Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts

Marcella Canton,1 Irina Neverova,2 Roberta Menabò, Jennifer Van Eyk,3 and Fabio Di Lisa1
1Dipartimento di Chimica Biologica and 2Sezione Biomembrane, Istituto di Neuroscienze del Consiglio Nazionale delle Ricerche, Università di Padova, 35121 Padova, Italy; and 3Departments of Physiology and Biochemistry, Queen’s University, Kingston, Ontario, Canada K7L 3N6

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Canton, Marcella, Irina Neverova, Roberta Menabò, Jennifer Van Eyk, and Fabio Di Lisa. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. Am J Physiol Heart Circ Physiol 286: H870–H877, 2004; 10.1152/ajpheart.00714.2003.—Although the contribution of reactive oxygen species to myocardial ischemia is well recognized, the possible intracellular targets, especially at the level of myofibrillar proteins (MP), are not yet fully characterized. To assess the maximal extent of oxidative degradation of proteins, isolated rat hearts were perfused with 1 mM H2O2. Subsequently, the MP maximally oxidative damage was compared with the effects produced by 1) 30 min of no-flow ischemia (I) followed in other hearts by 3 min of reperfusion (I/R); and 2) I/R in the presence of a potent antioxidant N-(2-mercapto-3-pionyl)glycine (MPG). Samples from the H2O2 group electrophoresed under nonreducing conditions and probed with actin, desmin, or tropomyosin monoclonal antibodies showed high-molecular mass complexes indicative of disulfide cross-bridges along with splitting and thickening of tropomyosin and actin bands, respectively. Only these latter changes could be detected in I/R samples and were prevented by MPG. Carbonyl groups generated by oxidative stress on MP were detected by Western blot analysis (oxyblot) under optimized conditions. The analyses showed one major band corresponding to oxidized actin, the density of which increased 1.2-, 2.8-, and 6.8-fold in I, I/R, and H2O2 groups, respectively. The I/R-induced increase was significantly reduced by MPG. In conclusion, oxidative damage of intracellular targets, especially at the level of myofibrillar proteins (MP), is not yet fully characterized. To assess the maximal extent of oxidative degradation of proteins, isolated rat hearts were perfused with 1 mM H2O2. Subsequently, the MP maximally oxidative damage was compared with the effects produced by 1) 30 min of no-flow ischemia (I) followed in other hearts by 3 min of reperfusion (I/R); and 2) I/R in the presence of a potent antioxidant N-(2-mercapto-3-pionyl)glycine (MPG). Samples from the H2O2 group electrophoresed under nonreducing conditions and probed with actin, desmin, or tropomyosin monoclonal antibodies showed high-molecular mass complexes indicative of disulfide cross-bridges along with splitting and thickening of tropomyosin and actin bands, respectively. Only these latter changes could be detected in I/R samples and were prevented by MPG. Carbonyl groups generated by oxidative stress on MP were detected by Western blot analysis (oxyblot) under optimized conditions. The analyses showed one major band corresponding to oxidized actin, the density of which increased 1.2-, 2.8-, and 6.8-fold in I, I/R, and H2O2 groups, respectively. The I/R-induced increase was significantly reduced by MPG. In conclusion, oxidative damage of MP occurs on reperfusion, although at a lower extent than in H2O2-perfused hearts, whereas oxidative modifications could not be detected in ischemic hearts. Furthermore, the inhibition of MP oxidation by MPG might underlie the protective efficacy of antioxidants.

actin; tropomyosin; oxidative stress; protein carbonylation; free radicals

Among the factors involved in the ischemic damage of the heart, few have received as much attention as the formation of reactive oxygen species (ROS). Once considered as major determinants of myocardial damage (13, 30), ROS are attracting novel interest owing to their possible roles in signal transduction (14, 19). The potential activation of several kinases by ROS has been proposed as part of a complex set of processes by which cells responding to stresses activate self-defense mechanisms. In this respect a slight increase in ROS formation has been proposed to contribute to myocardial preconditioning (34, 42, 51) and hibernation (6). Thus a large production of ROS is likely detrimental as opposed to the beneficial action exerted by a mild oxidative stress.

Besides the multifaceted relationship with the maintenance of cell viability, ROS formation has been proposed as a pivotal mechanism underlying contractile dysfunction, such as myocardial stunning (4). This reversible failure of contraction occurring on postischemic reperfusion has been proposed to depend on covalent changes of myofibrillar proteins (MP) (4). Indeed, the contractile defect can also be detected in chemically skinned myocytes and is attributed to a reduced sensitivity of the MP to Ca2+ (4, 21, 23, 25). Several lines of evidence support the involvement of ROS in stunning (5, 31, 40, 46). For instance, in keeping with a crucial role of MP alterations, the addition of the superoxide anion to isolated myofilaments was found able to reduce the maximal calcium-activated force (29). However, scarce information is available concerning both the identity of the affected proteins and the modalities of their modifications.

It is well known that the oxidative processes might affect the side chains of most amino acid residues (12). Cysteine and methionine residues are particularly sensitive to oxidation by ROS. Inter- and intramolecular disulfides generated from cysteine and sulfoxide from methionine are the only oxidative modifications that can be enzymatically repaired (3). The S-thiolation of myocardial proteins during ischemia and reperfusion has been elegantly addressed by using biotinylated glutathione and cysteine in recent papers (15, 16). Besides these reversible modifications, several irreversible modifications have been described to occur at the level of other residues, such as dityrosine formation, protein carbonylation, and nitrotyrosylation (12). In addition, proteins can be covalently modified by products of lipid peroxidation as demonstrated in ischemic hearts (17, 18).

The most widely studied modification induced by oxidative stress is the formation of carbonyl groups, which especially affects lysine, arginine, and proline (36). The importance of carbonylation in the ischemic-reperfused (I/R) heart is further highlighted by a recent study that ruled out the increase of protein nitrotyrosylation (35).

The detection of carbonyls is based on their reaction with 2,4-dinitrophenylhydrazine (DNPH), which produces the corresponding hydrazone; the oxidized protein can be detected by Western blot analysis with anti-dinitrophenyl (DNP) antibodies, a procedure known as oxyblot (27). Thus the assay of carbonyl groups in proteins provides a convenient technique for detecting and quantifying oxidative modification induced by physiological and pathological conditions (see Ref. 3 and

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references therein). However, the significance and the reliability of this method may be affected by conceptual and technical shortcomings (41). The present study was aimed at investigating the modalities of MP oxidation in rat hearts subjected to I/R protocols, 2) quantifying the extent of protein carbonylation, and 3) identifying the mostly affected proteins. The procedure for MP isolation and the oxyblot technique were optimized to minimize oxidation artifacts and coupled with the assessment of disulfide bonds using two-dimensional nonde- naturating/denaturing gels. The results indicate that actin undergoes major changes showing both reversible and irreversible modifications, as detected by the formation of carbonyl groups and the oxidation of cysteiny1 residues, which also affects tropomyosin. The degree of protein oxidation is inferior to that induced by H2O2 and is strongly attenuated by the antioxidant N-(2-mercaptopropionyl)glycine (MPG).

**MATERIALS AND METHODS**

Isolated heart perfusion. All aspects of animal care and experimentation were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and Italian regulations concerning the care and use of laboratory animals and were approved by the Ethical Committee of the University of Padova.

Male Wistar rats of 3 mo (180–200 g) were used. Hearts were rapidly excised and placed in cold (4°C) bicarbonate buffer, and the aorta was cannulated. The hearts were perfused with bicarbonate buffer gassed with 95% O2-5% CO2 at 37°C (pH 7.4). Perfusion was performed in the nonrecirculating Langendorff mode, as previously described (2). The bicarbonate buffer contained (in mM) 118.5 NaCl, 3.1 KCl, 1.18 KH2PO4, 25.0 NaHCO3, 1.2 MgCl2, 1.4 CaCl2, and 10.0 glucose. Hearts were not paced, and left ventricular pressure was monitored by means of a latex balloon inserted into the left ventricle and connected to a Statham transducer (P-2306) (2).

After a 10-min equilibration period, hearts were either perfused under normoxic conditions in the absence or presence of 1 mM H2O2 for 15 min or made ischemic by stopping the coronary flow for 30 min. During ischemia (I), hearts were maintained in a thermostatically controlled chamber filled with bicarbonate buffer at 37°C. In another group of hearts, ischemia was followed by 3 (I/R3) or 30 min (I/R30) of reperfusion.

The preischemic administration of the antioxidant MPG was examined in the I/R protocol. Antioxidant solutions were prepared immediately before use by dissolving MPG to a final concentration of 1 mM in bicarbonate buffer and maintained throughout the perfusion protocol.

The perfusion protocols were terminated by clamping the hearts with tongues precooled in liquid nitrogen. The samples were used immediately or stored under liquid nitrogen until analysis. Each perfusion protocol was repeated at least six times.

Myofibrillar protein extraction. MP were isolated in the presence of protease inhibitors, and the entire procedure was performed at 4°C. Briefly, 0.2 g of frozen ventricles were homogenized in 2 ml of ice-cold “antimyosin” buffer containing 25 mM imidazole and 5 mM EDTA (pH 7.2), added with an antiprotease mixture (10 μM leupeptin, 10 μM pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). The solutions were prepared with bidistilled water to limit free metal ion contamination. Just before use, the solution was stirred under vacuum and then bubbled with argon to maximally reduce the oxygen tension.

The classic procedure for MP extraction by means of Triton (33) was proven not to be adequate for DPNH derivatization, especially for the quantitation of the oxidative damage (data not shown). Indeed, Triton extraction generates a suspension, whereas the derivatization requires a homogenous solution. Thus we used an alternative method for MP extraction based on dropping the pH to 2.3 using 1% trifluoroacetic acid (TFA) (1, 32). The lower pH during the next derivatization step resulted in a very high background signal. Therefore, we further adapted the method by decreasing TFA concentration to 0.01%, which still allowed exclusive extraction of MP, but because the pH was maintained at >5.0, the derivatization process worked without increasing the background.

The protein suspension was centrifuged at 12,000 g for 10 min by means of a Beckman microcentrifuge, and the resulting pellet was resuspended with a Polytron homogenizer in 200 μl of a solution containing 0.01% TFA and protease inhibitors, previously bubbled with argon. The supernatant collected after a second centrifugation for 5 min was a protein solution enriched in MP (4–5 μg/μl). Aliquots of this extract were assayed for protein concentration (Bradford Bio-Rad Protein Assay, Bio-Rad Laboratories).

Oxyblot procedure. DPNH derivatization was carried out in a solution containing 6% SDS-1 mM DPNH-5% TFA, mixing 1 volume of protein solution in 6% SDS with 1 volume of DNP-H-TFA stock solution, as described by Levine et al. (27). The reaction was stopped immediately or after incubation times ranging from 2 to 50 min by neutralization with 2 M Tris base and 30% glycerol. The same samples were prepared in the absence of DNP-H as a control.

One-dimensional electrophoresis was promptly carried out on 12% SDS-polyacrylamide resolving gels at 4°C with 6.5 μg of derivatized-protein loaded per lane. Dinitrophenylated protein molecular mass standards were purchased from Intergen. Proteins were transferred to 0.45-μm pore-size nitrocellulose membranes at 400 mA constant current for 2 h (49) and were then stained with Red Ponceau S (Sigma Chemical). The membranes were blocked by incubation with 3% BSA for 1 h and then incubated for 90 min at room temperature with anti-DNP (1:100, Intergen). Blots were washed three times for 10 min and were subsequently incubated for 1 h with peroxidase-labeled anti-rabbit immunoglobulins (at 1:300 dilution). Blots were developed by using a chemiluminescence detection system. Densitometry was performed on scanned gels by using the IPLab Gel computer program for the Macintosh (Signal Analytics).

To quantitate the amount of oxidation and allow the comparison between the various samples, we defined the actin oxidation index (AOI). This parameter results from the ratio between densitometric values of the oxyblot bands and those of the corresponding bands stained with Red Ponceau S. To compare different experiments, AOI is normalized by setting this index equal to 1 for the control samples at time 0.

Immunoblots in one-dimensional and two-dimensional nonreducing-denaturing electrophoresis. MP (4–5 μg/μl) were denatured by boiling in 2% SDS, 5% glycerol, and 125 mM Tris-HCl (pH 6.8) in the presence of 1% β-mercaptoethanol. This procedure, referred to as the reducing condition, was compared with the nonreducing condition obtained by avoiding the addition of β-mercaptoethanol. To avoid artifacts due to the oxidation of thiol groups in vitro, nonreducing conditions were performed in the presence of 45 mM iodoacetamide.

The formation of disulfide bonds was further investigated by means of two-dimensional nondenaturing-denaturing electrophoresis (43). The first-dimensional electrophoresis was performed as single-dimen- sional electrophoresis under nonreducing conditions. After electrophoresis, the gel containing the separated polypeptides was excised and soaked in loading buffer with 5% β-mercaptoethanol at room temperature for 30 min and then subjected to SDS-PAGE in the second dimension at right angles to the first under reducing conditions and stained with Coomassie blue or subjected to immunoblotting. With this technique the proteins forming disulfide linkages before the reduction step (i.e., the second dimension) are identified as spots falling off the diagonal.

For immunoblotting analyses, 12 μg of protein per lane were loaded on 12% SDS-polyacrylamide resolving gels. After electroblotting was completed, the membranes were probed with the following monoclonal antibodies: 1) anti-tropomyosin CH1 clone (Sigma...
Among these modifications, other oxidative modifications affect proteins irreversibly. Among these modifications, the most characterized is by far the formation of carbonyl groups, which can be detected by means of nonreducing electrophoresis (Fig. 3).

**Effect of H₂O₂ perfusion on the carbonyl content of MP.** Besides the potentially reversible oxidation of cysteinyl residues, other oxidative modifications affect proteins irreversibly. Among these modifications, the most characterized is by far the formation of carbonyl groups, which can be detected by means of oxyblot. This procedure involves the covalent addition of DPNH to any available carbonyl groups (27). However, the specificity of such a simple reaction can be lost when the assay conditions are not properly controlled. Indeed, the prolongation of the reaction for periods of time exceeding 10 min might result in underestimation of the differences between control and modified samples or in the appearance of bands not representative of carbonyl formation in the oxyblots (Fig. 4A). To determine which signals are spurious, it is thus necessary to follow the kinetic of DPNH reaction of each sample. At time 0 (i.e., the reaction stopped by the neutralizing solution immediately after DPNH addition), any signal observed is probably indicative of unspecific binding. During the first 10 min the reaction proceeds gradually, increasing the density of the protein band in the oxyblot (Figs. 4A and 5A). Prolonging the duration of derivatization, we have found that side reactions other than hydrazone linkage occur, as shown by the appearance of multiple bands visible after 50 min (Fig. 4A). Additional sources of errors might be contributed by the extraction procedure of MP. We found that artificial MP oxidation is

### Table 1

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Fig. 1. Detection of disulfide cross-bridges in actin, tropomyosin, and desmin induced by H₂O₂ perfusion. Myofibrillar proteins (MP) were extracted from rat hearts that were perfused in the absence [control (CT)] or in the presence of 1 mM H₂O₂ (H₂O₂) for 15 min. Isolated myofibrillar samples were denatured in the absence (nonreducing condition) or in the presence (reducing condition) of β-mercaptoethanol and then analyzed by Western blot probed with antibodies to actin, tropomyosin, and desmin. High-molecular mass bands were detected by Western blot analysis after H₂O₂ perfusion (arrows) and can be attributed to disulfide cross-bridges because they were present only in nonreducing conditions. In addition, H₂O₂ perfusion induced thickening of actin band and splitting of tropomyosin band.
markedly decreased by the combination of nitrogen bubbling
and EDTA addition to the extraction buffer (Fig. 4B) as well as
the use of lower concentrations of TFA to solubilize the
proteins (not shown).

The oxyblot analysis shown in Fig. 5A demonstrates that
perfusion with 1 mM \( \text{H}_2\text{O}_2 \) for 15 min induces an irreversible
modification of MP in the isolated rat heart due to carbonyl
formation. The perfusion with 0.1 mM \( \text{H}_2\text{O}_2 \) for the same
duration did not produce any detectable change, whereas the
prolongation of the perfusion time or the increase of \( \text{H}_2\text{O}_2 \)
concentration did not induce further augmentation of MP
oxidation (data not shown) as no additional MP were modified.

To quantitate the level of oxidation of a particular protein,
we developed an index score, termed the oxidative index,
which was suitable and adaptable for comparison among dif-
ferent samples and experimental conditions (i.e., \( \text{H}_2\text{O}_2 \) vs. I/R).
To this aim the densitometric analysis of the oxyblot bands
(Fig. 5A) was normalized to the densities of the corresponding
bands of the same samples in the Red Ponceau-stained blots
(Fig. 5B). This procedure gives a ratio of densities, which is not
influenced by the amount of proteins loaded in the gels and
takes into account minimal differences in protein loading.
These ratios were further normalized to the value of time 0 in
the control samples made equal to 1. This additional normal-
ization was proven to be necessary, because the absolute values
of densities were slightly different among the various blots,
whereas multiple lanes performed with the same sample or
different samples from the control group did not show appreci-
able variations in density values (not shown). Because only
one band corresponding to actin (identified by immunoblot, not
shown) was visible in the oxyblot, the final ratio was termed
“actin oxidation index” (AOI, Fig. 5C). The values of this
index increased in the samples from \( \text{H}_2\text{O}_2 \)-perfused hearts
depending on the duration of the reaction with DPNH, whereas
no increase was observed in the control samples, indicating
that the low level of density in the oxyblot was due to
unspecific reactions (Fig. 5C).

**MP carbonylation during reperfusion.** Figure 6A illustrates
the formation of carbonyls in MP samples extracted from rat
hearts subjected to ischemia and posts ischemic reperfusion. In
most of the experiments, the duration of reperfusion was
limited to 3 min because the burst of ROS production occurs
in the very early phase of posts ischemic reperfusion (4). From
the kinetics of DPNH derivatization shown in Fig. 5, the analysis
was carried out by performing the time 0 and a derivatization
time of 10 min for each sample. Figure 6A, left, shows typical
eXamples of the oxyblots obtained after derivatization of the
samples for 10 min and demonstrates that ischemia without
reperfusion did not produce major changes in carbonyl forma-
tion, whereas after 3 min of reperfusion, a substantial increase
in the oxyblot densities could be detected. Similar to what was
observed in \( \text{H}_2\text{O}_2 \)-perfused hearts, in the reperfused hearts only
one band was visible corresponding to actin, the density of
which slightly increased by prolonging the reperfusion dura-
tion to 30 min (Fig. 6C). The AOI assessment (Fig. 6A, right,
and C) indicates that the increase produced by reperfusion is
less than half that caused by \( \text{H}_2\text{O}_2 \) perfusion. Importantly, the
carbonyl formation associated with reperfusion was almost
completely prevented when the hearts were perfused in the
presence of 1 mM MPG, an antioxidant, starting 5 min before

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Fig. 3. Effect of MPG pretreatment on actin and tropomyosin after ischemia (I) and reperfusion (R). MP were extracted from rat hearts that were normoxically perfused (CT) and subjected to 30 min of no-flow ischemia followed by reperfusion for 3 min in the absence (IR3) or in the presence of mercapto-
propionylglycine (IR3 + MPG). After MP extraction, aliquots of each sample
were denatured in the absence (nonreducing condition) or in the presence
(reducing condition) of \( \beta \)-mercaptoethanol and then analyzed by Western blot
analysis probed with tropomyosin monoclonal antibody or actin monoclonal
antibody. Of note, MPG inhibited MP oxidative changes.
the onset of ischemia (Fig. 6B). The data on protein carbonylation are summarized in Fig. 6C, indicating that AOI increased 2.8-, 3.3-, and 6.8-fold in I/R3-, I/R30-, and H<sub>2</sub>O<sub>2</sub>-perfused hearts, respectively, whereas AOI in ischemic or MPG-treated hearts was not significantly different from that of normoxic hearts.

According to a well-established notion, postischemic reperfusion was associated with a marked reduction in left ventricular developed pressure (LVDP, 31.6 ± 10.2% of preischemic values, n = 6). MPG perfusion hearts significantly increased the degree of LVDP recovery (60.2 ± 15.5%, P < 0.01, n = 6), confirming the ability of this antioxidant compound to afford myocardial protection (47). Supporting the concept of a maximal oxidative stress, H<sub>2</sub>O<sub>2</sub> perfusion induced a gradual decrease in LVDP concomitant with a rise in diastolic pressure, which starting after 5 min, reached a plateau (105 ± 10 mmHg, n = 6) after 13 min. At 15 min, when the hearts were removed for biochemical analyses, LVDP was no longer detectable.

**DISCUSSION**

The present results demonstrate that postischemic reperfusion causes the oxidation of MP, which appears to specifically involve actin and tropomyosin. The oxidation of these proteins involves reversible and irreversible processes and occurs to a lesser extent than that caused by H<sub>2</sub>O<sub>2</sub>. In all cases, the...
Fig. 6. MP carbonylation and contractile dysfunction induced by ischemia and postischemic reperfusion and protection by antioxidant N-(2-mercaptopropionyl)glycine (MPG). A: MP were extracted from rat hearts that were normoxically perfused (CT), subjected to 30 min of no-flow ischemia (I), or reperfused for 3 min after 30 min of ischemia (IR3). Myofibrillar extracts were DNPH derivatized for 10 min to detect protein carbonylation. Oxylot analysis shows a carbonyl increase only in samples from reperfused hearts. Carbonyls were detected in a band corresponding to actin (left) and quantitated as described in Fig. 5 (right). B, right, typical result of six different experiments. The average data and the statistics are shown in C. B: rat hearts were reperfused for 3 min after 30 min of ischemia in the absence (IR3) or in the presence of mercaptopropionylglycine (IR3 + MPG) and compared with normoxically perfused hearts (CT). Myofibrillar extracts were DNPH derivatized for 10 min. The oxylot (left) demonstrates that MPG (1 mM) attenuates the formation of protein carbonyls. Right, quantitative analysis that illustrates a typical result of six different experiments. Average data and the statistics are shown in C. C: changes in actin oxidation induced by the various perfusion protocols and normalized to control hearts. Values are means ± SE of 6 experiments. *P < 0.05 statistical difference with respect to control hearts.

oxidative modifications are largely attenuated by the presence of antioxidants.

Potentially reversible oxidations. Our results confirm and then extend previous studies performed in vitro and in situ indicating actin as a major target of oxidative modifications among MP (8–10, 35, 39). In particular, experiments performed on isolated actin exposed to H₂O₂ or HOCl display a wide array of biochemical and functional modifications (10). However, the modifications observed in vitro may differ from the changes occurring in situ. In fact, actin is in extensive contact with other proteins, and some of these interactions change depending if muscle is relaxed or contracted (26).

Besides these differences between in vitro and in situ processes, the identification of MP oxidation in situ is hampered by various technical problems. Because several biochemical assays or procedures that can be performed on isolated proteins would provide uninterpretable or meaningless results if applied to protein mixtures, such as MP, the number of possible assays is dramatically reduced (11).

We adapted classic procedures of electrophoresis that were never used for the study of the ischemic injury to characterize oxidative changes affecting cysteiny l residues. In particular, according to well-established protocols (38), SDS-PAGEs were performed under reducing and nonreducing conditions. Along with modifications of actin, this procedure allowed us to provide the first demonstration of tropomyosin oxidation in situ, as demonstrated by the appearance of a second band (doublet) under nonreducing SDS-PAGE of samples from reperfused and H₂O₂ perfused hearts, which was not present in control hearts. Furthermore, this latter protocol was associated with the appearance of additional changes in nonreducing SDS-PAGE, especially high-molecular mass bands, that were again not detected in samples from ischemic and reperfused hearts. Thus, as also confirmed by the quantitative assessment of the carbonyl formation, these qualitative changes suggest that postischemic reperfusion is far from producing a maximal degree of oxidation of MP.

The fact that in samples from reperfused hearts the native bands were modified without the appearance of high-molecular mass complexes rules out the formation of protein-protein disulfides and rather suggests the generation of mixed disulfides, i.e., protein glutationylation. This kind of covalent change, which may also explain the variation in the affinity for the antibodies displayed by the immunoblots in Fig. 3, has been recently investigated by using a cell-permeant biotinylated form of glutathione in isolated cell studies (45). As far as tropomyosin is concerned, the formation of mixed disulfides appears highly probable, because the sequence indicates the presence of a single cysteine residue (28), thus excluding the possibility of intramolecular disulfide bridges.

Irreversible oxidations and methodological issues. Besides potentially reversible oxidations, irreversible modes of MP oxidation were also investigated. The generic detection of carbonyl formation is the most used procedures for the detection of protein oxidation in situ (3, 20, 27).

Initially this procedure was applied to the study of whole heart homogenates by means of spectrophotometric detection (37). Although this procedure allowed the detection and quantitation of “general” protein oxidation in the postischemic reperfused hearts, the proteins affected could not be identified. This issue was addressed only recently exploiting the oxylot technique (35, 39). However, there are technical challenges involved with oxylot. For instance, carbonyl derivatives are not only generated by amino acid oxidation but can be also formed as a consequence of secondary reactions of some amino acids with 4-hydroxynonenal or reducing sugars. In addition, DPNH could give rise to unspecific reactions even with nonoxidized proteins by means of hydrophobic interactions or addition to glycosyl residues (3, 44). In addition, we found that a significant amount of DPNH binds independently of protein oxidation as can be shown by stopping the reaction immedi-
ately after DPNH addition (Fig. 5). On the other hand, the detection procedure could be flawed by prolonging the incubation of the proteins with the reactant (Fig. 4A). When these sources of errors are overlooked, the degree of oxidation in untreated samples can be emphasized, the differences between control and damaging conditions can be reduced, and proteins stained unspecifically can be taken into account. We suspect that examples of these pitfalls in the procedure for carbonyl staining may be recognized in recent papers that addressed the oxidation of proteins in hearts exposed to I/R protocols (35, 39). In fact, not only the increase in actin staining induced by reperfusion was less than twofold (35), but also actin was stained together with several other bands. However, the density of these bands was only slightly modified by reperfusion, indicating the occurrence of unspecific reaction(s) (39).

According to previously suggested guidelines (41), by limiting the incubation time to 10 min, we obtained oxyblots showing a large prevalence of actin along with the appearance of very few other bands faintly stained. Therefore, we documented an increase in the degree of actin oxidation determined by postischemic reperfusion that was more than threefold and was related to the maximal oxidation (sevenfold) produced by the administration of H$_2$O$_2$ in the perfusion buffer. It has to be pointed out that the quantitative assessment of a given process can hardly provide useful information on the severity of that same process if it is not related to its maximal extent. To our knowledge, for the first time in our study the extent of oxidative degradation of proteins in the I/R heart is compared with the effects produced by an oxidant at high doses to override the endogenous antioxidant defenses of the cardiac myocyte. This strategy is amenable not only to establish the maximal extent of the oxidative damage but also to characterize the type of oxidative changes induced by a given condition.

In reperfused hearts, we observed that the degree of actin carboxylation is slightly increased with prolonging the reperfusion duration from 3 to 30 min (Fig. 6C). It is tempting to speculate that such an increase might be ascribed to a lack of removal of damaged proteins owing to a possible failure of the proteasome, which has been reported to occur on reperfusion (7).

**Significance and consequences of myofibrillar protein oxidation.** The present results show that in the reperfused heart reversible types of oxidative changes, such as those involving cysteinyl residues, are concomitant with irreversible modifications, such as carbonyl formation, which require the proteolytic removal followed by the resynthesis of the affected protein for the recovery of its function. Both actin and tropomyosin modifications are likely to cause mechanical dysfunction as expected from their critical location in the contractile machinery and as suggested by the pathologies associated with their mutations (24, 50). The oxidative modifications that affect several functional features of actin in vitro (10) are likely to hamper the contractile function in situ, supporting the increased formation of oxyl radicals as a causative mechanism underlying myocardial stunning (4). The proposal of this mechanism is mostly based on evidence obtained through a pharmacological approach whereby the duration of stunning is greatly reduced by the administration of antioxidants. The protection afforded by antioxidants could result from actions exerted at sites different from MP, i.e., membranes and proteins involved in energy metabolism and tonic homeostasis.

The decrease in actin oxidation induced by MPG administration while supporting the involvement of protein oxidation in stunning suggests that antioxidants may accelerate the recovery of contraction on reperfusion by acting directly at the level of MP.

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**GRANTS**

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