SUMMARY OF DOCTORAL (PH.D.) THESIS

SULFHYDRYL OXIDATION OF MYOFIBRILLAR PROTEINS AND ITS EFFECT ON THE CONTRACTILE FUNCTION IN HUMAN CARDIOMYOCYTES

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1. Introduction

The functional and molecular effects of oxigen derived free radicals were extensively studied int the last decades. The physiological function of oxigen derived free radicals produced in small quantities was established, but it also become obvious that the pathological production of these substances (e.g. oxidative stress) had a significant role in the development of cardiovascular diseases. The most important pathological conditions leading to the excess formation of oxidative radicals are the ischaemic reperfusion injuiry and the heart failure. The deleterious effect of oxidative stress on contractile function was investigated in numerous experiments. Oxigen derived free radicals can react with nucleic acids, lipids and proteins impairing their function. These effects often lead to loss of function of these macromolecules in an irreversible manner. However, the oxidaton of sulfhydryl (SH) groups in cysteinyl residues of proteins are reversible. Accordingly, SH groups contributes to the redox regulation of physiological and pathophysiological functions, and highlight the importance of antioxidant defense in oxidative stress. SH groups of proteins can also be oxidized by nitrogen derived free radicals, like peroxinitrite.

In this work we studied the sulfhydril group oxidation of myofibrillar proteins and its effects on the contractile function in isolated cardiomyocytes of human heart. Here we tested the effects of the SH specific oxidant dithiodipyridine (DTDP) and the endogenous oxidant peroxinitrite in isolated cardiomyocytes. The mechanical and biochemical alterations were investigated under same conditions. An effort was made to investigate the reversion of SH oxidation, which can be used in the prevention and therapy as well.

1.1. Free radicals and the oxidative and nitrosative stress

Various oxigen and nitrogen derivatives are produced by enyzmatic and not enyzmatic manner in the cells. Some of them contain one or more unpaired electron and therefore highly reactive. These free radicals include reactive oxigen species (ROS) and reactive nitrogen species (RNS), respectively. ROS are typically generated by tightly regulated enzymes of

mitochondrial electron transport chain such as xanthine oxidase, NAD(P)H oxidase, NO synthase (NOS), cytochrome p450, ciclooxigenases, lipoxigenases and monoxigenases. Excess generation of ROS can cause cellular damage. Accordingly, there are several protective mechanisms against ROS mediated damage in the cells. Among the best characterized enzymatic pathways are catalases and glutathione peroxidase, superoxid dismutases (SODs) and thioredoxin. In addition, several antioxidant molecules constitute antioxidant buffer, like vitamin C, vitamin A, vitamin E, ubiquinone, gluthatione and the SH groups of intracellular proteins.

An excessive and sustained increase in ROS production damages the macromolecules and causes morphological and functional alterations. This phenomenon is called oxidative stress. The ROS induced protein changes range from protein fragmentation to varoius posttraslational protein modifications. The most important oxidant related modifications of amino acid side chains of proteins are the SH oxidation of cysteine, carbonylation of lysin, arginin, proline amino acids, and the nitration of tyrosine side chains. Irreversible oxidative modification of macromolecules has been linked to mutagenesis, carciogenesis, alteration of metabolic pathways and inactivation of proteins. Increased production of ROS has been implicated in the pathogenesis of certain diseases, like ischaemic reperfusion injuiry, heart failure, atherosclerosis, tumor, Alzheimer disease, aging, apoptosis, and myocardial hypertrophy.

1.2. Sulfhyrdyl (SH) groups

Cysteinyl residues of intracellular proteins have a diverse role in the biology of eukaryotic cells, being involved in enzyme mediated catalysis, electron transport, determination of structure of proteins. Additionally, SH groups are involved in the antioxidant cell defense and the maintanence of optimal intracellular redox state.

SH groups can be modified in different ways during oxidative or nitrosative stress. SH groups of proteins (P) reacting with each others form inter and intramolecular disulfide bonds (PSSP). This rection is reversible like the generation of mixed disulfides, the S-thiolation as well. The S-thiolation is the linking of low molecular weight thiols (cysteine, homocysteine, glutatione etc.) to the SH groups of proteins, the most important being S-glutationylation (PSSG). The reaction between NO and the SH groups (PSNO) is the reversible S-

nitrosylation. The irreversible modification of SH groups results in formation of sulfinic (PSO_2H) or sulfonic acid (PSO_3H).

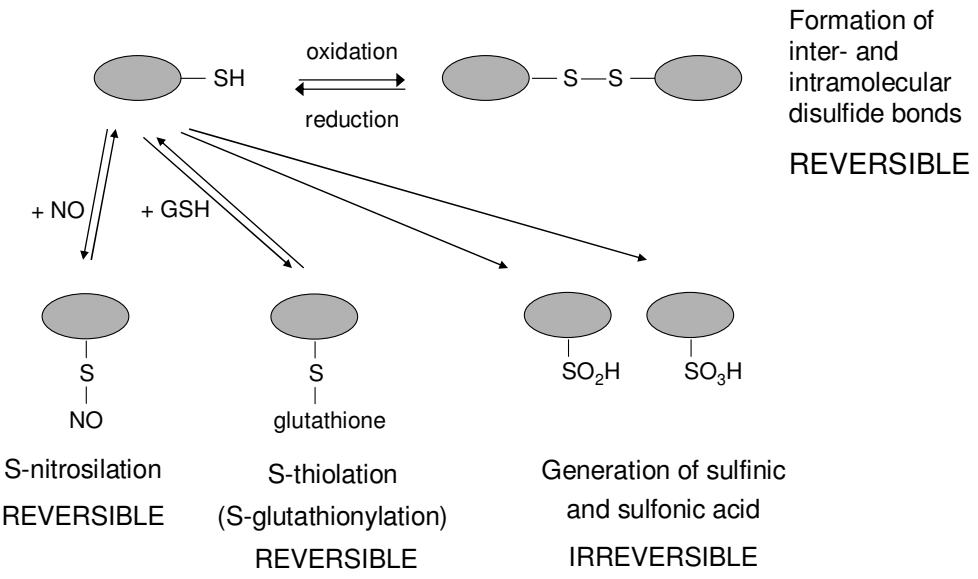

Figure 1. Oxidative modification of SH groups

Previous studies revealed that oxidative modification of cysteinyl residues plays both physiological and pathological roles, depending on the conditions. The functions of many enzymes are influenced by redox regulation. Under physiological conditions most cysteinyl residues are in a fully reduced state. Increase in the reversible conversion of SH groups to disulfides is one of the earliest sign of radical-mediated oxidation of proteins.

Myocardial protein SH content decreases characteristically during ischaemia and reperfusion, and SH oxidation of proteins may thereby contribute to the contractile dysfunction of the postischaemic stunned myocardium. Some of the most important reactive species, including superoxide, H_2O_2 and peroxynitrite have been shown to directly cause SH oxidation in ischaemic reperfusion injury.

1.3. Peroxynitrite

Increased level of peroxynitrite contributes to the development of ischaemic reperfusion injuiry and congestive heart failure. Peroxynitrite is a strong oxidative agent, produced by the reaction of NO and superoxide. It is relatively stable in alkaline pH, but its half life significally shorter in neutral pH (decomposes in seconds). Nitrotyrosine formation as a result of the reaction with tyrosine side chains is considered to be the hallmark of peroxinitrite formation. In addition, peroxinitrite can react with the SH groups of proteins. Although nitrotyrosine generation was proposed to contribute to the peroxynitrite-induced mechanical dysfunction, we have limited information regarding the peroxinitrite effects on protein SH groups.

1.4. Ischaemic reperfusion injuiry and heart failure

Postischaemic dysfunction (myocardial stunning), is the mechanical dysfunction that persists after reperfusion despite absence of irreversible damage and almost physiological coronary blood flow. It is a syndrome that has been observed in a wide variety clinical settings like in instable angina, in early reperfusion of acut myocardial infarct by thrombolysis or coronary intervention, during coronary artery bypass operation with cardioplegia etc. Nevertheless, reversible alterations in stunning result in the impairment of myocardial pump function.

The pathogenesis of myocardial stunning has not been definitively established, although two major mechanisms were proposed: it is mediated by the generation of oxigen–derived free radicals and by a transient calcium overload upon reperfusion. The culprit of contractile depression appears to be a decreased responsiveness of contractile filaments to calcium. Several studies demonstrated the increased production of ROS during reperfusion injury. The amount of generated free radicals was proportional with the severity of ischaemia. The oxidation of SH groups is suggested to contribute to the development of contractile dysfunction.

Several definitions of heart failure have been outlined over the years, although none has been generally satisfying. This reflects the complexity of this syndrome, which is characterized by reduced cardiac output and increased venous return, and is accompanied by

molecular abnormalities that cause progressive deterioration of the failing heart. Human heart failure is considered as a cardiocirculatory disorder. In accordance, heart failure is characterized by increased neurohumoral activation and citokin production, damaged endothel function in addition to the changes in contratility.

1.5. Antioxidant therapy

Based on the results of the studies regarding oxidative stress in the last decades the antioxidant therapy of the oxidative stress related pathologies seemed to be promising. Nevertheless the results of experimental researchers, epidemological studies and interventional trials with antioxidants were rather contradictory. Numerous studies have evaluated the influence of ascorbate, vitamin E, vitamin A, N-acetyl-L-cysteine and other reductive agents and antioxidants in the prevention and in the therapy of cardiovascular diseases. Among these there are studies reporting beneficial, neutral and negative outcomes as well. These studies demonstrate that the reversion and the prevention of oxidative modifications at the level of organism are determined by complex factors.

1.6. Aims

In our study the following aims were defined:

1. To investigate the influence of SH oxidation of myofibrillar proteins on the contractile function in human permeabilized cardiomyocytes.
2. To study the reversibility of protein SH oxidation with reducing agents, such as dithiothretol, reduced glutathion and N-acetyl-L-cysteine.
3. To identify the SH oxidized proteins responsible for the mechanical alterations.
4. To determine the SH oxidative component of peroxinitrite mediated contractile depression.

2. Methods

2.1.Ethical approval

The experiments on human tissues complied with the Helsinki Declaration of the World Medical Association and were approved by the Hungarian Ministry of Health (No. 323- 8/2005-1018EKU) and by the Institutional Ethical Committee at the University of Debrecen, Hungary (No. DEOEC RKEB/IKEB 2553-2006).

2.2. Mechanical measurements

Frozen human myocardial tissue samples were defrosted and mechanically disrupted. A demembranated single cardiomyocyte was mounted between two thin insect needles with silicone adhesive while viewed under an inverted microscope. The compositions of the relaxing and activating solutions used during force measurements were calculated as described by Fabiato (1979). Isometric force was measured after the myocyte had been transferred from the relaxing solution to a Ca^{2+}-containing solution. Peak isometric force (F_{total})) and the rate constant of force redevelopment (k_{tr}) at various [Ca^{2+}] levels were measured. The passive force component ($F_{passive}$) was determined in relaxing solution following the Ca^{2+} contractures. The Ca^{2+}-activated isometric force (F_o) was calculated by subtracting the $F_{passive}$ from the F_{total}. F_{active} at submaximal levels of activation was normalized to that at maximal activation (F_{max}) in order to characterize the Ca^{2+}-sensitivity of isometric force production (pCa_{50}).

In this study we applied dithiodipiridine (DTDP) and peroxinitrite as an oxidative agent and dithiothretol (DTT), reduced glutathion (GSH), N-acetyl-L-cysteine (NAC) as reducing agent.

2.3. Biochemical measurements

SH content was determined by incubation the permeabilized cardiomyocytes with the SH-sensitive Ellman's reagent. To analyze the sensitivity of certain myocardial proteins to SH oxidizing agents SH specific biotinylation and Western blot analysis were performed. Immunoprecipitation assays were used to separate myosin light chain 1 (MLC1) and cardiac troponin I (cTnI) from biotinylated cardiac protein mixtures to identify the protiens responsible for the contractile alteations.

3. Results

3.1. The mechanical consequenses of myofibrillar protein SH oxidation

The effects of SH oxidation on both active and passive forces and on the cross-bridge-sensitive rate constant of force redevelopment (k_{tr}) were investigated during repeated isometric Ca^{2+} contractures in permeabilized left ventricular cardiomyocytes of human hearts. Figure 1B illustrates that exposure to increasing concentrations of the oxidative DTDP decreased the maximal Ca^{2+}-activated force (F_o) in a graded fashion. However, even after the application of the highest DTDP concentration (10 mM in this case which eventually diminished force to zero), 10 mM DTT restored 77.6% of the control F_o, which was suggestive of reversible DTDP-evoked protein modification. Interestingly, neither the reduction nor the restoration of the force production was paralleled by any alteration in the light microscopic cross-striation pattern of the cardiomyocytes.

The effect of the DTDP concentration on the maximal Ca^{2+}-activated force was contrasted with that on the myocardial SH content by using the SH-sensitive Ellman's reagent. This assay revealed that increasing concentrations of DTDP decreased the SH content in the myocardial protein homogenates. Most of the force decline appeared at relatively low SH levels. DTDP-evoked SH oxidation was reversed by DTT. Alone, the reducing agent, 10 mM DTT, did not change the oxidative status.

Next, the SH-dependent changes in the myofibrillar mechanics were tested in detail. To this end, force recordings were performed at various submaximal [Ca^{2+}] levels before and after application of a single DTDP concentration (2.5 mM), which caused a reduction of about

50% in the maximal force. To visualize possible differences in the Ca^{2+} - force relations, the peak contractile forces were normalized to their respective maxima before and after the DTDP. This analysis revealed a reduction in the Ca^{2+} sensitivity of force production in response to DTDP, *i.e.* a rightward shift in the Ca^{2+} - force relationship (ΔpCa_{50}=0.22±0.02, P<0.01 vs. the control). Application of the reducing agent DTT (10 mM) sequentially after DTDP restored both F_o and its Ca^{2+} sensitivity to a large degree. The application of DTT alone did not alter either the active force or its Ca^{2+} sensitivity. The passive force assessed in relaxing solution did not change upon DTT treatment, but it was increased slightly by DTDP and remained elevated when DTT was applied after DTDP. The turnover rate of the actin-myosin cycle at maximal Ca^{2+} activation ($k_{tr,max}$) decreased from a control value of 1.07±0.04 s^{-1} to 0.8±0.05 s^{-1} at 2.5 mM DTDP (P<0.05), but a subsequent DTT treatment induced a full reversion in this parameter ($k_{tr,max}$= 1.11±0.05 s^{-1}). Similarly as for all other mechanical parameters, DTT alone did not modulate $k_{tr,max}$.

The Ellman's assay indicated that 2.5 mM DTDP resulted in a robust decrease in the myocardial free SH content, which was reversed partially by GSH or NAC when the antioxidants were all used at the same 10 mM concentration. SH content was 34±8% after 2.5 mM DTDP + 10 mM GSH, and it was 30±4% after 2.5. mM DTDP + 10 mM NAC (for all P<0.05 *vs.* 2.5 mM DTDP). Moreover, 100 mM GSH or 100 mM NAC reduced 2.5 mM DTDP-oxidized myocardial proteins more effectively than 10 mM GSH or 10 mM NAC. SH content after 2.5 mM DTDP + 100 mM GSH was 79±3%, whereas it was 68±15% after 2.5 mM DTDP + 100 mM NAC (for both P<0.05 *vs.* 2.5. mM DTDP).

As a matter of the different effectiveness for the employed reducing agents other proofs were also found. While 10 mM DTT seemed to be suitable to reverse SH oxidation and contractile mechanics, 10 mM GSH or 10mM NAC worsened the mechnaical effects of 2.5 mM DTDP: the elevations in $F_{passive}$ were further elevated, the decreased F_o was further decreased, and the $k_{tr,max}$ was slower after GSH and NAC treatments than before.

3.2. Investigation of sensitivity of contractile proteins againts SH oxidation and identification of proteins responsible for the contractile alteraions

As the next step, screening was performed to identify SH changes at the level of individual proteins upon oxidation (by DTDP) and subsequent reduction (by DTT) using

myocardial homogenates prepared similarly to that for the measurements of contractile force. Proteins containing SH groups were biotinylated and visualized by Western immunoblotting. The staining intensities decreased in parallel with the progression of S-thiolation at increasing DTDP concentrations (0-30 mM) in a qualitatively similar fashion with the results obtained with the Ellman's reaction. The concentration dependent change in the staining pattern provided evidence of differences in the susceptibilities of myocardial protein SH oxidation. The signal intensities of all the bands recovered when DTDP treatment was followed by DTT, suggesting that the effect of DTDP was reversible in all the proteins visualized. It was noteworthy that oxidation of several myofilament proteins appeared between DTDP concentrations of 0.1 mM and 1 mM where force did not decrease to a large degree. Moreover, treatment with higher DTDP concentrations (1-10 mM) where most of the contractile alterations were detected, the SH oxidation involved four prominent proteins, with apparent molecular weights of 130, 54, 45 and 26 kDa. Furthermore, complete oxidation of the proteins with molecular weights of 45 and 26 kDa was observed at these critical DTDP concentrations, while oxidation of the other two proteins with higher molecular weights were only partial even at 30 mM DTDP. The complete oxidation of the 45 kDa and 26 kDa proteins together with the large drop in F_o suggested a close relationship between the oxidative status of these proteins and myofibrillar force production.

The apparent molecular weight of the above two proteins was close to those of cTnI or MLC1. Therefore, immunoprecipitation assays were performed to reveal whether the decreasing staining intensity at 26 kDa protein was due to the oxidation of cTnI or MLC1. The bands developed by antibodies against cTnI or MLC1 illustrate that separation of cTnI and MLC1 from all the other proteins was successful by immunoprecipitation. However, when these immunoprecipitated proteins were visualized by our SH sensitive method (*i.e.* based on SH biotinylation) it was apparent that cTnI was completely oxidized at the lowest concentration of DTDP applied here (0.1 mM), in contrast with the MLC1 where the concentration dependent disappearance of signal intensity resembled that of the oxidation of the 26 kDa band in the previous assays. Hence these tests indentified MLC1 as a protein with a relatively high resistance against SH oxidation and potential SH dependent influence on Ca^{2+}-activated force production.

The 45 kDa protein which possessed similar DTDP susceptibility with MLC1 was identified by Western immunoblot analyses and silver-stained gels as actin. On the basis of their molecular weights and previous findings, the protein bands at of 130 kDa and 54 KDa might well reflect the myosin binding C-protein and desmin, respectively.

3.3. The conntribution of the SH oxidation to the peroxynitrite mediated contractile depression

Both peroxynitrite and DTDP decreased F_o to zero in permeabilized human ventricular cardiomyocytes in a concentration-dependent manner. In case of peroxynitrite the force diminution was noticed in the μM concentration range ($EC_{50,peroxynitrite}$ = 49 μM), while DTDP exerted its mechanical effect at higher concentrations ($EC_{50,DTDP}$ = 2.75 mM). To elucidate the SH-oxidizing effect of peroxynitrite and DTDP, parallel biochemical assays were performed with the SH-sensitive Ellman's reagent. Of note, the experimental conditions, including temperature for the Ellman's assays and mechanical measurements were comparable. Increasing concentrations of DTDP decreased the SH content of the myocardial proteins to zero along with the diminution of force. On the contrary, peroxynitrite-evoked significant SH oxidatation only at very high concentrations: the fraction of reduced SH groups decreased to 58±7% (P<0.05) of that of the untreated control (100%) following the application of 1 mM peroxynitrite.

Having established that SH oxidation associates with the deterioration of contractile force, the parameters of cardiomyocyte mechanics (F_o, $k_{tr,max}$ and $F_{passive}$) were investigated at a single peroxynitrite concentration (50 μM) resulting in about half maximal reduction in F_o. F_o declined to 56±4% of the control after the application of 50 μM peroxynitrite, however $F_{passive}$ and $k_{tr,max}$ did not change significantly. To estimate the relative contribution of SH oxidation besides other types of potential protein modifications to the reduction in F_o, SH specific reducing agents were employed to reverse SH oxidation. Indeed both 10 mM DTT and 10 mM NAC were able to evoke partial, although significant increases in F_o in peroxynitrite-treated cardiomyocytes, suggesting that this increase was specific for the reduction of protein SH groups. F_o was increased to 69±4% of the control by 10 mM DTT (P<0.05 vs. $F_{o,peroxynitrite}$), and the relative increase in F_o was to 71±7% of the control by 10 mM NAC (P<0.05 *vs.* $F_{o,peroxynitrite}$). $F_{passive}$ did not change following 10 mM DTT or 10 mM NAC in peroxynitrite-treated cardiomyocytes. Of note, $k_{tr,max}$ decreased slightly following 10 mM DTT or 10 mM NAC applications in peroxynitrite-treated cardiomyocytes. However, based on control experiments with repeated activations in the absence of any chemicals this small decrease in $k_{tr,max}$ was probably related to preparation run-down.

The partial contribution of SH oxidation to the peroxynitrite-evoked force reduction was confirmed by biochemical methods. Results of Ellman's assays showed that 1 mM peroxynitrite (a peroxynitrite concentration with maximal SH oxidative effect,) decreased the free SH content to 58±7%, which was completely reversed by 10 mM DTT. SH content after 1 mM peroxynitrite + 10 mM DTT was 94±5% (P<0.05 *vs.* 1 mM peroxynitrite). Surprisingly, when applied at the 10 mM concentration the effects of GSH or NAC did not reach significance on the overall free SH content of the myocardial protein preparations. SH content after 1 mM peroxynitrite + 10 mM GSH was 65±6%, and SH content after 1 mM peroxynitrite + 10 mM NAC was 64±6% (P>0.05 *vs.* 1 mM peroxynitrite). However, when the same reducing agents were employed at a concentration of 100 mM, both of them reduced protein SH groups effectively. Protein SH content after 1 mM peroxynitrite + 100 mM GSH was 105±15% and SH content after 1 mM peroxynitrite + 100 mM NAC was 118±14% (P<0.05 *vs.* 10 mM peroxynitrite). An effort was also made to identify whether certain proteins undergo selective oxidation-reduction cycles during the incubations with the various drug combinations. SH-specific biotinylation, however suggested a uniform decrease in the free SH group-specific staining intensity of myocardial proteins with different molecular weights at the highest peroxynitrite concentration (1mM). Moreover, these changes were fully reversed by 10 mM DTT, and only to a smaller degree by 10 mM GSH or 10 mM NAC. Therefore, the results of these assays were reminiscent of the results of the Ellmans's test, but failed to identify peroxynitrite-specific selective myocardial protein oxidation.

4. Discussion

This study reveals the significance of myofilament SH oxidation in the Ca^{2+}-regulated force production of the human heart. Our findings indicate that S-thiolation of a selected group of myofilament proteins, including MLC1 and actin, but not cTnI, reduces the Ca^{2+}-activated force and its Ca^{2+} sensitivity through alterations in the kinetics of actin-myosin cross-bridge transitions. These data allow us to propose a plausible molecular mechanism for the contractile depression in association with myocardial SH redox changes. To the best of our knowledge, this is the first study in which the relationship between selective SH oxidation and myofibrillar mechanical function has been demonstrated in human myocardial preparations.

In this study we focused on the functional consequences of the SH-dependent component of oxidative myocardial injury, and tested the relationship between the extent of protein SH group oxidation and Ca^{2+}-regulated force production. The alteraions in the cardiomyocyte mechanics evoked by DTDP proved to be reversible by normalizing the SH status.

Our primary aim was to discriminate between the protein constituents of permeabilized cardiomyocytes with structural or regulatory roles from the aspect of their SH oxidation-dependent influence on Ca^{2+}-activated force production in the human heart. The reversibility of the observed alterations following the sequential application of DTDP and DTT verified the SH specificity of our model approach.

Exposure of human myocardial samples to DTT without prior oxidative interventions failed to increase the amount of protein SH groups. Thus, the results of this study confirm the findings of earlier animal experiments and extend them to the human heart by demonstrating that the contractile proteins are predominantly present in their reduced form under physiological conditions.

In rat postischemic cardiac hearts, proteins of the cytosol, membrane, and myofilament/cytoskeletal compartments have all served as major substrates for S-thiolation following ischemia and reperfusion. In full agreement with the results of our model approach, these *in vivo* data also suggested SH depenent alterations both in the structural and regulatory proteins of the myocardium. However, we did not observe any appreciable changes in the light microscopic images of permeabilized human cardiomyocyte preparations under conditions where the protein SH oxidation was up-titrated to a level where the active force production was zero. This finding is in clear contrast with those where the sarcomeric structure and force were both deteriorated by *in vitro* incubation with the nitrogen oxide metabolite peroxynitrite, or with the proteolytic enzyme μ-calpain in an irreversible fashion, suggesting that SH oxidation has the potential to suspend myofibrillar force production through alterations in the fine regulation of contractile force, but without major changes in the three-dimensional lattice structure of the sarcomere.

When the concentration - effect relation of DTDP on SH oxidation was compared with the effect on the maximal Ca^{2+}-activated force, we were surprised to see that more than 50% of the SH groups had to be oxidized for a sensible reduction in force. This finding can be interpreted either as a threshold phenomenon or in terms of the critical involvement of a fraction of myocardial proteins with distinct and relatively low susceptibilities to SH oxidation. We favour this latter possibility because our biochemical assays pointed to the

complete oxidation in only two contractile proteins (with apparent molecular weights of 45 and 26 kDa) at DTDP concentrations where the force decline was most prominent (*i.e.* between 1 and 10 mM). Pursuing the idea of the critical involvement of specific proteins, we made efforts to identify these two proteins. The molecular weight, the abundance and Western immunoblot results suggested that the protein at 45 kDa is actin. As a matter of fact, there is ample evidence in animal models for a possible role of oxidized actin upon ischemia and reperfusion. However, based on the same dose-dependent effect of DTDP on the protein at 26 kDa, our data also suggested a contribution by an additional protein. Immunoprecipitation assays in parallel with the detection of protein SH oxidation identified this protein as MLC1.

In this study, we determined the SH oxidation dependent component of peroxynitrite-evoked mechanical alterations in human cardiomyocytes and compared these effects with that of the SH-specific DTDP. 50 μM peroxynitrite decreased F_o approximatly to 50%. DTT or NAC increased F_o significantly following 50 μM peroxynitrite, and hence identified an SH sensitive component of the peroxynitrite-induced mechanical dysfunction. A possible explanation for the finding that 10 mM NAC apparently did not reverse peroxynitrite-induced SH oxidation but it did reverse peroxynitrite-reduced F_o values may relate to a lower signal to noise ratio for the Ellman's assay than that for the mechanical measurements. Of note, 2.5 mM DTDP evoked a robust reduction in protein SH content and hence allowed the recognition of changes in the SH-oxidative status of proteins following 10 mM NAC or 10 mM GSH exposures. However, at lower levels of protein oxidation, that occurred either after 1 mM peroxynitrite or after 0.1 mM DTDP the increase in protein SH content did not reach significance either after 10 mM NAC or after 10 mM GSH, but only after the application of 100 mM NAC, 100 mM GSH or 10 mM DTT. Taken together, these results suggest that NAC and also GSH are capable to reverse peroxynitrite-induced SH oxidation, and that F_o is more sensitive for this reversion than protein SH content as evidenced by our Ellman's assay.

Results of earlier investigations from our laboratory demonstrated that the peroxynitrite-evoked force reduction is best paralleled by the nitration of a structural sarcomeric protein, α-actinin. In contrast, the DTDP-evoked reduction in force was correlated with the oxidation of the sarcomeric actin and myosin light chain 1. The divergent involvement of sarcomeric protein modifications following peroxynitrite or DTDP applications may explain why cross-bridge kinetics was not altered following peroxynitrite exposures, while it was largely affected by DTDP. Our effort to identify a hypothetical group of proteins undergoing selective oxidation-reduction cycles by peroxynitrite and antioxidant exposures was precluded by the homogenous and the relatively small changes in signal

intensities even at maximal peroxynitrite concentrations in our biotinylation assays. Hence, in this study we could not ascribe the peroxynitrite-evoked SH oxidation dependent mechanical changes to one or another sarcomeric protein.

One of the salient aims of our present investigation was to compare the relative potentials of different reducing agents in reverting SH-dependent mechanical alterations in human cardiomyocytes. To this end, following peroxynitrite or DTDP administrations assays were performed with DTT, NAC and with the intracellular antioxidant GSH. In general, DTT possessed the highest potential in the reversion of the mechanical and biochemical effects. On the other hand, the results obtained with GSH and NAC were seemingly contradictious. After DTDP both of these latter reducing agents induced a modest increase in the SH content. Interestingly, however these resulted in further diminishion in F_o and in $k_{tr,max}$, and significant increases in $F_{passive}$. However, after peroxynitrite, NAC similarly to DTT evoked a partial reversion in F_o. Redox reactions in the cells are determined by many factors, among which are probably the most important the redox potential, the conformation and the molecular size of the reacting partners. Additionally, SH oxidizing agents can react in different ways with the thiol groups of the proteins, generating intra- or intermolecular disulfide bridges or mixed disulfides. For example, DTDP besides generating disulfide bonds inside and among the proteins can also create a mixed disulfide between the cysteinil residues and one half of the DTDP molecules, thereby liberating the other halves of DTDP molecules as thiopyridones. DTT, due to its low redox potential, can reduce disulfides very rapidly and with a high efficiency seemingly irrespectively of the presence or absence of mixed disulfides. Conversely, GSH and NAC, both having higher redox potentials than DTT, are less effective in reducing the different forms of oxidized SH groups. We assume, therefore, that these differences can contribute to the explanation of the complex interplay between oxidative and reducing agents included in our experiments. Although there are positive human studies with antioxidants, including NAC , the results of big clinical trials with antioxidants in preventing the initiation and progression of cardiovascular diseases are also variable. The human clinical trials ended with negative outcomes and the results of this study emphasize the significance of the proper application and choice of antioxidants.

In conclusion, results of this model investigation revealed that the contribution of SH oxidation to the peroxynitrite-mediated contractile depression is inferior to other peroxynitrite-evoked biochemical effects in human cardiomyocytes. Our data also illustrated that Ca^{2+}-activated active force, Ca^{2+}-independent passive force and the kinetics of the actin-myosin cycle are in complex relations with myocardial protein oxidation. Different

combinations of the reduced and oxidized myocardial proteins may exert opposing effects on these parameters. Hence, the extent of myocardial protein oxidation, and the molecular characteristics of the oxidoreductive insults should be also considered when the SH-dependent mechanical alterations are evaluated in human cardiomyocytes.

LIST OF PUBLICATIONS

In extenso publications related to the thesis

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