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Original Research Communication

Oxidation of Myofilament Protein Sulfhydryl Groups Reduces the Contractile Force and Its Ca^{2+} Sensitivity in Human Cardiomyocytes

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ABSTRACT

This study sought to characterize the relation between the oxidation of protein sulfhydryl (SH) groups and Ca^{2+}-activated force production in the human myocardium. Triton-permeabilized left ventricular cardiomyocytes from donor hearts were exposed to an oxidative (2,2′-dithiodipyridine, DTDP) agent *in vitro*, and the changes in isometric force, its Ca^{2+} sensitivity, the cross-bridge–sensitive rate constant of force redevelopment at saturating [Ca^{2+}] ($k_{tr,max}$), and protein SH oxidation were monitored. DTDP (0.1–10 m*M* for 2 min) oxidized the myocardial proteins and diminished the Ca^{2+}-activated force with different concentration dependences ($EC_{50,SH}$ = 0.17 ± 0.02 m*M* and $EC_{50,force}$ = 2.46 ± 0.22 m*M*; mean ± SEM). The application of 2.5 m*M* DTDP decreased the maximal Ca^{2+}-activated force (to 64%), its Ca^{2+} sensitivity (ΔpCa_{50} = 0.22 ± 0.02), and the steepness of the Ca^{2+}-force relation (n_{Hill}, from 2.01 ± 0.08 to 1.76 ± 0.08). These changes were paralleled by reductions in the free SH content of the proteins (to 15%) and in $k_{tr,max}$ (to 75%). SH-specific labeling identified SH oxidation of myosin light chain 1 and actin at DTDP concentrations at which the changes in the contractile parameters occurred. Our data suggest that SH oxidation in selected myofilament proteins diminishes the Ca^{2+}-activated force and its Ca^{2+} sensitivity through an impaired Ca^{2+} regulation of the actin–myosin cycle in the human heart. *Antioxid. Redox Signal.* 10, 1175–1184.

INTRODUCTION

The excess production of reactive oxygen species (ROS) has been implicated in myocardial injury during ischemia and reperfusion in both animal (39) and human (21) hearts. ROS may influence the molecular signaling in the cardiomyocytes and evoke structural changes in targeted lipids and proteins (3, 13, 14, 29, 34, 36). In particular, modifications of myofibrillar proteins have been linked to reversible alterations during myocardial stunning (4). The ROS-induced changes range from protein fragmentation to various posttranslational protein modifications (8, 15, 16, 45). In general, in consequence of the multitude of parallel oxidative protein changes in the postischemic heart, it is difficult to constitute cause–effect relations for one or another type of chemical alteration for any myocardial protein. The oxidation of sulfhydryl (SH) groups (*i.e.*, S-thiolation) in contractile proteins is of special interest, because myocardial ischemia and reperfusion are associated with drastic changes in the thiol redox state of the cardiomyocytes (9, 10, 17, 53). Moreover, the reversible nature of myocardial redox regulation theoretically offers a means of preventing the depression of the postischemic myocardial contractile function. To this end, the molecular mechanism by which S-thiolated myocardial proteins impair the contractile function must be resolved. However, previous model studies in animals failed to reach a consensus on the molecular mechanism and mechani-

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cal consequence of contractile protein oxidation (8, 9, 17, 24, 32, 33, 48), and hence little can be directly extrapolated from these investigations for the human heart.

Modulation of the SH status is not restricted to a single myocardial protein. We therefore hypothesize that the mechanical dysfunction of the heart will depend on the extent of SH oxidation in a set of myocardial proteins performing functional or structural roles or both in the contractile process.

Hence, the relation between myocardial SH oxidation and Ca^{2+}-activated force production was investigated here. To evoke SH-specific oxidations in contractile proteins, 2,2′-dithiodipyridine (DTDP) was used in permeabilized human left ventricular cardiomyocytes *in vitro*, as earlier in skeletal muscle fibers of the rat and toad (30, 42, 43), and dithiothreitol (DTT) was used to test reversibility. The force at saturating [Ca^{2+}] (F_o), the Ca^{2+} sensitivity of isometric force production (pCa_{50}), the steepness of the Ca^{2+}-force relation (n_{Hill}), the passive force component, and the cross-bridge-specific rate constant of force redevelopment (k_{tr}) (6) (all at a sarcomere length of 2.2 μm) were used as parameters of the myofibrillar function. These were determined before and after exposure to SH-specific oxidative and reducing agents. Additionally, SH-specific labeling and immunoprecipitation assays were used to pinpoint contractile proteins directly responsible for the SH-specific contractile alterations.

These data illustrate selected myofibrillar proteins as potential mediators of the SH-dependent contractile dysfunction in the human heart.

MATERIALS AND METHODS

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).

Human left ventricular tissue samples, permeabilized cardiomyocytes

Human donor hearts obtained from five general organ-donor patients (a 50-year-old man, a 41-year-old woman, a 56-year-old man, a 46-year-old woman, and a 37-year-old woman) were explanted to obtain pulmonary and aortic valves as homografts for cardiac surgery. The donors did not reveal any sign of cardiac abnormalities and had not received any medication except short-term dobutamine and furosemide. The cause of death was cerebral contusion and cerebral hemorrhage due to accidents, or subarachnoid hemorrhage due to stroke. All biopsies were transported in cardioplegic solution (pH, 7.4; in m*M*): NaCl 110, KCl 16, $MgCl_2$ 1.6, $CaCl_2$ 1.2, $NaHCO_3$ 5, and kept at 4°C for ~1–4 h before being frozen in liquid nitrogen and stored at −80°C.

Frozen tissue samples were defrosted and mechanically disrupted in isolation solution (composition in m*M*: $MgCl_2$ 1, KCl 145, EGTA 2, ATP 4, imidazole 10; pH 7.0). The resultant suspension was incubated in isolation solution supplemented with 0.5% Triton X-100 (Sigma, St. Louis, MO) for 5 min to permeabilize all the membranous structures. The preparations were washed 3 times (centrifugation with 1,000 rpm for 1 min) and subsequently kept at 4°C until the following experiments.

Mechanical properties of cardiomyocytes, in vitro *applications of oxidative and reducing agents*

Permeabilized single-cardiomyocyte preparations were mounted between two thin needles with silicone adhesive (Dow Corning, Midland, MI) while viewed under an inverted microscope (Axiovert 135; Zeiss, Germany) (40, 50). The advantage of these preparations is that they present negligible diffusion obstacles, allowing almost instantaneous equilibration of oxidoreductive agents between the bathing medium and the proteins of the cardiomyocytes. One needle was attached to a force transducer (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Ontario, Canada). The force measurements were performed at 15°C, and the average sarcomere length was adjusted to 2.2 μm, as described previously (20).

The compositions of the relaxing and activating solutions used during force measurements were calculated as described previously (19). 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. All the solutions for force measurements contained (in m*M*): Mg^{2+} 1, MgATP 5, phosphocreatine 15, and *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid 100. The ionic equivalent was adjusted to 150 m*M* with KCl resulting in an ionic strength of 186.

To modulate the SH status of the myocardial proteins *in vitro*, cardiomyocytes were incubated in 200-μl relaxing solutions supplemented with various concentrations of DTDP (Sigma, St. Louis, MO) for 2 min or 10 m*M* DTT (Eastman Kodak Company, Rochester, NY) for 30 min at 22°C. Control experiments and literature data suggested that these durations were sufficient to reach steady-state changes in the SH status of our preparations.

The isometric force production was measured in a set of Ca^{2+}-containing solutions. When a steady force level was reached, the myocyte was reduced in length by 20% within 2 msec and then quickly restretched. As a result, the force first dropped from the peak isometric level to zero (difference = total peak isometric force, F_{total}) and then started to redevelop (Fig. 1A). The force redevelopment after the restretch was fitted to a single exponential to estimate the rate constant of force redevelopment ($k_{tr,max}$) at pCa 4.75 under both control (before DTDP) and test conditions (*i.e.*, after exposure to various concentrations of DTDP or 10 m*M* DTT or both). About 6 sec after the onset of force redevelopment, the cardiomyocyte was returned to the relaxing solution, where a shortening to 80% of the original length with a long slack duration (8 sec) was performed to determine the passive force level ($F_{passive}$). The Ca^{2+}-activated isometric force (F) was calculated by subtracting the passive force from the total peak isometric force, determined at each Ca^{2+} concentration.

After the first activation at pCa 4.75, the resting sarcomere length was readjusted to 2.2 μm, if necessary. The sec-

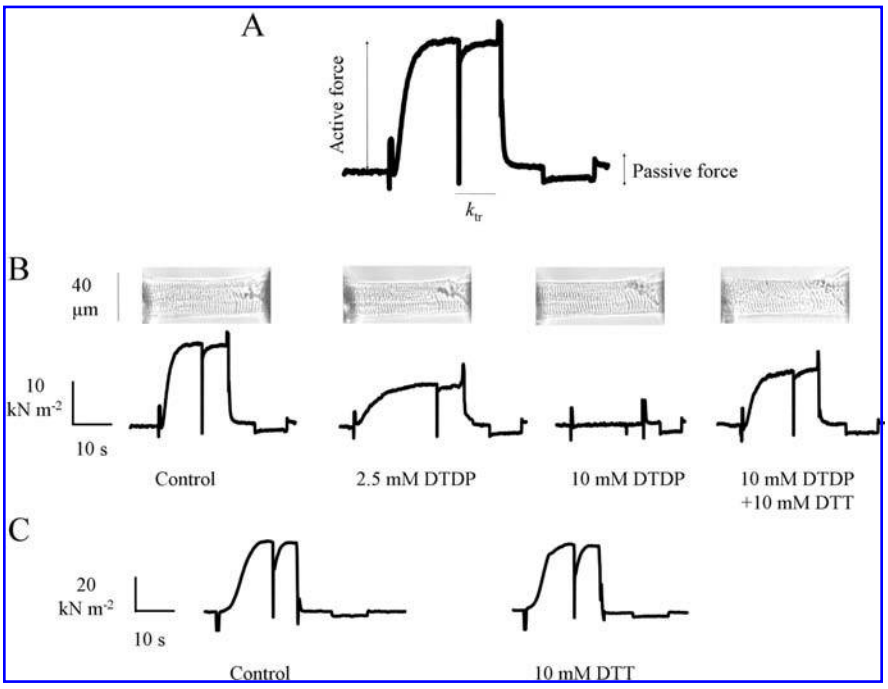

FIG. 1. Reversible force reductions on protein SH oxidation in permeabilized cardiomyocytes of human hearts. (**A**) Recording of a single Ca^{2+} contracture in an isolated cardiomyocyte for illustrative purposes during maximal Ca^{2+} activation. Ca^{2+}-activated active force and the rate constant of force redevelopment (k_{tr}) after unloaded shortening and restretch were recorded at *p*Ca 4.75; passive force was assessed at *p*Ca 9. (**B**) Active force in the absence of the SH oxidant DTDP (control) and after the sequential applications of 2.5 m*M* DTDP and 10 m*M* DTDP and 10 m*M* DTT in a single cardiomyocyte. (DTDP was applied for 2 min, and DTT for 30 min, both at *p*Ca 9.) The light-microscopic images above the force tracings illustrate the preserved cross-striation pattern in the same preparation. (**C**) DTT did not affect the force production in freshly isolated cardiomyocytes.

ond activation at *p*Ca 4.75 was used to calculate the maximal isometric force (F_o). The cells were subsequently exposed to a series of solutions with various concentrations of DTDP at *p*Ca 9.0, and subsequently to *p*Ca 4.75 without DTDP, to assess the concentration dependence of DTDP on F_o. To determine the mechanical consequences of protein SH oxidation on the Ca^{2+} sensitivity of isometric force production, the Ca^{2+}-force relations were also assessed at intermediate *p*Ca both before (control) and after 2.5 m*M* DTDP. The Ca^{2+}-activated force at submaximal levels of activation was normalized to that at maximal activation to characterize the Ca^{2+} sensitivity of isometric force production. When reexposure to *p*Ca 4.75 at the end of the test protocols yielded a value below 80% of the initial value, the measurements were rejected. To test reversibility, the preparations were also exposed to the reducing agent, DTT. In some cases, myocytes were treated only with DTT.

Determination of the SH status of myofilament proteins

To determine the SH content, permeabilized cardiomyocytes (prepared similarly to the mechanical measurements) were treated with increasing concentrations of DTDP or 10 m*M* DTT in isolation buffer or both at a protein concentration of 5 mg/ml at 22°C. Then SH content was determined by incubation with the SH-sensitive Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid); Sigma] for 15 min at 25°C (46). The absorbance of the solutions at 412 nm was considered to be proportional to their SH contents. The samples were assessed *via* calibration curves (standards: *N*-acetyl-L-cysteine and reduced glutathione; both from Sigma) fitted to a single exponential, and the SH contents of the cardiac samples were calculated.

Identification of SH groups oxidized in myofilament proteins

To identify myocardial proteins mediating the SH-dependent changes in the mechanical function, permeabilized cardiomyocytes were incubated in the presence of increasing concentrations of DTDP (0–30 m*M*) and/or 10 m*M* DTT in relaxing solution. Then the reagents were removed by three washing steps, and the protein concentrations were adjusted to 5 mg/ml. Subsequently, preparations were incubated in the presence of 60 μM (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (Pierce, Rockford, IL) at 25°C for 90 min to biotinylate the SH groups of the proteins. After biotinylation, the preparations were washed in isolation buffer 3 times and boiled in SDS-PAGE loading buffer (Sigma). The protein concentrations were tested by a dot-blot–based method; thereafter, 25 μg protein homogenates was applied to 6–18% gradient gels (Biorad, Hercules, CA) and subsequently transferred to nitrocellulose membranes. The membranes were blocked in 5% milk powder (1 h) and then incubated with a streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, CA) for 30 min. Bands representing biotinylated proteins at their free (reduced) SH groups were recorded on autoradiographic films, resulting in dark signals (Primax RTG-B, Berlin, Germany). Signal intensities were considered to be proportional with the free SH group contents of the respective proteins. In parallel experiments, gels were subjected to silver staining (26), as described previously (2), to facilitate the identification all of the proteins. The apparent molecular weights of proteins were determined by using prestained molecular weight standards (Fermentas, Burlington, Canada).

Immunoprecipitation

Immunoprecipitation assays were used to separate myosin light chain 1 (MLC1) and cardiac troponin I (cTnI) from bio-

tinylated cardiac protein mixtures prepared as described earlier. First these homogenates were diluted to 0.1 mg/ml in 1 ml of immunoprecipitation buffer (0.1% Triton X-100 in TBS). For MLC1 immunoprecipitation, diluted homogenates were incubated with 1 μg anti-MLC1 antibody (Sigma) or with the same amount of purified mouse IgG (control; Zymed Laboratories, San Francisco, CA) in the presence of 30 μl protein A/G agarose resin (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature. The resin-bound complexes were washed 3 times with the immunoprecipitation buffer and separated by centrifugation (1,800 rpm, 1 min). The immunocomplexes (washed pellets) were boiled in SDS sample buffer (Sigma) for 10 min, and each sample was divided into two to determine the biotinylated protein content and the efficiency of the immunoprecipitation. To detect MLC1 precipitation, the same anti-MLC1 antibody was diluted to 1:50,000 and used in Western immunoblotting. For the detection of the biotinylated proteins, the previously mentioned method (streptavidin-peroxidase conjugate) was used.

For the cTnI immunoprecipitation, 20 μl iron-conjugated anti-cTnI antibody (capture antibody, Liaison kit; Diasorin, Saluggia, Italy) was added to the homogenates. After incubation for 1 h at room temperature, the cTnI-antibody complexes were separated above a magnetic plate and washed 3 times with an appropriate washing buffer (wash buffer, Liaison kit; Diasorin). The magnetic particle–bound proteins were boiled in SDS sample buffer and divided into two to determine the biotinylated protein content and the efficiency of the immunoprecipitation, as described earlier for the MLC1 assay. To detect cTnI immunoprecipitation, an anti-cTnI antibody (Clone 16A11; RDI, Concord, MA) was used (dilution, 1:10,000) in Western immunoblotting.

Data analysis

Ca^{2+}-force relations were fitted to a modified Hill equation:

$$F = F_o\,[Ca^{2+}]^{nHill}/(Ca_{50}{}^{nHill} + [Ca^{2+}]^{nHill})$$

where F is the steady-state force at a given $[Ca^{2+}]$, whereas F_o, n_{Hill} and Ca_{50} (or pCa_{50}) denote the maximal Ca^{2+}-activated force at saturating $[Ca^{2+}]$, the slope and the midpoint of the sigmoidal relation, respectively. The results of the measurements for each cardiomyocyte were fitted individually, and the parameters were averaged. Western immunoblot assays were performed in triplicate, and typical results are shown.

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) and, where applicable, by Student's t test. Values are given as mean ± SEM. The number of experiments in each group varied between three and 31 from three to five different hearts. Statistical significance was accepted at $p < 0.05$.

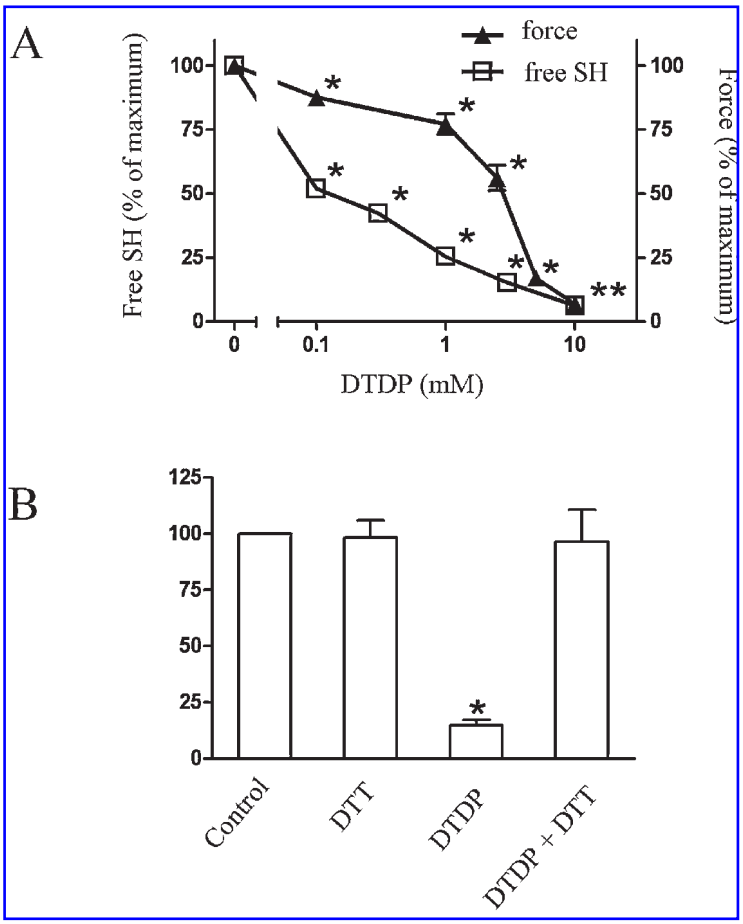

FIG. 2. Parallel changes in maximal Ca^{2+}-activated force and free (reduced) SH contents in permeabilized human cardiomyocytes. (**A**) The DTDP concentration dependences of the maximal Ca^{2+}-activated force and the amount of free SH groups relative to their control (0 m*M* DTDP). Error bars illustrate SEM when they are bigger than symbol size of the means. (**B**) The column bars illustrate the changes in free SH contents relative to the control (100%) in cardiomyocytes after 10 m*M* DTT, 2.5 m*M* DTDP, or 2.5 m*M* DTDP, followed by 10 m*M* DTT treatments, respectively, from *left* to *right*. The bars indicate the averages of $n = 3$ determinations. *$p < 0.05$ *vs.* the control.

RESULTS

The effects of SH oxidation on both active and passive forces and on the cross-bridge–sensitive rate constant of force redevelopment (k_{tr}) (see Fig. 1A) were investigated during repeated isometric Ca^{2+} contractures in permeabilized left ventricular cardiomyocytes of human hearts. Fig. 1B illustrates that exposure to increasing concentrations of the oxidative DTDP for 2 min decreased the maximal Ca^{2+}-activated force (F_o) in a graded fashion. However, even after the application of the highest DTDP concentration (10 m*M* in this case, which eventually diminished force to zero), 10 m*M* DTT (30 min) 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 (Fig. 1B). Moreover, DTT alone did not change F_o (27 ± 5 kN/m^2 before and 25.6 ± 4.6 kN/m^2 after DTT exposure; $n = 5$; Fig. 1C).

The effect of the DTDP concentration on the maximal Ca^{2+}-activated force was compared with that on the myocardial SH content by using the SH-sensitive Ellman's reagent in myocardial protein homogenates (Fig. 2). This assay revealed that increasing concentrations of DTDP decreased the SH content in the myocardial proteins along with the DTDP-evoked reduction in force. Of note, experimental conditions that included experimental temperature were largely comparable in protein homogenates and in cardiomyocyte preparations. Figure 2A shows that most of the force decline appeared at relatively low SH levels. DTDP-evoked SH oxidation was reversed by DTT: the SH content after 2.5 m*M* DTDP was 14.8 ± 2.4%, and after subsequent 10 m*M* DTT exposure, was 96.6 ± 14.1% (both expressed relative to the control in the same preparations). Alone, the reducing agent, 10 m*M* DTT, did not change the oxidative status, as the SH content after 10 m*M* DTT was 98.2 ± 7.6% of that of the control (Fig. 2B).

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 m*M*), which caused a reduction of ~50% in the maximal force (Fig. 3A). 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 exposure (Fig. 3B). This

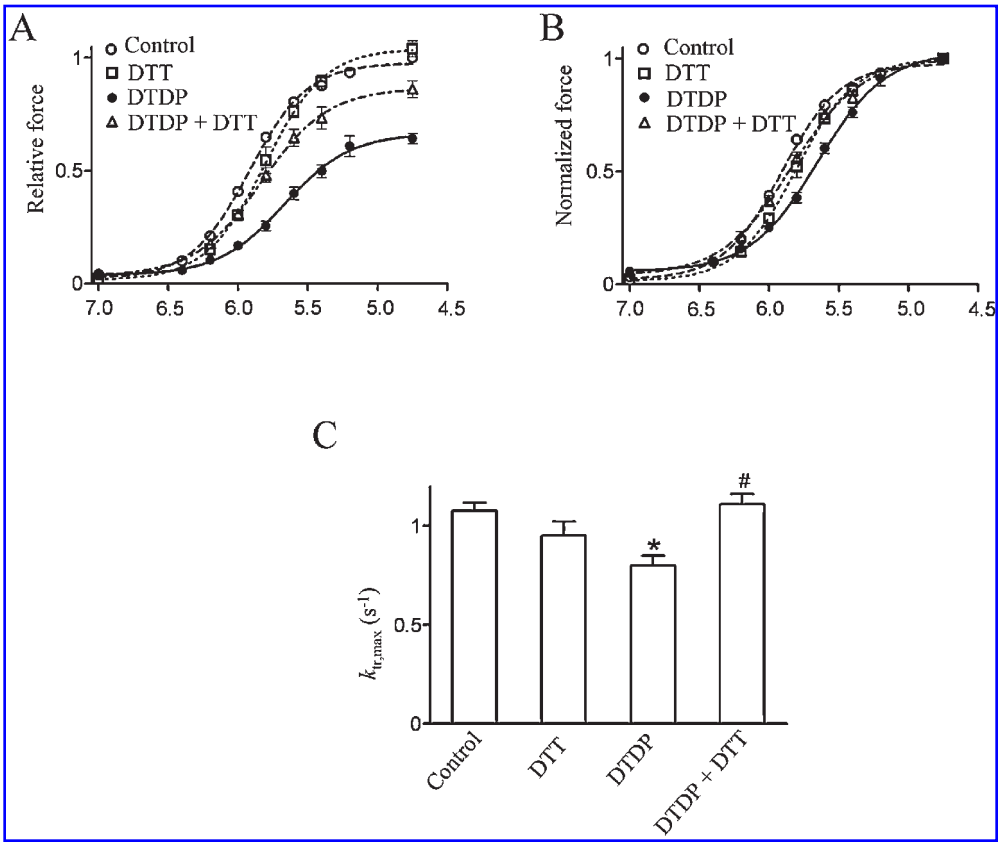

FIG. 3. The effects of SH group oxidation and reduction on the myofibrillar mechanics. The relative (**A**) and normalized (**B**) Ca^{2+}-force relations indicate that 2.5 m*M* DTDP decreased the Ca^{2+}-activated force at all applied Ca^{2+} concentrations, and the Ca^{2+} sensitivity of force production. The 10 m*M* DTT did not have a significant effect on the Ca^{2+}-sensitivity curve in the control cardiomyocytes, whereas it restored the force and its Ca^{2+} sensitivity to a large degree after DTDP. The influences of 10 m*M* DTT, 2.5 m*M* DTDP, or 2.5 m*M* DTDP followed by 10 m*M* DTT on the maximal turnover rate ($k_{tr,max}$) of the actin–myosin cycle (**C**). *$p < 0.05$ *vs*. the control: #$p < 0.05$ *vs*. DTDP. Results were obtained from $n = 31$ cardiomyocytes.

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 relation ($\Delta pCa_{50} = 0.22 \pm 0.02$; $p < 0.01$ *vs*. the control)]. Application of the reducing agent DTT (10 m*M*) sequentially after DTDP restored both F_o (to 86.1 ± 0.1% of the control F_o; $p < 0.05$ *vs*. DTDP) and its Ca^{2+} sensitivity ($\Delta pCa_{50} = 0.06 \pm 0.02$ *vs*. the control; $p < 0.05$ *vs*. DTDP) to a large degree (Fig. 3A and B). The application of DTT alone did not alter either the active force or its Ca^{2+} sensitivity. The Hill coefficients of the normalized Ca^{2+}-sensitivity curves were 2.01 ± 0.08 for the control cardiomyocytes, 2.07 ± 0.16 after DTT, 1.76 ± 0.08 after incubation with 2.5 m*M* DTDP ($p < 0.05$ *vs*. the control), and 1.72 ± 0.12 after 2.5 m*M* DTDP followed by 10 m*M* DTT ($p < 0.05$ *vs*. the control). The passive force assessed in relaxing solution (control: $F_{passive} = 2.6 \pm 0.3$ kN/m^2) did not change on DTT treatment, but it was increased slightly by DTDP and remained elevated when DTT was applied after DTDP ($F_{passive,DTDP} = 2.8 \pm 0.2$ kN/m^2, and $F_{passive,DTDP+DTT} = 3.5 \pm 0.5$ kN/m^2; both significant *vs*. the control). 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 per second to 0.8 ± 0.05 per second at 2.5 m*M* DTDP ($p < 0.05$), but a subsequent DTT treatment induced a full reversion in this parameter ($k_{tr,max} = 1.11 \pm 0.05$ per second). In addition, as for all other mechanical parameters, DTT alone did not modulate $k_{tr,max}$ (Fig. 3C). Control mechanical experiments in the absence of DTDP and DTT ($n = 3$) suggested that the observed reversible changes in the mechanical parameters could not be associated with preparation rundown, as pCa_{50}, $k_{tr,max}$, n_{Hill}, and $F_{passive}$ did not change during repeated Ca^{2+}-force relation determinations. However, the partial recovery in F_o after DPDT and DTT was probably complicated by this factor, because the same magnitude of F_o reduction was observed after similar long protocols in the absence of DTDP and DTT (not shown).

As the next step, screening was performed to identify SH changes at the level of individual proteins on oxidation (by DTDP) and subsequent reduction (by DTT) by using myocardial homogenates prepared similarly to that for the measurements of contractile force. Proteins containing SH groups were biotinylated and visualized by Western immunoblotting (Fig. 4). The staining intensities decreased in parallel with the progression of S-thiolation at increasing DTDP concentrations (0–30 m*M*) in a qualitatively similar fashion with the results obtained with the Ellman's reaction (see Fig. 2A). 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 and 1 m*M*, where force did not decrease to a large degree (Fig. 2A). Moreover, treatment with higher DTDP concentrations (1–10 m*M*) where most of the contractile alterations were detected, the SH oxidation involved four prominent proteins, with apparent molecular masses of 130, 54, 45, and 26 kDa. Furthermore, complete oxidation of the proteins with molecular massess of 45 and 26 kDa was observed at these critical DTDP concentrations, whereas oxidation of the other two proteins with higher molecular masses were only partial, even at 30 m*M* DTDP. The complete oxidation of the 45- and 26-kDa proteins together with the large decrease in F_o suggested a close relation between the oxidative status of these proteins and myofibrillar force production.

The apparent molecular mass of these 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 (Fig. 5). The bands developed by antibodies against cTnI

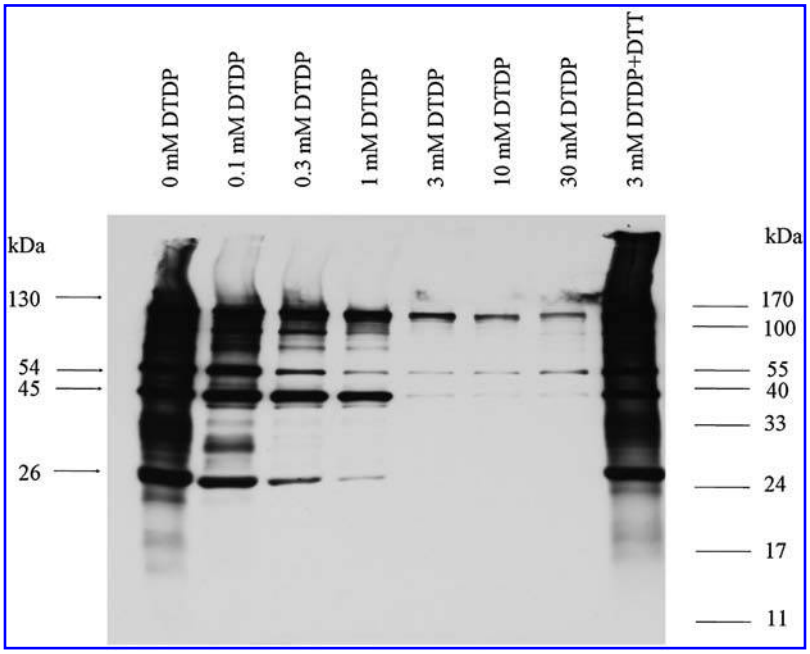

FIG. 4. Western immunoblot analysis of human myocardial proteins after exposure to increasing concentrations of DTDP. The decrease in staining intensity is proportional to the extent of oxidation of the protein SH groups. 10 m*M* DTT after the application of 3 m*M* DTDP restored the oxidized SH in all proteins. *Arrows*, Proteins whose oxidation coincided with the development of contractile dysfunction. Three independent assays provided identical results.

(Fig. 5B) or MLC1 (Fig. 5D) 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) (Fig. 5A and C), it was apparent that cTnI was completely oxidized at the lowest concentration of DTDP applied here (0.1 m*M*), 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 (Fig. 4). 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 that possessed DTDP susceptibility similar to that of MLC1 was identified by Western immunoblot analyses and silver-stained gels as actin (not shown). On the basis of their molecular masses and previous findings, the protein bands at 130 and 54 kDa might well reflect the myosin-binding C-protein and desmin, respectively (18, 22, 41).

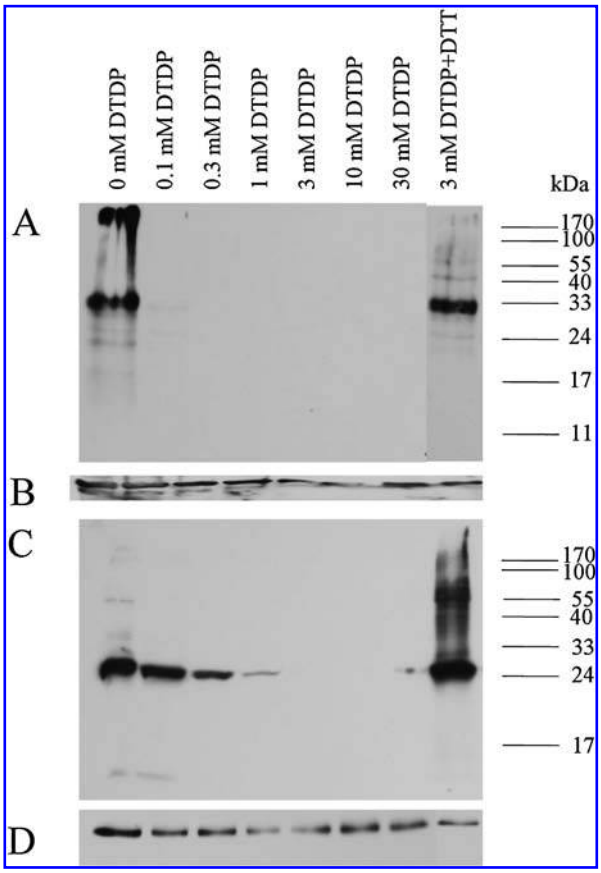

FIG. 5. Identification of the 26-kDa protein as MLC1. Comparable amounts of DTDP-treated biotinylated proteins were first immunoprecipitated by antibodies against either cTnI or MLC1, and then subjected to gel-electrophoresis and Western immunoblotting. Successful immunoprecipitations were verified by using antibodies against cTnI (**B**) or MLC1 (**D**). The results in (**A**), where antibodies against the biotinylated SH groups were used in parallel, indicate that the SH groups of cTnI were fully oxidized by 0.1 m*M* DTDP. In contrast, the DTDP concentration-dependent SH oxidation of the immunoprecipitated MLC1 (**C**) was reminiscent of the concentration dependence at the 26-kDa protein level in Fig. 4. The 10 m*M* DTT restored the native SH status of both cTnI and MLC1. Immunoprecipitation assays were performed in triplicate.

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 robust alterations in the oxidative status of several myocardial proteins are devoid of a direct effect on maximal Ca^{2+}-activated force. However, 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. Based on these data, we propose a plausible molecular mechanism for the contractile depression in association with myocardial SH redox changes.

Myocardial ischemia/reperfusion injury is characterized by oxidative stress and decreased contractility. Previous model studies of oxidative injury in animal hearts demonstrated that numerous myocardial proteins serve as targets with more than a single type of chemical alteration at their amino acid residues (17). It is noteworthy that the carbonylation of lysine, arginine, and proline is considered to be irreversible, whereas the formation of inter- and intramolecular disulfides is considered to be reversible. If they occur in contractile proteins or in proteins regulating intracellular Ca^{2+} homeostasis (28, 35) or both, these modifications (16) are of direct relevance as concerns the myocardial mechanics. The significance of carbonyl group formation in combination with SH oxidation in actin and tropomyosin was particularly emphasized in recent studies on postischemic hearts of rats, pigs, and dogs (8, 9, 17, 44). Moreover, biochemical evidence suggests that oxidative modifications modulate the architecture of actin filaments (37) and of myosin (52), which implies a role for oxidized actin and myosin in the postischemic myocardial dysfunction.

In this study, we focused on the functional consequences of the SH-dependent component of oxidative myocardial injury and tested the relation between the extent of protein SH group oxidation and Ca^{2+}-regulated force production. 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 after the sequential application of DTDP (7) and DTT verified the SH specificity of our model approach. To the best of our knowledge, this is the first study in which the relation between selective SH oxidation and myofibrillar mechanical function has been demonstrated in human myocardial preparations. 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 are compatible with the findings of earlier animal experiments (10, 17) and stress that the contractile proteins are predominantly present in their reduced form under physiologic conditions.

In rat postischemic cardiac hearts, proteins of the cytosol, membrane, and myofilament/cytoskeletal compartments have all served as major substrates for S-thiolation after ischemia and reperfusion (17). In full agreement with the results of our model approach, these *in vivo* data also suggested SH-dependent 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 in which the protein SH oxidation was uptitrated to a level at which the active force production was zero. This finding is in clear contrast with those previous results of our group in which the sarcomeric structure and force were both deteriorated by *in vitro* incubation with the nitrogen oxide metabolite peroxynitrite (5), or with the proteolytic enzyme μ-calpain (41) in an irreversible fashion. These distinctions thus suggest 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 >50% of the SH groups had to be oxidized for a sensible reduction in force. Our biochemical assays pointed to the complete oxidation in only two contractile proteins (with apparent molecular masses of 45 and 26 kDa) at DTDP concentrations at which the force decline was most prominent (*i.e.*, between 1 and 10 m*M*). Pursuing the idea of the critical involvement of specific proteins, we made efforts to identify these two proteins. The molecular mass, the abundance, and Western immunoblot results suggested that the protein at 45 kDa is actin. Ample evidence exists in animal models for a possible role of oxidized actin on ischemia and reperfusion (8, 17, 37, 44). 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.

Similar to our findings, a reduction in the Ca^{2+} sensitivity of force production was also noted in permeabilized skeletal muscle fibers of the rat and the toad after DTDP exposures (30, 43). We point out, however, that depending on the chemical nature of the SH oxidant and on the affected myofilament proteins, other types of mechanical responses may also develop. For example, with rodent cardiac preparations with intact membranes, it was recently documented that after the applications of nitroxyl (HNO), the one-electron product of nitric oxide, contractile performance increased through SH oxidation (12, 49). Besides decisive alterations in the Ca^{2+} transients, these studies also suggested an increase in the maximal Ca^{2+}-activated force. It can be argued, however, that HNO most probably targeted proteins bearing only highly reactive thiols and that the extent of HNO-evoked SH oxidation was small. In our hands, the DTDP-evoked decrease in Ca^{2+}-activated force and in its Ca^{2+} sensitivity developed together with a marked reduction in the actin–myosin turnover rate and in the amount of protein SH groups. It is noteworthy that all of these changes were effectively reversed after the normalization of the protein SH status, suggesting a close relation between all these variables (6). It should be also acknowledged, however, that the relatively small DTDP-evoked changes in passive force and *n*Hill could not be reverted by DTT. These implied partial restoration in some of the DTDP-affected protein conformations that were associated with the elastic properties and the cooperative force-generating actin–myosin interactions within the myofilaments. One of the possible explanations for this finding could relate to the giant trans-sarcomeric elastic protein titin. We speculate that titin oxidation affected passive tension and contractile activation, and that restoration of the conformation of this large molecule by DTT was partial within the sarcomere. The molecular weight of titin was, however too large to resolve on our SDS-polyacrylamide gels; hence, titin oxidation was not investigated here.

Taken together, our data suggest that besides actin, MLC1 may also contribute to the redox-sensitive regulation of myofibrillar force in the human heart. This supposition is corroborated by the present view on myofibrillar mechanics that signifies the role of MLC1 in the fine-tuning of the myosin motor function through the modulation of the magnitude of force production and cross-bridge kinetics (27, 31, 51). Conversely, the involvement of actin and MLC1 are based only on indirect correlative data. In this respect, identification and mutation analysis of DTDP- and DTT-susceptible cysteinyl residue(s) in actin and MLC1 are awaited to reveal the molecular mechanism of the physiologic effects. Moreover, we acknowledge that we cannot exclude the contribution of several other proteins, most notably myosin-binding protein C and desmin. It may be of interest that a reduction in the Ca^{2+}-activated force and its Ca^{2+} sensitivity has been proposed as a characteristic feature for the reversible postischemic myocardial depression, also known as myocardial stunning (24, 25). Proteolytic fragmentation of cTnI (23, 38), together with oxidative myofilament protein alterations (4), appear to be the most probable mechanisms to explain this myocardial pathology. Here, we report that SH oxidation in a few myofilament proteins, other than cTnI, also has the potential to impair Ca^{2+}-activated force and its Ca^{2+} sensitivity, and hence this mechanism may potentially represent another facet of postischemic myocardial dysfunction.

The conversion of SH groups to disulfides and other oxidized species is one of the earliest events during the radical-mediated oxidation of proteins (16), and the myocardial protein SH content decreases characteristically during ischemia and reperfusion. It is of interest that protein-SH content was reduced by >50% in rabbit hearts exposed to 60 min of low-flow ischemia (10). Moreover, clinical data suggested that the severity of the ischemic period and the oxidative stress during reperfusion can be linked with a delay in postoperative recovery of cardiac function in patients with coronary artery disease who are subjected to heart surgery (21). Accordingly, clinical observations indicated that *N*-acetylcysteine might be useful in the treatment of ischemic and reperfusion injury in acute myocardial infarction (1, 11, 34, 47). The intimate relation between the Ca^{2+} regulation and the myofilament SH status suggests that the preservation of effective antioxidant mechanisms is of direct relevance for the actin–myosin interaction in the human heart. Our data suggest that the SH oxidation in a few contractile proteins is of paramount significance in the preservation of Ca^{2+}-regulated force production during myocardial oxidative injury.

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ABBREVIATIONS

[Ca^{2+}], Ca^{2+} concentration; cTnI, cardiac troponin I; DTDP, 2,2′-dithiodipyridine; DTT, dithiothreitol; EC_{50}, concentration evoking half-maximal effect; F, isometric force; F_o, force at saturating [Ca^{2+}]; $F_{passive}$, passive force level; F_{total}, total peak isometric force; $k_{tr,max}$, rate constant of force redevelopment at saturating [Ca^{2+}]; MLC1, myosin light chain 1; n_{Hill}, Hill coefficient; pCa, $-\log[Ca^{2+}]$; $p$$Ca_{50}$, the Ca^{2+} sensitivity of isometric force production; SEM, standard error of the mean; SH, sulfhydryl; ROS, reactive oxygen species; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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