largest family. On the basis of amino acid sequence similarities, substrate specificity, and immunological cross-reactivity, seven classes of cytosolic GSTs have been identified in mammals. These classes are designated by the names of the Greek letters α (alpha), μ (mu), π (pi), σ (sigma), θ (theta), ω (omega), and ζ (zeta).

The ability of GST to alter levels of cellular glutathione in response to production of ROS has been implicated in protection of cells from ROS-inducing agents.

1.2. ISCHAEMIC/REPRFUSION INJURY

Myocardial reperfusion is the restoration of blood flow to an ischemic heart. Early reperfusion minimizes the extent of damage of heart muscle and preserves the pumping function of the heart. However, reperfusion has been referred as a „double edged sword" because after a prolonged period of ischemia reperfusion produces a marked damage in myocardium rather than restoration of normal cardiac function. This reoxygenation injury is mediated by a burst of ROS production. Thus, ischemia–reperfusion (I/R) injury could be defined as the damage to heart when blood supply is restored after a prolonged period of ischemia resulting in oxidative damage, inflammation and cardiac dysfunction. Reperfusion injury is an integrated response to the restoration of blood flow after ischaemia involving mechanical, extracellular and intracellular processes.

1.3. ISCHAEMIC POSTCONDITIONING

To protect the heart against ischaemia and reperfusion injury ischaemic preconditioning (IPC) is a well known strategy. However, unpredictability of clinical acute myocardial infarction precludes the application of preconditioning.

Postconditioning is controlled reperfusion, defined as a series of brief interruptions of ischemia/reperfusion applied at the very onset of reperfusion can protect the myocardium from ischaemic/reperfusion injury. It may have greater clinical potential than preconditioning because of the usebility in cases of unintended interventions. Heusch described ischemic postconditioning as "old wine in a new bottle". The concept of postconditioning was first revealed in 2002 by Vinten-Johansen and colleagues. The first studies published by Zhao et al., (2002).

2. AIMS AND HYPOTHESIS

In the first part of our investigations we aimed to identify the biological role of GST in cardiomyocytes under oxidative stress conditions. Principally, our aim was to evaluate the effect of GST inhibition (using its potent inhibitor, ethacrynic acid [EA]) on cardiomyocyte apoptosis and on the alteration of proteins and MAP kinase pathways.

In the second part of our study the main objective was to identify the role of MAPKs (JNK, p38 and ERK/p42-44) on the viability and apoptosis of cardiomyocytes when cells are exposed to various stress components of ischaemia and reperfusion using parallel GST and MAPKs inhibitors.

Finally, in the third we targeted to investigate whether inhibition of GST (by it potent inhibitor ethacrynic acid) can abolish the cellular mechanisms and benefit of ischaemic postconditioning (IPoC) in vitro ischaemic/reperfusion injury by assessing the cell viability and apoptosis in rat cardiomyocyte culture in addition on alteration of activities of mitogen activated protein (MAP) kinase pathways.

3. THE ROLE OF GLUTATHIONE S-TRANFERASE IN CELL VIABILITY AND SIGNAL PATHWAYS IN CARDIAC MYOCYTES UNDER OXIDATIVE STRESS CONDITIONS

3.1. INTRODUCTION

A large collection of experimental data support the presence of apoptosis in a variety of cardiovascular diseases. It has also been well investigated that oxidative stress is a major apoptotic stimulus in many cardiac diseases. Among numerous defence mechanisms against oxidative injury, glutathione S-transferase (GST) plays a crucial role. The GST family, which comprises a relatively high amount of total cytosolic protein, is responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates. Thus, glutathione homeostasis is essentially regulated by GST activity, and the glutathione redox status is critical for various biological events. Recently, novel roles for glutathione homeostasis and GST in signal transduction, gene expression, apoptosis, protein glutathionylation, nitric oxide metabolism and inflammation have been discussed.

It is important to note that alterations of cellular-reduced glutathione (GSH) metabolism and activity of GST can influence several signalling pathways. Certain types of GST play a key role in regulating mitogen-activated protein (MAP) kinase pathways involved in the cellular response to stress, apoptosis and proliferation, thus altering activity of apoptotic signal-regulating kinase-1 (ASK1) and influencing the decision regarding cell fate.

At least five of the human GST genes display functional polymorphisms. These polymorphisms are likely to contribute to interindividual differences in response to xenobiotics and clearance of oxidative stress products and, therefore, may determine susceptibility to various inflammatory pathologies including cancer, and cardiovascular and respiratory diseases. In addition, some GST polymorphisms have also been associated with increased risk of lung adenocarcinoma. Recent studies highlight the potentially unique roles of GST enzymes as crucial determinants of the development of ischemia-reperfusion (I/R). An association was found between different donor GST genotypes and primary graft dysfunction in patients following heart and lung transplantation. Other studies described the damaging effect of GST inhibition on peripheral and central motor neurons, cerebral astrocytes, isolated hepatocytes and vascular smooth muscle cells. Although the effect of GSH depletion in cardiomyocytes has been well described to be a result of different pathological states, the exact role of GST activity on cardiomyocyte apoptosis and alteration of signalling cascades of cardiomyocytes has not been determined.

3.2. AIMS

In present study we aimed to investigate the biological role of GST in cardiac myocytes under different stress conditions. Therefore we tried to identify the effect of GST inhibition via administration of EA when cells were exposed to various stress components of I/R. Since GST activity is a major determinant for survival and adaptive response to oxidative stress in the heart, thus we hypothesized that its pharmacologic inhibition with EA might exacerbate the severity and outcome of I/R and oxidative injury.

3.3. MATERIALS AND METHODS

3.3.1. Isolation of cardiac cells from neonatal rats

Primary culture of neonatal rat cardiomyocytes was prepared as described previously. Briefly, cells were obtained from ventricular myocytes of 2-4 day-old Wistar rats (Charles-River ltd., Hungary), using collagenase (Gibco™ Collagenase Type II, Invitrogen Corp., Carlsbad, CA, USA). Isolated cells were plated on collagen I-coated 24-well plates (BD Falcon) at the density of $2x10^5$ cells/ml. Cells were incubated in DMEM/F12 medium (Sigma–Aldrich, USA) supplemented with 10 % of fetal bovine serum (Gibco, USA). The following day, when the cells attached to the plate firmly, the medium was replaced with complete serum free medium (CSFM).

3.3.2. Ethacrynic acid is a potent inhibitor of GST

Present study utilised EA for pharmacological inhibition of GST. EA has been shown to be a substrate of majority of GST isozymes furthermore nonenzymatic GSH conjugation of EA also exists. Moreover it was shown that EA-SG was an inhibitor of the GSTs due to its greater affinity for the enzymes, whereas EA itself inhibits GST through reversible covalent interactions.

3.3.3. Experimental protocol

Cultured cardiomyocytes were randomly assigned to one of six experimental groups (Figure 1.): control group of cells that were incubated in CSFM without treatment (group 1). In the second group isolated cells treated with 150 μM of EA alone (group 2). In the third group cells exposed to 1 mM of H_2O_2 (group 3). In the fourth group cells treated with 1 mM of H_2O_2 together with 150 μM of EA (group 4). In the fifth group cells exposed to I/R (group 5). In the last group cells exposed to I/R and 150 μM of EA (group 6).

To mimic the ischaemic stimuli cell cultures were exposed to ischaemic buffer as described previously. During the ischaemic stress insult cardiac cell cultures were incubated in cell incubator in an atmosphere of 95 % air and 5% CO2, on 37 °C.

In groups receiving simulated I/R cells were exposed to 1,5 hours of ischemia using SI buffer, followed by 2,5 hours of reperfusion using normal CSFM. In group VI. (cells were exposed to both simulated I/R and EA) both ischemic buffer and reperfusion medium (CSFM) contained 150 μM of EA.

Based on our pilot experiments we chose to use a concentration of 150 μM and a treatment time of 4 hours.

Viability of cardiomyocytes was determined by colorimetric MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] to measure the absolute number of living cells in different groups. Ratio of apoptosis was evaluated after double staining with fluorescein isothicyanate (FITC)-labeled annexin V and propidium iodide using flow cytometry. The activation of JNK, p38, ERK/p42-p44 and Akt/PKB MAPKs were monitored with flow cytometry.

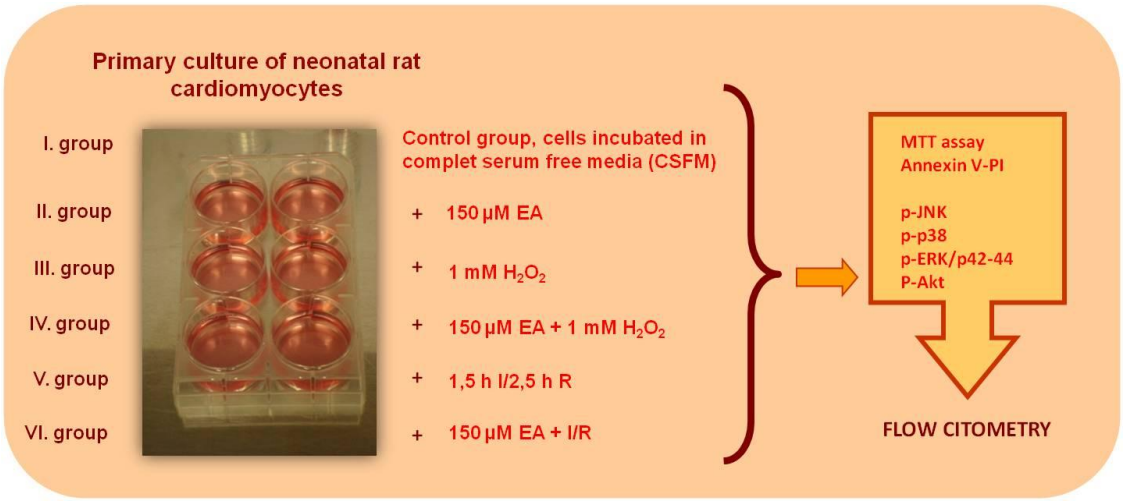

Figure 1. Experimental protocol, using GST inhibitor EA, oxidative stress (H_2O_2), simulated I/R and the combination of these. Viability of cells were measured by MTT assay, the amount of apoptotic cells were assessed by flow cytometry following annexin V-FITC/propidium iodide double staining. The activation of JNK, p38, ERK/p42-p44 and Akt MAPKs were determined by flow cytometric assay.

3.3.4. Statistical analysis

All data are presented as mean ± standard error of the mean (S.E.M). Differences between groups were assessed with one-way ANOVA and Student's t test and were considered significant when P-value was less than 0.05.

3.4. RESULTS

3.4.1. MTT assay results

An MTT assay was performed to measure the absolute number of living cells in the groups. In the control group, the amount of living cells was increased to 100%. EA alone reduced the ratio of living cells to 43.41±11.15%, measured by MTT assay. Both I/R and H_2O_2 alone caused a marked reduction in the amount of living cells. The effect of cell death was significantly stronger on EA administration in groups treated with H_2O_2 or exposed to I/R. (Figure 2.)

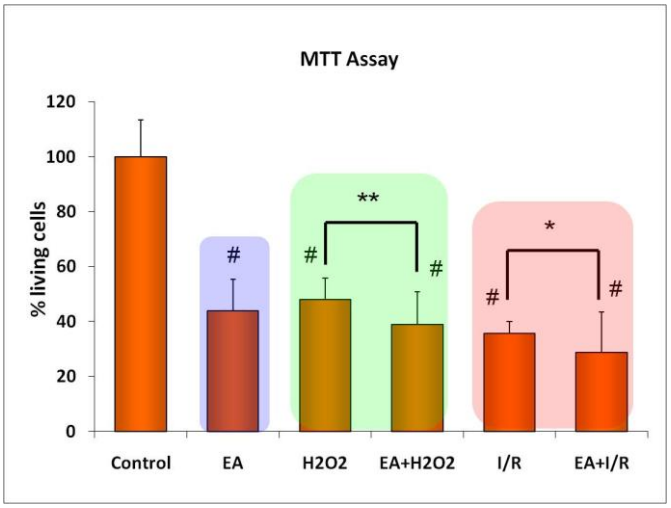

Figure 2. Viability of cardiomyocytes as measured by the MTT assay. #p<0,05 compared with the control group. *p<0,05; **p<0,01 compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion

3.4.2. Ratio of apoptosis

The control group had 85.7±1.94% of intact, living cells (annexin V and PI negative) and 4.6±0.82% of cells in the early phase of apoptosis (annexin V positive and PI negative). EA administration decreased the amount of living cells, and increased the percentage of apoptotic cells. A significant increase in the amount of apoptotic cells was observed in both the H_2O_2-treated and I/R groups, with a lower number of living cells. When EA was added in groups treated with H_2O_2 or I/R, the quantity of apoptotic cells was further increased and the amount of living cells was decreased. Interestingly, EA increased the amount of necrotic cells (annexin V negative and PI positive) during I/R and decreased the number of living cells. (Figure 3.)

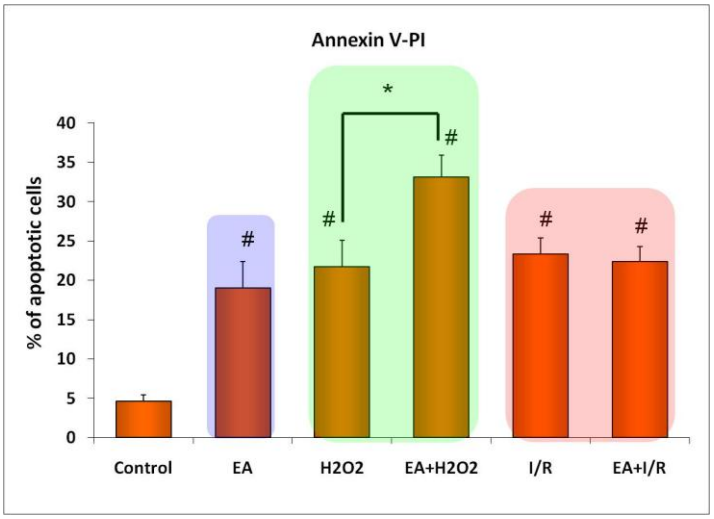

Figure 3. The mean percentage of apoptotic cells. Data are expressed as mean percentage ± SEM. #p<0,05 compared with the control group. *p<0,05 compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion

3.4.3. Phosphorilation of mitogen activated protein kinases (MAPKs)

c-Jun N-terminal kinase (JNK) activation increased markedly on EA administration to cardiomyocytes. H_2O_2 treatment increased the level of activated JNK; however, this difference was not significant. I/R caused a noticeable increase in JNK activation. On the other hand, EA was capable of augmenting the activation of JNK significantly when cells were cotreated with H_2O_2 or when cells were exposed to I/R. (Figure 4.)

GST inhibition led to a significant increase in p38 activation related to nontreated cells. Both H_2O_2 incubation and I/R resulted in a significant increase in p38 MAP kinase activation. EA administration during I/R increased p38 activity to 357.57±5.39% of control values. Likewise, when cells were incubated with H_2O_2 together with EA, the level of phophorylated p38 markedly increased; however, this difference was not statistically significant compared with the group treated with H_2O_2 alone.

Extracellular signal-regulated kinase (ERK/p42-44) phosphorylation increased in GST-inhibited groups (incubated with EA) that were either treated with H_2O_2 or exposed to I/R,

without any statistically significant difference. ANOVA failed to evaluate significant differences between groups. Moreover, the analysis of difference (using Student's *t* test) between the group receiving I/R and the group incubated with EA during I/R revealed statistically significant divergence.

Both administration of EA, H_2O_2 and I/R caused nonsignificant reduction of Akt activity. On the other hand, H_2O_2 treatment resulted in a more pronounced decrease (40.49±5.68%) of Akt phosphorylation when GST was inhibited by EA. There was no significant difference among groups as evaluated by ANOVA.

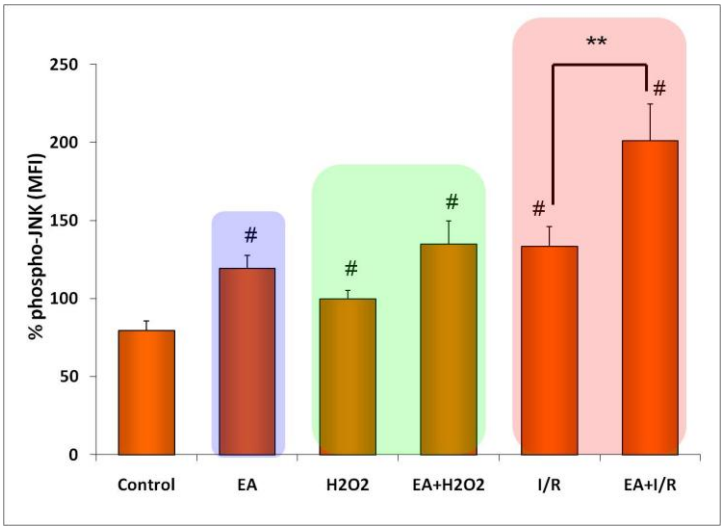

Figure 4. Phosphorylation of c-Jun N-terminal kinase (JNK) is demonstrated in cultured cardiomyocytes. #p<0,05 compared with the control group. **p<0,01 compared the linked groups to each other. EA Ethacrynic acid; I/R Ischemia and reperfusion; MFI Mean fluorescence intensity

3.5. CONCLUSION

Pharmacological inhibition of GST could markedly exaggerate oxidative stress-induced apoptosis in cardiomyocytes. GST inhibition was associated with increased activation of MAP kinases under stress conditions.

In our experiments administration of EA resulted in a marked increase of apoptotic cells, principally when cells were cotreated with H_2O_2. The amounts of necrotic cells were elevated following EA treatment and in the group receiving I/R and EA simultaneously. The increased level of reactive oxygen species and a more unfavourable glutathione state may exaggerate the intensity of insult and may explain the increased amount of necrotic cells in GST-inhibited groups during I/R.

On the other hand, GSTs associate with members of the mitogen activated protein kinase (MAPK) pathways involved in cell survival and death signaling. GSTπ was among the first isoenzymes found to inhibit c-Jun N-terminal kinase (JNK) through direct protein–protein interaction thus influencing cellular stress response and apoptosis. We have found that pharmacological inhibition of GST augments JNK activity itself. This could be explained by elimination of JNK sequestration within a protein complex with GST, and inhibition of S-glutathionylation.

The signalling pathway through p38 MAPK is activated by oxidative stress and is associated with cellular damage, mediation stress response and cytokine production. We

found that oxidative injury and I/R cause noticeable induction of p38 activity in cardiomyocytes, which is further increased by EA administration. Our results regarding p38 activation on GST inhibition can be explained by the above-described processes.

According to our results, ERK is activated on GST inhibition in the presence of H_2O_2 administration or during reperfusion. The level of phosphorylated ERK of GST-inhibited cells receiving I/R exceeded the ERK phosphorylation level of cells that have undergone I/R alone. These findings may represent the association between ERK and GST.

Although similar relationships between the synthesis of GST and Akt have been well investigated, the effect of GST inhibition on Akt-mediated cellular survival has not been fully described. Our results failed to show any further association between GST inhibition and Akt activity. The hindered antioxidant, antitoxic defense of cells treated with EA may explicate the results described by us.

4. INFLUENCE OF SELECTIVE MAPK AND GST INHIBITORS ON THE VIABILITY AND APOPTOSIS OF CARDIOMYOCYTES FOLLOWING OXIDATIVE STRESS

4.1. INTRODUCTION

It has been well investigated that oxidative stress is a major apoptotic stimulus in many cardiac diseases.

Among numerous defence mechanism against oxidative stress and ischaemic/reperfusion injury, the endogenous antioxidant enzyme glutathione S-transferase (GST) are crucially involved in cellular response to stress, apoptosis and proliferation.

GST is responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates.

GSTs play an important role as antioxidant enzyme modulating mitogen activated protein kinase (MAPK) pathways. The GSTs function to sequestering the kinase in a complex, serve a regulatory role, preventing cytotoxic ligands from interacting with their targets. Many studies have suggested that the mitogen activated protein kinases (MAPKs) may be important regulators of apoptosis in response to myocardial ischaemia/reperfusion.

Three major MAPKs, namely c-Jun NH_2-terminal protein kinase (JNK), p38 and extracellular signal-regulated protein kinase (ERK/p42-44) are activated in response to a wide variety of stimuli including growth factors, G protein-coupled receptors, and environmental stresses thus play a pivotal role in the transmission of signals from cell surface receptors to the nucleus. The pathways regulated by p38 and JNK contribute importantly to apoptosis. The mechanisms by which p38 and JNK induce apoptosis are largely cell and stimulus specific. ERK/p42-44 activation are protective against apoptotic cell death.

Our pilot study has been conducted to examine the biological role of GST in cardiac myocytes under oxidative stress conditions. We found that that pharmacological inhibition of GST by EA augments the apoptosis as a result of oxidative stress and simulated ischaemic-reperfusion (sI/R) injury. The study showed that GST inhibition was associated with increased activation of MAP kinases under stress condition.

4.2. AIMS

The main objective of this study was to verify our previous results and identify the effect of MAPK (JNK, p38 and ERK) inhibitors regarding GST inhibition (with administration of

ethacrynic acid) on the viability and apoptosis of cardiomyocytes when cells are exposed to various stress components of ischaemia and reperfusion (I/R).

4.3. MATERIALS AND METHODS

4.3.1. MAPK inhibitors

JNK inhibitor:	SP600125	Sigma-Aldrich (S5567)
p38 inhibitor:	SB239063	Sigma-Aldrich (S0569)
ERK/p42-44 inhibitor:	U0126	Sigma-Aldrich (U120)

4.3.2. Experimental protocol

Primary culture of neonatal rat cardiomyocytes was prepared and divided in six experimental groups according to different exposers: control group of cells incubated in CSFM without treatment. In Group II, cells incubated in medium containing 150 µM ethacrynic acid alone. In Group III, cells treated with 1mM H_2O_2 to simulate the oxidative stress. In group IV, cells exposed to 1mM H_2O_2 together with 150 µM ethacrynic acid. In Group V, cells exposed to swapping ischemic buffer to CSFM to simulate ischaemic-reperfusion injury. In Group VI, cells were treated to both sI/R and ethacrynic acid (EA). To antagonize the effect of JNK, p38 and ERK/p42-44 MAPKs, 10 µM JNK inhibitor (SP600125), 10 µM p38 inhibitor (SB239063) and 1 µM ERK inhibitor (U0126) was added simultaneously in every group. (Figure 5.)

Experimental groups	Without treatment	JNK inhibitor	p38 inhibitor	ERK/p42-44 inhibitor
I. Control (Cells were incubated in CSFM without treatment)	+			
		+		
			+	
				+
II. EA (Cells treated with 150 µM ethacrynic acid)	+			
		+		
			+	
				+
III. H2O2 (Cells treated with 1 mM H_2O_2)	+			
		+		
			+	
				+
IV. H2O2+EA (Oxidative stress + GST inhibition – double stress)	+			
		+		
			+	
				+
V. I/R (Cells exposed to 1,5 h ischaemia followed by 2,5 h reperfusion)	+			
		+		
			+	
				+
VI. I/R+EA (Simulated I/R + GST inhibition – double stress)	+			
		+		
			+	
				+

Figure 5. Experimental groups. Cells treated with 150 µM EA for GST inhibition. Cells treated with 1mM H_2O_2 to simulate the oxidative stress. In groups receiving simulated I/R, cells were exposed to 1,5 hours of ischemia using ischaemic buffer followed by 2,5 hours of reperfusion using normal CSFM. Cells were exposed to mentioned concentration of chemicals for 4 hours. EA – Ethacrynic acid; H_2O_2 – Hydrogen-peroxide; I/R – Ischaemia and reperfusion.

4.3.3. Determine the ratio of apoptosis

Ratio of apoptosis was evaluated after double staining with fluorescein isothiocyanate (FITC)-labeled annexin V (BD Biosciences, Pharmingen, USA) and propidium iodide (BD Biosciences, Pharmingen, USA) using flow cytometry.

4.3.4. Statistical analysis

Up to four different cardiac myocyte preparations were studied. Data of the experiments are expressed as means±SE. Differences between the means were compared using two sample Student's t-test for significance. $p<0,05$ was considered to be statistically significant.

4.4. RESULTS

4.4.1. MTT assay results

Viability of cardiomyocytes was determined by colorimetric MTT assay. In the control group without treatment, the amount of living cells was increased to 100%. JNK, p38 and ERK/p42-44 did not cause any significant changes in control groups without any stress effect. Ethacrynic acid, H_2O_2 and sI/R alone significantly reduced the ratio of living cells compared to control groups and only the p38 MAPK inhibition could significantly increase the viability of cells in H_2O_2-treated group. EA administration significantly enhanced the reduction of the viability of cells treated with H_2O_2 or exposed to sI/R compared to non treated cells and MAPK inhibitors could not increase significantly the percentage of living cells.

4.4.2. Ratio of apoptosis

The non-treated control group had 92,4±1,47% of intact, living cells (annexin V and PI negative) and 4.23±1,25% of cells in the early phase of apoptosis (annexin V positive and PI negative). Control groups treated with different MAPK inhibitors had no significant differences compared to non-treated control group. EA, H_2O_2 administration and simulated I/R significantly decreased the amount of living cells (p_{EA}= 0,0001; p_{H2O2}= 5,72E-09; $p_{sI/R}$= 2,65E-09) and increased the percentage of apoptotic cells (p_{EA}= 7,78E-05; p_{H2O2}= 1,68E-08; $p_{sI/R}$= 1,12E-08) in non-treated control group. The inhibition of proapoptotic JNK and p38 MAPKs significantly ameliorates the cell viability and attenuates the rate of apoptosis in EA- and H_2O_2-treated groups. On the other hand in these groups the inhibition of antiapoptotic ERK/p42-44 MAPK was significantly decreased the percentage of living cells (p_{EA}= 0,035; p_{H2O2}= 2,92E-05) and increased the amount of apoptotic cells (p_{EA}= 0,05; p_{H2O2}= 1,36E-05). In group, exposed to sI/R, only the p38 MAPK inhibitor was able to increase the percentage of viable cells ($p_{sI/R}$= 0,022) and diminish the rate of apoptosis ($p_{sI/R}$= 0,018) significantly. A significant increase in amount of apoptotic cells was observed in both groups, exposed to double stress, H_2O_2+EA or sI/R+EA, with a lower number of living cells compared to non-treated group or groups exposed to H_2O_2 or sI/R. The administration of JNK or p38 MAPK inhibitors elevated the level of living cells and reduced the rate of apoptosis but not significantly in groups treated with H_2O_2 or exposed to sI/R supplemented with EA. When the antiapoptotic ERK/p42-44 MAPK was inhibited in groups treated with double stress, the amount of living cells was further decreased and the quantity of apoptotic cells was further increased.

4.5. CONCLUSION

The present study showed that pharmacological inhibition of proapoptotic JNK and p38 could significantly decrease the oxidative stress-induced apoptosis while the inhibition of antiapoptotic ERK/p42-44 markedly reduces the cell viability in cardiomyocytes. This effect of these MAPK inhibitors could not be observed in case of double stress (oxidative stress + GST inhibition).

To evaluate the significance of JNK, p38 and ERK/p42-44 and the possible link of GST to these MAPKs during oxidative stress, three specific inhibitors (SP600125, SB239063 and U0126) were used respectively. The SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK. SB239063 is a novel p38 inhibitor that exhibits improved kinase selectivity and *in vivo* activity compared with the other p38 inhibitors. To influence on ERK/p42-44 activity, we used U0126, which is a potent inhibitor of MEK1/2, an upstream regulator of the phosphorylation of ERK/p42-44. According to Olli Tenhunen et all. administration of 1 μM U0126 significantly reduced the level of phospho-ERK. On the other hand, treatment with 1μM U0126 had no effect on the levels of phosphorylated p38 kinase and, similarly, the administration of SB239063 did not affect the level of phospho-ERK/p42-44.

Oxidative stress-induced apoptotic cell death during reoxygenation in cultured cardiomyocytes and *in vivo* hearts during reperfusion has been linked to an increased expression in JNK and p38 MAPKs. We have found that pharmacological inhibition of the proapoptotic JNK and p38 MAPK significantly increase the cell viability and decrease the ratio of apoptosis in groups receiving GST inhibition or oxidative stress compared to groups without JNK and p38 inhibitors. On the other hand in groups treated with double stress (GST inhibition together with oxidative stress or I/R injury) this protective effect of JNK and p38 inhibitor was lost. These findings may represent the association between JNK, p38 MAPK and GST.

In many cell types the ERK/p42-44 cascade appears to mediate specifically cell growth and survival signals. According to our results, the antiapoptotic ERK/p42-44 inhibitor treatment significantly decrease the cell viability and increase the ratio of apoptosis in groups receiving GST inhibition or oxidative stress compared to groups for lack of ERK/p42-44 inhibitor. In our previous study we demonstrated that antiapoptotic ERK/p42-44 is activated on GST inhibition, in the presence of H_2O_2 administration or during reperfusion. But in our present study we could not observed elevation in the percentage of living cells following oxidative stress or GST inhibition, likely because of the ERK/p42-44 inhibitor treatment. But among ERK/p42-44 inhibited groups exposed to GST inhibition, oxidative stress and simulated I/R injury, the ratio of living cells was the highest in case of GST inhibition. These findings may represent the association between ERK/p42-44 and GST. On the other hand in our preceiding study we also demonstrated that the level of phosphorylated ERK/p42-44 of GST-inhibited cells receiving sI/R exceeded the ERK/p42-44 phosphorylation level of cells that have undergone sI/R alone. Accordingly in this present examinations presumably because of ERK/p42-44 inhibition, in groups treated with double stress (GST inhibition together with oxidative stress or simulated I/R injury) the cardiomyocyte cell viability was diminished and the ratio of apoptosis was elevated but not significantly compared to groups in absence of ERK/p42-44 inhibitor. However a significant increase in amount of apoptotic cells was observed in both groups, exposed to double stress, H_2O_2+EA or sI/R+EA, with a lower number of living cells compared to groups exposed to H_2O_2 or sI/R alone.

5. THE ROLE OF GST IN MECHANISMS OF ISCHAEMIC POSTCONDITIONING

5.1. INTRODUCTION

Oxidative stress can lead to apoptotic, necrotic disorders in cells, after ischaemic/reperfusion injury in any organs. Oxygen free radicals are highly reactive molecules with an unpaired electron, and are associated widely with ischemic/reperfusion injury. In large quantities they overwhelm the endogenous antioxidant systems or, if the antioxidant system is insufficient or damaged, they accelerate the oxidative stress.

A promising approach to cardioprotection termed “ischaemic postconditioning” (IPoC) has been described by Vinten-Johansen et al. It contains short series of repetitive cycles of brief reperfusion and reocclusion of the coronary artery applied immediately at the onset of reperfusion, can be effective against reperfusion injury. They showed that the mechanisms involved in postconditioning protection take place within the first minutes of reperfusion.

5.2.AIMS

Postconditioning is controlled reperfusion, defined as a series of brief interruptions of ischemia/hypoxia applied at the very onset of reperfusion can protect the myocardium from ischaemic/reperfusion injury. The antioxidant glutathione S-transferase is crucially involved against oxidative stress and ischaemic/reperfusion injury. So we were concerned whether the pharmacologic inhibition of GST can influence the function and benefit of ischaemic postconditioning (IPoC) in vitro ischaemic/reperfusion injury. We examined the efficiency of ischaemic postconditioning by assessing the cell viability and apoptosis in rat cardiomyocyte cell culture and the activition of mitogen activated protein (MAP) kinase pathways.

5.3. MATERIALS AND METHODS

5.3.1. Cell culture model

Primary culture of 2-4 day-old neonatal Wistar rat cardiomyocytes was prepared and transferred onto 24 and 96-well plates (BD Falcon) at the density of 200 000 cells/ml as described previously in part 3. In each well 2 ml cell solution was seeded. After 24 hours from plating, cell media was substituted with CSFM.

5.3.2. Experimental protocol

In our experiments neonatal cardiac cell cultures were listed in 6 groups: Group I, control group of cells, incubated in CSFM without treatment; Group II, cells exposed to simulated ischemia-reperfusion (I/R); Group III, cells treated with simulated ischemia-reperfusion together with ischaemic postconditioning (IPoC); Group IV, cell were treated with 150 μM ethacrynic acid (EA) alone; Group V, cells exposed to simulated ischemia-reperfusion with 150 μM ethacrynic acid; Group VI, cells exposed to simulated ischemia-reperfusion with ischaemic postconditioning and 150 μM ethacrynic acid.

5.3.3. Ischemic postconditioning of the myocardium

Primary cardiomyocytes were utilized in a well established cellular model of ischemic postconditioning. Briefly, cells were exposed to simulated ischemic (SI) buffer for 1,5 hours

followed by 5 minutes reperfusion and then another 5 minutes ischemic insult before the 2,5 hours reperfusion. While cardiomyocytes were under hypoxic conditions in SI buffer, control cells were incubated in complete serum free medium (CSFM) such as during reperfusion period. (Figure 6.)

Figure 6. Experimental protocol and effects of postconditioning with simulated ischemia/reperfusion and the glutathione S-transferase (GST) inhibitor EA treatments on cardiomyocytes. Cardiomyocytes were incubated in complete serum-free medium (CSFM) or with EA followed by 1,5 hours ischaemia and brief period of ischemia before 2,5 hours long reperfusion (R)

5.4. RESULTS

5.4.1. MTT assay results

MTT assay was performed to measure the absolute number of living cells in different groups. In a control group the amount of living cells was taken to 100 %. Both EA and simulated I/R alone caused marked reduction in amount of living cells. The effect of cell death was significantly stronger upon administration of EA in groups exposed to simulated I/R (15,97±2,64). Ischaemic postconditioning could significantly increase the percentage of living cells in case of simulated I/R ($p=0,023$), but this change was not observed when it is combined with EA treatment ($p=0,0015$).

5.4.2. Ratio of apoptosis

The control group had 83.54±2.31 % of intact, living (annexin V and PI negative) cells and 7.8±2.05 % of cells in early phase of apoptosis (annexin V positive and PI negative). A significant increase of apoptotic cells was observed in both the EA-treated and simulated I/R groups with a lower number of living cells. When EA was added in simulated I/R groups the quantity of apoptotic cells was further increased with reduced amount of living cells. Ischaemic postconditioning resulted significant increase in the percentage of living cells and a significant decrease in the ratio of apoptotic cells in simulated I/R group while we could not detected this protective effect in simulated I/R group when EA co-treatment was applied.

5.4.3. Phosphorilation of mitogen activated protein kinases (MAPKs)

JNK activation increased markedly upon administration of EA to cardiac myocytes and in simulated I/R group. EA administration resulted in further increase in JNK activation in cells exposed to simulated ischaemia and reperfusion. Ischaemic postconditioning decreased the JNK phosphorilation significantly in simulated I/R group while this change could not be

detected in EA-treated simulated I/R group. Among the postconditioned groups the level of phosphorylated JNK was significantly higher when cardiomyocytes were treated with GST inhibitor EA. (Figure 7.)

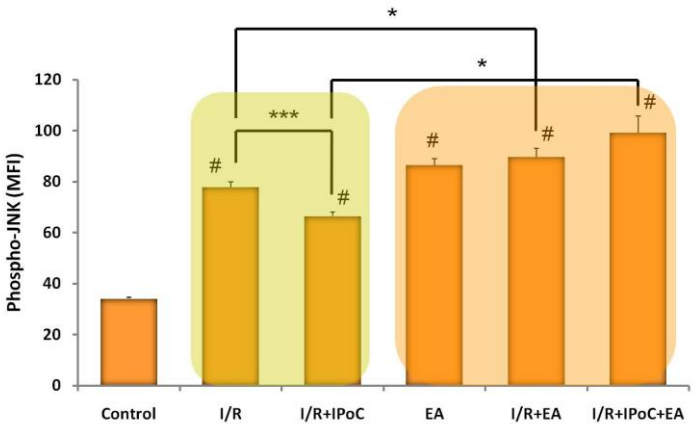

Figure 7. Phosphorylation of c-Jun N-terminal kinase (JNK) is demonstrated in cultured cardiomyocytes. $p^{\#}<0,05$ compared with the control group. $p^{***}<0,001$ compared the linked groups to each other. EA – Ethacrynic acid; I/R – Ischaemia and reperfusion; IPoC – Ischaemic postconditioning; MFI – Mean fluorescence intensity.

Both, simulated I/R and GST inhibition led to significant increase in p38 activation related to non-treated cells. EA administration during simulated I/R resulted in further elevation in phosphorylation of p38. A significant decrease in p38 activation was detected in ischaemic postconditioned group compared to I/R group (p=0,0037). However, we could not observe similar, significant decrease between these groups if the cells treated with EA (p=0,213). Ischaemic postconditioning reduced significantly the level of phospho-p38 compared to same group receiving EA administration (p=0,0072).

ERK/p42-44 phophorylation significantly increased in simulated I/R (p=2,52E-10) and GST inhibited (p=1,14E-08) groups. When cardiomyocytes were treated with both sI/R and EA we observed further elevation in the level of phosphorylated ERK/p42-44 compared to only GST inhibited group (p=0,039). The level of phosphorilated ERK/p42-44 of GST inhibited cells receiving simulated I/R was similar than the phopho-ERK/p42-44 level of cells undergone simulated I/R alone. Ischaemic postconditioning could significantly enhance the activation of ERK/p42-44 in simulated I/R group (p=0,0012) but we did not find significant change in case of double stress when simulated I/R combined with EA treatment using ischaemic postconditioning (p=0,53). The level of phosho-ERK/p42-44 was significantly higher in simple postconditioned group compared to the EA-treated group after postconditioning (p=0,0009).

The phosphorylation of GSK-3β which causes the inhibition of this protein kinase itself and therefore prevents the opening of the mitochondrial permeability transition pore (mPTP), a mechanism preventing apoptotic cell death was significantly lower in simulated I/R group compared to control group (p=0,0019). Significantly increased GSK-3β inactivation was observed in ischaemic postconditioned group compared to simulated I/R group (p=0,0393). We measured significant decreasing in GSK-3β inactivation in EA-treated group compared to control (p=0,00047) and simulated I/R (p=0,0016) groups. Further reduction in the level of phospho-GSK-3β could be detected in groups treated with double stress (simulated I/R and GST inhibitor EA) compared to simulated I/R (p=4,7E-07) or EA treatment (p=7,94E-05)

alone. We did not measure significantly higher phospho-GSK-3β level in postconditioned group treated with double stress compared to I/R+EA group (p=0,059). GSK-3β phosphorylation and thus the inactivation was significantly lower in case of EA administration (p=0,0006) compared the postconditioned groups to each other.

5.5. CONCLUSION

Present study showed that GST inhibition could markedly attenuate the protective effect of ischaemic postconditioning and resulted in increasing apoptosis in cardiomyocyte cell culture. GST inhibition was associated with different activation of MAP kinases and GSK3β protein kinase in the process of ischaemic postconditioning.

In our experiment administration of GST inhibitor EA resulted in marked increase of apoptotic cells, principally when cells were co-treated with simulated I/R. While ischaemic postconditioning could decrease the ratio of apoptosis in simulated I/R group, this positive effect could not be detected in GST inhibited group receiving simulated I/R. The increased level of reactive oxygen species and more unfavourable glutathione state may exagravate the intensity of insult and may explain the increased amount of apoptotic cells in GST inhibited group during simulated I/R and IPoC.

GSTs have been shown to form protein:protein interactions with members of the mitogen activated protein (MAP) kinase pathway thereby serving a regulatory role in the balance between cell survival and apoptosis. By interacting directly with MAP kinases, including c-Jun N-terminal kinase 1 (JNK1), GSTs function to sequester the ligand in a complex, preventing interactions with their downstream targets. We have found that pharmacological inhibition of GST augments JNK activation by itself and abolish the protective effect of ischaemic postconditioning. This could be explained by elimination of the GST - JNK protein complex.

The MAPK p38 is a signaling protein that plays a critical role in coordinating cellular responses to stress, including oxidative stress that is characterized by the accumulation of increased levels of reactive oxygen species (ROS) within the cell. We found that simulated I/R treatment cause noticeable induction of p38 activation in cardiomyocytes, which was further increased by administration of EA. Consistent with the literature ischaemic postconditioning was able to decrease significantly the phosphorylation of p38 in sI/R group while we observed similar but not significant decrease in sI/R group treated with GST inhibitor EA, accordingly the relationship is supposed between GST and p38 which led to abolished effect of IPoC.

According to our results ERK/p42-44 activation increased upon GST inhibition during reperfusion, like in ‘Conclusion’ of third chapter. This may presume the induction of this protective pathway due to oxidative stress. In our experiment the level of antiapoptotic phospho-ERK/p42-44 was significantly higher following IPoC – consistent with previous studies – but this change was not shown in the presence of GST inhibitor ethacrynic acid. Although the activation of ERK/p42-44 increased due to GST inhibition or IPoC the elevation of this antiapoptotic MAPK was lost in case of double stress, probably cause of the extended stress.

Glycogen synthase kinase-3β (GSK-3β), a protein kinase linked to the regulation of a variety of cellular functions including glycogen metabolism, gene expression, and cellular survival, could either be considered as a specific downstream target of the RISK pathway or indeed as a component of the RISK pathway. We detected significant reduction in the level of phospho-GSK-3β following simulated I/R treatment showing the harmful effect of I/R, but coincidently with the literature the inactivation of GSK-3β increased in case of IPoC. In case

of double stress (sI/R+EA) we observed significant reduction in the phosphorylation of GSK-3β, additionally IPoC was not able to significantly enhanced the inactivation of GSK-3β. This result may presume the association between GSK-3β and GST. The enhanced phosphorylation of GSK-3β and ERK/p42-44, which is found in postconditioning under control conditions and is hypothesized to be involved in the signal transduction pathway of postconditioning, is lost in the metabolic syndrome. This data may represent the fact that the protective effect of ischaemic postconditioning is lost in case of double stress.

6. DISCUSSION

Glutathione is the major intracellular low-molecular-weight thiol that plays a critical role in cellular defense against oxidative stress in cells. When cells are exposed to increased levels of oxidative stress, GSSG will accumulate and the ratio of GSH to GSSG will decrease. Therefore, the determination of the GSH/GSSG ratio and the quantification of GSSG are useful indicators of oxidative stress. GSTs catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of compounds in order to inactivate them and facilitate their excretion from the body. In general, the reactions catalyzed by GSTs are considered detoxifying, and serve to protect cellular macromolecules from damage caused by oxidative stress and cytotoxic agents.

In the first series of our experiments we found that ethacrynic acid, sI/R and H_2O_2 alone caused significant reduction in amount of living cells and significant increase in the ratio of apoptotic cells. GST inhibition by EA administration further decreased significantly the amount of living cells and further increased significantly the percentage of apoptotic cells in groups treated with H_2O_2 or exposed to sI/R.

GSTs play an important role as antioxidant enzyme modulating mitogen activated protein kinase (MAPK) pathways that control cell proliferation and cell death (apoptosis). The GSTs function to sequestering the kinase in a complex, serve a regulatory role, preventing cytotoxic ligands from interacting with their targets. The result of this action is a regulation of pathways that control stress response to I/R injury, cell proliferation and apoptotic cell death. GSTπ was among the first isoenzymes found to inhibit c-Jun N-terminal kinase (JNK) through direct protein–protein interaction thus influencing cellular stress response and apoptosis.

In our experiment JNK activation increased markedly by EA exposure to cardiac myocytes and significantly augmented the activation of JNK both when cells were co-treated with H_2O_2 or when cells were exposed to I/R. Inhibition of GST led to significant increase in p38 activation versus non-treated cells. Both H_2O_2 incubation and ischaemia-reperfusion resulted in significant increase of p38 MAP kinase activation. EA administration during I/R or incubation together with H_2O_2 increased markedly the level of phosphorilated p38. ERK/p42-44 phosphorylation increased in GST inhibited groups (incubated with EA) either treated with H_2O_2 or exposed to I/R. H_2O_2 treatment resulted in pronounced decrease of Akt phosphorilation when GST was inhibited by EA.

Three major MAPKs, namely c-Jun NH_2-terminal protein kinase (JNK), p38 and extracellular signal-regulated protein kinase (ERK/p42-44), play a pivotal role in the transmission of signals from cell surface receptors to the nucleus. Although there are conflicting reports on the role of MAPKs in death or survival after stress, it is commonly agreed that JNK and p38 MAPKs appear to be pro-apoptotic in many cell types while ERKs are the modulators of cell survival after reperfusion.

In the second series of our experiments we found that pharmacological inhibition of proapoptotic JNK and p38 MAPKs diminish apoptosis while the inhibition of antiapoptotic ERK/p42-44 MAPK augments apoptosis as a result of GST inhibition even as oxidative injury and simulated I/R. Thus this apoptotic signaling is presumably mediated by JNK, p38 and ERK/p42-44 MAPKs pathways. The protective effect of the inhibiton of proapoptotic JNK and p38 MAPKs was lost in groups, exposed to double stress (GST inhibition together with oxidative stress or simulated I/R injury), so GST activity is required for survival of cultured cardiomyocytes under stress conditions.

A promising approach to cardioprotection termed "ischaemic postconditioning" (IPoC) has been described by Vinten-Johansen et al. It contains short series of repetitive cycles of brief reperfusion and reocclusion of the coronary artery applied immediately at the onset of reperfusion, can be effective against reperfusion injury. They showed that the mechanisms involved in postconditioning protection take place within the first minutes of reperfusion.

In the third series of our experiments GST inhibition markedly attenuate the protective effect of IPoC which resulted in increasing apoptosis and decreasing cardiomyocyte cell viability. The proapoptotic JNK activation was significantly elevated and the proapoptotic p38 phosphorilation was not be able to significantly decrease in GST inhibited group despite of IPoC. Among same experimental conditions, ischaemic postconditioning could significantly enhance the antiapoptotic ERK/p42-44 activity in simulated I/R group but we did not find significant change in case of double stress when simulated I/R combined with EA treatment. The ratio of phospho-GSK-3β was significantly higher in IPoC group compared to I/R group, but we could not observed similar significant elevation between these groups if the cells treated with EA.

In sum, present study showed that inhibition of GST by EA augments the apoptosis as a result of simulated I/R furthermore abolish the protective effect of ischaemic postconditioning and this is presumably mediated by JNK, p38, ERK/p42-44 and GSK-3β signaling pathways because the activities of these kinases change on this way during ischaemic postconditioning. GST activity is required for survival of cultured cardiomyocytes under stress conditions. These findings highlight the important role of GST in protection against oxidative stress likely not only in experimental conditions but in different pathological disorders in human beings.

7. NOVEL FINDINGS

1) Firstly we demonstrated that the pharmacological inhibition of GST could markedly exaggerate oxidative stress-induced apoptosis in isolated cardiomyocytes, so GST activity is required for survival of cultured cardiomyocytes under stress conditions. Furthermore GST inhibition was associated with different activation of MAP kinases regulating these pathways under stress conditions.

2) We detected, using MAPK inhibitors, that GST plays crucial role among pro- and antiapoptotic MAPKs in the process of oxidative stress-induced apoptosis in cardiomyocyte cell culture.

3) We are the first who demonstrated that GST inhibition could markedly attenuate the protective effect of ischaemic postconditioning and resulted in increasing apoptosis in isolated cardiomyocytes. It was clarified that in the process of ischaemic postconditioning GST inhibition is in the close association with activation of different MAP kinases and protein kinases.

8. PUBLICATIONS AND PRESENTATIONS

8.1. Publications related to the thesis

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9.4. Presentations

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