THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph.D.)

Investigation of the cellular and molecular mechanisms of contractility in human ventricular cardiomyocytes

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1.INTRODUCTION

Cardiovascular disease (including ischemic heart disease, CHD) is the leading cause of premature death worldwide. Coronary heart disease (CHD) is responsible for more than a half of Hungarian premature death, having CHD as the major contributor. Ichemia results in myocardial injury and cell death via insufficient O_2 and glucose supply combined with accumulation of anaerobic metabolic end products. In addition, a reperfusion injury can be observed during re-canalization of the occluded artery, characterized by ATP depletion, and increased intracellular Ca^{2+} concentrations. Therefore, iscemia-reperfusion results in a decrease of myocardial contractility.

Many possible mechanisms have been proposed in the progression of cardiomyopathy. One of the most important is the increased oxidative and nitrosative stress related irreversible deterioration. Free radicals are highly reactive compounds, which are able to cause cytopathological alterations. They evoke alterations of cytoskeletal structure and affect the viability of the cells. Myocardial cell death is often related to the activation of poly(ADP-ribose)polymerase-1 (PARP-1). In general, PARP-1 acts as a molecular switch between apoptosis and necrosis. PARP-1 mediated apoptotic pathways are usually involves activation of proteases of caspase family.

The regulation of intracellular Ca^{2+} concentration is important in the intracellular signaling and contraction and also in the survival of the cells upon ischemia-reperfusion. Increased intracellular Ca^{2+} concentration can activate a series of deleterious effects, including disruption of mitochondrial membrane potential, uncoupling of electron transport in terminal oxidation, activation of apaptotic pathways (release of cytochrome c) and activation of protein kinase C (PKC) enzymes. Among these changes, PKC has a central role in regulation of proliferation and differentiation, in apaptosis, in intercellular signaling and in myocardial contractility. Interestingly a variety of effects are observed upon PKC activation. Activation of PKCδ upon ischemia increases myocardial contractile dysfunction, while activation of PKCε results in cardioprotection. Accordingly, there are controversial data regarding the role of PKC in the regulation of cardiac contractility, and only limited information is available about human physiology.

2. AIMS

Our aims were to investigate the following topics:

1. The role of protein kinase C in the modulation of contractility in human heart

What is the role of protein kinase C in cardiac ischaemia-reperfusion injury evoked contractile alterations?

2. Markers of myocardial damage in chronic heart failure

Are there significant synthesis of oxidative and nitrosative radicals in human heart failure?
What is the contribution of mithocondrial caspase-dependent and -independent cell death to the myocardial injury?

3. METHODS

3.1. Left Ventricular Tissue Samples

Healthy human hearts were obtained from 5 general organ donor patients whose hearts were explanted to obtain pulmonary and aortic valves for transplant surgery (donor hearts). The donors did not show any sign of cardiac abnormalities and did not receive any medication except plasma volume expanders, dobutamine, and furosemide. The causes of death included cerebral contusion due to accidents and cerebral hemorrhage or subarachnoid hemorrhage due to stroke. Failing hearts were obtained from 8 explanted end-stage failing hearts (NYHA class IV). Both the failing and the donor hearts were kept in cardioplegic solution until their arrival to the laboratory. A detailed summary of the pretransplant data and drug therapy is shown on. The experiments complied with the Helsinki Declaration of the World Medical Association and were approved by the Albert Szent-Györgyi Medical University Ethical Review Board (no. 51–57/1997. OEj). Left ventricular wall samples were obtained from the base. All biopsies were stored in cardioplegic solution and kept at 4 °C for approximatelly 6 to 8 h before being frozen in liquid nitrogen. Subsequently, the tissue samples were stored at –80 °C.

3.2. Markers of myocardial damage in chronic heart failure

Detection of Poly-ADP-Ribosylation, oxidized protein, PARP-1, caspase-9, and nitrotyrosine

To detect the carbonyl groups caused by oxidation of protein side-chains, an OxyBlot oxidized protein detection kit was used according to the manufacturer's instructions.

Myocardial proteins were separated by SDS-PAGE and blotted to nitrocellulose membranes. These membranes were probed with anti-PAR antibody, rabbit anti-PARP-1 antibody, rabbit anti-caspase-9 antibody, or rabbit anti-nitrotyrosine antibody. The immunoreactive proteins were visualized using an ECL detection reagent and X-ray autoradiographic film.

Immunohistochemical analysis of Poly(ADP-Ribose) (PAR) and apoptosis inducing factor (AIF)

Human ventricular tissue samples were frozen in Tissue-Tek OCT compound. Sections (5 μm thick) were made in a cryostat and were placed on adhesive slides and fixed in acetone for 10 min. Endogenous peroxidase activity was suppressed by treating the slides with 0.3% H_2O_2 in methanol for 30 min. Primary antibody against PAR polymers (1:100) and AIF (1:100) were applied.

3.3. The role of protein kinase C in modulation of contractility in the human heart

Reconstitution of human cardiomyocytes

Human cardiomyocytes were reconstituted by adding the cytosol to the demembranated myocytes at a dilution of ~1 mg myocyte protein/ml. In case of stimulation, reconstituted cardiomyocytes were incubated in the presence or absence of PMA (10 μmol/L), Ca^{2+} and PKC inhibitors GF 109207X and Gö 6976 (10 μmol/L) for 10 or 30 min (physiological and biochemical assays, respectively).

In vitro phosphorylation

The ability of PKA and PKC isoforms to phosphorylate myofibrillar regulatory proteins was tested by *in vitro* phosphorylation experiments. Human ventricular heart muscle homogenates were incubated with recombinant protein kinase C (PKC) α, γ, δ, ε and η in the presence of [γ-^{32}P]ATP. In parallel to the phosphorylation of myocardial proteins kinase activities were measured using 1 mg/ml histone IIIS substrate.

Western immunoblot

PKC α, δ and ε protein expression was determined from human ventricular heart muscle homogenates. Protein concentrations were determined by BCA assay using BSA as standard and the concentration of the homogenates were adjusted to 4 mg/ml. 50 μg proteins was loaded onto 10% SDS polyacrylamide gels and after separation transferred to nitrocellulose membranes. Membranes were probed with antibodies against PKCα (1:5 000)

PKCδ and PKCε (both dilution 1:1 000) and the signal was detected by a peroxidase conjugated anti-rabbit IgG specific antibody (1:50 000). Recombinant human PKC isozymes were loaded on the same gels/membranes as control proteins. The bands were visualized by ECL and evaluated by Image J software. Only bands on the same membrane were used to avoid alterations due to different transfer efficiency or other technical reasons.

Investigation of the translocation of PKC isozymes

Experiments were performed to investigate the translocation of endogenous PKCα, β1, δ and ε from the cytosol to the contractile protein machinery. Demembranated myocytes were incubated with cytosol (1 mg/ml concentration, 450 µl/tube) in the presence or absence of Ca^{2+} (5 mmol/L) and PMA (10 µmol/L) for 30 min. Then the myocytes were washed three times with isolation solution (centrifugation by 1 000 *g*, 2 min, supernatants were discarded) containing the same concentration of Ca^{2+} and PMA, as it was used during the incubations to avoid dissociation of the translocated proteins. After the last washing, supernatants were carefully removed and the myocytes (pellet) were boiled with 60 µl of 2X SDS sample buffer for 5 min. 30 µl of this solution was subjected to western blot analysis with antibodies against PKCα, β1, δ and ε. Bands were visualized by peroxydase conjugated secondary antibodies and ECL reaction. Intensities of the bands were quantitated by ImageJ, and results were expressed as optical densities (AU).

Determination of free Ca^{2+} concentrations in the presence of cytosol fraction

Demembranated cardiomyocytes were incubated with the cytosol and 0, 0.001, 0.01, 0.1, 1, 5 or 10 mmol/L Ca^{2+} was added to the supernatant to estimate the Ca^{2+}-dependency of PKCα association. The free Ca^{2+} concentrations were determined using a Fluo-3 calibration curve, measured by a BMG NOVOstar fluorescent plate reader.

Gel overlay assay for PKCα-binding proteins in demembranated myocytes

The interactions of PKCα and its putative anchoring proteins were further explored by overlay assays. Proteins from isolated and demembranated cardiomyocytes were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with 2µg/ml purified, recombinant PKCα in the presence of 10 µmol/L phorbol myristate acetate (PMA) and 5 mmol/L Ca^{2+} in 1% milk powder containing TBS for 2 h, followed by three rinses with TBS, supplemented with 5 mmol/L Ca^{2+}. Membranes were then

processed for Western blotting with an anti PKCα antibody (1:20,000) as described above, except that all of the solutions were supplemented with 5mmol/L Ca^{2+}. Controls were incubated with 1 % milk powder containing TBS and 5 mmol/L Ca^{2+} without the recombinant PKCα.

In vitro binding of recombinant human PKCα to recombinant cardiac TnI or troponin complex

In vitro binding assays, using purified, recombinant human TnI and purified recombinant PKCα verified the Ca^{2+}-dependent interaction between these two molecules. Recombinant, purified PKCα (0.1μg) was incubated with recombinant, purified TnI (0.1 μg/ml TnI) or reconstituted troponin complex (0.3 μg/ml, 1:1:1 stochiometry of TnI, TnC and TnT) in the presence or absence of Ca^{2+} (5 mmol/L) for 2 hours at room temperature. TnI was precipitated with an anti-TnI antibody (clone 16A11) or with mouse IgG (control), then pulled down with a protein A sepharose CL-4B resin. The precipitates were washed 5 times with TBS containing 0.1% Triton-X-100 and the respective concentration of Ca^{2+}. Precipitates were analyzed by Western immunoblot for PKCα as detailed above.

Immunohistochemistry

Aceton fixed consecutive sections (10 μm thick) were made from donor and failing hearts. These were immunolabeled with monoclonal antibody against PKCα (1:100) and TnI (1:100).

Measurement of physiological properties

Demembranated single cardiomyocytes were mounted between two thin needles with silicone adhesive while viewed under an inverted microscope. One needle was attached to a force transducer element and the other to an electromagnetic motor. The measurements were performed at 15 °C and the average sarcomere length was adjusted to 2.2 μm as described previously. The pCa (-log[Ca^{2+}]) values of the relaxing and activating solutions (pH 7.2) were 9 and 4.75, respectively. Solutions with intermediate free Ca^{2+} levels were obtained by mixing activating and relaxing solutions. Isometric force was measured after the preparation had been transferred from the relaxing solution to a Ca^{2+}-containing solution. When a steady force level was reached, the length of the myocyte was reduced by 20% within 2 ms and then quickly restretched (release-restretch maneuver). As a result, the force first dropped from the peak isometric level to zero (difference = total peak isometric force) and then started to redevelop.

The passive force component was determined in relaxing solution following the Ca^{2+} contractures. Ca^{2+}-activated isometric force was calculated as the difference of passive and maximal active isometric force. After the first maximal activation at pCa 4.75, resting sarcomere length was readjusted to 2.2 μm, if necessary. Then cells were subsequently exposed to a series of solutions with intermediate pCa to construct a force-pCa relationship. After obtaining the first (control) force-pCa the myocytes were incubated in the presence of a Ca^{2+} concentration which evoked ~50% of maximal force for 10 minutes and then the functional parameters (force-pCa relationship and passive tension) were measured again to reveal the effects of the Ca^{2+} stimulation. Ca^{2+}-force relations were fitted to a modified Hill equation: $F=F0[Ca^{2+}]nHill/(pCa_{50}nHill + [Ca^{2+}]nHill)$ where F is the steady-state force, Fo is the steady isometric force at saturating Ca^{2+} concentration, the Hill coefficient nHill is a measure of the steepness of the relationship, and pCa_{50} is the midpoint of the relation. Values are given as means±S.E.M. for n myocytes. Differences were tested by means of Student's paired t-test comparing the values before and after treatments. The level of significance was $P<0.05$.

4. RESULTS

4.1. Markers of myocardial damage in chronic heart failure

Effects of oxidative stress-induced myocardial damage

Overall, we set out to investigate some of the pathological events occurring during human heart failure that lead to apoptosis of the ventricular myocytes, and therefore, to the progression of disease. To determine the levels of oxidized proteins in donor and failing heart samples, tissues were screened for carbonyl adducts. Compared with donor heart samples, strong immunoreactivity for oxidized protein end-products was readily detected in failing hearts. As assessed by semiquantitative densitometry, the level of carbonyl adducts was increased approximately 3-fold.

The higher production of superoxide and NO leads to the formation of peroxynitrite, which reacts with protein tyrosine residues to generate nitrotyrosine. Earlier, it was found that myocardial proteins (such as alfa-actinin) could be nitrated by peroxynitrite treatment, *in vitro*, with a consequent decrease in the contractile force. Via the same approach, failing and donor hearts were tested for nitrated proteins: no difference was found. It should be noted that in vitro peroxynitrite treatment resulted in a robust signal, indicating that the experimental system was sensitive to protein tyrosine nitration. Additionally, no significant nitrotyrosine

staining was found in donor or failing hearts, confirming the absence of nitrotyrosine-producing (nitrosative) stress.

Oxidative stress-induced effects of mitochondria and PARP-1 network

Oxidative stress is associated with the activation of PARP in many cells and tissues. To determine the level of PARP activation, donor and failing heart samples were screened for poly-ADP ribosylated proteins. As assessed by semiquantitative densitometry, the level of poly-ADP ribosylated proteins was increased 2.3-fold in the failing hearts compared with the donor hearts. Investigation of the subcellular pattern of poly-ADP ribosylated proteins demonstrated an obvious nuclear localization in the failing hearts. Relative to the donor hearts, the PAR-positive nuclei were 3.1-fold more abundant. The nominal difference between the western immunoblot technique (a 2.3-fold elevation in poly-ADP ribosylated proteins) and the immunohistochemical data (a 3.1-fold increase in PAR-positive nuclei) may suggest a higher sensitivity of the latter approach.

Activation of PARP-1 often results in AIF-mediated apoptosis. To test the initiation of the apoptotic program, the localization of AIF was also tested; no translocation (indicative of AIF activation) was found in donor or failing hearts.

An elevation in poly-ADP ribosylation may be due either to a higher expression of PARP-1 or to catalytic activation of the enzyme. PARP-1 expression was tested in the donor and in the failing heart samples with a PARP-1–specific antibody, and no significant difference was found. Interestingly, a high level of degradation of PARP-1 was detected in both failing and donor hearts. This is in accordance with the finding that PARP-1 activation is often followed by the poly-ADP ribosylation of PARP-1 and by proteolytic degradation and inactivation. It is not clear what is the relevance of the variability in expression or in degradation of PARP-1 in the donor samples. It is possible that variability in the donor sample pool or differences in the collection of the samples may contribute to the diversity in both donor and failing hearts.

Caspases are well-known proteases capable of PARP cleavage and the promotion of apoptosis. The activation of one of the upstream caspases (caspase-9) was analyzed in our samples. Procaspase-9 (not cleaved, inactive caspase-9) expression was approximately 3-fold lower in the donor hearts than in the failing hearts, as determined by densitometry of caspase-9–specific bands using Western immunoblot.

4.2. The role of protein kinase C in modulation of contractility in the human heart

Effects of endogenous protein kinase C on the Ca^{2+} activated contractile force of human ventricular cardiomyocytes

Incubation of demembranated human cardiac myocytes with a Ca^{2+} concentration evoking about half maximal force production (pCa 5.8, active force during the incubation: 62±1 % of the maximum) for 10 min resulted in a 37±5 % reduction in the maximal Ca^{2+}-activated (active) force determined at pCa 4.75. In contrast, demembranated cardiac myocytes stimulated with Ca^{2+} (active force during the incubations: 56±8 % of the maximum) in the presence of the cytosol were completely protected from the decrease in the contractile force (2±3 % increase in the maximal active force). In parallel, the structural effects of the maintained contractions were also recorded as a means of light microscopy. Apparently, the maintained contractions evoked some deterioration of the cross striation pattern of the myocytes, both in the presence and in the absence of cytosol. Next, the possible contribution of cytosolic proteins (probably with Ca^{2+}-regulated activity) was investigated. From the many candidates, our attention has been attracted to the protein kinase C (PKC) due to its ability (i) to regulate contractility, (ii) to translocate from the soluble fractions to the particulate fractions, and (iii) to be activated by Ca^{2+} (classical PKC isoforms). The administration of the PKC activator PMA (10 min application) apparently affected neither the contractility (maximal active force: 96±1 % of the maximal active force obtained before the treatment), nor the cytosol mediated protection upon prolonged Ca^{2+} contractions (maximal active force: 98±1 % of the maximal active force obtained before the treatment). In contrast, inhibition of PKC by GF 109203X and by Gö 6976 (10 μmol/L) partially antagonized the cytosol mediated protection (a decrease of active force by 15±5 %, $p<0.05$, and by 9±2 %, $p<0.05$). In addition, control experiments with GF 109203X showed no effects on the active force in the absence of Ca^{2+} incubations (2±3 %increase in the maximal active force).

Effects of endogenous protein kinase C on the passive tension of human ventricular cardiomyocytes

Isolated cardiac myocytes possess a Ca^{2+} independent passive tension (force) at a sarcomere length of 2.2 μm. In contrast to the Ca^{2+} dependent (active) force, the passive force of the myocytes was elevated (passive force: 302±46%, $p<0.05$) after prolonged Ca^{2+} contractions. The addition of cytosol significantly antagonized this elevation (passive force: 146±9 %, $p<0.05$). As the matter of the contribution of PKC to these changes, PKC activation

by PMA or inhibition by GF 109203X and by Gö 6976 was without effect on this parameter both in the absence and in the presence of Ca^{2+}.

Effects of endogenous protein kinase C on the Ca^{2+} sensitivity of force development in human ventricular cardiomyocytes

There was no apparent relationship between PKC activation (PMA) or inhibition (GF 109203X and Gö 6976) and the pCa50 or Hill coefficient. In general, pCa50 values tended to be higher after the treatments, while the Hill coefficients were not affected.

Expression of protein kinase C isozymes in human ventricular cardiomyocytes

Expression levels of three PKC isoforms (α, δ and ε) were quantified in human left ventricular tissue samples. These assays suggested that PKCα is an abundant isoform in the human heart with an expression level about 20 times higher than those for PKCδ or PKCε (expression was 189±31, 7±3 and 7±2 ng/mg protein for PKCα, PKCδ and PKCε).

Phosphorylation of human ventricular proteins by protein kinase C isozymes

The ability of PKC isoforms to phosphorylate myofibrillar regulatory proteins was checked by *in vitro* phosphorylation experiments. The Ca^{2+} dependent PKCα and γ showed similar activity (4.3±0.9 and 4.4±2.1 pmol/min, respectively) and substrate specificity, while the Ca^{2+} independent isoforms produced a rather unique pattern. PKCδ possessed the highest activity (6.6±3.3 pmol/min) and selectively phosphorylated a protein with a molecular mass of26 kDa. PKCε showed the lowest overall activity (1.6±0.3 pmol/min) and seemed to be specific to phosphorylate a protein with a molecular mass of 60 kDa. PKCη (activity: 2.7±0.8 pmol/min) selectively phosphorylated two proteins with molecular masses of >200 kDa and 48 kDa. Overall, the expression and *in vitro* phosphorylation data suggest the dominance of PKCα over PKCδ and ε in the human heart (calculated relative activities (expression multiplied by the activity) are: 813, 46 and 11, respectively).

Intracellular targeting of protein kinase Cα in the human ventricular cardiomyocytes

As a matter of *in vivo* regulation, experiments were performed to investigate the possible translocation of endogenous PKCα, β1, δ and ε from the cytosol to the contractile protein machinery. In accordance with a predominantly cytosolic localization under unstimulated conditions, only a low level of association of PKC isozymes were found in the absence of Ca^{2+}. Importantly, in the presence of Ca^{2+} the level of PKCα bound to the

contractile system was selectively increased. Interestingly, the widely employed PKC activator PMA alone was without significant effects on the interaction between PKC isozymes and myofilaments, although it evoked significant translocation when applied together with Ca^{2+} (PKCα and ε), suggesting a decisive role for Ca2+ in the regulation of translocation. When the Ca^{2+}-dependency of PKCα translocation was assayed, an EC_{50}=645 nmol/L for the free [Ca^{2+}] was obtained.

Ca^{2+} regulated interaction of human cardiac TnI and protein kinase Cα

The interactions of PKCα and its putative anchoring proteins were further explored by overlay assays. Five potential PKCα binding proteins were found in the myofibrillar system of human cardiomyocytes. Moreover, one of the most prominent bands co-migrated with the thin filament regulatory protein TnI. *In vitro* binding assays, using purified, recombinant human TnI and purified recombinant PKCα verified the existence of the Ca^{2+}-dependent interaction between these two molecules. In the cardiac myocytes, TnI functions as a member of the troponin complex, consisting of TnT, TnC and TnI in equimolar concentrations. Hence, the association of PKCα to TnI may be influenced by other constituents of the troponin complex. *In vitro* reconstitution of the troponin complex, however, did not affect the Ca^{2+}-dependent interaction between TnI and PKCα.

Colocalization of human cardiac TnI and protein kinase Cα in the human ventricle

In accordance with the biochemical data, we found a significant colocalization of PKCα with TnI in human ventricular tissue samples, although the majority of PKCα was expressed in the cytosol, consistent with its cytosolic location under low Ca^{2+} conditions.

5. DISCUSSION

5.1. Markers of myocardial damage in chronic heart failure

Experimental and clinical studies have demonstrated the increased production of reactive oxygen species (ROS) (superoxide, H_2O_2, and hydroxyl radical) in the pathogenesis of acute and chronic heart failure. Plasma malondialdehyde-like activity, a marker of lipid peroxidation, is increased in patients with ischemic and nonischemic dilated cardiomyopathy; it correlates with the severity of the symptoms, and exhibits an inverse relationship with the ejection fraction and the exercise capacity. The pericardial concentration of 8-iso-PGF_2 (a marker for ROS production) correlates closely with the end-systolic and end-diastolic diameters of the left ventricle and with the functional severity of heart failure. Furthermore,

there is a significant positive correlation between myocardial ROS production and left ventricular contractile dysfunction in experimental models.

Myocardial ROS generation is triggered by repetitive episodes of ischemia and reperfusion, by inflammatory cytokines, by catecholamine auto-oxidation, and by prostaglandin biosynthesis. Impaired antioxidant defense mechanisms (superoxide dismutase, catalase, and glutathione peroxidase) or reduced concentrations of endogenous antioxidants (vitamin E, ascorbic acid, and cysteine) can contribute to an increased ROS production within the myocardium. Sources of ROS in the failing myocardium include xanthine oxidoreductases, cyclooxygenases, the mitochondrial electron transport chain (complex I), activated neutrophils, NOS, and the auto-oxidation of certain tissue metabolites and NAD(P)H oxidoreductases. The current results are consistent with these findings and demonstrate the presence of significant oxidative stress in failing human heart samples, whereas the control donor hearts displayed low levels of oxidative stress. The latter finding is consistent with the view that the collection of the hearts and the storage and the processing of the samples during our procedures do not trigger a significant degree of oxidant generation.

The cardiomyocytes, the endocardial endothelium, the coronary endothelium, and the cardiac nerves are sites of NO production by Ca^{2+}-dependent NOS. NO serves a number of important physiological roles in the regulation of cardiac function, including coronary vasodilation, inhibition of platelet and neutrophil adhesion and activation, modulation of the cardiac contractile function, and inhibition of cardiac oxygen consumption. Although NO is essential in cardiac physiology, at higher levels, or in the presence of reactive oxygen species, NO can also exert cytotoxic effects. Many of the toxic actions of NO are not due to NO directly, but are mediated via the production of the highly reactive oxidant peroxynitrite, the reaction product of NO and superoxide.

Animal models of heart failure have furnished ample evidence of both the overproduction of NO and the generation of peroxynitrite (at least as evidenced by its footprint, nitrotyrosine). NO is overproduced in the failing myocardium as a consequence of the increased expression and activity of iNOS. There is a correlation between the chronic overexpression of iNOS and peroxynitrite generation and cardiac enlargement, conduction defects, sudden cardiac death, and less commonly, heart failure in mice. Myocardial iNOS is induced in rats with volume-overload heart failure, and increased iNOS activity leads to a loss of myocardial contractility and β-adrenergic hyporesponsiveness. Neuronal NOS-derived NO production has also been demonstrated in the failing human heart. Although peroxynitrite generation has been reported in various forms of acute and chronic heart failure in many

animal models and in some human studies, we failed to detect differences in tyrosine nitration in the failing and donor hearts, even though the evidence of massive tyrosine nitration in the positive controls demonstrates that the assay used was adequate. It is possible, however, that low, subthreshold levels of nitrotyrosine are present in our samples, and it is also conceivable that peroxynitrite formation and tyrosine nitration follow a particular time course and our samples were taken from patients where tyrosine nitration was no longer present.

Three particular pathways initiated by ROS and reactive nitrogen species in the failing heart are related to the activation of PARP: activation of caspases, activation of MMPs, and DNA strand breaks. As far as PARP activation is concerned, it has been demonstrated in ischemia-, banding-, diabetes-, and cardiotoxic drug–induced heart failure in murine studies. There is multiple evidence of the activation of caspases, and this issue has been widely investigated and debated in light of the relevance or nonrelevance of apoptosis in the context of chronic heart failure. The activation of MMPs also been revealed in heart failure. It is important that both caspases and MMPs have the ability to induce the cleavage of PARP-1 in heart failure.

The present study has yielded evidence of PARP activation in myocardial samples from patients with heart failure. Thus, the current study adds a further example of a human disease in which PARP activation has been demonstrated. Previous work has demonstrated PARP activation, among others, in human diabetic microvessels. The current findings are also consistent with a recent report by Pillai, DiNapoli and colleagues of increased poly(ADP-ribosyl)ation in human failing hearts. However, in contrast with Pillai, DiNapoli and colleagues, we were unable to detect the upregulation of PARP-1 protein in the heart failure samples, which suggests that the increased poly(ADP-ribosyl)ation in our samples was most probably due to activation of the catalytic function of PARP. This can occur via two principal mechanisms: breaks in the DNA strands or increased intracellular calcium concentration. Both of these mechanisms have been demonstrated in failing hearts, and in theory, both mechanisms could contribute to PARP activation.

The activation of caspases may potentially be triggered by two different mechanisms. In one of these, initiator caspases such as caspase-8 or -9 are activated in a multimeric complex (for example, caspase-8 in the death-inducing signaling complex and caspase-9 at the apoptosome). Alternatively, caspases are activated by catalytic processing of the zymogens at specific cleavage sites. Because of the central role of caspase-9 in the activation of PARP-1–degrading enzyme pathways, an effort was made to detect caspase-9 in our system; our data demonstrated that it is over-expressed (a higher expression of procaspase).

Activated caspases may trigger the cleavage of PARP, and may also act upon a variety of intracellular targets to promote apoptosis. The relationship between PARP activation and PARP cleavage, apoptosis vs. necrosis, is complex, but accumulating evidence suggests that PARP activation contributes to cell necrosis, while PARP cleavage (leading to a decreased PARP-1 activity) may serve as a protective mechanism (to prevent necrosis by cellular energy exhaustion) and thereby permit apoptosis. Interestingly, it was found that cardioplegic arrest induces apoptosis signaling pathways in myocardial endothelial cells and cardiac myocytes. In our case, however, we did not find significant translocation of AIF, suggesting that these apoptotic pathways were not activated in the donor or in failing hearts studied here.

Caspase-9 expression were higher in failing hearts than in donor hearts. The relevance of this finding is not clear. On the one hand, caspases can initiate the degradation of PARP-1, which degradation was indeed observed in the case of failing hearts. On the other hand, caspase activation can promote apoptosis, which is in contrast with the apparent lack of AIF translocation in both donor and failing hearts. It is therefore possible that caspase-9 overexpression and higher activation is a bystander effect of PARP-1 activation/degradation or apoptosis.

It is clear that further experiments (using biopsies obtained at different stages of heart failure) are required to clarify the relationship between apoptosis and PARP-1 activation. For example, such endocardial biopsies were taken and evaluated for apoptotic markers in the case of dilatative cardiomyopathy. Interestingly, controversial results were obtained, suggesting that the therapy of the patients or methodical difficulties (obtaining endocardial biopsies from the same locations) affect the results of the apoptotic tests. It should be also taken into account that a rather diverse set of human heart samples were analyzed. Therefore the etiology of the heart failure could have contributed to some of the differences seen (coronary heart disease, which is ischemic and would be expected to lead to enhanced oxidative stress, vs. a dilated cardiomyopathy, which could be from alcohol or a virus).

Our data suggest that PARP-1 activation did not result in the initiation of apoptosis in failing human hearts, according to the absence of AIF translocation. It is therefore possible that PARP-1 is activated by a limited number of DNA breaks as an important element of the DNA repair system. Additionally, it should be considered that our samples were from end-stage failing hearts, which may have successfully adapted to the higher level of oxidative stress and to the consequent PARP-1 activation. Altogether, it cannot be ruled out that acute PARP-1 activation results in apoptosis in the case of other pathologies (for example, in case of ischemia-reperfusion).

In animal studies, potent antioxidant compounds and potent PARP inhibitors exhibit significant efficacy in preventing myocardial dysfunction, reducing hypertrophy, and improving myocardial efficacy, suggesting that oxidative stress and PARP activation are related. It is certainly important to perform clinical studies to determine the potential causative role of the oxidative/nitrosative stress/PARP pathway in the pathogenesis of human heart failure. Overall, the current study has clearly provided evidence of oxidative stress and PARP activation in human failing heart samples, confirming earlier animal results in human tissue samples and supporting the design of future clinical trials.

5.2.The role of protein kinase C in modulation of contractility in the human heart

PKC is involved in the regulation of force generation of human ventricular cardiomyocytes and implicated in the pathomechanism of various cardiovascular disorders, including ischemic heart disease, congestive heart failure, myocardial hypertrophy, hypertension and atherosclerosis. PKC can phosphorylate several amino acid residues in TnI, TnT, myosin light chain 2, myosin binding protein C and desmin. As a result of these phosphorylations decreased Ca^{2+}-sensitivity of the MgATPase activity, prolongation of isovolumetric relaxation time, increased afterload, decreased myosin sliding speed, decreased contractility and reduced Ca^{2+} sensitivity of contraction has been observed. Our data adds to the range of these effects, suggesting that PKC is also involved in the maintenance of contractile force in case of prolonged increased intracellular Ca^{2+} concentrations, a condition which generally occurs upon ischemia-reperfusion. This beneficial effect of PKC was totally unexpected in light of the wealth of evidence for decreased contractility upon PKC activation. In particular, Belin et al. recently reported that heart failure is accompanied by increased PKC dependent myofibrillar protein phosphorylation and decreased contractility in the rat. Moreover, this effect of PKC was probably mediated by increased phosphorylation of troponin proteins. The differences in the experimental setups provide a plausible explanation for the controversial findings (increase versus decrease in contractility). In the earlier reports the effects of PKC were tested under conditions, where PKC activity was several times higher than the control, like heart failure or transgenic models, or phosphorylation of myofibrillar proteins by *in vitro* kinase treatments, or target proteins altered by site directed mutagenesis. In contrast, we used reconstituted cardiomyocytes containing physiological levels of PKC isoforms, in addition to the endogenous mixture of myofibrillar substrates and the respective targeting proteins. It is therefore possible that the development of heart failure is characterized by a dysregulation of PKC pathway, leading to the disruption of its physiological targeting and pathological phosphorylation of myofibrillar proteins, which may conceal the

physiological effects, revealed in this report. It needs to be also noted, that PKCα not only regulates myofibrillar response to Ca^{2+}, but have a pivotal role in the regulation of intracellular Ca^{2+} concentrations. Although this latter feature is important determinant of cardiac contractility, it was not investigated here. Besides to the differences in the experimental conditions, a striking difference was found in the PKC isoform expression between rodent and human hearts. For example, while PKCε seems to be the predominant isoform in rodent hearts, PKCα expression is the highest in human. In particular, we found about 20 fold higher PKCα expression than that of PKCδ and PKCε in donor human hearts. Moreover, although an increase in PKC expression is a hallmark of heart failure independently of the species, significant differences were found in the isoform expression pattern in human ventricular samples in end stage dilated cardiomyopathy and in severe aortic stenosis, again highlighting the complexity of PKC pathway in the heart. In this context, PKC isoform selectivity can be achieved by at least three ways, namely (i) by selective expression, (ii) by different substrate specificities (iii) or by different targeting of the enzyme. Here an effort was made to investigate all of these possibilities. As a matter of substrate selectivity, *in vitro* phosphorylation assays were performed on human ventricular myocytes and confirmed earlier data, suggesting a pivotal role of intracellular targeting in the determination of the apparent PKC isoform selectivity, *in vivo*. In particular, although differences in kinase activities and specificities were clearly recognized, all of the studied PKC isozymes were able to phosphorylate the main myofibrillar targets of PKC, such as TnI, TnT, *in vitro*. In accordance, the intracellular targeting of PKC isozymes and in particular PKCα wasinvestigated in detail. First of all, increase in Ca^{2+} concentration selectively induced the translocation of PKCα to the particulate fraction in the rat heart, which was confirmed in this study using human ventricular tissue samples. This pinpointed PKCα as the most possible mediator of the sarcomeric effects, resulting in depression of contractility or in protection of contractile force (this report), depending on the conditions. Moreover, PKCα appears to be apromising therapeutic target to improve cardiac contractility. To exploit this potential, the ubiquitous expression and the importance of PKCα in various physiological processes should be taken in account. The optimal drug candidate would act selectively on the heart muscle located PKCα and specifically modulate its effects on Ca^{2+} handling or on thin filament mediated regulation of sarcomeric sensitivity to Ca^{2+}. We made an effort to reveal the molecular mechanism of PKCα targeting to sarcomeric protein machinery. The Ca^{2+} concentration required for half maximal translocation of PKCα to the contractile system (645 nmol/L) was in the physiological range. Next, the sarcomeric PKCα anchoring proteins were

investigated. The thin filamental regulatory TnI was identified as a potential binding protein. Importantly, the interaction between TnI and PKCα was modulated by Ca^{2+} and was not affected by interaction of TnI with the other members (TnT and TnC) of the troponin complex. Moreover, immunohistochemical analysis revealed PKCα and TnI colocalization in the human ventricle. These data suggest that pharmacological modulation of TnI-PKCα interaction may be a strategy to regulate PKCα effects selectively on the sarcomeric proteins. In the biochemical point of view PKC binding proteins are classified as substrates that interact with C-kinase (STICKs), receptors for inactive C-kinase (RICKs) and receptors foractivated C-kinase (RACKS). In general, PKC activation involves the binding of DAG (or experimentally its stable analogue PMA) to the soluble PKC which then anchors to membranous structures. While TnI apparently one of the STICKs in the human heart, the interaction between TnI and PKCα seems to be regulated by Ca^{2+} alone, independently of lipids. One of the proteins with similar properties is the sdr protein, which targets PKCα to the caveolae in a Ca^{2+} dependent manner, in the absence of DAG or its analogues. This finding suggested that Ca^{2+} evokes a conformation change in PKC, revealing new interaction sites for nearby proteins. Analysis of the binding of PKCα to sdr suggested that while Ca^{2+} facilitates the sdr-PKC interaction, it is stabilized by phosphatidyl serine. In contrast, we found an apparently stable interaction between TnI and PKCα in the absence of lipids, although it may be further stabilized by phosphatidyl serine, *in vivo*. It was suggested that temporal and spatialchanges in intracellular free Ca^{2+} concentrations regulate the localization of PKCα in vascular smooth muscle cells. The same concept may be applied for ventricular cardiomyocytes. They have similar localized changes in the free intracellular Ca^{2+} concentrations; moreover, they spontaneously produce a rise in intracellular Ca^{2+} concentrations during the contractions. It is an exciting possibility that PKCα moves between the cytosol and the thin filament, where TnI temporarily anchors it to the thin filaments, during the contraction-relaxation cycle of cardiac myocytes and probably contributes to the maintenance of contractile force upon ischemiareperfusion. On the other hand, dysregulation of PKCα expression/targeting apparently leads to pathological phosphorylation of sarcomeric proteins and to a decrease in contractility. In summary, our data suggest that PKC plays a role in the maintenance of contractile force in human ventricular cardiomyocytes. The proposed mechanism of the PKC mediated protection is that PKCα translocates to the contractile protein machinery in a Ca^{2+} dependent manner, where it is anchored to the TnI. A practical application of these findings may be the pharmacological modulation of PKCα targeting in ischaemia/reperfusion to improve human cardiac contractility.

6. SUMMARY

The current results demonstrate the presence of significant oxidative stress in failing human heart samples. In contrast, no differences were found in tyrosine nitration in the failing and donor hearts. This study has yielded evidence of PARP activation in myocardial samples from patients with heart failure, adding a further example of a human disease in which PARP activation has been demonstrated. Nevertheless, upregulation of PARP-1 protein in the heart failure samples was not observed, suggesting that the increased poly(ADP-ribosyl)ation in our samples was most probably due to activation of the catalytic function of PARP. PARP activation did not result in the initiation of apoptosis in failing human hearts, according to the absence of AIF translocation. It is therefore possible that PARP-1 is activated by a limited number of DNA breaks as an important element of the DNA repair system. Additionally, it should be considered that our samples were from end-stage failing hearts, which may have successfully adapted to the higher level of oxidative stress and to the consequent PARP-1 activation.

PKCα moves between the cytosol and the thin filament, where TnI temporarily anchors it to the thin filaments, during the contraction-relaxation cycle of cardiac myocytes and probably contributes to the maintenance of contractile force upon ischemia/reperfusion. Analysis of the binding of PKCα to TnI suggested that Ca^{2+} facilitates the TnI-PKC interaction. It is an exciting possibility that PKCα moves between the cytosol and the thin filament, where TnI temporarily anchors it to the thin filaments, during the contraction-relaxation cycle of cardiac myocytes and probably contributes to the maintenance of contractile force upon ischemia/reperfusion. On the other hand, dysregulation of PKCα expression/targeting apparently leads to pathological phosphorylation of sarcomeric proteins and to a decrease in contractility. In summary, our data suggest that PKC plays a role in the maintenance of contractile force in human ventricular cardiomyocytes. The proposed mechanism of the PKC mediated protection is that PKCα translocates to the contractile protein machinery in a Ca^{2+} dependent manner, where it is anchored to the TnI. A practical application of these findings may be the pharmacological modulation of PKCα targeting in ischaemia/reperfusion to improve human cardiac contractility.

LIST OF PUBLICATIONS:

***In extenso* publications related to the thesis:**

Molnár A, Tóth A, Bagi Z, Papp Z, Édes I, Vaszily M, Galajda Z, Papp G, Varró A, Szüts V, Domokos G, Lacza Z, Szabó C Activation of the poly(ADP-ribose) polymerase pathway in human heart failure. Mol Med. 2006;12(7-8):143-52. IF: 2,708

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Molnár A., Szilágyi S., Borbély A**.,** Vaszily M., Édes I., Papp Z., Tóth A. Translocation and substrate specificity of protein kinase C alpha isozyme in human myocardium. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred, Hungary. 2005

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