Oxidative Modification and Inactivation of the Proteasome during Coronary Occlusion/Reperfusion*

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Restoration of blood flow to ischemic myocardial tissue results in an increase in the production of oxygen radicals. Highly reactive, free radical species have the potential to damage cellular components. Clearly, maintenance of cellular viability is dependent, in part, on the removal of altered protein. The proteasome is a major intracellular proteolytic system which degrades oxidized and ubiquitinated forms of protein. Utilizing an in vivo rat model, we demonstrate that coronary occlusion/reperfusion resulted in declines in chymotrypsin-like, peptidylglutamyl-peptide hydrolase, and trypsin-like activities of the proteasome as assayed in cytosolic extracts. Analysis of purified 20 S proteasome revealed that declines in peptidase activities were accompanied by oxidative modification of the protein. We provide conclusive evidence that, upon coronary occlusion/reperfusion, the lipid peroxidation product 4-hydroxy-2-nonenal selectively modifies 20 S proteasome α-like subunits iota, C3, and an isosform of XAPC7. Occlusion/reperfusion-induced declines in trypsin-like activity were largely preserved upon proteasome purification. In contrast, loss in chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities observed in cytosolic extracts were not evident upon purification. Thus, decreases in proteasome activity are likely due to both direct oxidative modification of the enzyme and inhibition of fluorogenic peptide hydrolysis by endogenous cytosolic inhibitory protein(s) and/or substrate(s). Along with inhibition of the proteasome, increases in cytosolic levels of oxidized and ubiquitinated protein(s) were observed. Taken together, our findings provide insight into potential mechanisms of coronary occlusion/reperfusion-induced proteasome inactivation and cellular consequences of these events.

Restoration of coronary blood flow to previously ischemic cardiac tissue is often associated with declines in cardiac function, including myocardial stunning, ventricular arrhythmias, hemodynamic abnormalities, and, in the long term, development of heart failure (1, 2). This paradoxical phenomenon, broadly termed ischemia/reperfusion injury, is accompanied by dramatic increases in tissue levels of free radicals as well as byproducts of lipid peroxidation (1–9). Due to the high reactivity of these species (10–12), it has been proposed that free radical events play a key role in myocardial ischemia/reperfusion injury. Nevertheless, mechanisms by which free radicals alter cardiac function have not been fully elucidated. Free radical modification of protein can alter and, in many cases, inhibit, protein activity (10–15). In addition, oxidatively cross-linked protein(s) are resistant to proteolytic degradation (16–20). Thus, the presence of oxidized protein could impact cellular function by changing the catalytic and/or regulatory properties of specific proteins and by placing abnormal demands on finite cellular volume. Clearly, the level of oxidatively modified protein reflects the balance between free radical damage and proteolytic degradation. It is therefore important to investigate the response of proteases involved in the degradation of oxidized protein to coronary ischemia/reperfusion.

Oxidatively modified proteins are preferentially degraded in vitro by the 20 S proteasome in an ATP-independent fashion (18, 21–24), while covalent attachment of ubiquitin marks protein for ATP-dependent degradation by the 26 S proteasome (25–29). Both forms of the proteasome cleave substrate proteins at the carboxyl end of basic (trypsin-like), hydrophobic (chymotrypsin-like), and acidic (peptidylglutamyl-peptide hydrolase) amino acids (25–29). The 20 S proteasome is the catalytic core of the 26 S proteasome and is comprised of four heptameric rings arranged coaxially. The subunits of the 20 S core are classified into α and β subfamilies, with non-catalytic α-heptamers forming each of the two outer rings and catalytic β-heptamers forming the two inner rings (25–29). The 26 S proteasome is formed upon association of a multisubunit regulatory protein, termed PA700, with the α subunits on the 20 S proteasome. PA700 confers ATP/ubiquitin-dependent proteolytic properties to the 26 S proteasome (25–29). Proteasome function has been implicated in a diverse set of cellular processes, including turnover of oxidized (18, 21–24), gap junction (30), and myofilibrillar proteins (31), regulation of NFκB-dependent stress response pathways (32, 33), and, more recently, apoptosis (34, 35). Thus, alterations in proteasome activity during coronary occlusion/reperfusion would be expected to significantly impact a number of cellular events, thereby influencing the outcome of cardiac ischemia/reperfusion.

The current study was undertaken to characterize the effects of coronary occlusion/reperfusion on proteasome peptidase activities and to begin to assess how alterations in proteasome function may contribute to the development of ischemia/reperfusion injury. Our results demonstrate that in vivo coronary occlusion/reperfusion induced significant declines in proteasome function. This was accompanied by selective free radical derived modification of specific proteasome subunits. Increases in the cytosolic levels of oxidatively modified and ubiquitinated

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proteins occurred commensurate with declines in proteasome peptidase activities. Mechanisms by which oxidative modification of the proteasome leads to enzyme inactivation are discussed in light of previous in vitro findings. In addition, potential physiological consequences of loss of proteasome activity are related to observed increases in cytosolic levels of oxidized protein and declines in the ability of myocytes to degrade ubiquitinated protein.

**MATERIALS AND METHODS**

In *Vivo Model of Coronary Occlusion/Reperfusion*

In a modification of a previously described procedure (36), male Harlan-Sprague Dawley rats were anesthetized by intramuscular administration of a mixture of xylazine, ketamine/HCl, and acepromazine (3:3:1) (0.5–0.75 ml/kg). To ensure the stability of blood pH throughout the experiments, a solution composed of 0.9% NaCl, 8.4% NaHCO₃, 50% dextrose (4:3:3) was injected intraperitoneally (200–300 ml/kg) 30 min prior to administration of anesthesia. An endotracheal tube was inserted and used for ventilation with oxygen-supplemented room air using a positive pressure respirator (5–15 mm Hg). Body temperature was maintained at 37°C using a rectal probe attached to a thermostermometer and an appropriate heating lamp. The heart was exposed by performing a midline thoracotomy. After the animal had been allowed to stabilize for 15 min, a ligature was placed around the left anterior descending coronary artery close to its origin from the aorta. The ends were exteriorized and passed through polyethylene tubing. Coronary occlusion was achieved by pressing the tube against the heart muscle while pulling on the ligature followed by clamping the tube with a hemostat. This was accomplished by immediate transection of the left ventricular free wall and a marked drop in blood pressure. Reflow was initiated by releasing the ligature. This was accompanied by immediate hyperemia of the left ventricular free wall as well as a marked increase in heart rate and blood pressure. Experimental conditions tested were: 1) 90 min of control perfusion; and 2) 30 min of LAD coronary occlusion followed by 60 min of reperfusion (*n* = 5 rats for each procedure).

**Preparation of Cytosolic Fractions**

Cytosolic fractions were prepared from the left ventricular free wall following each experimental protocol. Briefly, hearts were excised and the left ventricular free wall rapidly removed and minced. Tissue was then immersed and rinsed in cold homogenization buffers (180 mM KCl, 5 mM MOPS, 2 mM EDTA, pH 7.25). This was followed by homogenization in 25 ml of homogenization buffer of ventricle with a Polytron homogenizer (low setting, 3 s). The homogenate was then centrifuged at 500 × g for 5 min at 4°C yielding a pellet corresponding to crude nuclear fraction. The 5000 x g supernatant was centrifuged at 5000 x g for 10 min at 4°C. The 5000 x g supernatant represents the crude cytosolic fraction. Protein determinations were made using the BCA system (Bio-Rad). Proteins were stained with silver nitrate and the gels were run for 650 V-h at 4°C. Polyacrylamide gels were used for separation of the cytosolic fractions and Western blot detection of particular proteins.

**Purification of the 20 S Proteasome**

The 20 S proteasome was purified from cytosolic extracts obtained from left ventricular homogenate as previously described, with minor modifications (18, 37, 39, 40). Prior to purification of the proteasome, respective cytosolic extracts prepared after 90 min of control perfusion (*n* = 5) or 30 min of coronary occlusion followed by 60 min of reperfusion (*n* = 5) were pooled. Briefly, pooled cytosolic fractions containing 5 mM 2-mercaptoethanol were subjected to an ammonium sulfate cut (35 and 60% saturation). The 60% ammonium sulfate pellet was resuspended in 20 mM Tris-HCl, 10 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.2, and dialyzed at 4°C against the same buffer. Purification was monitored by SDS-PAGE. Purified 20 S proteasome was prepared by ion exchange chromatography over a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech) and gel filtration chromatography on a Superose 6 column (Amersham Pharmacia Biotech) utilizing a Beckman Gold liquid chromatograph.

**One-dimensional SDS-PAGE and Western Blot Analysis**

Protein samples were resolved by SDS-PAGE (Novex Minigel II). For detection of protein-associated carbonyl groups, samples were treated with 2,4-dinitrophenylhydrazine, as summarized below, prior to resolution of protein(s) by SDS-PAGE. The polyacrylamide gel content of gels for particular experiments was as indicated in the figure legends. Typically, one gel of a series was stained with Coomassie Blue. The second gel was used to electrolot protein onto a nitrocellulose membrane. Blots were processed according to standard protocols with minor modifications, depending on the primary antibody utilized, as detailed below.

**Native Gel Electrophoresis and Western Blot Analysis**

Proteasome complexes present in cytosolic extracts were analyzed by native gel electrophoresis followed by activity staining or immunoblotting. Cytosolic proteins were resolved by polyacrylamide gel electrophoresis under non-denaturing conditions as described (41, 42). The proteins were then run on a runs of stacking and running gels of 8% and 10% acrylamide, respectively, and the gel was run for 650 V-h at 4°C. The gel was incubated in 50 mM Tris, 25 mM KCl, 10 mM NaCl, 1.0 mM MgCl₂, 1.0 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, at pH 7.8 for 15 min at 37°C. The gel was then mounted on a light box and bathed in the same solution containing 100 μM LLVY-MCA and 5.0 mM ATP. Activities of the proteasome complexes were visualized after exposure of the gel to substrate for 30.0 min. For Western blot experiments, proteins were electrolot blotted onto nitrocellulose membrane and probed with appropriate primary antibodies. For both activity and Western blot staining, assignment of proteasome complexes was accomplished by comparison with purified 20 S and 26 S complexes.

**Two-dimensional Gel Electrophoresis and Western Blot Analysis**

The first dimension was performed utilizing immobilines Drystrips (pH 3–10, length 13 cm) in a Mini-PROTEAN II focusing system (Amersham Pharmacia Biotech). A 15-μg aliquot of purified proteasome was diluted in sample buffer (9 μl urea, 2% Chaps, 2% Pharmalytes pH 3–10, 20 mM dithiothreitol, and bromphenol blue). The Drystrip was rehydrated in this solution in a Re-swelling Tray (Amer- sham Pharmacia Biotech) overnight at room temperature and then focused for 50,000 V-h (23 h). After focusing, the Immobiline Drystrips were equilibrated for 10 min in equilibration buffer (50 mM Tris-HCl, pH 6.8, 6 mM urea, 30% (v/v) glycerol, 1% w/v SDS) supplemented with 1% (v/v) dithiothreitol followed by 10 min in equilibration buffer containing 2.5% (v/v) iodoacetamide. The second dimension, SDS-PAGE, was performed using a 12% (v/v) gel and the Protease II electrophoresis system (Bio-Rad). Proteins were stained with silver nitrate and the gel digitized utilizing a JX-330 Sharp scanner. Spot detection and quanti-
Oxalblot kit. 2,4-dinitrophenylhydrazine utilizing Intergen reagents and protocols on protein samples with was a gift from Mathias Kroll (Institut Pasteur, Paris, France).

**Immunoblotting and Methods**

One- and two-dimensional Western blots were evaluated utilizing a series of primary antibodies, as indicated. Blocking buffer was composed of 0.2% (w/v) 1-block (Tropix) in PBS supplemented with Tween 20 (0.05% w/v). Washing buffer was comprised of phosphate-buffered saline/Tween 20. Primary antibody binding was visualized using an alkaline phosphatase or horseradish peroxidase based chemiluminescence assay (Tropix and Amersham). As indicated, control experiments were performed in which normal rabbit serum was utilized instead of primary antibodies.

**Anti-proteasome Antibodies**

Polyclonal antibody raised against purified 20 S proteasome has previously been prepared (rabbit host) (40). Antibodies specific to proteasome subunits C2 (MCP 20), C3 (MCP 21), XAPC7 (MCP 34), and Rpt1 (ATPase subunit of PA700) were obtained from Affiniti Research Products Limited. Antibody IIB5, which is specific for the iota subunit, was a gift from Mathias Kroll (Institut Pasteur, Paris, France).

**Anti-DNP Antibodies**—Prior to gel electrophoresis, protein-associated carbonyls were derivatized by treatment of protein samples with 2,4-dinitrophenylhydrazine utilizing Intergen reagents and protocols (Oxyblot kit).

**Anti-HNE Antibodies**—We have previously prepared and characterized polyclonal antibodies specific to 1:1 amino acid-HNE Michael adducts (rabbit host) (43).

**Anti-ubiquitin Antibodies**—Polyclonal antibodies (rabbit host) specific to ubiquitin were obtained from StressGen and Dako.

**RESULTS**

**Coronary Occlusion/Reperfusion-induced Alterations in Proteasome Peptidase Activities**—The effects of occlusion/reperfusion on peptidase activities of cardiac proteasome were evaluated utilizing an in vivo rat model in which coronary occlusion was induced by ligating the left anterior descending coronary artery and reperfusion was initiated by removal of the ligature. In this study, anesthetized rats were subjected to 90 min of instrumented perfusion or 30 min of coronary occlusion followed by 60 min of reperfusion. Upon completion of each experimental protocol, the left ventricle was homogenized and cytosolic fractions were prepared. Chymotryptsin-like, peptide- and tryptophyl-peptide hydrolase, and trypsin-like activities of the proteasome present in the cytoplasmic milieu were evaluated utilizing the fluorogenic peptides LLVY-MCA, LLE-NA, and LSTR hydrolyzing activities after perfusion were 934.0 ± 94.5, 831.0 ± 71.3, and 191.0 ± 38.0 pmol/min/mg, respectively. For all conditions tested, n = 5. Values represent the mean ± S.D. p values (t test) for occlusion/reperfusion versus perfusion are: * p ≤ 0.0004; † p ≤ 0.002; ‡ p ≤ 0.01.

of free radical production (1–9). It was therefore of interest to determine whether the proteasome is a target for oxidative modification(s). This was accomplished by evaluating changes in the relative levels of 2,4-dinitrophenylhydrazine-reactive carbonyl groups present on the proteasome purified from left ventricular homogenate following coronary perfusion and occlusion/reperfusion. Carbonyl functional groups can be introduced into proteins by a variety of oxidative processes, including direct oxidation of amino acid side chains and reaction of lipid peroxidation products with nucleophilic residues (10–12, 14, 15, 18, 43, 45, 46). Furthermore, HNE has been reported to increase in concentration during cardiac ischemia/reperfusion (9). Thus, reperfusion-induced free radical events are likely to be mediated, in part, by HNE-derived modifications to protein. Utilizing antibodies specific to 1:1 amino acid-HNE Michael adducts (43), we demonstrated increases in the HNE content of the 20 S proteasome following coronary occlusion/reperfusion (Fig. 2). As with protein carbonyls, HNE modification was selective to specific proteasome subunits. Thus, free radical-derived modifications to the proteasome occur during coronary occlusion/reperfusion.

**HNE-Derived Modifications to Specific Subunits of the 20 S Proteasome**—To gain further insight into potential mechanisms by which coronary occlusion/reperfusion induces declines in proteasome peptidase activities, the subunit composition of purified 20 S proteasome and the occurrence of HNE-derived modifications to specific subunits were evaluated by two-dimensional gel electrophoresis/Western blot analysis. Because detection of protein carbonyls requires prior derivatization with 2,4-dinitrophenylhydrazine, a process which alters the isoelectric properties of modified subunits, we chose to evaluate HNE-derived modifica-
tions to specific subunits. In these experiments, the identities of 20 S proteasome subunits were assigned based on two-dimensional Western blot analyses of proteasome purified from rats which had not undergone surgery, utilizing antibodies specific to distinct 20 S proteasome subunits. As determined by silver staining, 14 proteins were clearly visible for all 20 S proteasome preparations analyzed (Fig. 3, A and B). The subunit pattern appeared similar after both perfusion and occlusion/reperfusion. However, as estimated by densitometric analysis, coronary occlusion/reperfusion diminished the relative intensity of silver staining of three 20 S proteasome subunits, β-like subunit C10 and α-like subunits iota and C2 (level of significance set to 2-fold variation in intensity). An increase in the relative silver staining intensity of an isoform of the α-like subunit XAPC7 was also observed (level of significance set to 2-fold variation in intensity). Although changes in the silver staining intensities of four subunits were evident, the protocol utilized for 20 S proteasome purification has previously been shown to yield stoichiometric recovery of subunits and, for each preparation analyzed, equal amounts of purified 20 S complex were used (40). Therefore observed declines in the intensity of silver staining suggest coronary occlusion/reperfusion-induced modification to specific subunits. As depicted in Fig. 3C, three proteasome subunits were selectively modified by HNE upon coronary occlusion/reperfusion: iota, C3, and an isoform of XAPC7. Each of these HNE-modified proteins are α-like subunits which are known to interact with at least one of the catalytic β-like subunits X, Y, and/or Z (44). No antibody binding was detected in analyses of purified proteasome from perfused animals (not shown). These data suggest that alteration of proteasome subunits and, more specifically, selective modification of particular subunits by the lipid peroxidation product HNE may contribute to proteasome inactivation observed upon occlusion/reperfusion.

**Coronary Occlusion/Reperfusion-induced Alterations in Peptidase Activities of Purified 20 S Proteasome**—It has previously been shown that proteins are cross-linked by HNE and other free radical processes and can act as inhibitors of the proteasome (16–20). Thus, the presence of protein substrates and/or inhibitors in cytosolic extracts may block accessibility of catalytic sites to fluorogenic peptides thereby limiting measurable activity. If these factors contribute to declines in peptidase activities observed in cytosolic extracts, inhibition should be relieved upon proteasome purification. As shown in Fig. 4, occlusion/reperfusion-induced declines in chymotrypsin-like and peptidyglycyl-amidyl-peptide hydrolase activities were not evident in purified 20 S proteasome. In contrast, trypsin-like activity remained depressed (55%) upon protein purification. Thus, the respective catalytic activities of the proteasome are differentially susceptible to distinct mechanisms of inhibition.

**Coronary Occlusion/Reperfusion-induced Alterations in the Relative Distribution of 26 S and 20 S Proteasome Complexes**—In addition to events which affect the structure/function of the catalytic core, observed declines in proteasome peptidase activities could arise from dissociation of the 26 S complex. To test this possibility, cytosolic proteins were resolved by native gel electrophoresis followed by activity staining for 26 S and 20 S proteasome utilizing the fluorogenic peptide LLVY-MCA. As shown in Fig. 5, the proteasome was present primarily as the 26 S complex in extracts prepared from perfused tissue. Upon occlusion/reperfusion, a signal corresponding to 20 S proteasome became evident (Fig. 5A). These observations indicate that occlusion/reperfusion results in an increase in the ratio of 20 S to 26 S complexes. Activity staining analyses are, however, qualitative in nature since staining intensities are influenced by a number of factors including differential accessibilities of the fluorogenic peptide to the respective proteasome complexes. We therefore sought further information on the relative level of 26 S proteasome present in
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Fig. 4. Peptidase activities of purified 20 S proteasome. Peptidase activities of 20 S proteasome purified from left ventricular free wall (2.0 μg of purified enzyme per assay) after 90 min of sham-operated perfusion (P) or 30 min of occlusion followed by 60 min of reperfusion (R) were measured. Chymotrypsin-like, peptidylglutamyl-peptide hydrolase, and trypsin-like activities of proteasome were evaluated utilizing the fluorogenic peptides LLVY-MCA, LLE-NA, and LSTTR-MCA, respectively. Activities are presented as a percent of values obtained for proteasome purified from cardiac tissue after coronary perfusion (mean ± S.D., n = 4 for each experimental protocol). *, p ≤ 0.01 for trypsin-like activity (two-tailed t test).

Fig. 5. Evaluation of the relative distribution of proteasome complexes. Cytosolic extracts were prepared from left ventricular free wall after 90 min of sham-operated perfusion (P) or 30 min of occlusion followed by 60 min or reperfusion (R). As described under “Materials and Methods,” the extracts were evaluated by native gel electrophoresis and evaluated by activity staining with the fluorogenic peptide LLVY-MCA (100 μM) (Panel A) or Western blot analysis utilizing antibodies specific to Rpt1, a subunit of PA700 complex of the 26 S proteasome (Panel B). The data shown is representative of three separate cytosolic extracts each, for coronary perfusion or occlusion/reperfusion.

Extracts from perfused and occluded/reperfused cardiac tissue. As judged by Western blot analyses utilizing antibodies specific to Rpt1, a subunit of PA700 (44), no appreciable change in cytosolic levels of 26 S complex was observed (Fig. 5B). Thus, coronary occlusion/reperfusion appears to result in slight alterations in association of the 26 S proteasome complex.

Oxidative Modification and Ubiquitination of Cytosolic Protein—Direct oxidation is known to mark protein(s) for degradation by the 20 S proteasome in vitro (18, 21–24). One might therefore expect that the environment created during coronary occlusion/reperfusion coupled with declines in proteasome activity would result in the appearance of oxidized forms of protein in the cytosol. Thus, we sought information on relative alterations in the levels of oxidized cytosolic protein(s) due to coronary perfusion and occlusion/reperfusion. As shown in Fig. 6B, coronary occlusion/reperfusion induced a distinct increase in cytosolic levels of oxidatively modified protein. Oxidative modification was not global in nature, but appeared specific to distinct protein bands, some of which exhibited a greater degree of oxidation than others (Fig. 6B). In addition to in vitro degradation of oxidized protein by the 20 S proteasome (18, 21–24), it is well established that covalent attachment of ubiquitin marks protein(s) for degradation by the 26 S proteasome (25–28). Declines in the peptidase activities of the proteasome are likely to impair proteolysis of ubiquitinated protein(s). We therefore evaluated changes in the relative cytosolic levels and molecular weight distribution of ubiquitinated protein resulting from coronary occlusion/reperfusion. As judged by Western blot analysis using anti-ubiquitin antibodies, we observed a distinct increase in the cytosolic content of ubiquitinated protein (Fig. 6D). Although high molecular weight smears of ubiquitinated proteins were not observed, ubiquitination status was evaluated after a single duration of reperfusion (60 min) during which proteasome activity was not completely abolished (Fig. 1). Therefore the appearance of ubiquitinated protein likely reflects the combined effects of increased ubiquitination and diminished rates of degradation. No binding was observed when normal rabbit sera was used in place of ant ubiquitin antibodies. Thus, coronary occlusion/reperfusion is accompanied by increases in the levels of oxidatively modified and ubiquitinated cytosolic protein(s).

In vivo coronary occlusion/reperfusion results in declines in chymotrypsin-like, peptidylglutamyl-peptide hydrolase, and trypsin-like activities of the proteasome as assayed in cytosolic extracts. While inactivation does not appear to be due to global disruption of the 26 S proteasome complex, functional changes were accompanied by overall increases in free radical-derived modifications to purified 20 S proteasome. Two-dimensional gel electrophoresis of the 20 S complex indicated occlusion/reperfusion-induced alterations in the silver staining properties of β-like subunit C10 and α-like subunits iota, C2, and a isoform of XAPC7. As determined by Western blot analysis of two-dimensional gels, the lipid peroxidation product HNE selectively modifies 20 S proteasome α-like subunits iota, C3, and an isoform of XAPC7. Occlusion/reperfusion-induced declines in trypsin-like activity were largely preserved upon proteasome purification. In contrast, loss in chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities observed in cytosolic extracts were not evident upon purification. Thus, decreases in proteasome activity are likely due to both direct oxidative mod-

DISCUSSION

In vivo coronary occlusion/reperfusion results in declines in chymotrypsin-like, peptidylglutamyl-peptide hydrolase, and trypsin-like activities of the proteasome as assayed in cytosolic extracts. While inactivation does not appear to be due to global disruption of the 26 S proteasome complex, functional changes were accompanied by overall increases in free radical-derived modifications to purified 20 S proteasome. Two-dimensional gel electrophoresis of the 20 S complex indicated occlusion/reperfusion-induced alterations in the silver staining properties of β-like subunit C10 and α-like subunits iota, C2, and an isoform of XAPC7. As determined by Western blot analysis of two-dimensional gels, the lipid peroxidation product HNE selectively modifies 20 S proteasome α-like subunits iota, C3, and an isoform of XAPC7. Occlusion/reperfusion-induced declines in trypsin-like activity were largely preserved upon proteasome purification. In contrast, loss in chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities observed in cytosolic extracts were not evident upon purification. Thus, decreases in proteasome activity are likely due to both direct oxidative mod-

Fig. 6. Detection of oxidatively modified and ubiquitinated cytosolic proteins. Cytosolic extracts were prepared from sham-operated hearts 60 min after coronary occlusion/reperfusion. As judged by Western blot analyses utilizing antibodies specific to ubiquitin, we observed a distinct increase in the cytosolic content of ubiquitinated protein (Fig. 6D). Although high molecular weight smears of ubiquitinated proteins were not observed, ubiquitination status was evaluated after a single duration of reperfusion (60 min) during which proteasome activity was not completely abolished (Fig. 1). Therefore the appearance of ubiquitinated protein likely reflects the combined effects of increased ubiquitination and diminished rates of degradation. No binding was observed when normal rabbit sera was used in place of ant ubiquitin antibodies. Thus, coronary occlusion/reperfusion is accompanied by increases in the levels of oxidatively modified and ubiquitinated cytosolic protein(s).
ification of the enzyme and inhibition of fluorogenic peptide hydrolysis by endogenous cystolic inhibitory protein(s) and/or substrate(s). Concurrent with proteasome inactivation, relative increases in cystolic levels of oxidized and ubiquitinated protein(s) were observed. Taken together, our findings provide insight into potential mechanisms of coronary occlusion/reperfusion-induced proteasome inactivation and critical direction for future investigations on the cellular consequences of these events.

Results of previous in vitro studies support a role for free radical events and, more specifically, HNE modification in proteasome inactivation. Treatment of purified proteasome with free radical generating systems or HNE led to declines in peptidase activities (37, 42, 48). Both trypsin-like and peptidylglutamyl-peptidase hydrolyase activities were susceptible to metal-catalyzed oxidation, while HNE inactivation was selective to trypsin-like activity (37). In addition, administration of the pro-oxidant Fe3+-nitroacetate into mice induced a decrease in trypsin-like and peptidylglutamyl-peptide hydrolyase activities of the proteasome. Interestingly, these activities recovered over time, commensurate with disappearance of HNE modification(s) to the proteasome (49). We have shown that occlusion/reperfusion induced declines in trypsin-like activity are largely preserved upon purification of the 20 S proteasome, indicating post-translational modification as a mechanism of enzyme inactivation. During coronary occlusion/reperfusion, HNE modifies specific α-like subunits and therefore does not appear to exert its effects by directly interacting with catalytic β-like subunits. HNE modifications to α-like subunits may, however, interfere with the accessibility of the catalytic core to proteasome substrates and/or impact catalytic activities by altering interaction(s) between regulatory α- and catalytic β-like subunits. Furthermore, we observed an overall increase in the carbonyl content of the proteasome. Since relative increases in protein-associated carbonyls are a general index for the occurrence of oxidative damage to protein, it is likely that as yet unidentified free radical-mediated modifications are present on other proteasome subunits, some of which may be catalytic β-like subunits.

Formation of inhibitory proteins is an additional mechanism by which free radicals may affect proteasome function during coronary occlusion/reperfusion. We and others have previously shown that proteins cross-linked by bifunctional HNE and other free radical processes are resistant to degradation by the proteasome and can act as potent inhibitors of the enzyme (16–20). Declines in chymotrypsin-like and peptidylglutamyl-peptide hydrolyase activities were fully abolished upon purification of the 20 S proteasome, while partial return of trypsin-like activity was observed. These results suggest that cytosolic factors, such as inhibitory and/or substrate proteins, are removed during purification. Importantly, although only trypsin-like inactivation appears due, in large part, to the occurrence of post-translational modification of the enzyme, inhibition of any given peptidase activity would be expected to affect overall catalysis by the proteasome. Proteasome-dependent proteolysis involves theconcerted actions of multiple peptidase activities and the subsequent release of relatively small peptide products (25–29). Therefore inactivation of trypsin-like activity is likely to result in delayed release of products from the catalytic core resulting in proteasome inhibition and/or inefficient degradation of protein substrates. Ongoing studies seek to identify specific sites of free radical-derived modification(s) to proteasome subunits and determine which oxidatively modified and ubiquitinated proteins are substrates and/or inhibitors of the enzyme.

Proteasome inactivation would be expected to result in the build-up of oxidized and ubiquitinated protein. Free radical-mediated modifications can significantly alter and, in many cases inhibit, protein activity (10–15). Thus, the inability to efficiently remove modified forms of protein may impact a variety of cellular pathways. Accumulation of oxidized protein within cells would also compromise intracellular dynamics by placing abnormal demands on an already finite cellular volume. Furthermore, declines in proteasome function would contribute to a build-up of ubiquitinated protein(s), known substrates of the 26 S proteasome. This in turn could impact ubiquitin-dependent cell signaling pathways such as degradation of IκB and subsequent activation of NFκB (32, 33). Furthermore, recent results of in vitro studies with cells in culture implicate proteasome inactivation in the induction of apoptosis (34, 35, 50). Thus, declines in proteasome function may impact the outcome of cardiac ischemia/reperfusion.

In summary, our results provide support for the hypothesis that free radicals generated upon coronary occlusion/reperfusion mediate declines in proteasome function, thereby altering the balance between protein oxidation, ubiquitination, and degradation. The potential relevance of these processes to other organs is indicated by a previous study demonstrating HNE modification to and declines in peptidase activities of the proteasome in the brain of mice exposed to cerebral ischemia/reperfusion (51). Future studies must identify sites and forms of oxidative modification to proteasome subunits in order to fully characterize specific mechanisms by which the proteasome is inactivated. In addition, experiments utilizing varying durations of occlusion and reperfusion will establish when in the sequence of events critical alterations take place and address the potential for proteasome activation under certain conditions. Ongoing efforts to identify oxidized and ubiquitinated substrates and inhibitors of the proteasome, which are not efficiently degraded following coronary occlusion and reperfusion, will enable elucidation of the long-term consequences of proteasome inhibition on specific cellular and cardiac functions.

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REFERENCES
1. Bolli, R., and Marban, E. (1999) Physiol. Rev. 79, 609–634
2. Reimer, K. A., and Jennings, R. B. (1992) in The Heart and Cardiovacular System (Pozzard, H. A., Haber, E., Jennings, R. B., Katz, A. M., and Morgan, H. E., eds) 2nd Ed., pp. 1875–1873, Raven Press, Ltd., New York
3. Ferranti, R. (1996) J. Cardiovacular Pharmacol. 28, S1–S9
4. Zweier, J. L. (1988) J. Biol. Chem. 263, 1353–1357
5. Ambrosio, G., Zweier, J. L., Diulio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., and Chiariello, M. (1993) J. Biol. Chem. 268, 18532–18541
6. Das, D. R., George, A., Liu, X. K., and Rao, P. S. (1989) Biochem. Biophys. Res. Commun. 165, 1004–1009
7. Ambrosio, G., Flaherty, J. T., Diulio, C., Tritto, I., Santoro, G., Elia, P. P., Condorelli, M., and Chiariello, M. (1991) J. Clin. Invest 87, 2056–2064
8. Kramer, J. H., Misalk, V., and Weglicki, W. B. (1994) Ann. N. Y. Acad. Sci. 723, 180–196
9. Blasig, I. E., Grune, T., Schonheit, K., Rohde, E., Jakstadt, M., Haseloff, R. F., and Siems, W. G. (1995) J. Biol. Chem. 270, 1224–1224
10. Berlett, B. S., and Stadtman, E. R. (1995) J. Biol. Chem. 272, 20313–20316
11. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Free Radic. Biol. Med. 11, 81–128
12. Szewda, L. I., and Stadtman, E. R. (1993) Arch. Biochem. Biophys. 301, 391–395
13. Szewda, L. I., and Stadtman, E. R. (1993) J. Biol. Chem. 268, 3342–3347
14. Uchida, K., Tsai, L., and Stadtman, E. R. (1993) J. Biol. Chem. 268, 3342–3347
15. Uchida, K., and Stadtman, E. R. (1993) J. Biol. Chem. 268, 6388–6393
16. Frigeret, B., Stadtman, E. R., and Szewda, L. I. (1994) J. Biol. Chem. 269, 21639–21643
17. Frigeret, B., and Szewda, L. I. (1997) FEBS Lett. 405, 21–25
18. Frigeret, B., Szewda, L. I., and Stadtman, E. R. (1994) Arch. Biochem. Biophys. 311, 168–173
19. Sitte, N., Huber, M., Grune, T., Ladhoff, A., Doecke, W.-D., von Zglinicki, T., and Davies, K. J. A. (2000) FASEB J. 14, 1490–1498
20. Sitte, N., Merker, K., Von Zglinicki, T., Grune, T., and Davies, K. J. (2000) FASEB J. 14, 2495–2502
21. Pacifici, R. E., Kono, Y., and Davies, K. J. A. (1993) J. Biol. Chem. 268, 15405–15411
22. Rivett, A. J. (1985) J. Biol. Chem. 260, 300–305
23. Davies, K. J., and Goldberg, A. L. (1987) J. Biol. Chem. 262, 8227–8234
24. Grune, T., Reinheckel, T., and Davies, K. J. (1997) FASEB J. 11, 526–534
25. Bochtler, M., Dittrich, L., Groll, M., Hartmann, C., and Huber, R. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 295–317
26. Coux, O., Tanaka, K., and Goldberg, A. L. (1987) J. Biol. Chem. 262, 8227–8234
27. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Cell. Dev. Biol. 14, 425–479
28. Voges, D., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 836–864
29. DeMartino, G. N., and Slaughter, C. A. (1999) J. Biol. Chem. 274, 22123–22126
30. Laing, J. G., and Beyer, E. C. (1995) J. Biol. Chem. 270, 26399–26403
31. Ebbe, M., Emod, M., Groll, M., Hartmann, C., and Davies, K. J. (1999) J. Biol. Chem. 274, 21848–21852
32. Powers, S. K., Demirel, H. A., Vincent, H. K., Coombes, J. S., Naito, H., Hamilton, K. L., Shanely, R. A., and Jessup, J. (1998) Am. J. Physiol. 275, E1468–E1477
33. Conconi, M., and Friguet, B. (1997) Mol. Biol. Rep. 24, 45–50
34. Conconi, M., Petropoulos, I., Emod, I., Turlin, E., Biville, F., and Friguet, B. (1998) Biochem. J. 333, 407–415
35. Conconi, M., Zwickl, P., and Baumeister, W. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 331–350
36. Uchida, K., Szweda, L. I., Levine, R. L., and Stadtman, E. R. (1993) Methods Enzymol. 186, 464–478
37. Levine, R. L., Williams, J. A., Stadtman, E. R., and Shachter, E. (1994) Methods Enzymol. 233, 346–357
38. Humphries, K. M., and Szweda, L. I. (1998) Biochemistry 37, 15835–15841
39. Okada, K., Wangpoengtrakul, C., Osawa, T., Toyokuni, S., Tanaka, K., and Uchida, K. (1999) J. Biol. Chem. 274, 23787–23793
40. Haynes, R. L., Szweda, L., Pickin, K., Welker, M. E., and Townsend, A. J. (2000) Mol. Pharmacol. 58, 788–794
41. Keller, J. N., Huang, F. P., Zhu, H., Yu, J., Ho, Y.-S., and Kindy, M. S. (2000) J. Cereb. Blood Flow Metabol. 20, 1467–1473
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