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

PhD Thesis

By

Barbara Cserepes MD

Head of the Doctoral School: Judit Nagy MD, PhD, Dsc.

Supervisor:Erzsébet Rőth MD, PhD, Dsc.

Consultant: Erzsébet Rőth MD, PhD, Dsc., Gábor Jancsó MD, PhD

University of Pécs, Faculty of Medicine
Department of Surgical Research and Techniques

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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.

ISCHAEMIC PRECONDITIONING

It was first described by Murry et al., 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. Ischaemic PC afford to the myocardium more resistance against infarction and reperfusion arrhythmias in all species tested so far, protect the postischaemic contractile dysfunction of stunned myocardium and also reduces the extent of apoptosis, thus it was seen to have considerable clinical relevance in treatment of patients with ischaemic heart disease, but the need for pretreatment has made it impractical in the clinical settings.
When was first described by Murry et al., 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. 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.

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 event. In contrast, a more promising approach to cardioprotection termed "ischaemic postconditioning (PS)" has been described by Vinten-Johansen et al. 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").
The mechanisms of postconditioning are divided into mechanical, cellular and molecular events. 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. Hence, postconditioning marshals a variety of endogenous mechanisms that operate at numerous levels and target a broad range of pathological mechanisms.

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. in 1995. CRF polypeptides play biologically different roles in the behavior and in response to stress mediating the action of the hypothalamic-pituitary-adrenal axis. 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, placenta, adrenal gland, gastrointestinal tract, ovaries, lung, lymphocytes and in the heart and vasculature. It has been shown, that Ucn immunoreactivity in the human failing heart is more intense than in the normal myocardium.
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 CRF. This effect was found to be obligated to a fall in total peripheral resistance, and other studies demonstrated a direct relaxant effect of Ucn on rat arterial segments. Ucn also produces dose-dependent and prolonged increase in heart rate and cardiac output, and recent studies in the isolated rat heart demonstrate Ucn produces direct positive inotropic effects as well as coronary vasodilation. 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 injury.
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} channels.

OVERALL AIM OF THE PROJECTS

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.

ISCHAEMIC PRECONDITIONING ON CULTURED CARDIOMYOCYTES
(First part of the study)

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 hypoxia.
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., which was similar as Simpson and Savion made in 1982 with some modification. In a pilot study Gordon et al. 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 cardiomyocytes. 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. Therefore we suspected that this new model should prove useful in unravelling the molecular alterations underlying I/R injury and myocardial apoptosis.

Materials and methods

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 previously. After decapitating the pups, we immersed briefly the carcasses in 70-96% ethanol to desinfect 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 $Cacl_2$- 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 glas 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 Cº with shaking.
After making the filtering units to 25 mm filter holder containing 100 μm nylon mesh attached on Falcon tube. 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), 100 IU/ml penicillin, 0,1 mg/ml streptomycin, 1,28% 200mM L-Glut supplemented with 10 % fetal bovine serum - 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.
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 °C) to reduce the number of contaminating non-muscle cells.

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 at the density of 200000 cells per/ml and incubated in cell incubator.
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; 1µM insulin; 5,64 µg/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.

Experimental protocol. To mimic the ischaemic stimuli and test ischaemia cell cultures were exposed to a previous described ischaemic buffer (simulated ischemia (SI) buffer) that contained 137 mM NaCl, 3.5 mM KCl, 0.88 mM $CaCL_2 \cdot 2H_2O$, 0.51 mM $MgSO_4 \cdot 7H_2O$, 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% CO_2, 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).

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 spectophotometry at 340 nm.

Cell viability. Cardiomyocytes were picked up by using 0.25% trypsin, 0.2 % EDTA solution at 37 ºC, 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 mm^2 area in Bürker hemocytometer. and cell viability was defined by comparing the number of live cardiomyocytes to total cell amount.

Results

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±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±3,2 IU/l. Preconditioning with 10 min long ischaemic insult (group 3) significantly decreased the LDH release from cardiomyocytes to 95±6,8 IU/l, while preconditioning with 20 min long ischaemic stimulus (group 4) resulted 103±7,5 IU/l LDH content in the cell culture medium.

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

Discussion

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 negligible. 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-contracture 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-workers. Our aim was to define an optimal ischaemic preconditioning protocol in this model using the ischaemic buffer solution of Gordon et al., 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.

Conclusion. Our results showed that preconditioning with ischamic 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.

UROCORTIN RELEASE DURING HYPOXIA IN CARDIOMYOCYTES
(Second part of the study)

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 injury. 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 vasodilation. In addition, exogenous Ucn may protect myocardial cells during ischaemia, as it increases the survival of cultured cardiomyocytes exposed to simulated ischaemia and also reduces the infarct area after ischaemia/reperfusion injury in rat heart.
Ucn mRNA is expressed in the isolated rat cardiac cell cultures, whereas CRF R2ß 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 R2ß mRNA expression is markedly depressed in left ventricular hypertrophy compared with normal left ventricle.
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 function. 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.

Materials and methods

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, cell solution was filtered with 100 μm nylon mesh in a medium consisting of DMEM/F-12, 100 IU/ml penicillin, 0,1 mg/ml streptomycin, 1,28% 200mM L-Glut supplemented with 10 % fetal bovine serum. 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% CO_2, 37 °C).
Than the suspension was transferred onto poly-D-lysine covered 8-well culture slides (BD Falcon 8-well culture slides). 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 2x 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.

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 (group6). Thereafter cell supernatants were removed from plates and cardiac cells were washed and fixed in paraformaldehyde solution for studying Ucn expression.

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 ºC. Then cells were fixed in buffered 4 % paraformaldehyde solution for at 37 ºC. 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; and fat free milk powder 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 ºC. 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 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.

Results

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 epression 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).

Discussion

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. 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 increased. These datas 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 81-82. Interestingly, the 22-kDa Ucn precursor protein was detected in rat cardiac myocytes by Western blot analysis. 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 40amino 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 precursor. There is another possible proteolytic processing site Arg-Arg at position 64-65 in the human Ucn precursor, 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 hypothised 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 it's 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.

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.

PRECONDITIONING WITH UROCORTIN ON NEONATAL CULTURED CARDIOMYOCYTES- DOES IT HAVE ANY EFFECTS? (Third part of the study)

Background

Many years ago, Murry and his collegues 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 size, impoved recovery of contractile function, reduced number of arrhythmias, and reduction in LDH level. However, clinical applications of ischaemic PC have been limited by ethical concerns.

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, the mechanism of this cardioprotection may occur via protein kinase C and activation of mitochondrial K_{ATP} channels, but there is no definitive evidence that during ischaemia A1 receptor activation is associated with either of these events. Recently, adenosine receptor agonists have been shown to activate p38 MAPK activity in rat myocardium and isolated ventricular myocytes.
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 cardiomocytes against simulated ischaemia. 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 tissue. In an other study it was demonstrated, that Ucn has immediate and delayed cardiopotective actions that mimic ischaemic preconditioning via PKC and K_{ATP} channels.

Methods

Cultured neonatal ventricular rat cardiomyocytes were transferred onto 12-well plates 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.

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 (10μM) in group 4 and Ucn (0,1μM, 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.

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. Propidium iodide (PI) is a standard flow cytometric viabily 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 apoptosis.
After cell solution was resuspend 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.

LDH level measurements. Following experimental protocol LDH enzyme activity was determined from snap frozen culture mediums by using spectrophotometry at 340 nm.
Cell death analysis. Cardiomyocytes were picked up with 0.25% trypsin, 0.2 % EDTA solution at 37 ºC, centrifugated 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 mm^2 area in Bürker hemocytometer and cell death was determined by trypan blue positive cells in %.

Results

Exposure of cell culture for 3 h simulated ischaemia followed by 2 h recovery in normal medium (ischaemic, group 2) markedly increased **LDH level** to 27,71±3,15 IU/l medium. When preconditioned the cells with Ucn (group 5), LDH level was reduced to 8,71±0,6 IU/l, which protective action was comparable with that observed with adenosine (group 4) (7,85±0,508 IU/l) or in ischaemic (group 3) (6,28±0,64 IU/l) preconditioned groups.
Preconditioning with adenosine (group 4), ischemia (group 3) and UCN (group 5) caused a marked decrease by 44,69±0,105%; 47,02±0,12%; and 45,69±0,87 % in the **number of trypan blue positive cells** compared with control cells (group 2) (57,057±1,209 %).
The number of myocytes stained with AnnexinV-FITC alone (no PI), which demonstrates the **extent of apoptosis** was 84,43±1,98 % in the ischamic group (group 2), which was significantly diminished by 40,81±0,99 % (group 4); 38,9±1,68 % (group 3); and 46,54±1,11 % (group 5) after the different preconditioning methods.
In addition the percentage of PI positive cardiomyocytes, which demonstrates the **degree of necrosis** was also markedly reduced by 24,5±3,33 % after the ischaemic PC (group 3); 23,34±1,99 % in adenosine preconditioned group (group 4), and 24,14±1,57 % in Ucn treated group(group 5) as opposed to the increased value of 40,27± % in the control samples(group 2).

Discussion

Although a number of in vitro models for the study of ischaemia/reperfusion have previously been described, 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 injury.
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 conclusion, we have 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.

"PHARMACOLOGICAL" POSTCONDITIONING USING UROCORTIN
(Fourth part of the study)

Introduction

Postconditioning is defined as a series of brief interruptions of reperfusion applied at the onset of reperfusion. 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 reperfusion. 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.
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). In this study cardiac myocytes were exposed to incubation of Ucn in normoxic environment at the point of reoxygenation after simulated ischaemia/reperfusion insult.

Materials and methods

Cell culture modell. Cultured neonatal ventricular rat cardiomyocytes were transferred onto 12-well plates 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.
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.

Results

LDH production by neonatal rat cardiomyocytes exposed to 30 minutes ischaemia followed by 2 h reperfusion in normal serum free medium was 10,14±0,73 IU/l (group A/2); whereas LDH level was significantly decreased by 3,57±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±1,47 IU/l). In non treated control cardiomyocytes (group A/1) LDH level was 3,42±0,36 IU/l.
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±0,75 and 3,42±0,61 IU/l compared with cardiac cells which were exposed 60 min ischaemia alone (group B/2) (11,71±0,68 IU/l). In non treated cells (group B/1)LDH release was 3,42±0,36 IU/l.
The **percentage of trypan blue positive cells** in non treated contol cardiomyocytes (group A/1) was 36,16±2,78 %. After 30 min ischaemia and 2 h reperfusion period in normal medium (group A/2) the percentage was 55,42±1,89 %; while postconditioning with both ischaemia (group A/3) (39,21±0,76 %) and Ucn (group A/4) (41,33±2,39 %) caused a marked diminution in the number of trypan blue stained cells.
In non treated contol group (group A/1) the **percentage of trypan blue positive cells** was 36,16±2,78 %. Ischaemic postconditioning following 60 min ischaemic stress decreased the percentage of trypan blue positive cells by 42,7±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±1,99 % (group B/4).
Extent of apoptosis (myocytes only stained with Annexin V-FITC) in non treated (group A/1) cardiac cells was 22, 67±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±0,78 % (group A/2), which was markedly reduced by 36,91±0,69 % (group A/3) and 45,64±1,01 % (group A/4) after the different postconditioning methods.
Extent of apoptosis (myocytes only stained with Annexin V-FITC) in non treated (group B/1) cardiac cells was 22, 67±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±0,68 % (group B/3) and to 34,87±0,76 % (group B/4) in ischaemic and Ucn induced PS in comparison with cardiomyocytes exposed to 60 min ischaemia alone (83,19±0,93 %) (group B/2).
Moreover the **ratio of PI positive cardiomyocytes** which demonstrates the **extent of necrosis** was 5,17±0,54 % in non treated control group (group A/1). After 30 min ischaemic stress the ratio was markedly reduced by 8,75±0,61 % in ischaemic postconditioned group (group A/3) as opposed to the increased value of 11,44±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±1,49 %) (group A/4).
Range of necrosis was 5,17±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±0,53 % in Ucn treated (group B/4) and 8,52±0,73 % in ischamic postconditioned (B/3) group, while after 60 min ischaemia alone the rate of PI positive myocardial cells was 14,44±0,6 % (group B/2).

Discussion

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 model, since then it has been observed in in vivo rabbit and rat heart and in rat myocardial cell culture. In isolated perfused rat heart was confirmed that ischaemic PS protects the myocardium against ischaemia-reperfusion injury by activating the PI3-kinase-Akt pathway, which is in close connection with the reperfusion injury salvage kinase (RISK)-pathway.

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. Beyond urocortin, given of insulin, bradykinin, atorvastatin during the first few minutes of reperfusion also upregulates the RISK pathway, 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.

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 determinating 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 event.

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 stimuli.

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 ischaemia.

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