

CELL PROTECTIVE EFFECT OF STRESS-
REGULATORY PEPTIDE UROCORTIN IN THE
ENDOGENOUS ADAPTATION MECHANISMS OF
MYOCARDIUM

PhD Thesis

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ABBREVIATIONS

| | |
|--------------------------------|---------------------------------------|
| AP-1 | activator protein-1 |
| Akt | protein kinase B |
| BNP | brain natriuretic peptide |
| cAMP | cyclic AMP |
| CK | creatin kinase |
| CRF | corticotrophin-releasing factor |
| CSFM | complete serum free medium |
| DMEM | Dulbecco's Modified Eagle' Medium |
| DAG | diacyl-glycerol |
| ERK | extracellular signal-regulated kinase |
| Gi | inhibitory G protein |
| Gq11 | a GTP binding protein |
| GPCR | G-protein coupled receptor |
| GSK 3β | glycogen synthasebkinasr-3 β |
| HO-1 | haemoxigenase-1 |
| HSP | heat shock protein |
| iNOS | inducible nitric oxide synthase |
| IP3 | inosiltol-triphosphate |
| I/R | ischaemia/reperfusion |
| K_{ATP} channel | ATP-sensitive potassium channel |
| LAD | left anterior descending artery |
| LDH | lactate dehydrogenase |
| MAPK | mitogen aktivated protein kinase |

| | |
|--------------------------------|---|
| MEK-1 | mitogen activated protein extracellular signal-related kinase |
| MnSOD | manganese superoxide-dismutase |
| mPTP | mitochondrial permeability transition pore |
| NF-κB | nuclear factor-kappaB |
| NO | nitrogen oxide |
| PC | preconditioning |
| PCR | polymerase chain reaction |
| PI | propidium iodide |
| PS | postconditioning |
| PI 3 kinase | phosphatidylinositol triphosphate kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PMN | polymorphonuclear cell |
| RISK | reperfusion injury salvage kinase |
| ROI | reactive oxygen intermediates |
| SOD | superoxide dismutase |
| SWOP | second window of protection |
| Ucn | urocortin |

1. INTRODUCTION

Several natural stimuli can be potentially malign and cause irreversible cell injury or death. However, in the case of reversible injury, cellular processes can be activated to counteract the effects of the noxious stimulus. Also, endogenous intracellular defence mechanisms can appear to induce alterations, which can increase cellular resistance to injury.

Ischaemia/reperfusion (I/R) is an example of a detrimental stress-stimulus. It was the most intensively examined question in the last decade of cardiovascular research, however the mechanisms underlying the pathogenesis of I/R injury are not fully clarified.

The notion „preconditioning” (PC) refers to a general adaptive response of the organism whereby pretreatment with different noxious stress–stimulus (hypoxia, physical and pharmacological agents) can augment cellular tolerance against subsequent lethal stress-insult.

1.1. ISCHAEMIC PRECONDITIONING

It was first described by Murry et al.¹, that short periods of myocardial ischaemia resulted in reduction in infarcted area during a long-lasting coronary artery occlusion. This phenomenon was called “preconditioning with ischaemia”. Following these initial studies, the protection was further characterized both in terms of time course and various end-points of I/R injury. Soon, a variety of preconditioning stimuli were exposed including heat stress², hypoxia³, rapid cardiac pacing^{4,5}, stretch stress⁶ and different pharmacological agents^{7,8}. Ischaemic PC afford to the myocardium more resistance against infarction and reperfusion arrhythmias in all species tested so far^{9,10,11,12}, protect the postischaemic contractile dysfunction of stunned myocardium and also reduces the extent of apoptosis^{13,14}, thus it was seen to have considerable clinical relevance in treatment of patients with ischaemic heart disease¹⁵, but the need for pretreatment has made it impractical in the clinical settings.

When was first described by Murry et al.¹, this process was to be seen to appear as an immediate response lasting no more than a few hours. In 1993 two similar studies by Kuzuya et al. and Marber et al. recognised that in addition to the initial phase, a second wave of protection can be exhibited 24 to 72 hours following the preconditioning protocol^{16,17}. Also the evoked cardioprotection is biphasic: the *classic or early PC*

appears immediately after the PC stimuli and lasts for 2-3hours, while in the *delayed PC* the protective response obtains 24 hours later.

1.1.1. Signaling pathways of classic preconditioning

It is believed that here are emerging views behind this form of protection. Protein kinase C (PKC) phosphorylates other kinases, including p38 mitogen activator protein kinase (p38 MAPK), and eventually leads to the opening of the mitochondrial ATP- sensitive potassium (K_{ATP}) channels⁷. It is followed by the influx of potassium ions reducing the influx of calcium ions into the cell.

1.1.2. Signaling pathways of delayed preconditioning

Delayed PC can be induced beside the brief ischemic-reperfusion cycles by other trigger factors, such as adenosine throughout A1 receptor activation^{18,19,20}, bradykinin^{21,22,23}, opioids²⁴, and bacterial endotoxin²⁵. All of these triggers can lead to the phosphorylation of PKC and other kinase systems including tyrosine kinase and MAPK cascades which results in activation of an ubiquitous transcription factor, nuclear factor- kappaB (NFkB). This factor is seen to play a crucial role in the regulation of I/R mediated gene expression and consecutive protein synthesis²⁶, involving manganese-superoxide dismutase (SOD)¹⁶, heat shock protein (HSP) 72¹⁷, catalase, inducible nitric oxide synthase (iNOS), haemoxigenase-1 (HO-1). **(Figure 1.)**

MYOCARDIAL PRECONDITIONING

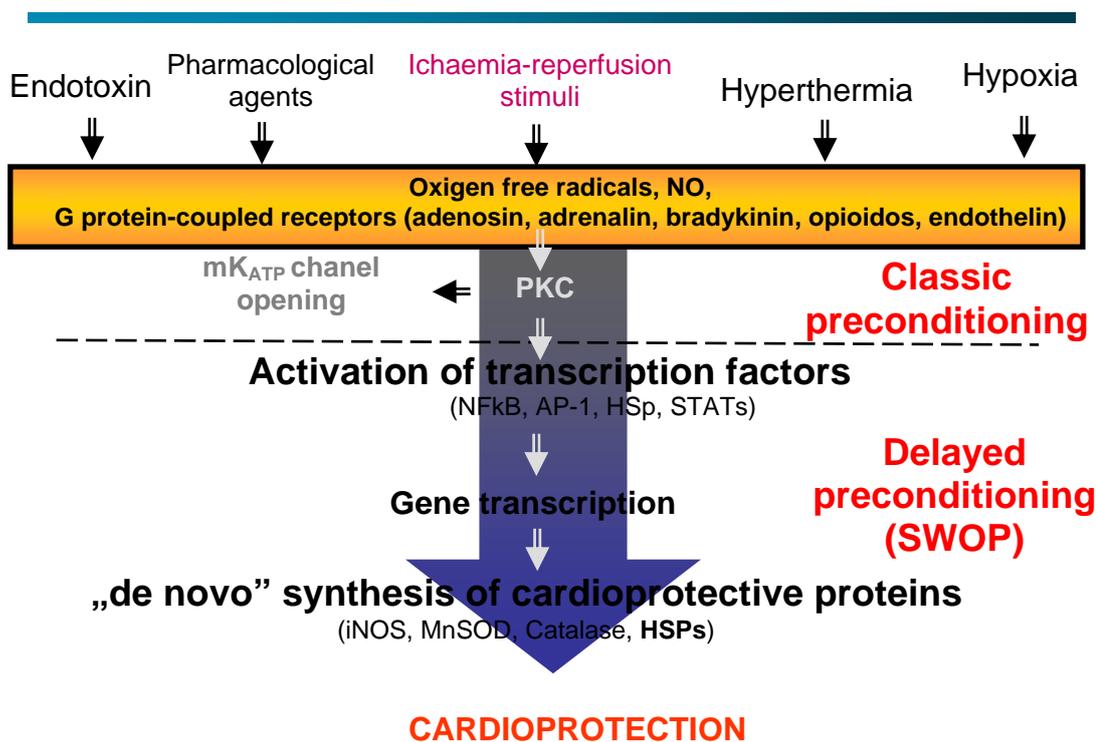


Figure 1. Signal transduction pathways of classic and delayed ischaemic preconditioning

(NO:nitrogen monoxid; mK_{ATP}: mitochondrial ATP dependent potassium channel; PKC: protein kinase C; NFkB: nuclear factor kappa B; AP-1: Activator protein-1; iNOS: inducible nitric oxide synthase; MnSOD: manganese superoxid dismutase; HSP: Heat shock protein; SWOP: second wave of protection)

1.2. ISCHAEMIC POSTCONDITIONING

Despite the powerful protective effects of PC, the clinical application of this phenomenon has been rather disappointing, mainly because it must be instituted before the ischaemic event²⁷. In contrast, a more promising approach to cardioprotection termed “ischaemic postconditioning (PS)” has been described by Vinten-Johansen et al.²⁸. It consists of short intermittent ischaemic stimuli started immediately at the onset of reperfusion after prolonged ischaemia. Unlike preconditioning, postconditioning theoretically allows unlimited application in the clinical settings, and thus has attracted much attention over the past years. Importantly, some pharmacological agents can

provide comparable protection when applied during early reperfusion (“pharmacological postconditioning”)^{29,30,31}.

1.2.1. How does it work- intracellular aspects of ischaemic postconditioning

The mechanisms of postconditioning are divided into mechanical, cellular and molecular events³². According to the *mechanical* explanation during perfusion pressure can increase water extravasation and cause edema, which is aggravated by microvascular injury. PS delays the washout of adenosine, the accumulation of which attenuates superoxide anion generation by neutrophils and endothelial cells, and activates mitochondrial K_{ATP} channels via adenosinergic G protein-coupled receptor activation. Regarding the *cellular* events: better endothelial function increases nitric oxide release by endothelial cells, which further attenuates superoxide anion levels and both neutrophil activation and adherence to the endothelial cells. Postconditioning decreases the intracellular buildup of oxidants and calcium in cardiomyocytes, which inhibits mitochondrial permeability transition pore (mPTP) opening, thereby inhibiting both apoptosis and necrosis. Conforming to the *molecular* events, in the process of PS, the activation of survival kinases, such as PI3 kinase, p-Akt, ERK1/2 and pro-apoptotic enzymes (Bcl2/Bax ratio) also reduces apoptosis, and potentially conversion to necrosis^{33,34,35,36}. Hence, postconditioning marshals a variety of endogenous mechanisms that operate at numerous levels and target a broad range of pathological mechanisms. **(Figure 2.)**

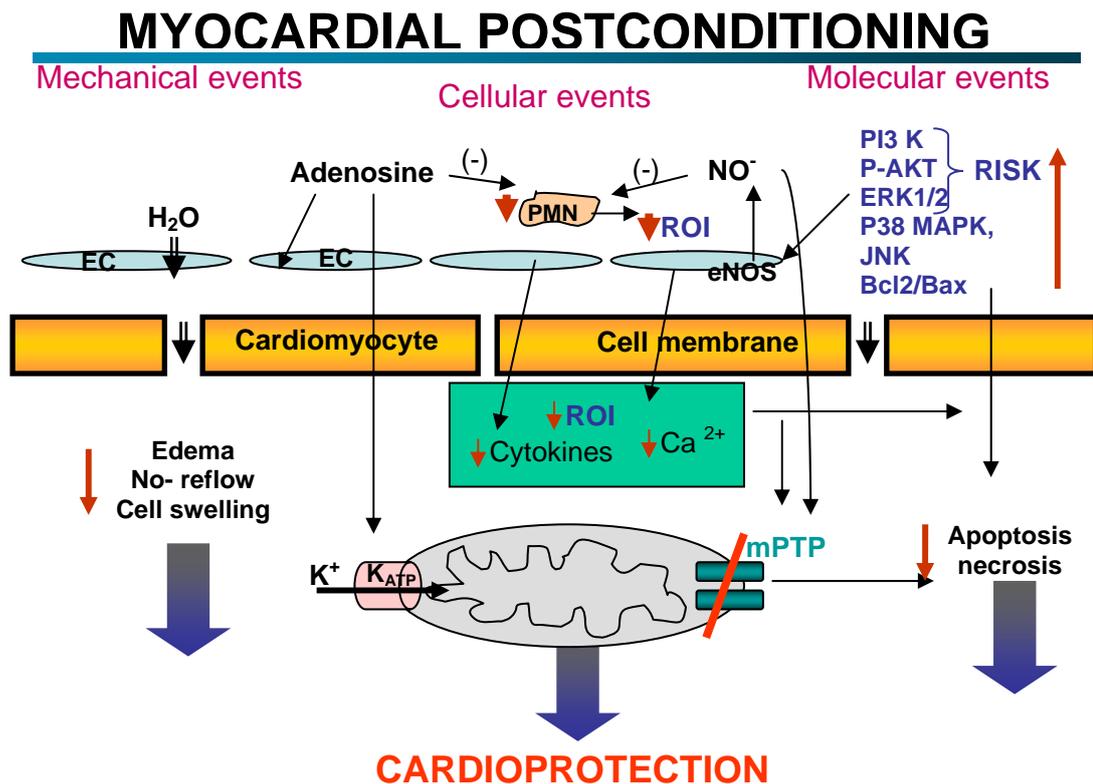


Figure 2. Signaling pathways of ischaemic postconditioning

(EC: endothelial cell; PMN: polymorphonuclear cell; ROI: reactive oxygen intermediates; NO: nitrogen monoxide; mK_{ATP}: mitochondrial ATP dependent potassium channel; eNOS: endothelial nitric oxide synthase; mPTP: mitochondrial permeability transition pore; ERK: extracellular signal-related kinase; MAPK: mitogen activated protein kinase; PI3 K: phosphatidylinositol-3-kinase; RISK: reperfusion injury salvage kinase)

1.3. UROCORTIN

Urocortin (Ucn), a 40 amino acid peptide belongs to the corticotrophin-releasing factor (CRF) family, was first identified in rat midbrain by Vaughan et al.³⁷ in 1995. CRF polypeptides play biologically different roles in the behavior and in response to stress mediating the action of the hypothalamic-pituitary-adrenal axis³⁸. Ucn shows 45% amino acid sequence homology to CRF, however it is more conserved than CRF across species.

Expression of human Ucn has been found to the present in the central nervous system^{39,40,41}, placenta, adrenal gland, gastrointestinal tract⁴², ovaries⁴³, lung,

lymphocytes⁴⁴ and in the heart⁴⁵ and vasculature⁴⁶. It has been shown, that Ucn immunoreactivity in the human failing heart is more intense than in the normal myocardium⁴⁷.

1.3.1. Signal transduction pathways of urocortin

The actions of all members of the CRF family are mediated through two subtypes of CRF receptor-CRF R1 and CRF R2 with variants R2a and R2b. Although CRF-R1 expressed predominantly in brain and pituitary, binds Ucn in like manner as CRF, Ucn binding affinity is approximately 40-fold higher for CRF-R2⁴⁸ and its distribution overlaps with that of the peptide in both the central nervous system and periphery, demonstrating strong expression throughout the heart and (myocardium, epicardium, arterioles)⁴⁵ and vasculature (endothelial and smooth muscle cells)⁴⁹.

Activation of CRF R2 elevates the cellular contents of cyclic AMP (cAMP) through the G protein- adenylate cyclase pathway⁵⁰; stimulating the cAMP- dependent protein kinase (PKA).

Other pathways that involve Ucn binding are reported to be mediated via activation of PKC and K_{ATP} channels⁵¹ and the MAPK⁵² and PI3 kinase/Akt pathways⁵³ and may involve increased expression and production of heat shock protein 90⁵⁴ and cardiotrophin-1⁵⁵ peptides shown to be cardioprotective against lethal stress.

1.3.2. Cardiovascular actions of urocortin and it's possible role in heart protection

Strong expression of both Ucn and its receptor within the heart and blood vessels suggests a role in cardiovascular regulation. Vaughan et al. reported that intravenous Ucn reduced blood pressure in rats more effective than CRF⁵⁶. This effect was found to be obligated to a fall in total peripheral resistance⁵⁷, and other studies demonstrated a direct relaxant effect of Ucn on rat arterial segments⁵⁸. Ucn also produces dose-dependent and prolonged increase in heart rate and cardiac output^{57,59}, and recent studies in the isolated rat heart demonstrate Ucn produces direct positive inotropic effects as well as coronary vasodilation⁶⁰. In addition to its effects on cardiac and vascular function, Ucn is reported to prevent cell death (necrotic and apoptotic) in cultured rat cardiac myocytes presuming administered before ischaemia or at the point

of reoxygenation via a MAPK dependent pathway and reduce infarct size in the intact heart following ischaemia/reperfusion injury⁵².

Furthermore, it has been also shown that Ucn has similar cardioprotective effects as the ischaemic PC in both early and delayed phases through activation of PKC and K_{ATP} channels⁵¹. (Figure 3.)

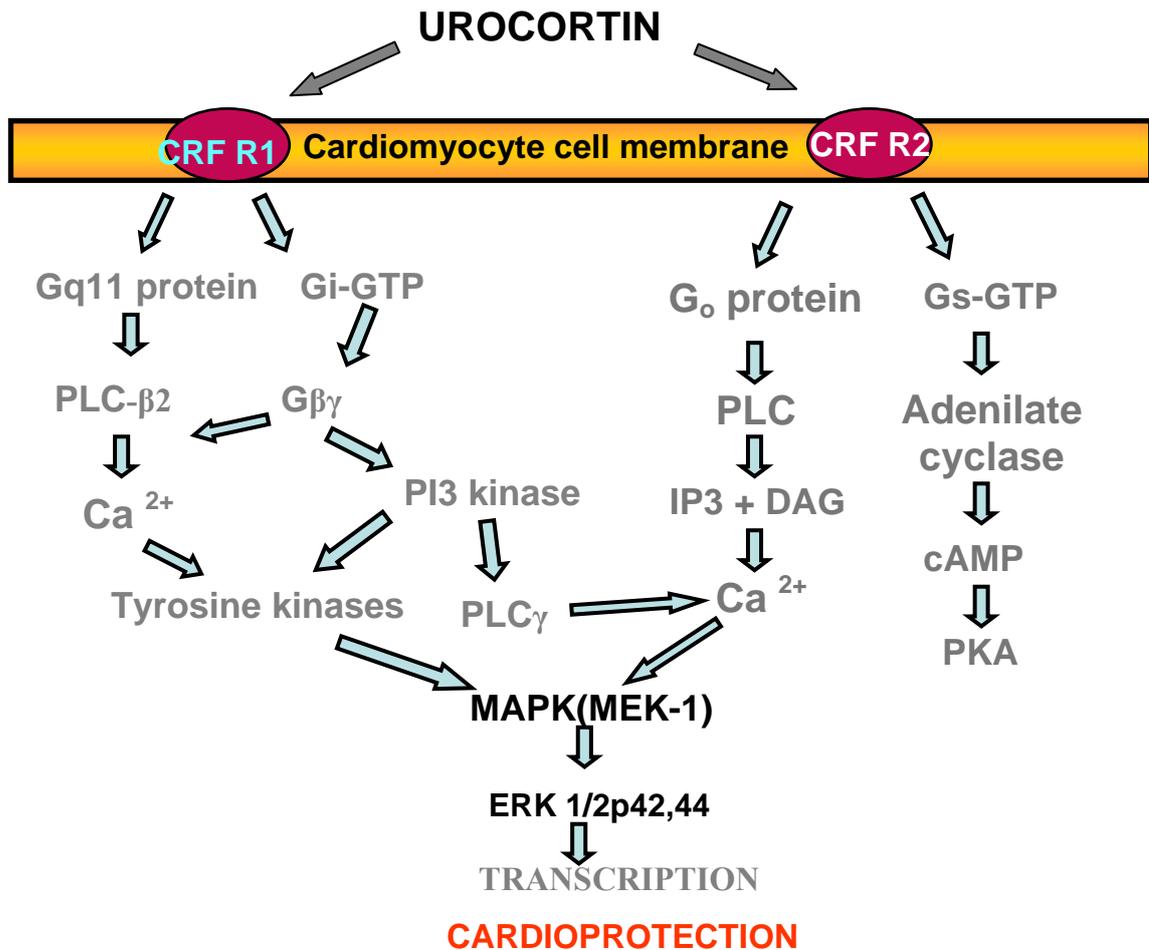


Figure 3. Sinaling cascade of urocortin via CRF R1 and R2 by Khan SQ. et al.⁶¹

(CRF R: corticotrophin releasing factor receptor; PLC: phospholipase C; IP3: inositol-triphosphate; PKA: phosphokinase A; DAG: diacyl-glycerol; cAMP: cyclic adenosine-monophosphate; Gi: inhibitory G protein; Gq11: a GTP binding protein; MEK-1: mitogen activated protein extracellular signal-related kinase; PI3 kinase: phosphatidylinositol 3 kinase)

2. AIMS AND HYPOTHESIS

Reperfusion injury is an integrated response to the restoration of blood flow after ischaemia, and is initiated at the very early moments of reperfusion, lasting potentially for days. However, rapidly initiating reperfusion is the most effective treatment to reduce infarct size resulting from myocardial ischaemia. The mechanical interventions of ischaemic preconditioning and postconditioning represent interventions with multiple and interacting components marshaled against myocardial ischaemia/reperfusion injury by endogenous cardioprotective mechanisms.

In the first part of our investigations we aimed to work out an ischaemic preconditioning model on cultured neonatal cardiomyocytes, applying different ischaemia/reperfusion cycles in cardiac myocytes. We aimed to measure cell viability by staining dead cardiac cells and to show the level of two necrosis enzyme: lactate dehydrogenase (LDH) and creatin kinase (CK) from cell culture medium to compare the cardioprotective effect of the various preconditioning stimuli against I/R injury.

In the second part of our study we also aimed to define the role and release of stress-protein urocortin in myocardial hypoxia. After a short ischaemic stimulus cardiac cells were stained with fluorescent immunohistochemistry in different time periods.

In the third part we targeted to investigate the effect of urocortin as a pharmacological preconditioning agent using on neonatal myocardial cell culture. We measured the level of apoptosis and necrosis, and cell viability after various preconditioning stimuli. Adenosine is a well known trigger of ischaemic preconditioning through activation of adenosine A1 receptors; we hypothesize that Ucn can also mimic the cell protective effects of ischaemic preconditioning as adenosine.

Finally we aimed to examine the actions of urocortin in the process of postconditioning. The literature is replete with studies of reperfusion pharmacotherapy in which agents were administered at reperfusion, including such materials as adenosine, opioids, insulin and analogs. We aimed to evaluate the cardioprotective effect of Ucn using at the onset of reperfusion after a longer ischaemic period compared with ischaemic postconditioning.

3. ISCHAEMIC PRECONDITIONING ON CULTURED CARDIOMYOCYTES

3.1. Neonatal cardiomyocytes- a model for studying cell protection against ischaemia/reperfusion injury

3.1.1. Introduction

The term preconditioning was first introduced to describe an experimental observation in dog hearts exposed to repetitive short periods of ischaemia. At present, this phenomenon has been expanded to include different stress-stimuli which can induce protection against different types of cellular dysfunction during hypoxia⁶². Since Murry and his coworkers¹ exposed the myocardium to a PC protocol by occluding the left anterior descending artery (LAD) in dogs, other studies have investigated ischaemic PC in mice, rabbits, pigs and in rats in vivo and in vitro perfused hearts^{63,64,11}.

Nowadays investigations tend to use well-characterized in-vitro cell culture models before applying in vivo animals in the basic researches. We therefore worked with an in-vitro neonatal cardiac cell culture model, using the method of Tokola et al.⁶⁵, which was similar as Simpson and Savion made in 1982⁶⁶ with some modification.

In a pilot study Gordon et al.⁵¹ compared two buffer solutions, which were performed to determine the optional conditions for simulating ischaemia/reperfusion injury. Simulated ischaemia causes both necrotic and apoptotic death of primary cultures of neonatal rat cardiomyocytes⁶⁷. In this environment, the cells were exposed to stresses of hypoxia, acidosis and stagnant incubation medium. The pO₂ and pH of the medium gradually decreased during the ischaemic insult and ultimately fell to a level of pH 6.5-6.8, respectively. Hypoxia triggered severe cell injury, including morphological degeneration, CPK release, beating impairment, ATP depletion and apoptosis, which can be evidenced by positive nuclear staining using Hoechst 33258⁶⁸. These results indicate that neonatal cardiomyocytes subjected to the new simulated ischaemia model exhibit several similarities to cardiac ischaemia, including the simultaneous appearance of necrosis, breakdown of cellular ATP, beating cessation and apoptosis. Therefore we suspected that this new model should prove useful in unravelling the molecular alterations underlying I/R injury and myocardial apoptosis.

3.1.2. Aims

In our study we aimed to find an adequate ischaemic preconditioning protocol on our cell culture model, by using ischaemic buffer solution to mimic hypoxic conditions. We exposed neonatal cardiac cells to ischaemic buffer as preconditioning stimulus for various time. Thereafter we controlled the cell protective effect of these insults measuring lactate dehydrogenase (LDH) enzyme level and cell viability (trypan blue staining) after exposing the cell cultures to a longer test ischaemia and reperfusion period.

3.2. Materials and methods

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

3.2.1. Isolation of cardiac cells from neonatal rats

Primary cultures of neonatal rat cardiac muscle cells are made by using collagenase dissociation method for heart of 2-4-day old Wistar rat pups as described previously⁶⁵. After decapitating the pups, we immersed briefly the carcasses in 70-96% ethanol to disinfect and moved them on a sterile Petri dish in a laminar hood. With sterile instrument set we removed both forelegs and skin covering the chest and to reveal the heart removed the chest with second set of sterile instruments avoiding damage of the heart. During aorta was clamping with forceps, we perforated the right atrium with injection needle to perfuse the heart with disaggregation medium I. -consisting Collagenase II (GIBCO) in PBS; 51 mM CaCl₂- by puncturing the apex of the ventricle. To get pure atrial and ventricular cell culture we removed the auricular appendages and apex of the heart and transferred them on separate glass Petri dishes containing disaggregation medium II (disaggregation medium I and PBS in ratio of 1:1). After dissecting all the pups we cut the tissues into 1-2 mm fragments and transferred the minces to conical flasks and incubated at 37 C° with shaking.

After making the filtering units to 25 mm filter holder containing 100 μm nylon mesh (BD Biosciences-Cell Strainer) attached on Falcon tube (BD Falcon). Then we filtered the supernatants and added culture medium I -consisting of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (GIBCO), 100 IU/ml penicillin, 0,1 mg/ml streptomycin (GIBCO), 1,28% 200mM L-Glut (GIBCO) supplemented with 10 % fetal bovine serum (FBS, GIBCO)- onto filtrates. To the tissue minces we added fresh disaggregation medium I., mixed them, and we repeated the cycle of incubation and collecting the supernatant until most of the tissues are disintegrated.



Figure 4. Cell incubator

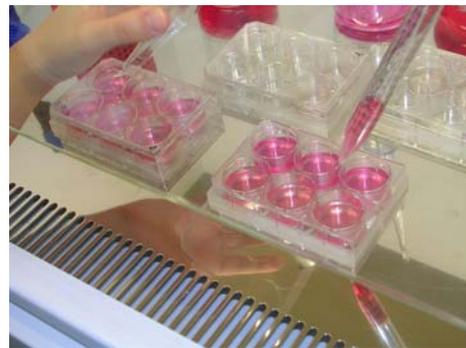


Figure 5. BD Falcon 12-well plates

Then the filtrates were washed by centrifugation at room temperature, medium was discarded and the ventricular cells were resuspended in DMEM/F-12 10%FBS medium. After the second centrifugation the suspension was seeded on 100 mm cell culture dishes and incubated for 45 minutes in cell incubator (95 % air, 5% CO_2 , 37 $^\circ\text{C}$) to reduce the number of contaminating non-muscle cells. **(Figure 4.)**

After gentle shaking, we collected the muscle cell-enriched suspension in cell culture bottle and counted the cells in Bürker hemocytometer taking 180 μl suspension and 20 μl trypan blue vital staining.

Thereafter the muscle cell-enriched fraction was seeded onto 12-well plates (BD Falcon) at the density of 200000 cells per/ml and incubated in cell incubator. **(Figure 5.)**

The cells were cultured in DMEM/F-12 10% FBS medium for 24 hours (h) from plating, and thereafter replaced with complete serum free medium (CSFM) – containing 2,5 mg/ml bovine serum albumin; 1 μM insulin; 5,64 $\mu\text{g/ml}$ transferrin; 32nM selenium; 2,8 mM Na-pyruvate; 0.1-1 nM T3; 100 IU/ml penicillin; 0.1 mg/ml streptomycin; 200 mM L-Glut; DMEM/F-12- to prevent the proliferation of non-muscle cells and to

standardise the experimental protocol. Within 2 days a confluent monolayer of spontaneously beating cardiomyocytes was prepared.

3.2.2. Experimental protocol

To mimic the ischaemic stimuli and test ischaemia cell cultures were exposed to a previous described ischaemic buffer (simulated ischemia (SI) buffer)⁵¹ that contained 137 mM NaCl, 3.5 mM KCl, 0.88 mM CaCl₂·2H₂O, 0.51 mM MgSO₄·7H₂O, 5.55 mM D-glucose, 4 HEPES, 2%FCS, 10 mM 2-deoxy-D-glucose and 20 mM DL-lactic acid (pH 6.2). 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 .

While cardiomyocytes were under hypoxic conditions in SI buffer, control cells were incubated in complete serum free medium such as during reperfusion period.

In our experiments neonatal cardiac cell cultures were listed in 4 groups. In control group (group 1) the isolated neonatal cardiac cells were incubated in SI buffer for 3 hours and then buffer was changed into CSFM medium for 2 hours. In the preconditioned groups cardiomyocytes were exposed to different ischaemic preconditioning stimuli before the longer 3 hours ischaemic insult and 2 hours reperfusion period. In the second group isolated cells were preconditioned with one 5 minutes long ischaemic stimulus (group 2); in the third group preconditioning insult was 10 minutes long (group 3); and in the fourth group the ischaemic stimulus lasted 20 minutes before test ischaemia (3 hours) and reperfusion (2 hours). (Figure 6.)

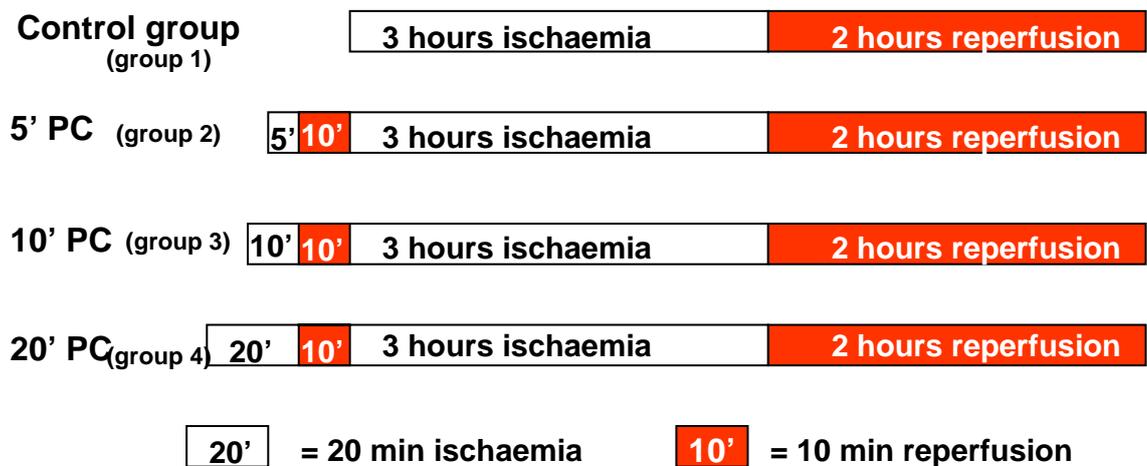


Figure 6. Different preconditioning protocols on neonatal cell cultures

3.2.3. LDH level measurements

Following the experimental protocol cell culture mediums of cardiomyocytes were collected and snap frozen under liquid nitrogen. LDH enzyme activity was determined by using spectrophotometry at 340 nm.

3.2.4. Cell viability

Cardiomyocytes were picked up by using 0.25% trypsin, 0.2 % EDTA solution at 37 °C, pelleted by centrifugation and resuspended in PBS.

To 45 µl cell suspension 5 µl trypan blue (dissolved in PBS) was added and both alive and trypan blue positive dead myocytes were counted on 1 mm² area in Bürker hemocytometer. and cell viability was defined by comparing the number of live cardiomyocytes to total cell amount.

3.2.5. 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. For LDH determinations the enzyme levels (in IU/l) were correlated to the number of the cells studied.

3.3. Results

3.3.1. LDH enzyme level of the cardiac cell culture medium

Exposure of cell culture for 3 h simulated ischaemia followed by 2 h recovery in normal medium as reperfusion period (control, group 1) resulted $131,7 \pm 7,3$ IU/l LDH level measured from the culture supernatant. When preconditioned the cells with 5 min ischemic stimulus (group 2), LDH level was reduced to $119 \pm 3,2$ IU/l. Preconditioning with 10 min long ischaemic insult (group 3) significantly decreased the LDH release from cardiomyocytes to $95 \pm 6,8$ IU/l, while preconditioning with 20 min long ischaemic stimulus (group 4) resulted $103 \pm 7,5$ IU/l LDH content in the cell culture medium. (Figure 7.)

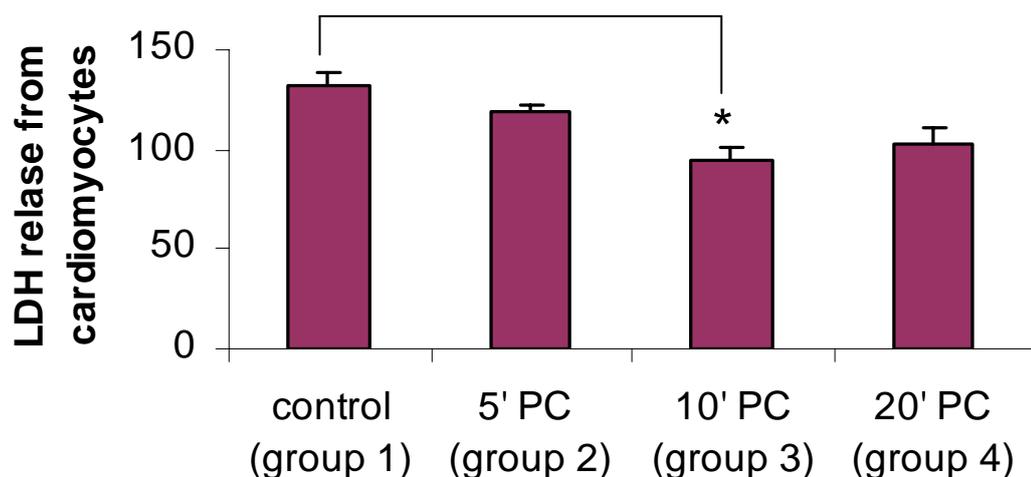


Figure 7. Lactate dehydrogenase enzyme release from cardiomyocytes to cell culture medium in U/l.

3.3.2. Cell viability using vital staining

Cell viability was determined by comparing the number of live cardiac cells to total cell (live and trypan blue positive dead cells) amount. In the control group (group 1) exposure of cell culture for 3 h simulated ischaemia followed by 2 h reperfusion

resulted $0,119\pm0,097$ % cell viability rate. After preconditioning with 5 min ischemic stimulus (group 2), cell viability was $0.625\pm0,189$ %, while preconditioning with 10 min long ischaemic insult (group 3) significantly increased the number of alive cardiomyocytes to $4,615\pm0,176$ %. 20 min long ischaemic preconditioning stimulus (group 4) resulted $1,846\pm0,54$ % in cell viability following 3 h test ischaemia and 2 h reperfusion period. **(Figure 8.)**

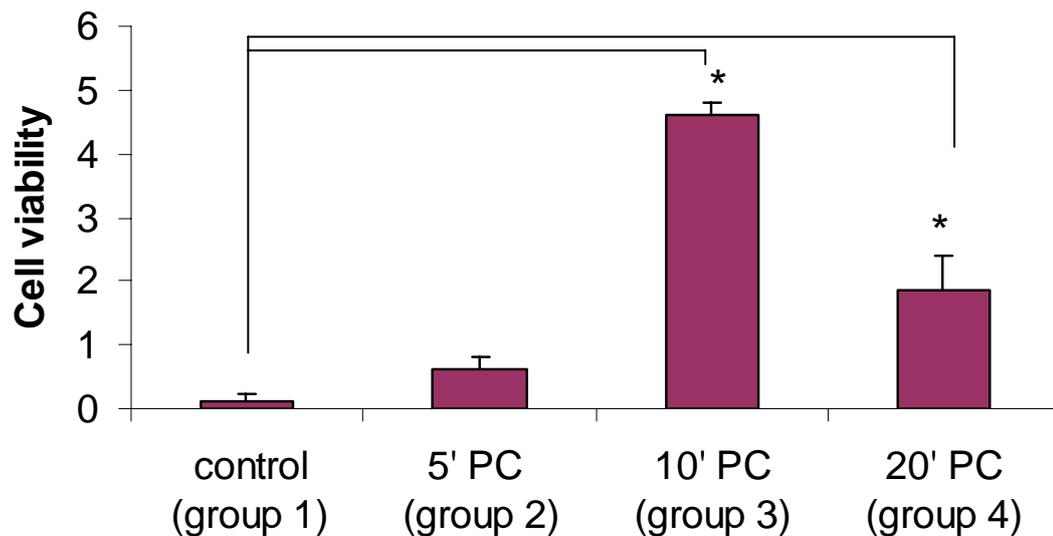


Figure 8. shows the cell viability after the different preconditioning protocol. The diagram demonstrates cell viability comparing the number of live cardiomyocytes to total (live and dead) cell amount.

3.4. Discussion

Acute myocardial infarction results in loss of working myocardium, which in turn leads to a clinical spectrum ranging from left ventricular dysfunction to advanced heart failure. Activation of the endogenous mechanism of preconditioning could confer protection to the ischaemic heart. Moreover one important feature of preconditioning is that it has protected in every species tested giving us confidence that it would surely protect the human heart as well although a direct test of that hypothesis has not been possible.

In our study we used an *in vitro* neonatal cell culture model, which contained mainly (in 95%) cardiomyocytes that provided special features opposite to *in vivo* heart models. The most obvious advantage of studying cardiomyocytes compared to the whole heart might be the elimination of other cell types, notably fibroblasts and endothelial cells, so that their influence is negligible⁶⁹. However, by separating cardiac cells from their syncytial neighbours and also from the blood vessels and surrounding extracellular matrix, important aspects of the pathophysiology of myocardial ischaemia/reperfusion injury in the whole heart are lost. Isolated cardiomyocytes are not capable of reproducing the mechanism of reperfusion hyper-contracture⁷⁰ in the whole heart so well, in which the myocardial cells exert so much force on each other that there are cytoskeletal and sarcolemmal disruptions which cause massive enzyme release and secondary influx of calcium ions into broken cells. The combination of contracture, interstitial edema and vascular plugging by platelets, leukocytes and fibrin can lead to no-reflow phenomenon, which process is unable to be studied on isolated cardiomyocytes.

In our experiments we used an *in vitro* neonatal cell culture model, which was first described by Tokola and his co-workers⁶⁵. Our aim was to define an optimal ischaemic preconditioning protocol in this model using the ischaemic buffer solution of Gordon et al.⁵¹, that contained 2-deoxy-D-glucose and lactic acid, thereby mimicing many of the well-documented conditions that are present during ischaemia *in vivo* including decreased pH (6,2), decreased utilization of substrates (2-deoxy-D-glucose, an analog of glucose that cardiac myocytes cannot utilize for energy), and elevated lactic acid. Our results showed that in our cardiac cell culture 10 minutes ischaemic stimulus followed

by 10 min reperfusion provided the most effective cell protective effect against sustained ischaemia and the following reperfusion.

Ischaemic preconditioning was demonstrated to be a potent protective mechanism in the pioneering studies of Armstrong et al⁷¹ in freshly isolated cardiomyocytes and Ikonomidis et al. in cultured cardiomyocytes⁷². Armstrong and his co-workers were the first to investigate ischaemic PC in isolated myocardial cells using an in vitro simulated ischaemic model⁷³ in which isolated adult cardiomyocytes were centrifugated at a very low speed into a pellet so that the fluid layer over the cells was limited to one-third the volume of the pellet. The supernatant was covered with an oil layer and incubated at 37 °C. Since then, several research groups have applied ischaemic palleting model as ischaemic preconditioning stimulus in various species of freshly isolated cardiomyocytes^{74,75,76}.

Myocardial cells in primary culture have been used by many research groups^{72,77,78} in which a variety of techniques have been used to simulate ischaemia in culture. Severe hypoxia, using nitrogen concentration between 95% and 100%, combined or without glucose-free medium and/or high potassium concentration and lactate.

In a recent study cultured cardiomyocytes were preconditioned by exposing them for 10 min to a hypoxic HEPES-based solution without glucose with physiological concentration of KCl without lactate, and balanced to a physiological pH to stimulate the mild ischaemic episode, followed by a 10 min reoxygenation as reperfusion period in culture medium 199. This group used during the long index ischaemia a solution with low pH (6,5) level, high K⁺ (12 mmol/L) and high lactate (20 mmol/L) concentration. The same researchers examined the ultrastructural changes after preconditioning cardiomyocytes with 10 minutes pelleting followed by 10 minutes of simulated reperfusion prior to the pelleting index simulated ischaemia, in which protocol reperfusion was provided by resuspending the cardiac cells from the pellet in an iso-osmotic physiological solution⁷⁹.

In our study we examined the cell protective effect of 10 min long ischaemic preconditioning stimulus by measuring the release of LDH enzyme into culture medium from cardiomyocytes, and with trypan blue staining method to determine cardiac cell viability. Trypan blue, a 961 kDa dye, will enter cardiomyocytes through a damaged cell membrane, staining the entire cardiomyocyte and indicating the cell to be

irreversibly injured⁸⁰. Evaluating cardiomyocyte viability by trypan blue staining has become the gold standard in ischaemic preconditioning studies either freshly isolated or cultured cardiomyocytes^{81,82,83}.

Our results showed that preconditioning with ischemic buffer solution was cardioprotective against ischaemia/reperfusion injury in our primary neonatal cell culture model, whereby this model provide an essential platform for these studies and should make a critically important contribution to future preconditioning research and, more generally, the mechanistic study of the pathophysiology of myocardial ischaemia and reperfusion.

4. UROCORTIN RELEASE DURING HYPOXIA IN CARDIOMYOCYTES

4.1. Effects of urocortin in the cardiovascular system – does it change during hypoxic conditions?

4.1.1. General background

Hypoxia/ischaemia is probably the main physiological stress to the heart. Reperfusion is the only means of salvaging ischaemic myocardium and limiting infarct development. However, it is paradoxically associated with cell death termed lethal reperfusion injury⁸⁴. Proving the existence of lethal reperfusion injury is difficult, and the only way to investigate this phenomenon is by the administration of possible modulators of such injury immediately before reperfusion and not before ischaemia, with the subsequent assessment of infarct size. Until now there is no experimental agent that has been shown to be effective in limiting lethal reperfusion injury both in animal models and the clinical settings.

Recent studies show, exogenous urocortin increases heart rate and cardiac output and also produces coronary vasodilation⁸⁵. In addition, exogenous Ucn may protect myocardial cells during ischaemia, as it increases the survival of cultured cardiomyocytes exposed to simulated ischaemia⁶⁷ and also reduces the infarct area after ischaemia/reperfusion injury in rat heart⁵².

Ucn mRNA is expressed in the isolated rat cardiac cell cultures, whereas CRF R2 β is predominantly expressed in cardiomyocytes compared with non cardiac cells. Ucn mRNA expression is higher in left ventricular hypertrophy than in normal left ventricle, whereas CRF R2 β mRNA expression is markedly depressed in left ventricular hypertrophy compared with normal left ventricle⁴⁷.

Given the high affinity of Ucn for CRF R2, Ucn at concentrations in picomolar and nanomolar ranges should have an important role as a local regulator of cardiac function⁸⁶. However, little is known about the exact cellular source of Ucn in the physiological conditions and additional second messengers that may have been also involved.

4.1.2. Aims

Nishikimi et al. have recently reported that the human myocardium was immunohistochemically positive for Ucn, and the staining was more intense in the failing heart. Therefore the purpose of the present study is to investigate whether Ucn immunoreactivity or expression shows any changes after ischaemic insult on neonatal cardiac cell culture line.

4.2. Materials and methods

4.2. 1. Isolated neonatal cell culture

Ventricular cardiac myocytes were prepared from the hearts of neonatal Wistar rats (*day 2-4*) as described previously in part 3. After digestion with collagenase II (GIBCO), cell solution was filtered with 100 µm nylon mesh (BD Biosciences Cell Strainer) in a medium consisting of DMEM/F-12 (GIBCO), 100 IU/ml penicillin, 0,1 mg/ml streptomycin (GIBCO), 1,28% 200mM L-Glut (GIBCO) supplemented with 10 % fetal bovine serum (FBS, GIBCO). The filtrates were washed by centrifugation and thereafter the ventricular cells were resuspended in DMEM/F-12 10%FBS medium. To reduce the number of contaminating non-muscle cells the suspension was seeded on 100 mm cell culture dishes and incubated for 45 minutes in cell incubator (5% CO₂, 37 °C).

Then the suspension was transferred onto poly-D-lysine covered 8-well culture slides (BD Falcon 8-well culture slides). **(Figure 9.)** Poly-D-lysine is used to enhance cell attachment to plastic and glass surfaces for many cell types including cardiomyocytes. In each well 500 µl cell solution was seeded at the density of 2×10^5 cells per ml solution. After 24 hours from plating, cell media was substituted with complete serum free medium (CSFM) to prevent the proliferation of non-muscle cells and to standardize the experimental protocol. Within 2 days a confluent monolayer of spontaneously beating cardiomyocytes was prepared. **(Figure 10.)**



Figure 9. BD Falcon 8-well culture slides

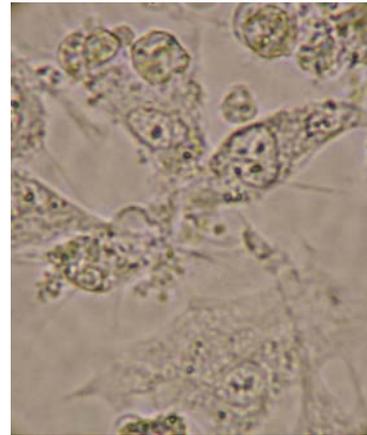
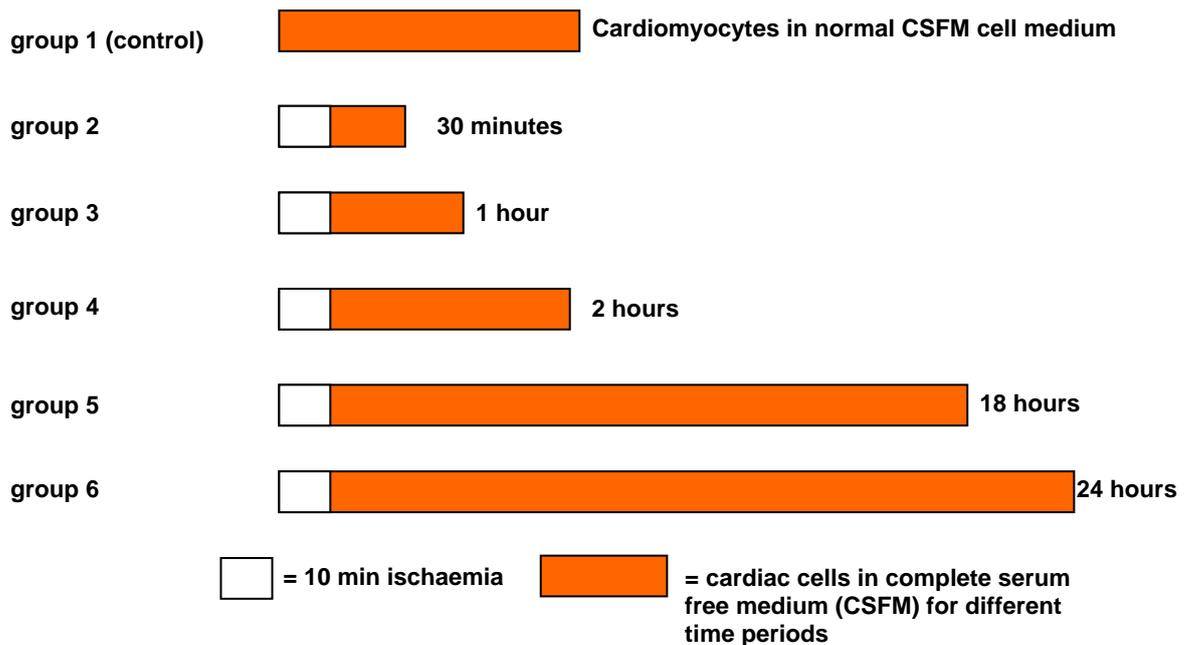


Figure 10. Cardiomyocytes

4.2.2. Experimental protocol

In this part of our study we aimed to investigate the change of urocortin expression in cardiac cell line after a short ischaemic stimulus. To mimic the ischaemic insult cardiomyocytes were exposed to the previous described ischaemic buffer (see in part 3) that contained anti-glucose metabolite 2-deoxy-D-glucose and DL-lactic acid.

Myocardial cell cultures were divided in 6 groups. In control group (group 1) cells were incubated in normal CSFM cell medium. In the rest groups neonatal cardiac cell cultures were subjected to ischaemic buffer for 10 minutes, than ischaemic medium was changed into normal CSFM cell medium for 30 minutes (group 2); 1 hour (group 3); 2 hours (group 4); 18 hours (group 5) and for 24 hours (group 6). Thereafter cell supernatants were removed from plates and cardiac cells were washed and fixed in paraformaldehyde solution for studying Ucn expression. **(Figure 11.)**



(Figure 11). Experimental groups in investigating the release of urocortin after hypoxic stress.

4.2.3. Immunohistochemical staining of urocortin on cardiac cells

After cultured neonatal cardiomyocytes were exposed to CSFM cell solution for various time periods, the supernatant of cultures were removed from each well, and cardiac cells were washed gently with phosphate buffered saline (PBS) at 37 °C. Then cells were fixed in buffered 4 % paraformaldehyde solution for at 37 °C. After 1 hour incubation, plates were washed gently twice for 5 min with PBS on a shaker, then PBS was changed into Tris buffered saline (TBS) to avoid cross-reaction with paraformaldehyde. Slides were washed three times 10 min with TBS solution. To enhance the membrane permeability cells were incubated in TBS/T (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.4) for 1 hour at room temperature. Non-specific binding places were blocked in 5% blocking serum in TBST/T solution (contained serum of secondary antibody-goat (Sigma); and fat free milk powder (MilkQuick)) for 1 h. Without washing primer antibody- rabbit anti-urocortin, Ig G fraction of antiserum (Sigma Aldrich)- was added to the blocking serum in a concentration of 1:200 and cardiomyocytes were incubated overnight at 4 °C. Then plates were washed for 10 min three times on shaker with TBS/T and fluorescence immunostaining was performed using Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (Invitrogen, Molecular Probes) in PBS as a

detection reagent, according to the manufacturer's instructions. After 1 hour incubation period with the secondary antibody, slides were covered with Fluoromount and were examined and photographed in fluorescent microscope (Canon).

4.3. Results

4.3.1. Urocortin immunoreactivity on cultured cardiomyocytes

In the control group (group 1) in which myocardial cells were incubated only in CSFM cell solution, Ucn specific staining was to be seen in some cardiomyocyte. In group 2 and group 3 –following 10 min ischaemic insult and 30 min or 1 hour reperfusion period- Ucn immunoreactivity was similar as it was in control group. However in group 4 after 2 hours reperfusion, Ucn expression was more expressed in many myocardial cell cytoplasm. Following 18 or 24 hours reperfusion in group 5 and group 6, Ucn immunostaining appeared only in some cardiac cell cytoplasm, such as in control group (group1). (Figure 12-17.)

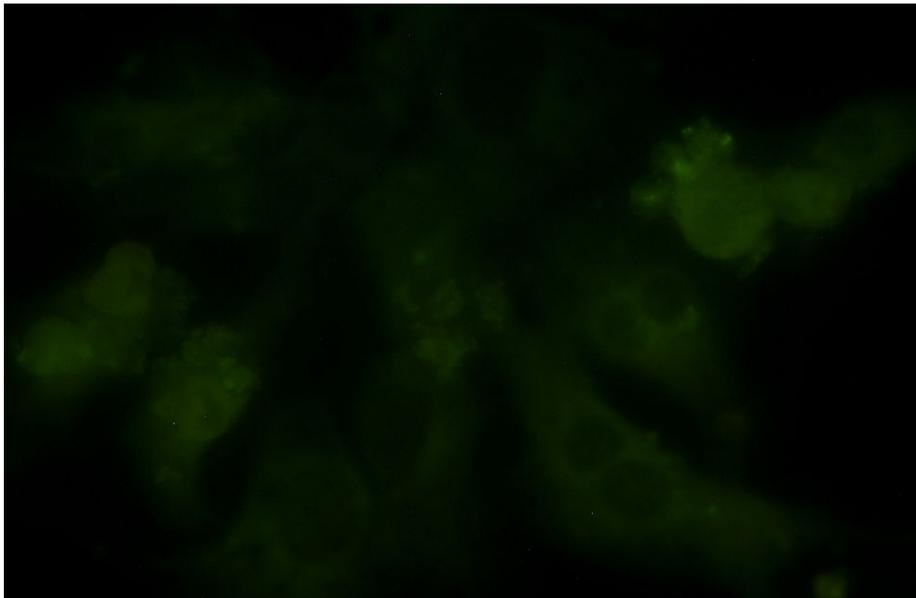


Figure 12. In Group 1, control cells were incubated in normal complete serum free medium.



Figure 13. In **group 2** cells were exposed to 10 min ischaemia and 30 minutes reperfusion.

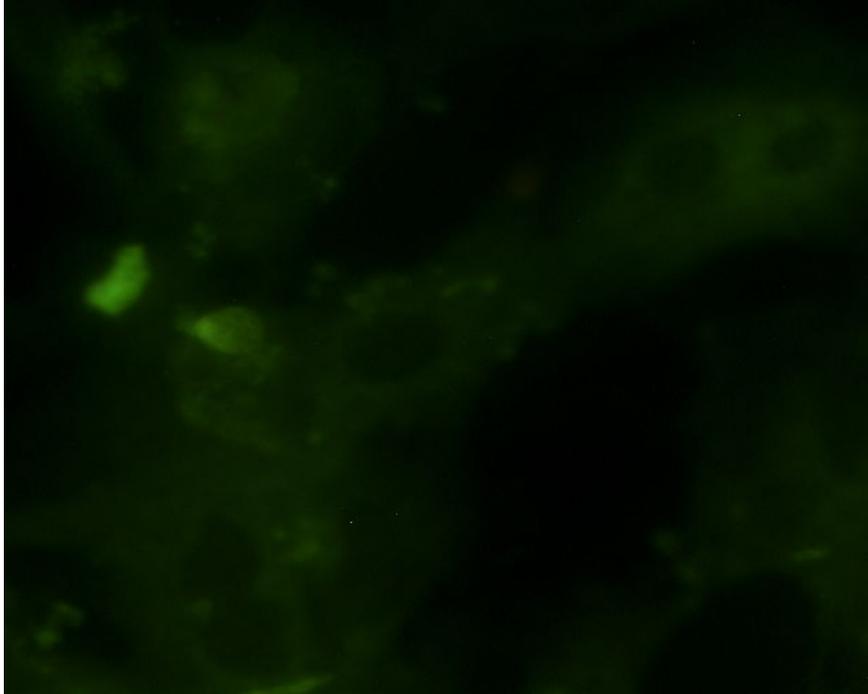


Figure 14. In **group 3** cardiomyocytes underwent in 10 min hypoxia and 1 hour recovery in CSFM.

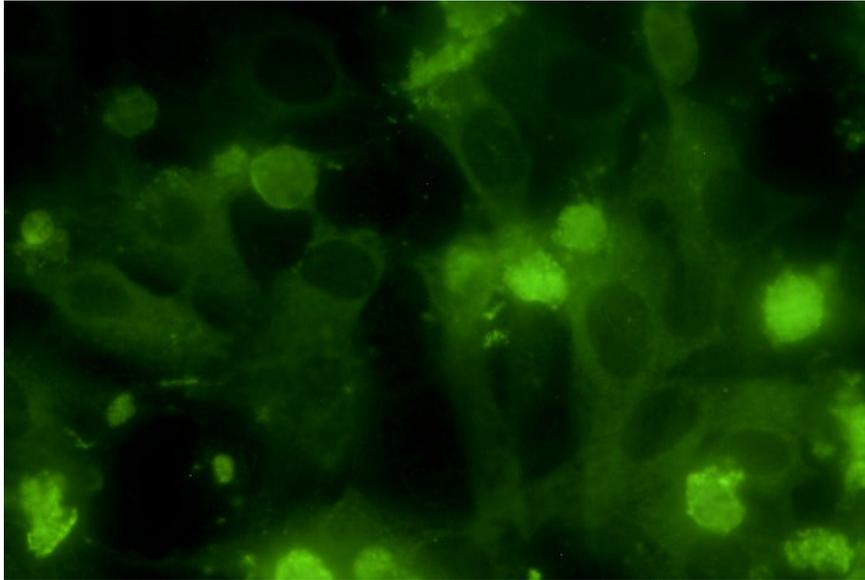


Figure 15. In **group 4** cell cytoplasm stained very intensive with urocortin after 10 minutes ischaemia and 2 hours recovery in normal cell solution.

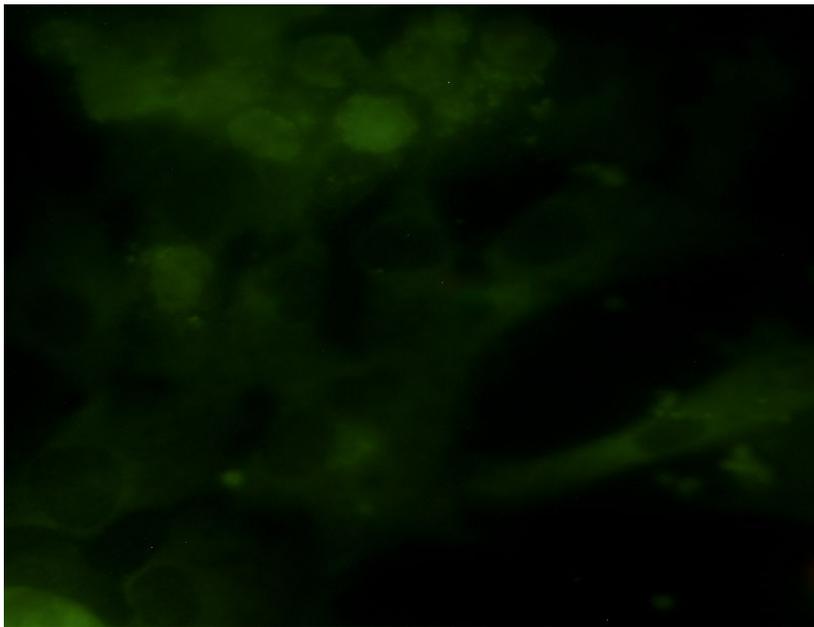


Figure 16. In **group 5** cells were 18 hours in CSFM after hypoxic insult.

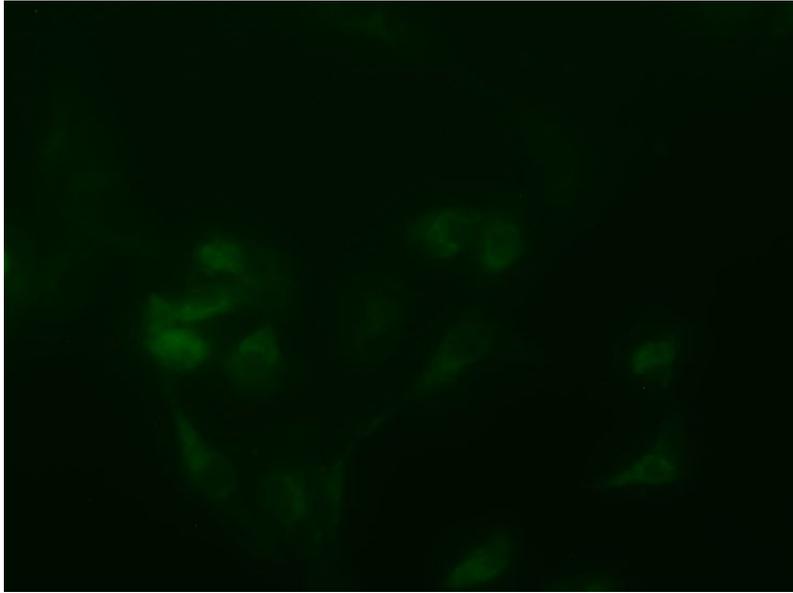


Figure 17. In **group 6** cardiac cells were incubated 24 hours in CSFM as recovery in normal cell environment after the short hypoxic stimulus.

4.4. Discussion-Intensive urocortin expression after short ischaemic stimulus-urocortin has a presynthetic form?

In our study we investigated Ucn immunoreactivity on neonatal rat cardiomyocytes after brief ischaemic stress stimulus. Our results showed that Ucn specific immunostaining appeared the most intensively in cell cytoplasm 2 hours after the ischaemic insult.

In a recent study of Okosi et al.⁸⁷ endogenous Ucn expression within the rat heart could be detected with polymerase chain reaction (PCR). Furthermore, the expression of Ucn mRNA in rat cardiac cell line or in primary cultures of cardiomyocytes was shown to increase 12-18 h after thermal injury. Following 4 hours simulated ischaemia and 18 hours reoxygenation Ucn mRNA expression was greatly increased⁶⁷. These data suggest that in cardiomyocytes the synthesis of new Ucn peptide is seemed to begin at least 18 h after injury. Chromatographic studies demonstrated that the main form of Ucn-like immunoreactivity was the larger molecular weight peptide in the human heart. Human Ucn is generated from a 124-amino acid Ucn precursor through posttranslational enzymatic processing of Arg-Arg at position 81-82⁸⁸. Interestingly, the 22-kDa Ucn precursor protein was detected in rat cardiac myocytes by Western blot analysis⁵². On the other hand, in column chromatograph study the main peak was detected between the elution positions for CNP-53 (a 53-amino acid peptide) and Ucn (a 40-amino acid peptide), which peak may be a peptide consisting of about 45-50 amino acids and may therefore represent a partially processed form of the Ucn precursor rather than the 22-kDa Ucn precursor⁸⁹. There is another possible proteolytic processing site Arg-Arg at position 64-65 in the human Ucn precursor⁸⁹, and the cleavage of this site may generate a 57-amino acid peptide containing the 40- amino acid Ucn sequence. In addition, two minor peaks were also detected in the larger molecular weight position. According to these issues, together with our results, it might be hypothesised that Ucn exists in a presynthetic form in the cardiac cell cytoplasm, where it can be transformed into an active form during a few hours to exert its cardioprotective effect by activating several signal transduction pathways. Further studies are required to clarify whether such an incompletely processed Ucn is present in the human heart as well as the mature type of Ucn consisting of 40 amino acids.

Our results are in positive correlation with a recently published neuroscientific study. It has been shown that 2 hours after exposing rats to various stress insults, Ucn like

immunoreactivity was to be seen in Edinger-Westphal nucleus, which nucleus plays an important role in the regulatory mechanisms of stress⁹⁰.

The increased expression of Ucn following thermal and ischaemic injury, and the protective effects of exogenous Ucn against cell death induced by ischaemia⁸⁸, suggest that this peptide may function as an endogenous cardioprotective agent.

Other cardiac proteins, such as heat shock proteins⁹¹ and cardiotrophin-1⁹², have also been shown to protect cardiac myocytes from hypoxic injury.

Ucn exerts a protective effect in primary cardiac myocyte culture exposed to lethal simulated ischaemia and this effect is rapid, occurring 30 min after Ucn administration⁵².

Ucn is also cardioprotective when added at the point of reperfusion.

These novel findings prompted Ng and his co-workers⁹³ to investigate Ucn plasma levels in humans with heart failure compared with age- and gender-matched normal controls. In this study, plasma Ucn was found to be elevated in heart failure, especially in its early stages. Moreover Ucn mRNA expression is higher in the hypertrophic hearts as compared to normal hearts, whereas CRF- R2 β mRNA expression is reduced in ventricular hypertrophy, suggesting that Ucn may have a negative regulatory effect on mRNA expression of CRF R2 β in the hypertrophic hearts⁹⁴.

All of these results suggested that Ucn is an endogenous cardiomyocyte peptide, which could play a crucial role in regulation of myocardium tolerance against ischaemic injury. Whether administration or replacement of Ucn in ischaemic heart disease or in defined heart failure could be used as a therapy, remains to be explored in humans.

5. PRECONDITIONING WITH UROCORTIN ON NEONATAL CULTURED CARDIOMYOCYTES- DOES IT HAVE ANY EFFECTS?

5.1. Introduction

5.1. 1. Background

Many years ago, Murry and his colleagues¹ demonstrated for the first time the protective effects of brief ischaemic stimuli on dog hearts. Nowadays, it is apparent that ischaemic preconditioning is a powerful endogenous adaptive tool for myocardial cell protection against ischaemic/reperfusion damage. Cardioprotection resulting from ischaemic PC is reflected in reduced infarct size⁹⁵, improved recovery of contractile function⁹⁶, reduced number of arrhythmias⁹⁷, and reduction in LDH levels⁹⁸. However, clinical applications of ischaemic PC have been limited by ethical concerns.

5.1.2. Pharmacological preconditioning with adenosine

Thus pharmacological PC has emerged as an ideal alternative to ischaemic PC. As pharmacological PC agent, presumed triggers of ischaemic PC could be employed, such as G-protein coupled receptor (GPCR) agonists, K_{ATP} channel openers, nitric oxide donors. Adenosine A1 receptor agonists are one type of GPCR agonist. Although adenosine A1 receptor agonist PC is well established in several species^{99,100}, the mechanism of this cardioprotection may occur via protein kinase C and activation of mitochondrial K_{ATP} channels^{96,7}, but there is no definitive evidence that during ischaemia A1 receptor activation is associated with either of these events¹⁰¹. Recently, adenosine receptor agonists have been shown to activate p38 MAPK activity in rat myocardium¹⁰² and isolated ventricular myocytes¹⁰³. In a study by Ballard-Croft et al.¹⁰⁴ it has been presented that acute adenosine A1 receptor PC involves mediation via p38 MAPK cascade in an in vivo model. This cardioprotection appears to be associated with modulation of p38 MAPK activation and distribution in discrete subcellular compartments. According to another in vivo study, adenosine receptor PC reduces myocardial infarct size via subcellular ERK signaling¹⁰⁵.

5.1.3. Urocortin can mimic ischaemic preconditioning

In the previous part of our study it has been evidenced that Ucn might have an important role in the answering mechanisms of hypoxic myocardium. As a member of the corticotrophin-releasing factor family, it has been recently shown, that Ucn protect an immature phenotype of cardiomyocytes against simulated ischaemia⁵². It is suggested, that the cardioprotective effects of Ucn depends on an action at CRF R2, which receptor is the only CRF receptor that has been detected in heart tissue¹⁰⁶. In an other study it was demonstrated, that Ucn has immediate and delayed cardioprotective actions that mimic ischaemic preconditioning via PKC and K_{ATP} channels⁵¹.

5.1.4. Aims

In this part of our study we aimed to investigate the action of Ucn against ischaemia/reperfusion injury on neonatal cardiac cell cultures using it before the sustained ischaemic stress insult as a possible pharmacological preconditioning agent. Furthermore we compared these effects with ischemic and adenosine induced pharmacological preconditioning.

5.2. Methods

Cultured neonatal ventricular rat cardiomyocytes were transferred onto 12-well plates (BD Falcon) at the density of 200000 cells per/ml as described previously in part 3. **(Figure 18.)** In each well 5 ml cell solution was seeded. After 24 hours from plating, cell media was substituted with CSFM.

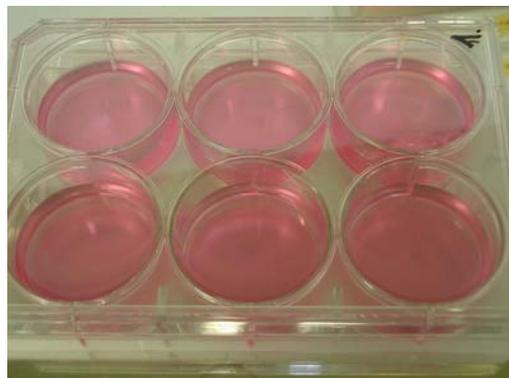


Figure 18. 12-well plates filled with cell solution

5.2.1. Preconditioning protocol

In this part of this study we aimed to investigate the effect of Ucn as a pharmacological preconditioning agent on cultured neonatal cardiac myocytes compared with the cardioprotective effects of ischaemic and adenosine generated preconditioning.

In group 1 (non treated cells) cell cultures were exposed to only complete serum free medium (CSFM). In group 2 (ischaemic group) cardiomyocytes underwent 3 h sustained ischaemia through incubation in ischaemic buffer (see part 3.), which period was followed by 2 h reperfusion in normal culture medium. To study the cardioprotective effects of ischaemic and pharmacological preconditioning with adenosine or urocortin; cardiomyocytes were exposed to ischaemic buffer (group 3), to adenosine supplemented normal medium (10 μ M) in group 4 and Ucn (0,1 μ M, Sigma) supplemented medium in group 5 for 10 minutes. After these preconditioning stimuli cardiac cells were removed in normal serum-free medium for 10 min immediately before the 3 h sustained ischaemic insult and the 2 h long recovery phase in normal medium. (Figure 19.).

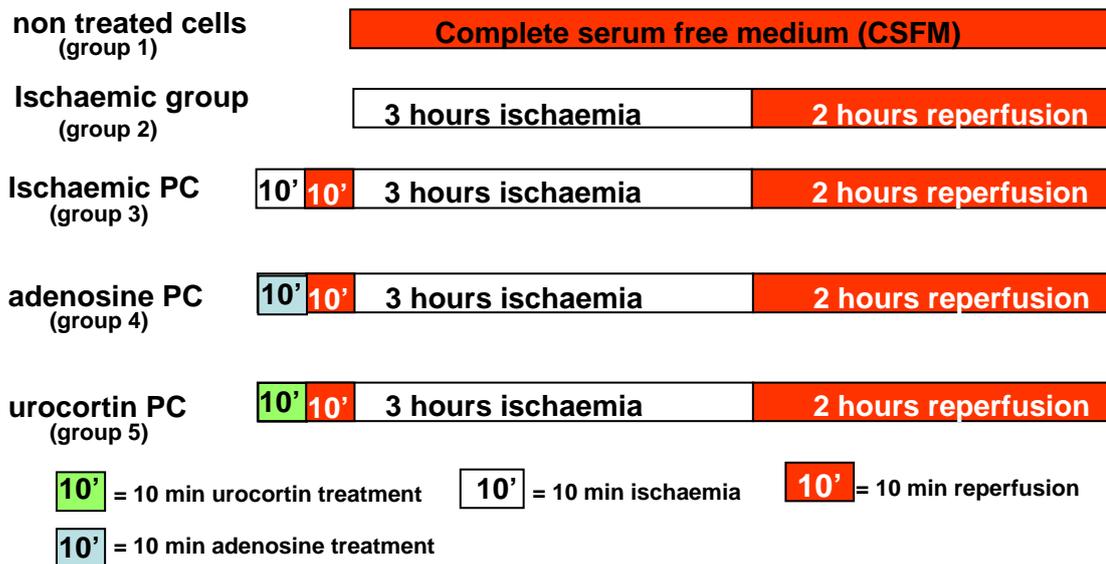


Figure 19. Different ischaemic preconditional protocol on neonatal cardiomyocyte culture model.

5.2.2. Level of apoptosis and necrosis using Annexin V-Propidium iodide staining and flow cytometry

Annexin V- FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phospholipid phosphatidylserine to the external environment.

Annexin V is a Ca^{2+} -dependent phospholipid binding protein that has a high affinity for phospholipid phosphatidylserine, and is useful for identifying apoptotic cells with exposed phospholipid phosphatidylserine. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas as the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing measurable apoptosis¹⁰⁷.

After cell solution was resuspended in binding buffer (contained 10 mM HEPES/NaOH; PH 7.4; 140 mM NaCl; 2.5 mM CaCl_2), Annexin V and PI, apoptosis/necrosis was evaluated by FACS Calibur flow cytometer (BD, USA). From each sample 5000 cells were counted and the number of stained cells was expressed as a percentage of all counted cardiac myocytes.

5.2.3. LDH level measurements

Following experimental protocol LDH enzyme activity was determined from snap frozen culture mediums by using spectrophotometry at 340 nm.

5.2.4. Cell death analysis

Cardiomyocytes were picked up with 0.25% trypsin, 0.2 % EDTA solution at 37 °C, centrifugated and resuspended in PBS.

To 45 μ l cell suspension 5 μ l trypan blue (dissolved in PBS) was added and both alive and trypan blue positive dead myocytes were counted on 1 mm² area in Bürker hemocytometer and cell death was determined by trypan blue positive cells in %.

5.2.5. Statistical analysis

Up to seven different cardiac myocyte preparations were studied. Datas of the experiments are expressed as means \pm 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. For LDH determinations the enzyme levels (in IU/l) were correlated to the number of the cells studied.

5.3. Results

Urocortin is such a cardioprotective agent against ischaemia-reperfusion injury as adenosine induced or ischaemic preconditioning.

Exposure of cell culture for 3 h simulated ischaemia followed by 2 h recovery in normal medium (control, group 1) markedly increased **LDH level** to $27,71 \pm 3,15$ IU/l medium. When preconditioned the cells with Ucn (group 4), LDH level was reduced to $8,71 \pm 0,6$ IU/l, which protective action was comparable with that observed with adenosine (group 3) ($7,85 \pm 0,508$ IU/l) or in ischaemic (group 2) ($6,28 \pm 0,64$ IU/l) preconditioned groups. **(Figure 20.)**

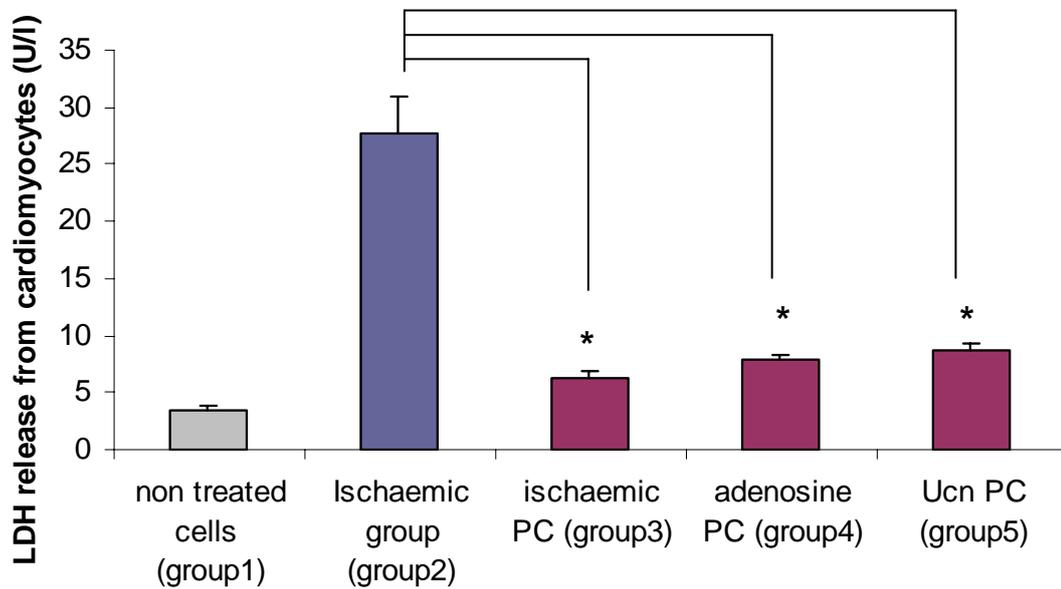


Figure 20. shows the lactate dehydrogenase enzyme release from cardiomyocytes to cell culture medium in U/l measured by spectrophotometry. Results are expressed as means \pm SE (* $P < 0.05$ vs. ischaemic group).

Preconditioning with adenosine (group 3), ischemia (group 2) and UCN (group 4) caused a marked decrease by $44,69 \pm 0,105\%$; $47,02 \pm 0,12\%$; and $45,69 \pm 0,87\%$ in the **number of trypan blue positive cells** compared with control cells (group 1) ($57,057 \pm 1,209\%$). **(Figure 21.)**

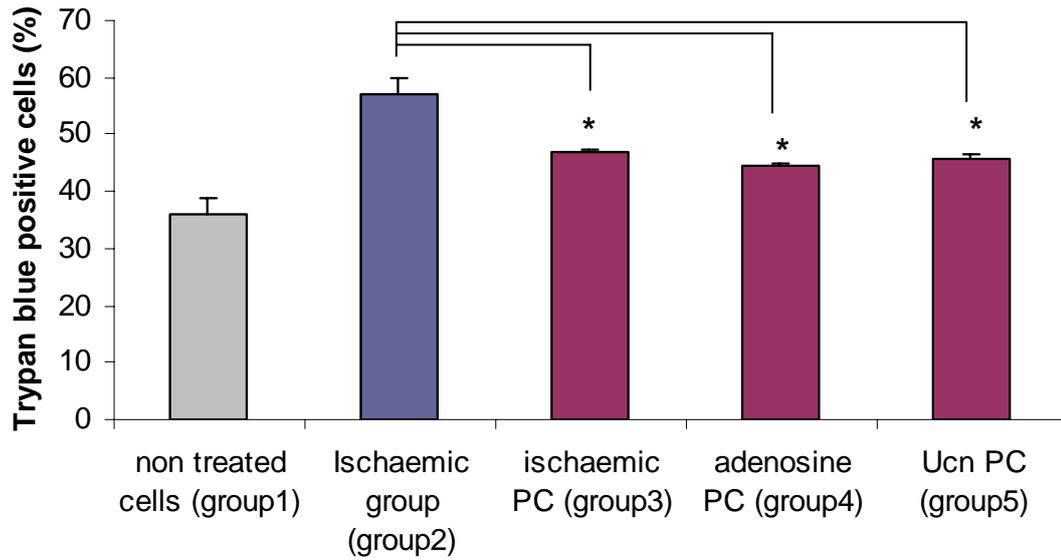


Figure 21. demonstrated the number of trypan blue positive dead cells in groups of preconditioning protocol. Results are expressed as means \pm SE (* P< 0.05 vs. ischaemic group).

The number of myocytes stained with AnnexinV-FITC alone (no PI), which demonstrates the **extent of apoptosis** was $84,43 \pm 1,98$ % in the control group (group 1), which was significantly diminished by $40,81 \pm 0,99$ % (group 3); $38,9 \pm 1,68$ % (group 2); and $46,54 \pm 1,11$ % (group 4) after the different preconditioning methods. (**Figure 22.**)

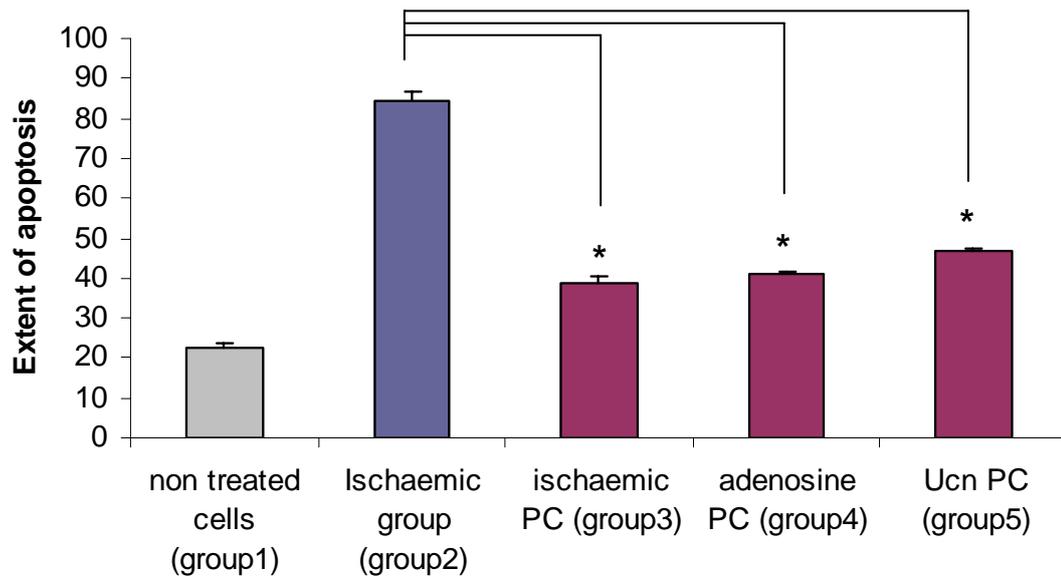


Figure 22. shows the percentage of Annexin V-FITC stained cells, which demonstrates the extent of apoptosis in our groups. (* P< 0.05 vs. ischaemic group)

In addition the percentage of PI positive cardiomyocytes, which demonstrates the **degree of necrosis** was also markedly reduced by $24,5\pm 3,33\%$ after the ischaemic PC (group 2); $23,34\pm 1,99\%$ in adenosine preconditioned group (group 3), and $24,14\pm 1,57\%$ in Ucn treated group (group 4) as opposed to the increased value of $40,27\pm$ % in the control samples (group 1). (**Figure 23.**)

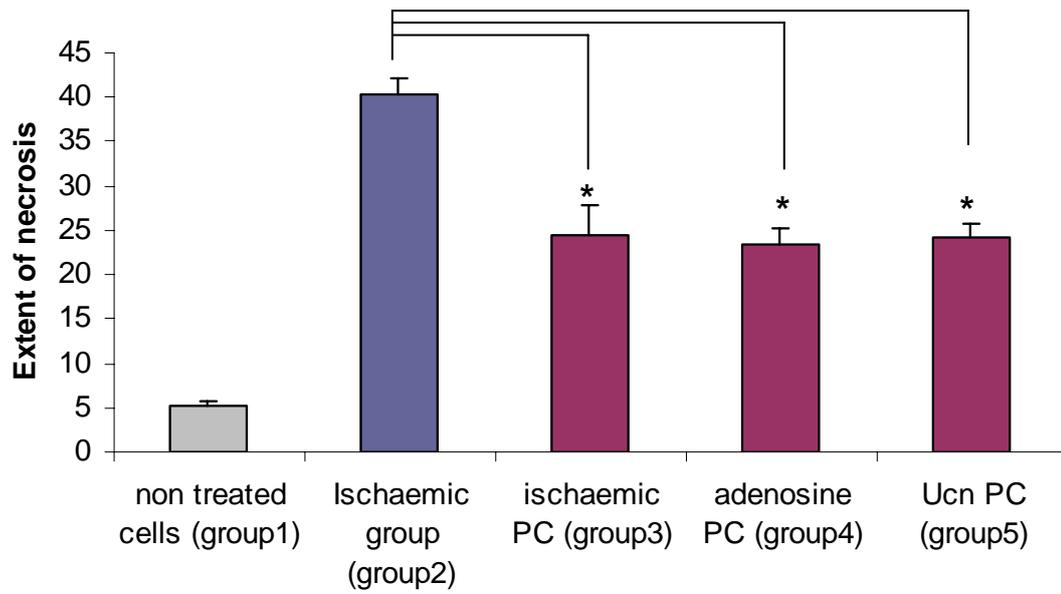


Figure 23. demonstrates the extent of necrosis by the percentage of propidium iodide positive cells. Results are expressed as means \pm SE (* $P < 0.05$ vs. ischaemic group)

Graphs of Figure 22. and 23. demonstrated the mean percentage of the ratio of Annexin V-FITC labelled apoptotised and with propidium iodide stained necrotised cardiac cells from 7 different cell cultures.

Examples of dot-plots as determined by flow cytometry following Annexin V and propidium iodide double-staining. Horizontal axis represents annexin V intensity and vertical axis shows propidium iodide staining. The lines divide each plot into four quadrants: lower left quadrant: living cells, lower right quadrant: early apoptotic cells, upper left quadrant necrotic cells, upper right quadrant: late apoptotic cells. **(Figure 24.)**

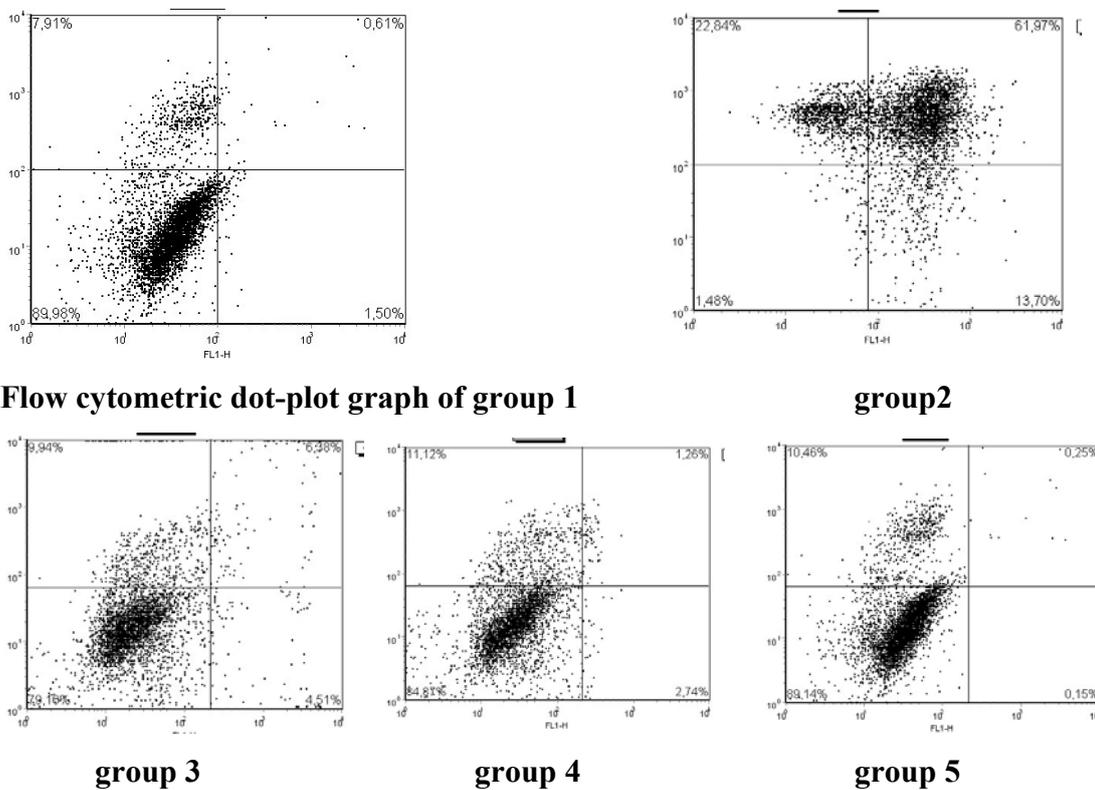


Figure 24. Five dot-plot diagrams shows the ratio of living, apoptotic and necrotic cardiomyocytes in all investigated groups.

5.4. Discussion- Ucn might be pharmacological agent as adenosine

Although a number of in vitro models for the study of ischaemia/reperfusion have previously been described^{108,109}, most of these findings cause an irreversible injury in cardiomyocytes. In our study, simulated ischaemia consisting of exposure of cardiac cells to a metabolic inhibition milieu (HEPES buffer supplemented with 2-deoxy-D-glucose and DL-lactic acid) for 4 h followed by recovery in serum free medium resulted in an optimal cellular injury⁵¹.

In this part of our study we demonstrated the cardioprotective effect of the CRF-related peptide urocortin against simulated ischaemia/reperfusion injury in neonatal rat cardiomyocytes. The cardioprotective effect of Ucn was observed when the cardiac cells were exposed to normal cell culture medium supplemented with Ucn for 10 min before prolonged ischaemic period (3 hours), which was followed by 2 hours of recovery in normal, defined complete serum free medium. After urocortin treatment cell culture medium was changed into ischemic buffer, whereby Ucn was not presented during hypoxic period in the ambience of cardiomyocytes. However, it was able to develop an antiapoptotic, and antinecrotic effect –represented as a reduction in Annexin V and propidium iodide staining- against ischemia/reperfusion trauma.

In a previous study, the effects of pretreating neonatal cardiomyocytes with Ucn for 30 min, 1 h, 2 h, and 24 h before a more severe, lethal hypoxic insult were determined⁵². In contrast with our findings, no cardioprotection was observed with a brief exposure (10 min) to Ucn immediately before lethal ischaemia. However, when Ucn was present for 30 min, or longer, cell death was reduced.

According to a study by Gordon et al., exposure of adult rat cardiomyocytes to simulated ischaemia buffer or to normal HEPES buffer supplemented with Ucn for a short period, also conferred a protective effect against cellular injury⁵¹. In their experimental protocol, adult cardiac myocytes were exposed to simulated ischaemic buffer for 5 min and recovery in normal medium for 10 min immediately before the 4 h ischaemic insult, to determine whether that model exhibits the immediate phase of ischaemic PC. The same time course was used to determine whether adult cardiomyocytes could be pharmacologically preconditioned with Ucn. For these investigations, cardiac cells were exposed to normal HEPES based buffer supplemented

with Ucn (0,1 μ M) for 5 min, followed by 10 min recovery in normal serum free medium before the 4 h ischaemic period.

Referring to these results, it has been shown that evoked cardioprotection by both ischaemic PC and pharmacological PC (adenosine or urocortin induced) depends on the different experimental models and types of various methods, protocols.

Several investigators reported that single-agent pharmacological PC was effective as ischaemic PC^{110,111,112}, however other studies^{113,114} showed only marginal cardioprotection by single-pharmacological PC strategy and demand a combined-pharmacological PC technique in which multiple agents are used to develop optimal cardioprotection. The memory of cardioprotection conferred by preconditioning and the underlying mechanisms have been a subject of extensive research for many years. It has been suggested that the mitochondrial K_{ATP} channel and PKC create a positive-feedback loop during the memory phase of ischaemic PC¹¹⁵. Adenosine stimulates A1, A2, A3 receptors, which are coupled with G proteins¹¹⁶. G protein activation causes membrane translocation and phosphorylation of PKC in a Ca^{2+} dependent and Ca^{2+} independent manner by generating the lipid second messenger DAG¹¹⁷. The heterotrimeric G proteins can also activate PI3 kinase via the transactivation of receptor tyrosine kinases through the generation of reactive oxygen species^{118,119}. Thereafter it is clarified, that adenosine alone can activate mitochondrial K_{ATP} channels and the endothelial isoform of NO synthase through the activation of PKC and PI3 kinase^{120,121}.

Among the end effectors of PKC- ϵ , mitochondrial K_{ATP} channels, sarcolemmal K_{ATP} channels, and mitochondrial voltage-dependent anion channels are the targets of PKC- ϵ responsible for cardioprotection^{122,123,124}. It has been demonstrated that PI3 kinase confers cardioprotection by phosphorylating Akt, the substrate of PI3 kinase, which then modulates the phosphorylation status of several downstream kinases, such as glycogen synthase kinase-3 β (GSK 3 β)¹²⁵. In many studies it is suggested, that inactivation of glycogen synthase kinase-3 β by Akt-dependent phosphorylation plays a cardioprotective role by inhibiting mitochondrial permeability transition¹²⁶. Akt also phosphorylates apoptosis-regulatory molecules such as Bad, procaspase-9, and cAMP-responsive element-binding protein in certain cell types^{127,128,129}.

Known cellular actions of urocortin include increases in cAMP production, stimulation of secretion of atrial and brain natriuretic peptide, activation of the PI3 kinase and

activation of MEK1-p42/p44 MAPK pathway in neonatal cardiomyocytes^{130,131,132}. It was also analyzed, that global changes appear in gene expression in cardiac myocytes after Ucn treatment using gene chip technology¹³³. They report that Ucn specifically induces enhanced expression of the Kir 6.1 cardiac potassium channel subunit, which showed that the cardioprotective effect of Ucn both in isolated cardiac cells and in the intact heart is specifically blocked by both generalized and mitochondrial-specific K_{ATP} channel blockers.

In adult rat cardiac cell culture it was demonstrated, that the PKC inhibitor chelerythrine abrogated the decrease in cellular injury that was induced by Ucn during simulated ischaemia. Exposure of cardiomyocytes to the K_{ATP} channel blocker 5-hydroxydecanoate during ischaemia also abolished the cell protective effects of Ucn⁵¹.

In conclusion, we have clearly demonstrated a cardioprotective action of urocortin against ischaemia/reperfusion injury in neonatal cardiac cell culture. Using urocortin before ischaemic period as a preconditioning agent conferred cardioprotective memory against both cardiac myocyte apoptosis and necrosis. The cell safekeeping effects is likely to be attributed to the extended signaling pathways of Ucn by its receptors. Urocortin may thus have potential as a protective agent during planned episodes of myocardial ischaemia such as coronary artery bypass graft surgery, percutaneous coronary intervention or cardiac transplantation.

6. “PHARMACOLOGICAL” POSTCONDITIONING USING UROCORTIN

6.1. Introduction

6.1.1. Ischaemic postconditioning- relevant clinical approaches

Preconditioning has provided a powerful paradigm for the experimental analysis of the signaling pathways, both of cardiomyocyte cell death and protection from it¹³⁴. Nevertheless, PC does not decrease ultimate infarct size but delays the development of infarction so that with timely reperfusion, the actual necrotised region is reduced¹³⁵. However, the clinical exploitation of the PC phenomenon has been rather disappointing, because PC must be instituted briefly before an ischaemic event, which is not possible in daily life, and also because in cardiac surgery, where it would be feasible, alternative powerful protective interventions are already being used, such as opioids, inhalation anesthetics, cardioplegia.

In 1986, a study by Buckberg¹³⁶ entitled “Studies of controlled reperfusion after ischaemia” was reported the concept of attenuating ischemia/reperfusion injury by controlling the conditions (flow rate, temperature) and composition (pH level, metabolic substrates, calcium-reducing agents, anti-oxidants) of the reperfusate.

Reperfusion is necessary for salvage of myocardium from infarction. However, reperfusion itself is associated with numerous events that extend infarct size and other manifestations of injury beyond that observed equivalent periods of ischaemia alone. These events are jointly called “reperfusion injury”. Many of these events take place in the very early moments of reperfusion, and have been linked directly to cell injury and death. The mechanism of reperfusion injury relate to the excess formation of free radicals¹³⁷, to calcium overload, and finally to hypercontracture¹³⁸.

Postconditioning is defined as a series of brief interruptions of reperfusion applied at the onset of reperfusion²⁸. This new cardioprotective phenomenon results in a significant reduction in infarct size in the canine heart model of ischaemia/reperfusion. Subsequent studies have demonstrated that ischaemic postconditioning also reduces other markers of reperfusion-induced injury, such as apoptotic cell death, endothelial dysfunction and neutrophil accumulation. Recent studies revealed that postconditioning exerts its protective effects through the recruitment of prosurvival kinases such as PI3 kinase-protein kinaseB/Akt and the p42/p44 extracellular signal-regulated kinase1/2 (ERK 1/2)

pathways, which is also termed as reperfusion injury salvage kinase (RISK) pathway, at the time of reperfusion¹³⁹. Interestingly, in this study it was demonstrated that recruitment of this protective RISK pathway, at the time of reperfusion, contributes to the protection of both ischaemic PC and the newly described phenomenon of ischaemic PS.

It is not clear whether it is the reperfusion intervals or the ischaemic episodes that are important to the cardioprotection induced by postconditioning³². These additional periods of postconditioning re-occlusion following index ischaemia may alter the production of endogenous autacoids such as adenosine, bradykinin or opioids¹⁴⁰. In addition, Kin et al reported, that in a rat model of coronary artery occlusion-reperfusion, blockade of adenosine receptors five minutes before reperfusion reversed the infarct reduction observed with postconditioning¹⁴¹.

6.1.2. Urocortin as pharmacological agent using in early reperfusion

Brar and his colleagues demonstrated for the first time, that after prolonged episode of ischaemia, the presence of urocortin, at the time of reoxygenation, prevented cell death, by an antiapoptotic action (represented as a reduction in Annexin V and TUNEL staining)⁵². In this study cardiac myocytes were exposed to incubation of Ucn in normoxic environment at the point of reoxygenation after simulated ischaemia/reperfusion insult.

6.1.3. Aims

The aim of this part was to investigate the effect of short one-cycle Ucn treatment at the beginning of reoxygenation after simulated hypoxic insult on cultured neonatal cardiomyocytes. We also examined the efficiency of Ucn -using it as a possible postconditioning agent- compared with ischaemic PS after different long hypoxic stimuli.

6.2. Materials and methods

6.2.1. Cell culture model

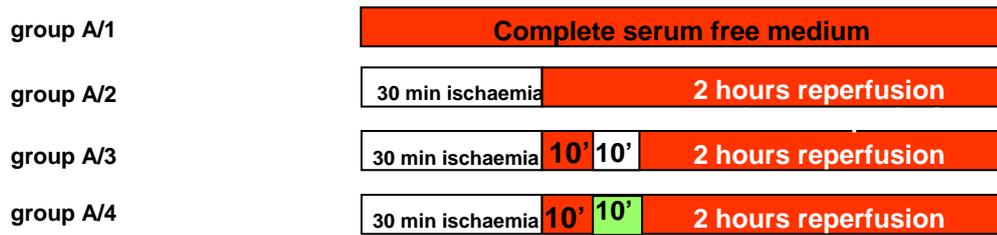
Cultured neonatal ventricular rat cardiomyocytes were transferred onto 12-well plates (BD Falcon) at the density of 200000 cells per/ml as described previously in part 3. In each well 5 ml cell solution was seeded. After 24 hours from plating, cell media was substituted with CSFM.

6.2.2. Postconditioning protocol

To investigate the efficiency of Ucn in the process of PS in comparison with ischaemic postconditioning stimuli isolated neonatal rat ventricular myocytes were subjected to the following protocols. In protocol A, control cardiac myocytes (group A/1) were incubated in normal cell culture medium. In ischaemic group (group A/2) cells underwent only 30 minutes ischaemia and 2 hours recovery in normal cell culture medium. In treated groups cells were exposed to 30 min ischaemia followed by ischaemic postconditioning (group A/3) or urocortin treatment for 10 min (group A/4) before the 2 hours recovery period.

In protocol B, control cells were under normal medium (group B/1). In ischaemic group (group B/2) cardiomyocytes were exposed to 60 min ischaemia and 2 h replacement in normal cell solution. In treated groups cells were exposed to 60 min ischaemia followed by ischaemic postconditioning stimulus (group B/3) or urocortin treatment for 10 min (group B/4) before the 2 h recovery interval. **(Figure 25.)**

Protocol A



Protocol B



10' = 10 min urocortin treatment **10'** = 10 min ischaemia **■** = reperfusion

Figure 25. Protocol of our groups by investigating cell protective effect of ischaemic and Ucn induced postconditioning protocols

6.2.3. Data analysis

Up to seven different cardiac myocyte preparations were studied. Data of the experiments are expressed as means±SE. Differences between the means were compared using Student's t-tests for significance. P<0.05 was considered to be statistically significant. For LDH determinations the enzyme levels (in IU/l) were correlated to the number of the cells studied.

6.3. Results

Urocortin protects myocardial cells from cellular injury in the process of postconditioning.

LDH production by neonatal rat cardiomyocytes exposed to 30 minutes ischaemia followed by 2 h reperfusion in normal serum free medium was $10,14 \pm 0,73$ IU/l (group A/2); whereas LDH level was significantly decreased by $3,57 \pm 0,64$ IU/l using 10 minutes ischaemic stimulus at the early reperfusion period (group A/3). A similar but nonsignificant trend was observed in Ucn postconditioned group (group A/4) ($8,28 \pm 1,47$ IU/l). In non treated control cardiomyocytes (group A/1) LDH level was $3,42 \pm 0,36$ IU/l. (**Figure 26.**)

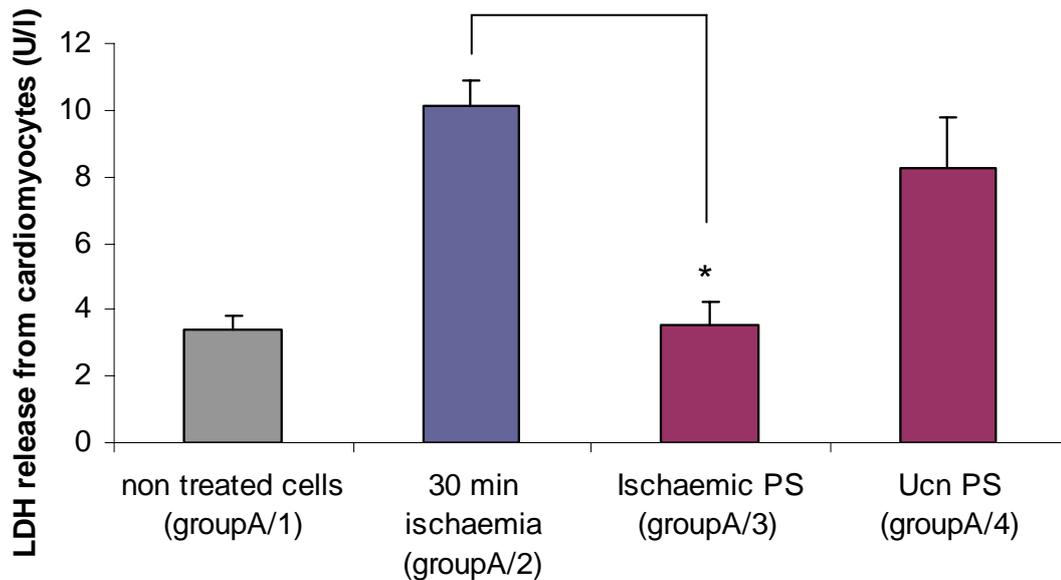


Figure 26. Lactate dehydrogenase level in cell culture medium in groups of experimental protocol A. After cardiomyocytes were exposed to 30 min ischaemia and 2 hours recovery, LDH production was significantly reduced only in the presence of ischaemic postconditioning stimulus (group A/2). Results are expressed as means \pm SE (* $P < 0.05$ vs. group A/2).

LDH levels were also reduced in both ischaemic (groupB/3) and by Ucn postconditioned samples (group B/4) after myocytes were exposed 60 minutes ischaemia by $6\pm 0,75$ and $3,42\pm 0,61$ IU/l compared with cardiac cells which were exposed 60 min ischaemia alone (group B/2) ($11,71\pm 0,68$ IU/l). In non treated cells (group B/1) LDH release was $3,42\pm 0,36$ IU/l. (**Figure 27.**)

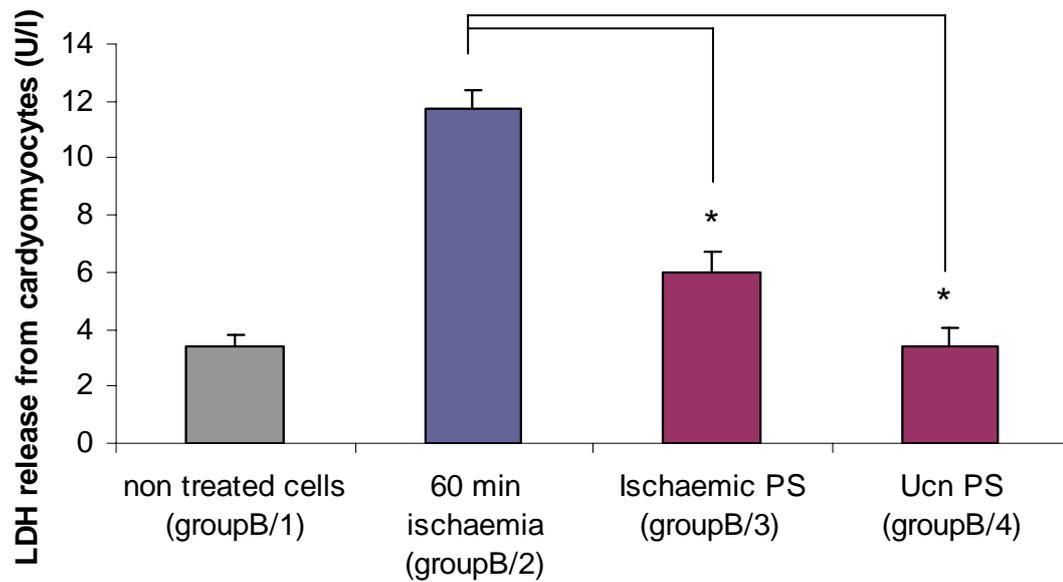


Figure 27. shows LDH levels after postconditioning in protocol B. Following 60 minutes ischaemia and 2 hour reperfusion, LDH release from cardiomyocytes was markedly decreased in both postconditioning groups versus non treated group B/2. Results are expressed as means \pm SE (* $P < 0.05$ vs. group B/2).

The **percentage of trypan blue positive cells** in non treated control cardiomyocytes (group A/1) was $36,16 \pm 2,78$ %. After 30 min ischaemia and 2 h reperfusion period in normal medium (group A/2) the percentage was $55,42 \pm 1,89$ %; while postconditioning with both ischaemia (group A/3) ($39,21 \pm 0,76$ %) and Ucn (group A/4) ($41,33 \pm 2,39$ %) caused a marked diminution in the number of trypan blue stained cells. **(Figure 28.)**

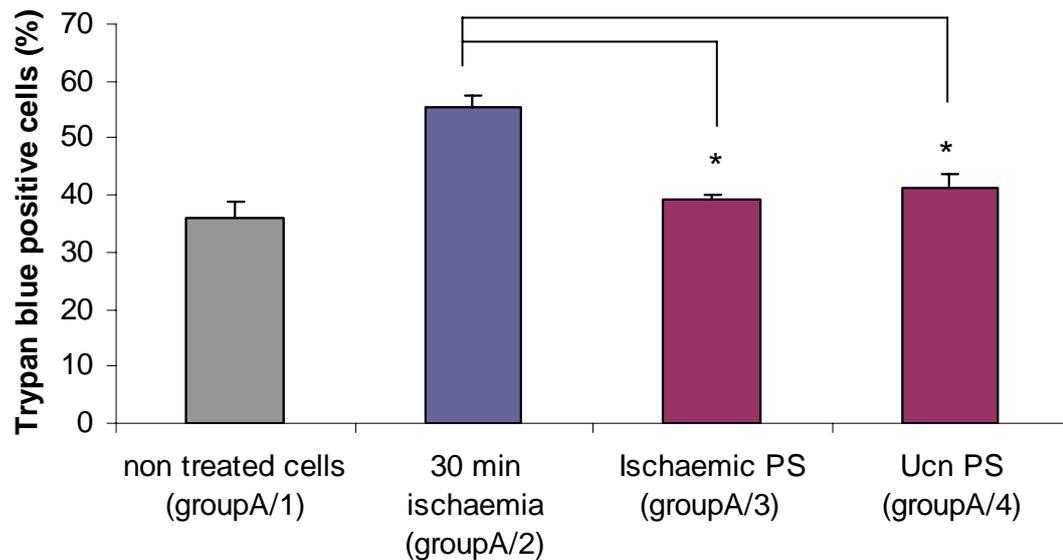


Figure 28. demonstrates the number of trypan blue positive dead cells in % in groups of postconditioning protocol A. Cell death was significant diminished in the presence of both ischaemic- and urocortin postconditioning stimuli compared to group A/2. Results are expressed as means \pm SE (* $P < 0.05$ vs. group A/2).

In non treated control group (group A/1) the **percentage of trypan blue positive cells** was $36,16 \pm 2,78$ %. Ischaemic postconditioning following 60 min ischaemic stress decreased the percentage of trypan blue positive cells by $42,7 \pm 1,93$ % (group B/3) compared with 60 min ischaemia alone (group B/2); using of Ucn as postconditioner agent decrease was more expressed: proportion of trypan blue positive cells was $36,02 \pm 1,99$ % (group B/4). (**Figure 29.**)

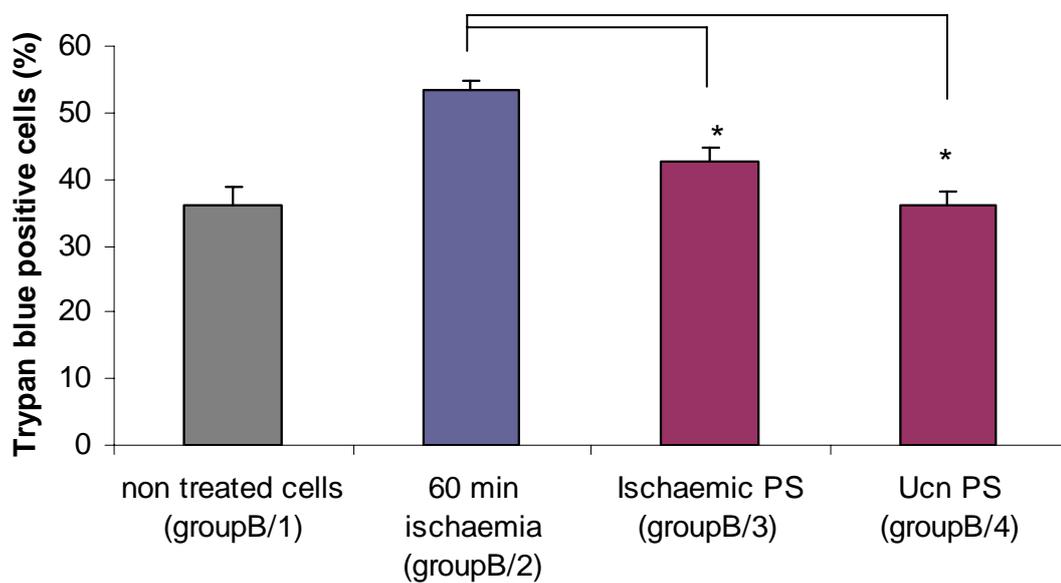


Figure 29. The number of dead cardiomyocytes stained by trypan blue in groups of experimental protocol B. Number of trypan blue positive dead cells was markedly reduced in group B/3 and B/4 versus “60 min ischaemia” (B/2) group. Results are expressed as means \pm SE (* $P < 0.05$ vs. B/2 group).

Extent of apoptosis (myocytes only stained with Annexin V-FITC) in non treated (group A/1) cardiac cells was $22,67 \pm 1,3$ %. After cardiac cells were exposed to 30 min ischaemia and 2 h recovery in normal serum free medium the amount of myocytes stained with AnnexinV-FITC (no PI) was $66,97 \pm 0,78$ % (group A/2), which was markedly reduced by $36,91 \pm 0,69$ % (group A/3) and $45,64 \pm 1,01$ % (group A/4) after the different postconditioning methods. **(Figure 30.)**

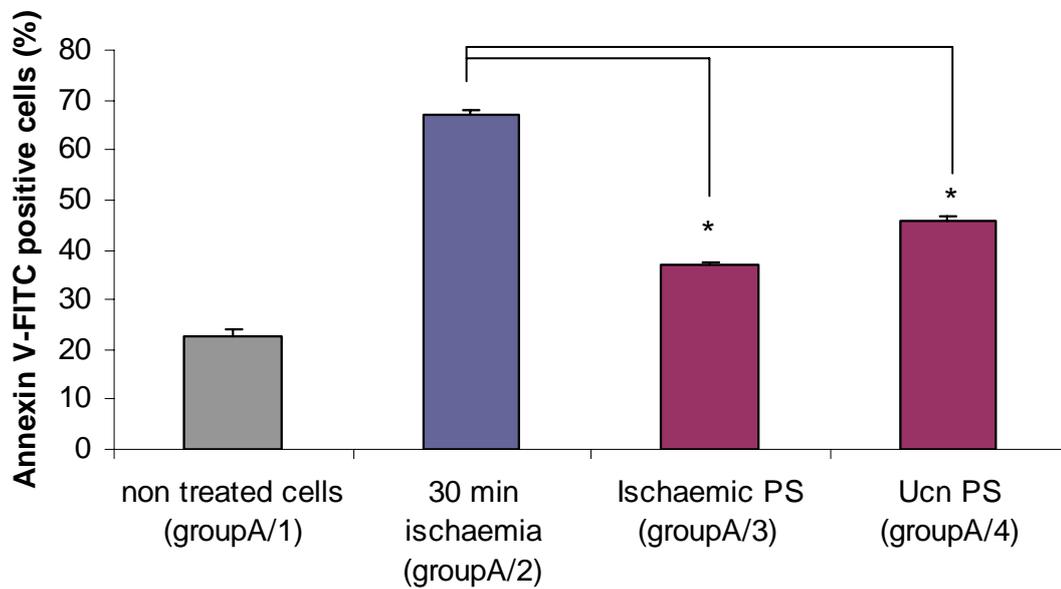


Figure 30. Level of apoptosis in protocol A. The rate of apoptosis was significantly decreased using different postconditioning protocols considering following 30 min ischaemia and 2 h reperfusion. Results are expressed as means \pm SE (* $P < 0.05$ vs. group A/2).

Extent of apoptosis (myocytes only stained with Annexin V-FITC) in non treated (group B/1) cardiac cells was $22,67 \pm 1,3$ %. As previously similar trend was noticed after 60 min long ischaemia: rate of Annexin V-FITC stained cells was decreased by $40,29 \pm 0,68$ % (group B/3) and to $34,87 \pm 0,76$ % (group B/4) in ischaemic and Ucn induced PS in comparison with cardiomyocytes exposed to 60 min ischaemia alone ($83,19 \pm 0,93$ %) (group B/2). (**Figure 31.**)

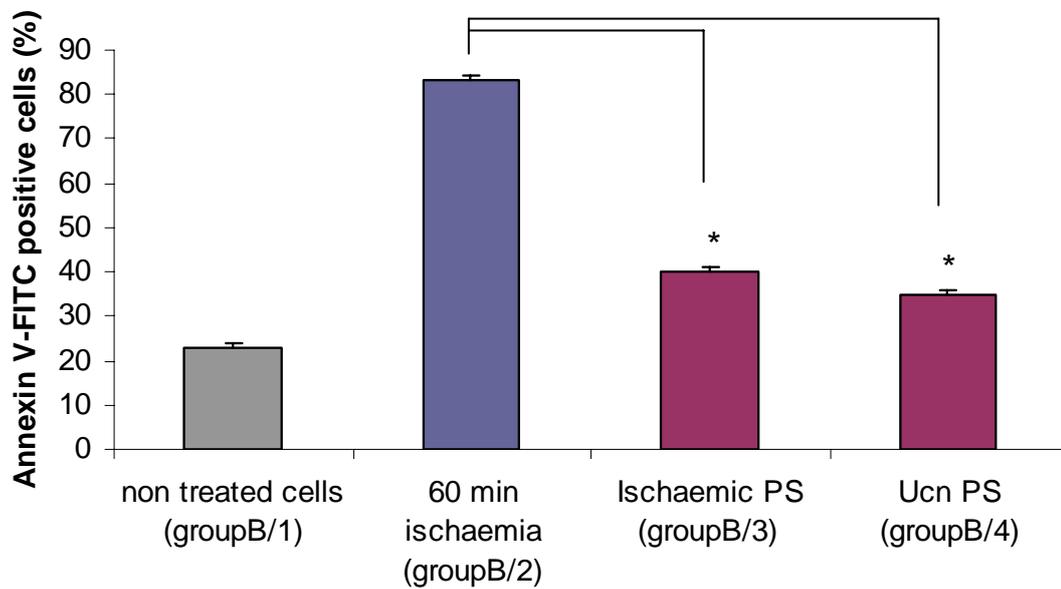


Figure 31. Extent of apoptosis in groups of postconditioning protocol B. The amount of Annexin V –FITC stained cardiac cells was markedly reduced using different postconditioning protocols considering following 30 min ischaemia and 2 h reperfusion Results are expressed as means \pm SE (* $P < 0.05$ vs. group B/2).

Moreover the **ratio of PI positive cardiomyocytes** which demonstrates the **extent of necrosis** was $5,17 \pm 0,54$ % in non treated control group (group A/1). After 30 min ischaemic stress the ratio was markedly reduced by $8,75 \pm 0,61$ % in ischaemic postconditioned group (group A/3) as opposed to the increased value of $11,44 \pm 0,54$ % in ischaemic group (group A/2). Following 30 min ischaemia cardioprotective actions of Ucn also tended to reduce the extent of necrosis, but the difference was not significant ($9,66 \pm 1,49$ %) (group A/4). (**Figure 32.**)

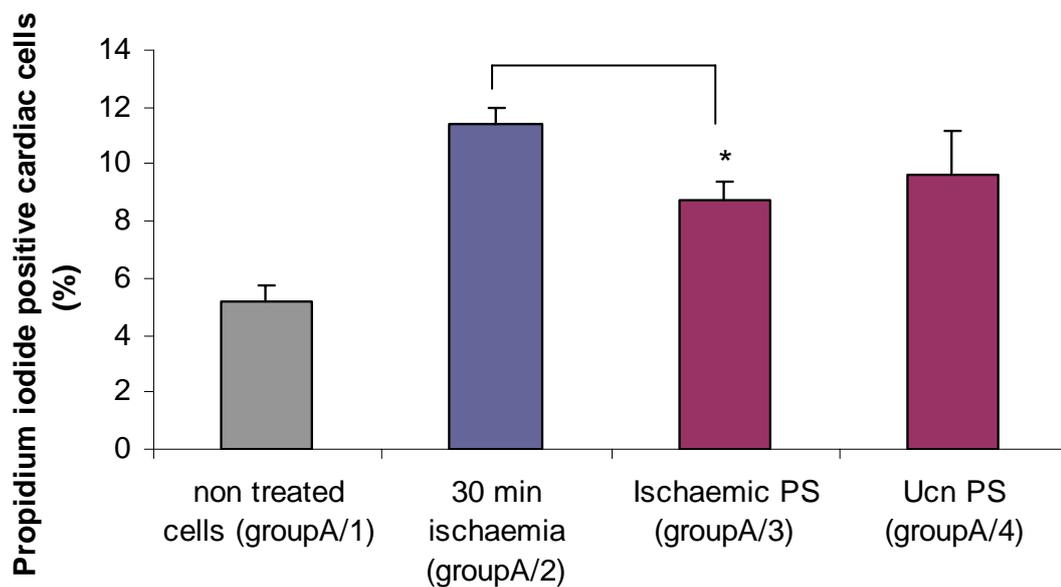


Figure 32. Extent of necrosis by counting propidium iodide positive cells in flow cytometer in groups of experimental protocol A. The amount of cardiomyocytes stained with propidium iodide was significantly decreased using ischaemic postconditioning protocol. Non significant decrease was to be seen in Ucn treated group. Results are expressed as means \pm SE (* $P < 0.05$ vs. group A/2).

Range of necrosis was $5,17 \pm 0,54$ % in non treated control group (group B/1). When cardiomyocytes were exposed to 60 min ischaemic stimulus, the Ucn induced PS was more effective against cell injury: percentage of PI positive cells was $6,29 \pm 0,53$ % in Ucn treated (group B/4) and $8,52 \pm 0,73$ % in ischemic postconditioned (B/3) group, while after 60 min ischaemia alone the rate of PI positive myocardial cells was $14,44 \pm 0,6$ % (group B/2). **(Figure 33.)**

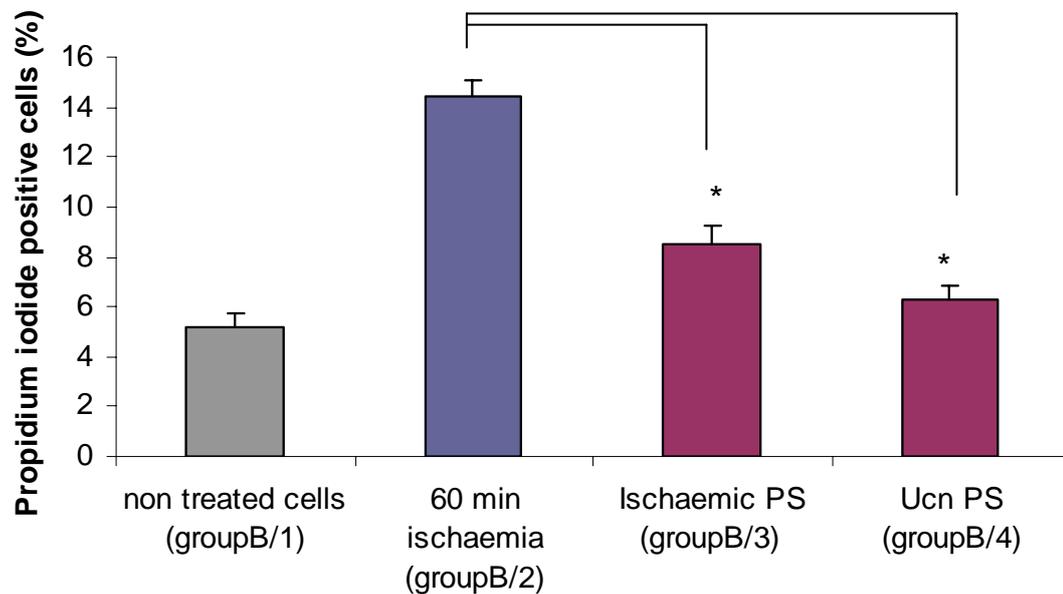
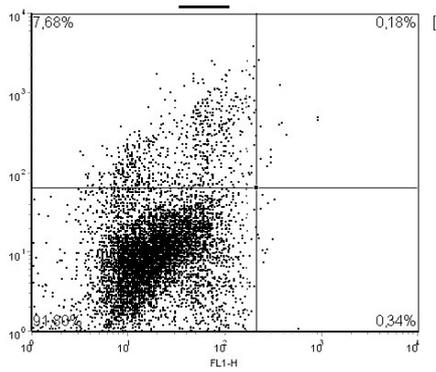
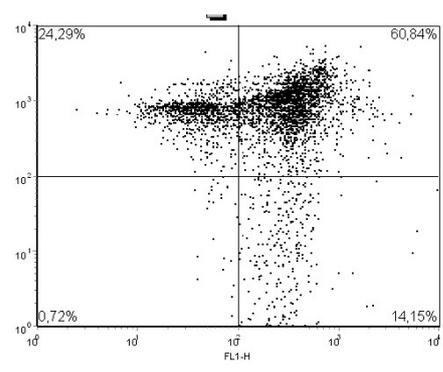


Figure 33. Extent of necrosis in groups of postconditioning protocol B. The rate of necrosis was significantly decreased using ischaemia and Ucn as postconditioning stimuli compared to non-postconditioned group. Results are expressed as means \pm SE (* $P < 0.05$ vs. group B/2).

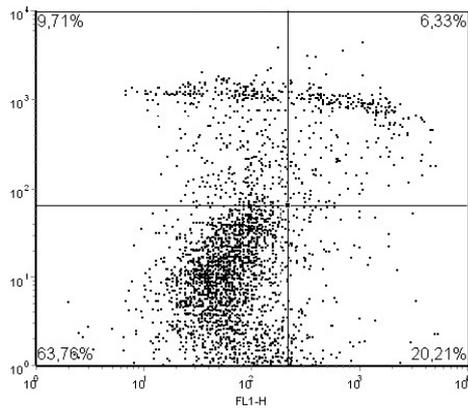
The next examples of dot-plots -determined by flow cytometry following Annexin V and propidium iodide double staining- demonstrates the rate of living, apoptotic and necrotic cardiac myocytes in the groups of protocol B. **(Figure 34.)**



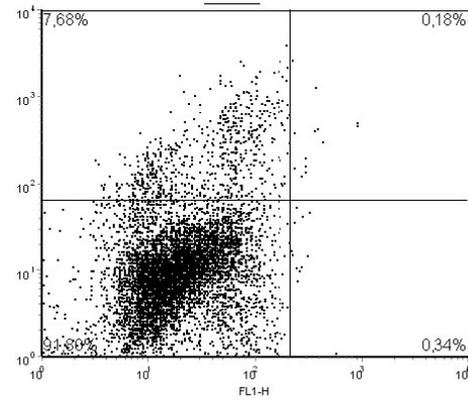
group B/1



group B/2



group B/3



group B/4

Figure 34. Horizontal axis represents Annexin V intensity and vertical axis shows propidium iodide staining. The lines divide each plot into four quadrants: lower left quadrant: living cells, lower right quadrant: early apoptotic cells, upper left quadrant necrotic cells, upper right quadrant: late apoptotic cells.

6.4. Discussion-after longer ischaemic period urocortin is more effective as ischaemic postconditioning

PC and PS have many common properties in respect to physiological effects¹⁴² and signal transduction pathways^{52,54}, but they have also relevant differences. The main difference is the impact of the timing of application: PC is able to adapt the myocardium's tolerance against ischaemia-reperfusion injuries which changes take place before or during ischaemia, while in PS the trigger stimulus must be active during early reperfusion.

We demonstrated for the first time the effectiveness of Ucn on isolated neonatal rat cardiomyocytes as a postconditioning factor compared with ischaemic PS. Ischaemic PS was originally described in an in vivo dog model¹⁴³, since then it has been observed in in vivo rabbit¹⁴⁴ and rat heart¹⁴⁵ and in rat myocardial cell culture¹⁴⁶. In isolated perfused rat heart was confirmed that ischaemic PS protects the myocardium against ischaemia-reperfusion injury by activating the PI3-kinase-Akt pathway¹⁴⁷, which is in close connection with the reperfusion injury salvage kinase (RISK)-pathway.

Urocortin, a peptide related to hypothalamic corticotrophin releasing factor, is released by myocytes in response to stressful stimuli such as ischaemia⁶⁷. Using also neonatal myocytes, Brar et al.⁵² demonstrated that after a prolonged episode of hypoxia, the presence of Ucn at the time of reoxygenation, prevented cell death, by an antiapoptotic action. In these studies, cardiomyocytes were exposed to lethal ischemic insult, and Ucn was added to the cardiac myocytes at the point of reoxygenation. In our experiments we used a non-lethal ischaemic injury, and after the Ucn treatment at the onset of recovery phase (reperfusion) we removed the peptide from cell culture medium, whereby it existed only nearby the cardiac cells for a short 10 min period. It was also shown, that the cardioprotection observed with giving Ucn at the point of reperfusion was mediated via the ERK 1/2 cascade. In addition, the infarct limiting effect associated with Ucn was abrogated in the presence of the MEK 1/2 inhibitor PD098059, and this effect was accompanied by a reduction in Ucn-induced ERK 1/2 phosphorylation^{148,52}. Furthermore, activation of the PI3 kinase- Akt cascade has also been implicated in Ucn-mediated protection⁵³. In rat myocardium, Ucn-mediated protection against hypoxia-reoxygenation injury was abrogated with the specific PI3 kinase inhibitors, wortmannin or LY294002.

Beyond urocortin, given of insulin, bradykinin, atorvastatin during the first few minutes of reperfusion also upregulates the RISK pathway¹⁴⁹, therefore, using of Ucn as a pharmacological postconditioning agent can be compared with ischaemic PS.

After 60 minutes hypoxic stress Ucn showed more expressed cardioprotective effect than after 30 minutes ischaemia and this defence was more powerful than ischaemic PS. However, it is still not clear which factors are responsible for that difference in the protective state, we confirmed that presumption that the possible involvement of activation of effectors and signal transduction pathways that are induced by G-protein coupled receptors make a pharmacological approach for attenuating reperfusion injury

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7. CONCLUSION

In our study we investigated the cardioprotective effect of a newly identified 40 amino-acids peptide, urocortin, affected also two endogenous adaptive mechanism of the myocardium, which could be useful in human against ischaemia-reperfusion induced cell injury.

PC is triggered by substances released from tissue during brief periods of ischaemia and reperfusion and the tissue need not even be myocardium as indicated by the cardioprotection realised through “remote preconditioning” involving, for example, tourniquet occlusions of the vascular beds of skeletal muscles or short occlusion and reflow of a mesenteric artery¹⁵¹. Specific receptors on the cardiac cell membrane, including adenosine A1 and A3, bradykinin B2, α 1-adrenergic, AT1 angiotensin II, muscarinic M2, and δ -opioid receptors, are stimulated by these substances¹⁵². The downstream signalling cascades from these receptors are complex and a detailed description is beyond the terms of reference for this review. However, it is clear that multiple protein kinases are involved, notably more isoforms of PKC^{153,154} and tyrosine kinases as Src, and PI3 kinase¹⁵⁵. There is a good evidence that multiple, redundant signaling pathways lead to cardioprotection. While it is relatively easy to block single cycle ischaemic PC by a variety of interventions, multi-cycle PC results in more potent protection, as it was demonstrated in vivo, which is more difficult to block¹⁵⁶. Thus, while PC is a threshold phenomenon, its protection is not as simple as all-or-non in the heart in vivo. Simultaneous pharmacological blockade by both PKC and tyrosine kinase activities have been demonstrated to be necessary to abolish the cardioprotection of multi-cycle ischaemic PC in rats¹⁵⁷ and single cycle PC in pigs¹⁵⁸. The involvement of these kinases suggests that the “memory” of classic ischaemic PC is embodied in the phosphorylation state of likely multiple end effector proteins which through the consequences of their level of activity achieve cardioprotection, specifically the prolongation of the sustained “test” ischaemic period which results in a specific amount of cardiomyocyte cell death.

Candidates for end effectors in ischaemic PC include channel proteins in the cardiomyocyte cell membrane, with the sarcolemmal K_{ATP} channel¹⁵⁹, Cl^- channels¹⁶⁰ including the CFTR channel¹⁶¹, the inward rectifier K^+ channel¹⁶² and connexin 43 related channels¹⁶³ as contenders. In the mitochondria, the mitochondrial K_{ATP} channel and the mPTP are candidates^{164,165}. Establishing the end effectors of ischaemic PC will

be essential to truly understand this fascinating phenomenon. It would seem intuitive that the study of isolated cardiomyocytes should be able to contribute valuable insights into the mechanistic understanding of PC, which cannot be achieved by a study limited to the whole heart, in vivo or in vitro.

Previous studies identified the survival kinases PI3 kinase-PKB/Akt and ERK 1/2 as key players in the protection afforded by PS in healthy myocardium¹⁶⁶. A study investigating pharmacological postconditioning by adenosine and bradykinin suggests that PKB/Akt is upstream of ERK 1/2 and that its downstream targets GSK3 β , eNOS, and p70S6K are markedly phosphorylated by PS not only in the healthy but also in the remodelled hearts¹⁶⁷.

Coronary artery disease is currently one of the leading causes of mortality and morbidity in the western world. The serious often fatal consequence of coronary artery disease is an acute myocardial infarction, which results from the acute occlusion of one of the major coronary arteries. Nowadays, it seems, that the best hope of limiting the size of the myocardial infarct is the timely restoration of coronary blood flow, by either thrombolysis or primary percutaneous coronary intervention. Despite the current reperfusion strategies, the mortality and morbidity associated with an acute coronary occlusion remains significant, necessitating the development of novel cardioprotective strategies, which can be used as adjunctive therapy to the current reperfusion strategies. Two of the most powerful mechanisms for protecting the myocardium against hypoxia are the ischaemic preconditioning, and postconditioning. However PC depends crucially on intervening before the ischaemic event, which is difficult, given the unpredictable onset of an acute coronary artery occlusion. More amenable approach to cardioprotection is to protect the myocardium after the acute coronary occlusion has occurred, as in the PS, where cardioprotective strategy can be applied at the time of reperfusion.

In this regard, the activation of prosurvival kinases, such as Akt and ERK 1/2, namely RISK pathway, at the time of reperfusion, has been demonstrated to confer powerful cardioprotection against myocardial ischaemia/reperfusion injury.

It is well certified that the cardioprotective phenomena of ischaemic preconditioning and the recently described postconditioning exert their cardioprotective effects through the recruitment of the RISK pathway, and that the protection in these settings is mediated through the inhibition of mPTP opening¹⁶⁸. Therefore, the pharmacologic

manipulation of this pathway at the time of reperfusion may enable one to harness the powerful cardioprotective benefits of both ischaemic PC and PS, and provide a novel approach to cardioprotection.

Ucn is suggested to be useful as adjuvant therapy for myocardial ischaemia or myocardial infarction. The possible therapeutic potential of Ucn is just beginning to be realised. It has potential uses in heart failure and acute heart infarction, both of which are prevalent disease in the western world. Heart failure is pathophysiological state that is characterised by haemodynamic features, neurohormonal activation and fluid retention. Current novel pharmacological therapies for heart failure include synthetic peptides that are analogous to BNP, which are clinically useful in acute decompensated heart failure. In animal models, Ucn has been shown to reverse the neurohormonal activation that occurs in heart failure, to improve haemodynamics associated with this disorder and to act as a positive inotrope, with additional diuretic and natriuretic effects¹⁴².

Ucn also acts as a coronary vasodilator. Many *in vivo* and *in vitro* models including our study, have demonstrated that Ucn protect cardiac myocytes from cell death induced by hypoxia. As Ucn acts on both CRF R1 and CRF R2, a more targeted approach to therapy might involve the use of specific CRF R2 receptor agonists, thus ensuring that the hypothalamic-pituitary axis is not stimulated.

Recently, two further members of the CRF peptide family were identified -Ucn II (stresscopin) and Ucn III (stresscopin related peptide)- due to their homology with Ucn^{169,170}. Ucn II and Ucn III exhibited more potent antinecrotic and antiapoptotic effects than Ucn in cardiomyocytes exposed to hypoxic/reperfusion injury¹⁷¹, although the vasodilator effects are reported to be less potent than that of Ucn in rat thoracic aorta¹⁷².

A potentially adverse effect of Ucn might be the deleterious effects of chronic elevation of cAMP on cardiac function. It is possible that chronic administration of Ucn might cause increased arrhythmogenesis and also have adverse effects on survival. This remains to be further evaluated, and whether the beneficial cardioprotection could be separated from this effect on cAMP requires other investigation.

As a trigger component of the RISK pathway, urocortin might also contribute to the powerful cardioprotective benefits of both ischaemic preconditioning and postconditioning, whereby providing a novel approach in heart protection.

8. NOVEL FINDINGS

Our result shows, that in a neonatal primary cardiomyocyte cell culture a short, 10 minutes long ischaemic preconditioning stimulus by using an ischaemic buffer solution was cardioprotective against ischaemia/reperfusion injury. Cardioprotection was to be demonstrated by measuring the release of necrosis enzyme lactate dehydrogenase from cardiomyocytes and by determining cell viability with trypan blue staining. The shorter (5 minutes) and longer (20 minutes) ischaemic insults did not result in such a significant protective effect as the optimal 10 minutes hypoxic one-cycle stimulus.

We have demonstrated that urocortin specific immunostaining appeared the most intensively in cardiac cell cytoplasm 2 hours after a short hypoxic stress insult. In the intact myocardium urocortin also exists in the cell cytoplasm; following ischaemic injury monitoring the expression of this peptide by a specific immunostaining on neonatal cardiac cell culture model resulted in only increased immunoreactivity 2 hours after ischaemic insult. Intensive staining was not to be seen neither 30, 60 minutes nor 18, 24 hours following the hypoxic event¹⁷⁴.

This is the first study to directly compare the beneficial effect of urocortin in neonatal rat myocardial cell culture with adenosine induced preconditioning and ischaemic preconditioning. The cardioprotective effect of Ucn was noticed when cardiomyocytes were exposed to Ucn supplemented medium for 10 min, which was followed by 3 h “test” ischaemia and 2 h of recovery in normal serum free medium. Cardioprotection was demonstrated by measuring necrosis enzyme release from cardiac cells, and by showing the antiapoptotic and antinecrotic effect of the different preconditioning stimuli¹⁷³.

Our results demonstrated for the first time the effectiveness of Ucn on isolated neonatal rat cardiomyocytes as a postconditioning factor compared with ischaemic PS. We investigated the cardioprotective effect of both ischaemic and Ucn induced postconditioning events after different long ischaemic insults. Following 60 minutes hypoxic stress Ucn showed more intensive cardioprotective effect than after 30 minutes ischaemia¹⁷³.

9. ACKNOWLEDGEMENTS

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