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Oxidized and Ubiquitinated Proteins May Predict Recovery of Postischemic Cardiac Function: Essential Role of the Proteasome

SAUL R. POWELL,[1] PING WANG,[1] HARVEY KATZEFF,[1] RESHMA SHRINGARPURE,[2] CHERYL TEOH,[2] IGOR KHALIULIN,[3] DIPAK K. DAS,[4] KELVIN J.A. DAVIES,[2] and HERZL SCHWALB[3]

ABSTRACT

This study examined the hypothesis that postischemic levels of oxidized and/or ubiquitinated proteins may be predictive of functional recovery as they may be indicative of activity of the 20S and/or 26S proteasomes, respectively. Subjecting isolated rat hearts to 15 min of ischemia had no effect on 20S- and 26S-proteasome activities; however, both were significantly ($p < 0.05$) decreased by 70% and 54%, respectively, following 30 min of ischemia and 60 min of reperfusion, changes associated with increased levels of protein carbonyls and ubiquitinated proteins. Preischemic treatment of hearts with the proteasome inhibitor, MG132, resulted in dose-dependent decreases ($p < 0.05$) in recovery of postischemic function [MG132 (μ*M*), heart rate × pressure product: 0, 11,158 ± 2,423; 6, 11,400 ± 3,009; 12, 5,513 ± 2,225; 25, 2,325 ± 992] and increased accumulation of ubiquitinated proteins. Preconditioning with repetitive ischemia (IP) or preischemic treatment with nicorandil (Nic) resulted in a significant increase in postischemic 20S-proteasome activity after 60 min of reperfusion (control, 95 ± 4; IP, 301 ± 65; Nic, 242 ± 61 fluorescence units). Only Nic had similar effects on 26S-proteasome activity. These results support the conclusion that a correlation exists between eventual recovery of postischemic function and levels of oxidized and/or ubiquitinated proteins, a phenomenon that may be dependent on activity of the 20S and 26S proteasomes. *Antioxid. Redox Signal.* 7, 538–546.

INTRODUCTION

Posttranslational processing plays an essential role in modifying activity and function of many proteins and has been implicated in regulating diverse functions, including tubulin assembly and disassembly (50), endocytosis (41), gene transcription (10), cell signaling (1, 7), immune function and antigen presentation (3), reproduction (47), and cell death (37, 52). Polyubiquitination is one modification that plays an essential role in termination of protein function and may act as a signal for protein degradation (51). Sequential addition of ubiquitins to the ε-amino group of a protein lysine involves a specific ubiquitin ligase and targets a protein for degradation by the 26S proteasome (43). The 26S proteasome is composed of the 20S proteasome as its "core" catalytic unit capped on each end by a 19S regulatory complex that confers ubiquitin specificity and requirement for ATP (39). The 20S proteasome is a cylindrical structure containing four concentric rings, each containing seven subunits. The proteolytic center is located inside the cylinder and has multiple protease activities (28).

Ubiquitin-dependent degradation of proteins has been implicated in turnover of important regulatory proteins, such as

[1]Department of Medicine, Long Island Jewish Medical Center Campus of the Albert Einstein College of Medicine, New Hyde Park, NY.
[2]Andrus Gerontology Center, University of Southern California, Los Angeles, CA.
[3]The Joseph Lunenfeld Cardiac Surgery Research Center, Hadassah–Hebrew University Medical Center, Jerusalem, Israel.
[4]Department of Surgery, University of Connecticut School of Medicine, Farmington, CT.

the cyclins and transcription factors (17, 18), plays an important role in antigen presentation (3) and an essential role in regulating several stress-responsive signaling pathways (4, 15), and maintains the balance between many cellular pro- and anti-death pathways (11, 29). Ubiquitin-dependent protein degradation represents only one facet of proteasome-mediated proteolysis. The 20S proteasome is capable of removing misfolded or otherwise damaged proteins, without the requirement for ubiquitin or energy (45, 46), and may act as a secondary line of defense by removing oxidatively modified proteins in mammalian cells exposed to an oxidant stress (12). Myocardial ischemia is associated with numerous post-translational modifications of proteins, many of which can be ascribed to oxidative phenomena. Indeed, we have demonstrated carbonylation of actin isoforms in the ischemic heart (35, 44), and examination of modifications of troponins and other myofilament elements is an ongoing area of research (49). One consequence of protein oxidation is increased vulnerability to proteolysis (14), and both actin and the troponins, and other myofilament elements, are lost following ischemia (16), although the role of the proteasome has not been examined.

Potential roles for the proteasomes in myocardial ischemia can be postulated in which the 20S proteasome facilitates recovery by removal of damaged proteins, and the 26S proteasome regulates the balance between pro- and anti-death signaling pathways. Interference with these processes might be catastrophic and lead to cell death, an event possibly preceded by accumulation of oxidized and/or ubiquitinated proteins. Yet the prospective roles of the proteasomes in cell death or recovery during myocardial ischemia have not been examined in any great detail. Most of the studies of proteasome in ischemic injury are in nervous tissue and demonstrate inhibition of the 20S proteasome associated with accumulation of oxidized and ubiquitinated proteins (2, 21, 22). One study in the heart (5) has demonstrated oxidative modification and inactivation of the 20S proteasome following 30 min of left anterior descending artery occlusion. To date, the effect of ischemia on 26S-proteasome activity has not been examined.

The current study examines the hypothesis that postischemic levels of oxidized and/or ubiquitinated proteins may be predictive of functional return as they may be indicative of 20S- and/or 26S-proteasome activities. We demonstrate increases in postischemic protein oxidation and ubiquitination associated with significant inhibition of the proteasomes. Further, we show that additional inhibition of the proteasome is associated with greater postischemic increases in ubiquitinated proteins and decreased recovery of postischemic function, whereas myocardial preconditioning, which improves postischemic function, partially preserves proteasome activity.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (225–275 g) were obtained from Charles River Laboratory, Inc. (Wilmington, MA, U.S.A.), Taconic Farms (Germantown, NY, U.S.A.), Harlan Laboratories (Jerusalem, Israel), or Hilltop Farms (Scottsdale, PA, U.S.A.), and allowed a 3-day in-house acclimatization period and *ad libitum* access to food (Ralston Purina Co., St. Louis, MO, U.S.A.) and water prior to experimental use. All protocols were approved by their respective Institutional Animal Care and Utilization Committee and were in compliance with the *NIH Guide for the Care and Use of Laboratory Animals* (revised 1996).

Chemicals and reagents

All chemicals and reagents were obtained from reputable sources. MG132 (Z-leu-leu-leucinal) was obtained from Peptides International (Louisville, KY, U.S.A.). Nicorandil (Nic) was kindly provided by Aventis Pharmaceuticals (Dublin, Ireland). Lactacystin was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.).

Perfused heart preparation

Rats were injected with sodium heparin (500 units, ip.) 30 min before being anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hearts were removed rapidly and then orthogradely perfused through the coronary arteries (25) as previously described (33) at a constant pressure of 95 cm H_2O. The perfusate was a modified Krebs–Henseleit (KH) buffer consisting of the following (in mmol/L): NaCl 118, KCl 6.1, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, HEPES 1.0, and glucose 11.1. Complete buffer was prepared the day of the experiment by mixing the proper amounts of concentrated stock solutions to which was added the appropriate quantity of glucose and $CaCl_2$. All concentrated solutions, with the exception of $MgSO_4$, were tested for the presence of adventitious metals, and treated with iminodiacetic acid chelating resin beads (50–100 mesh; Chelex 100®; Bio-Rad, Hercules, CA, U.S.A.) obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) if necessary, as previously described (32). When called for, Nic (1 mmol/L) was dissolved in saline and perfused through a side arm into the aortic cannula by a syringe pump (SE 400, Becton Dickinson, Paris, France) at a rate adjusted to 1/20 of heart coronary flow yielding a final concentration of 50 μmol/L.

Assessment of hemodynamic function and exclusion criteria

Left ventricular systolic pressure development and end diastolic pressure were determined by way of a latex balloon (0.1 ml) that was expanded to exert a physiologic end diastolic pressure of 5 mm Hg as previously described (33). Heart rate was calculated from the R to R interval of the electrocardiogram. Function was calculated as the rate $\times$ pressure product, which is expressed as mm Hg $\times$ min. Coronary flow was monitored and determined by timed collection of coronary effluent (data not shown). Hearts were excluded from the study if they failed to maintain a developed systolic pressure of at least 70 mm Hg, or a heart rate of at least 220 beats/min during the 20-min pretreatment equilibration period, or if a persistent arrhythmia was present during the equilibration period.

Protocols

The basic protocol perfused hearts for a 20-min preischemic equilibration period followed by 30 min of normothermic global ischemia and then 60 min of aerobic reperfusion. In the proteasome inhibitor experiments, hearts were perfused with buffer containing up to 25 μmol/L MG132 during the 20-min preischemic equilibration period only. During reperfusion, hearts were perfused with KH buffer without the inhibitor. When MG132 was added to the perfusate, it was first dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the KH buffer (control and treatment groups), when added, was 0.25%, which in preliminary experiments had no effect on pre- or postischemic hemodynamic function (data not shown). In the preconditioning experiments, control hearts were perfused for 38 min with KH preischemically, followed by 25 min of no-flow global ischemia at 37ºC (index ischemia), and then 60 min of reperfusion with KH. Ischemic preconditioned (IP) hearts were treated with two episodes of 3 min of global ischemia interrupted by 2 min of reflow and followed by 10 min of KH perfusion prior to ischemia. Pharmacologically preconditioned hearts were treated with 50 μmol/L Nic over 10 min followed by 10 min of washout prior to the index ischemia.

Proteasome activity

Proteasome activity was determined in cell lysate as described by Grune *et al.* (13). In brief, cardiac tissue was homogenized in HEPES buffer containing (in mmol/L) NaCl 137, KCl 4.6, KH_2PO_4 1.1, $MgSO_4$ 0.6, EDTA 1, dithiothreitol 1, digitonin 0.01%, without protease inhibitors, at 4°C and then centrifuged at 10,000 *g* to obtain the soluble fraction. Cell supernatant (100 μg of protein) was incubated in 50 mmol/L Tris HCl buffer, pH 7.8, containing (in mmol/L) KCl 20, $MgCl_2$ 0.5, and dithiothreitol 1, for 1 h with the proteasome-specific peptide, suc-LLVY-MCA (75 μmol/L; Biomol Research Labs, PA). Hydrolysis was stopped by addition of ice-cold ethanol and dilution with 0.125 mol/L sodium borate, pH 9.0. Fluorescence products were monitored at 380 nm excitation and 440 nm emission. The reaction was carried out in the absence and presence of the proteasome inhibitor, lactacystin (5 μmol/L; Biomol Research Labs), to differentiate between non–proteasome- and proteasome-mediated peptide hydrolysis, and in the absence and presence of 5 mmol/L ATP (with and without lactacystin) to differentiate between the 20S and 26S proteasomes, respectively. For the most part, results are expressed as percentage of control, necessary because storage of tissue samples, even at −80° C, can result in interassay variation, particularly with respect to 26S-proteasome activity. Care was taken to avoid freeze-thawing of tissue samples more than once and to match experimental samples with preischemic controls that had been stored under the same conditions and time intervals.

Immunoblot assays

Cardiac tissue was homogenized in HEPES buffer containing (in mmol/L) NaCl 137, KCl 4.6, KH_2PO_4 1.1, $MgSO_4$ 0.6, EDTA 1, digitonin 0.01%, plus a cocktail of protease inhibitors (leupeptin, 5 μg/ml; aprotinin, 5 μg/ml; pepstatin, 7 μg/ml; and phenylmethylsulfonyl fluoride, 40 μg/ml) at 4°C and then centrifuged at 10,000 *g* to obtain the soluble fraction. Cellular proteins (10–50 μg) were separated on 4–20% Tris-HCl gels (Bio-Rad Laboratories) using standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (24) with immunoblotting carried out using standard techniques and developed with an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences, Boston MA, U.S.A.) or directly on the membrane with the horseradish peroxidase system (Vectastain®; Vector Laboratories, Inc., Burlingame, CA, U.S.A.) using 3,3',5,5'-tetramethylbenzidine (TNB) as a substrate. Membranes were probed with a polyclonal (rabbit) antibody specific for ubiquitin (Sigma).

Protein oxidation

Cytosolic proteins were reacted with dinitrophenylhydrazine to tag carbonyl groups and then separated under reducing conditions using standard polyacrylamide gel electrophoresis (24). Protein carbonyls were then determined using an immunoblot technique and antibody specific for dinitrophenylhydrazine as previously described (44).

Statistical analysis

All results are expressed as means ± SEM. Statistical significance of differences between sample populations with equal variance was evaluated using one-way ANOVA followed by the Tukey test for *post-hoc* analysis. Analysis of differences between multiple groups was performed with a repeated measures of analysis of variance (RMANOVA) where the within factor was time. Statistical differences of $p < 0.05$ were considered to be significant. All statistics were performed using the SigmaStat statistical analysis package (Jandel Scientific, Chicago, IL, U.S.A.).

RESULTS

Effect of ischemia and reperfusion on proteasome activities

Proteasome activities were determined by measuring hydrolysis of the proteasome-specific peptide, suc-LLVY-MCA, to a fluorescent end product. Results of this series of experiments are expressed as percentage of control, where control 20S-proteasome activity was 953 ± 81 and control 26S-proteasome activity was 573 ± 94 fluorescence units/mg of protein/h. 20S-proteasome activity was not affected by 15 min of global ischemia and/or reperfusion (Fig. 1A). After 30 min of global ischemia alone, 20S-proteasome activity was decreased by 40% (not significant), and significantly ($p < 0.05$) decreased by 70% after 30 min of global ischemia and 60 min of reperfusion. 26S-proteasome activity was measured only after 30 min of global ischemia; it was depressed by 45% (not significant) by the end of ischemia, and significantly ($p < 0.05$) decreased by 54% by the end of 60 min of reperfusion (Fig. 1B).

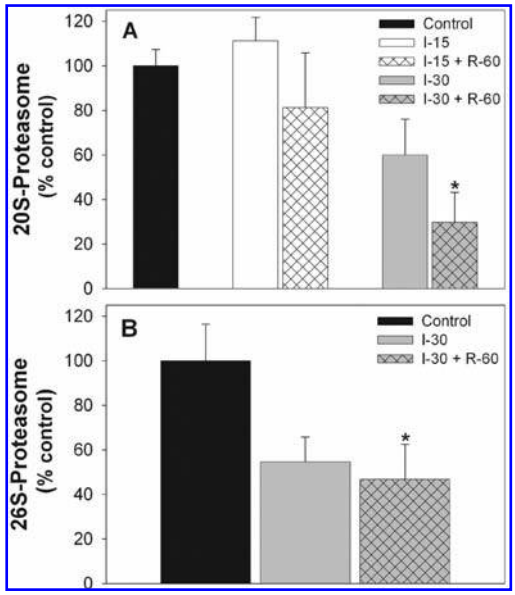

FIG. 1. Proteasome activity is decreased following ischemia and reperfusion. Isolated rat hearts were equilibrated for 20 min (control) and then subjected to either 15 min of global ischemia (I-15) followed by 60 min of reperfusion (I-15 + R-60), or 30 min of global ischemia (I-30) followed by 60 min of reperfusion (I-30 + R-60). At the indicated time points, hearts were analyzed for 20S-proteasome (**A**) or 26S-proteasome (**B**) activities. The values represent the means ± SEM of five to 10 individual hearts in each group. *$p < 0.05$ (ANOVA; Tukey) when compared with the control group.

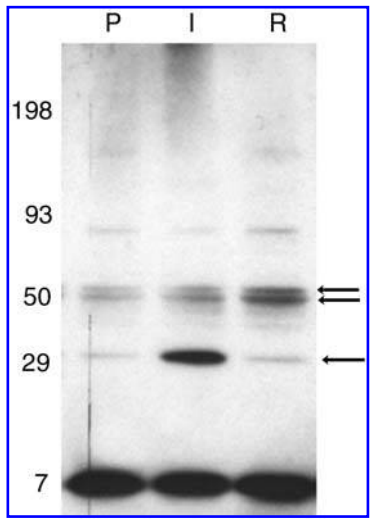

FIG. 2. Accumulation of ubiquitinated proteins in ischemic myocardium. Isolated hearts were equilibrated for 20 min, then subjected to 30 min of normothermic global ischemia followed by 60 min of reperfusion. After equilibration (P), ischemia (I), and reperfusion (R), hearts were processed for immunoblot determination of ubiquitin-conjugated proteins. Arrows indicate bands with accumulations of ubiquitinated protein(s) at 34, 50, and 55 kDa. The membrane depicted is representative of four separate experiments.

Effect of ischemia and reperfusion on myocardial ubiquitinated proteins

Ubiquitinated proteins were assessed using western blot techniques and ubiquitin-specific antibodies. As illustrated by Fig. 2, 30 min of ischemia and 60 min of reperfusion result in accumulation of ubiquitinated proteins within myocardial tissue. This was particularly apparent in bands of molecular masses of 34, 50, and 55 kDa (see arrows, Fig. 2), thus confirming decreased activity of the 26S proteasome. The identities of these proteins were not studied as part of these experiments, but were in a related experiment. Potential identities are offered in the Discussion.

Effect of ischemia and reperfusion on myocardial protein carbonyls

The overall effect of varying times of ischemia on myocardial protein carbonyls was assessed. As illustrated by Fig. 3, 15 min of global ischemia produced marginal increases in protein carbonyls after ischemia and/or reperfusion. However, after 30 min of ischemia and 60 min of reperfusion, a large increase in protein carbonyls was detected over a wide range of molecular masses, an observation generally consistent with what we have published previously (33, 44). We have previously characterized the broad band at ~45 kDa as containing a mixture of β- and γ-actin isoforms (44). Although it is tempting to suggest that these results conclusively demonstrate that myocardial proteins are increased following ischemia and reperfusion, in light of decreases in 20S-proteasome activity, this result needs to be interpreted cautiously.

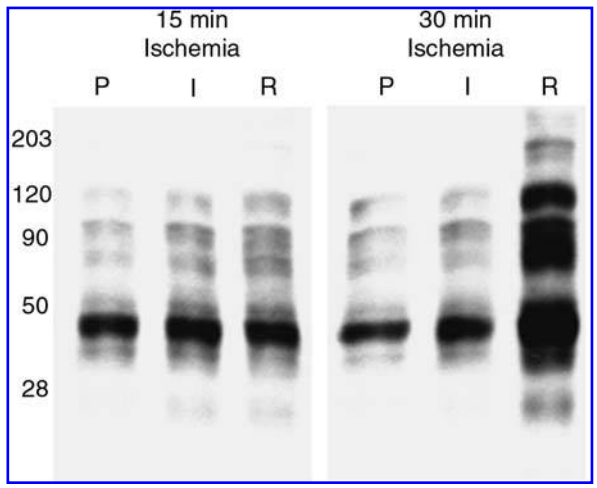

FIG. 3. Oxidation of myocardial proteins during and following ischemia. Isolated hearts were equilibrated for 20 min preischemically, and then subjected to 15 or 30 min of global ischemia, followed by 60 min of reperfusion. After equilibration (P), ischemia (I), and reperfusion (R), hearts were processed for immunoblot determination of protein carbonyl. The membrane depicted is representative of five separate experiments.

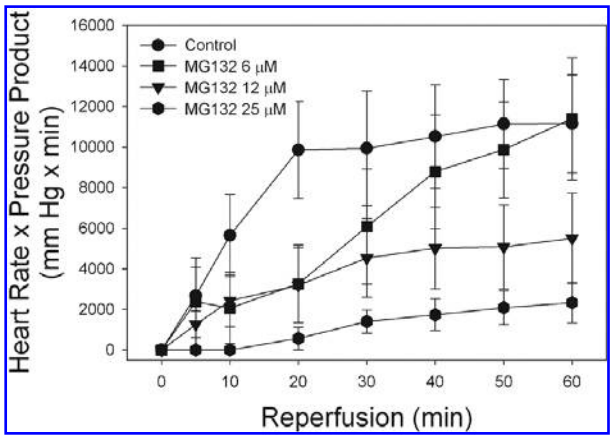

FIG. 4. Proteasome inhibition decreases postischemic recovery of function. Isolated hearts were perfused with up to 25 μmol/L MG132 for 20 min prior to 30 min of normothermic global ischemia. After ischemia, hearts were perfused with buffer alone and allowed to recover for 60 min. Function was determined as the heart rate $\times$ pressure product. The values represent the means ± SEM of a minimum of six hearts per group.

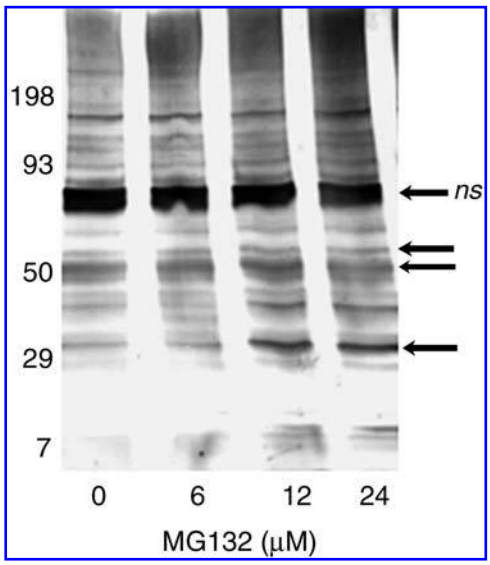

FIG. 5. Myocardial ischemia and proteasome inhibition lead to enhanced accumulation of ubiquitinated proteins. Isolated hearts were perfused with up to 25 μmol/L MG132 for 20 min prior to 30 min of normothermic global ischemia. After ischemia, hearts were perfused with buffer alone and allowed to recover for 60 min. At the end of reperfusion, hearts were processed for immunoblot determination of ubiquitin-conjugated proteins. For this series of experiments, development of color was done on the membrane using TNB as a substrate. Arrows indicate bands with accumulations of ubiquitinated protein(s) at 34, 50, and 55 kDa. The arrow marked *ns* indicates a band that represents nonspecific binding of the secondary antibody. The membrane depicted is representative of three separate experiments.

Effect of proteasome inhibition on postischemic recovery of function and ubiquitinated proteins

The effect of additional inhibition of the proteasome on recovery of postischemic function was assessed by treating isolated hearts with the proteasome inhibitor, MG132, which was included in the buffer during the preischemic period only. Preischemic treatment with up to 25 μmol/L MG132 resulted in a concentration-dependent decrease in recovery of hemodynamic function in the postischemic period (Fig. 4). Most of the 12 μmol/L time points and all of the 25 μmol/L time points were significantly ($p < 0.05$, RMANOVA) less than their respective control values. To confirm that MG132 was providing additional inhibition of the proteasome over and above ischemia alone, ubiquitin-conjugated proteins were assessed. Changes in function correlated with a dose-dependent increase in accumulation of ubiquitin-conjugated proteins measured at the end of reperfusion (Fig. 5). Most intriguing was the observation of dose-dependent accumulation of ubiquitin-conjugated proteins in bands at 34, 50, and 55

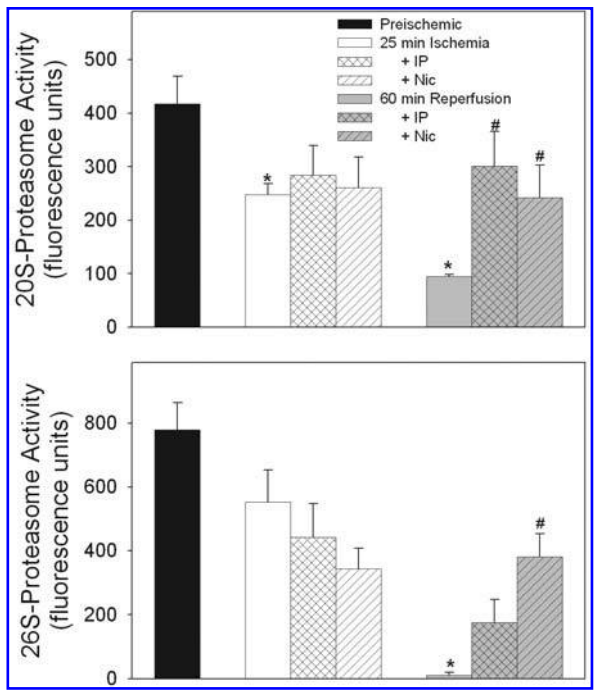

FIG. 6. Nic and ischemic preconditioning improve postischemic 20S- and 26S-proteasome activity. Isolated rat hearts were equilibrated for 38 min preischemically, followed by 25 min of global ischemia (index ischemia), and then 60 min of reperfusion (control). Hearts were preconditioned with two episodes of 3 min of global ischemia interrupted by 2 min of reflow and followed by 10 min of KH buffer perfusion prior to ischemia (IP); or preconditioned pharmacologically with 50 μmol/L Nic infused over 10 min followed by 10 min of washout prior to ischemia (Nic). Values represent the means ± SEM of four hearts per group. $^{*}p < 0.05$ (ANOVA; Tukey) compared with control; $^{\#}p < 0.05$ (ANOVA; Tukey) compared with corresponding control.

kDa that match bands observed after 30 min of ischemia (Fig. 2, see arrows). Interpretation of these results requires some caution as we have shown that pharmacologic inhibition of the proteasome results in loss of contractile function and cardiomyocyte apoptosis in a perfused heart preparation (34). The doses used in the current study generally do not have significant effects on function and apoptosis in the nonischemic heart within the 20-min preischemic "loading" interval.

Preliminary evidence that preconditioning preserves activity of the proteasomes

To assess the effects of preconditioning, isolated hearts were subjected to intermittent ischemia (IP) or treated with the mitochondrial K_{ATP} channel opener, Nic (42). Figure 6 illustrates the effect of IP and Nic on 20S-proteasome (top) and 26S-proteasome (bottom) activities. 20S-proteasome activity was significantly ($p < 0.05$) decreased by 39% following the index ischemia and 75% by the end of reperfusion. IP and Nic had no effect on 20S-proteasome activity following the index ischemia, but both treatments significantly ($p < 0.05$) increased activity after reperfusion. 26S-proteasome activity was decreased following the index ischemia, but not significantly, and IP and Nic had no effect on this. However, after reperfusion, 26S-proteasome activity was drastically (significance, $p < 0.05$) decreased to the point that it was almost not detectable. It is not clear why the index ischemia had such an extreme effect in these samples, but nonetheless, at least Nic significantly ($p < 0.05$) increased 26S-proteasome activity by the end of reperfusion.

DISCUSSION

The current study examines the hypothesis that postischemic levels of oxidized and/or ubiquitinated proteins may be predictive of functional return as they may be indicative of activity of the 20S and/or 26S proteasomes. A series of correlations between postischemic activity of the proteasomes, levels of oxidized and/or ubiquitinated proteins, and recovery of hemodynamic function were developed. The established relationship between ischemia duration and postischemic formation of oxidative species (31), and evidence that the proteasomes are vulnerable to oxidative damage and inactivation (5, 40), provide the rationale for the initial correlative study examining the effect of varying durations of ischemia on activities of the proteasome. We observed that 15 min of global ischemia had no effect on 20S-proteasome activity, but that 30 min resulted in significant loss, a result in general agreement with that published by Bulteau *et al.* (5). However, we also demonstrate decreased activity of the 26S proteasome following 30 min of ischemia and 60 min of reperfusion. The observed proteasomal inhibition correlates with past determinations of degree of postischemic recovery of function, as 15 min of global ischemia generally results in function not significantly different from that of control hearts, but after 30 min of ischemia, function is significantly depressed by 50% or more (33, 35). The relationship between recovery of function and proteasome activity is further strengthened by results of the inhibitor and preconditioning studies. Inhibition of the proteasome with MG132 resulted in a dose-dependent decrease in postischemic recovery. Preconditioning of the myocardium with ischemia or Nic improves postischemic function (23), and partially preserved activity of the 20S proteasome and, to a lesser degree, the 26S proteasome.

Inhibition of the 20S proteasome correlated with increases in protein carbonylation, as little accumulation was observed after 15 min of global ischemia, but large increases were detected after 30 min of global ischemia and 60 min of reperfusion. Numerous studies (5, 23, 33, 35, 44) have demonstrated increases in protein oxidation following myocardial ischemia and have generally suggested this result as indicative of increased oxidation of proteins. At any point in time, the level of protein oxidation products is dependent on rates of formation and degradation. In the current study, we observed increased protein carbonyls following 30 min of ischemia and 60 min of reperfusion, corresponding to a time when activity of the 20S proteasome was significantly decreased. After 15 min of ischemia and reperfusion, little protein oxidation was detected at a time when 20S-proteasome activity was not decreased. In light of a recent study (46) that concluded that the 20S proteasome can degrade oxidatively modified proteins without the need for ubiquitin or ATP, the interpretation of these past studies may require further analysis, particularly in light of the effects of preconditioning on 20S-proteasome activity. We have previously shown that preconditioning decreases postischemic levels of carbonyl products of cytosolic (44) and mitochondrial proteins (23). We theorized that one mechanism was decreased production of oxidative species, which decreases downstream protein oxidation, an effect that could explain preservation of proteasome activity in the preconditioned heart. It is probable that our previous observations of decreased protein oxidation (23, 44) result from decreased formation *and* improved removal of oxidized proteins in the preconditioned heart. We remain convinced that increased production of oxidative species during ischemia leads to oxidation of myocardial proteins, but it can no longer be assumed that increases are due merely to increased formation without assessment of degradation.

Postischemic inhibition of the 26S proteasome also led to accumulation of modified proteins, in this case, ubiquitinated proteins. We have reported an association between inhibition of the proteasome, loss of contractile function, and cardiomyocyte apoptosis in nonischemic hearts (34). Ubiquitin-dependent protein degradation by the 26S proteasome regulates numerous redox-sensitive signaling pathways, including the c-Jun N-terminal kinase (48), nuclear factor-κB (NFκB) (7), and JAK-STAT (53) pathways, and plays an essential role in maintaining the balance between numerous pro- and antiapoptotic proteins, such as Bax (26), p53 (19), $p27^{kip1}$ (30), and hypoxia-inducible factor-1α (20). When the 26S proteasome is inhibited, these proapoptotic proteins tend to accumulate as a result of decreased degradation, a phenomenon known as dysregulation. Numerous studies (for reviews, see 11, 29) in a variety of cell lines have shown that pharmacologic inhibition of the proteasome results in apoptosis coincident with dysregulation of several pro-death proteins. The presence of three bands at 34, 50, and 55 kDa containing in-

creased levels of ubiquitinated proteins suggests that ischemia might result in a similar phenomenon. These bands were not identified as part of this study, but in a related study (36) we partially immunoprecipitated the 34-kDa band with a $p27^{kip1}$-specific antibody and the 50- and 55-kDa bands with a Bax-specific antibody, suggesting accumulation of ubiquitinated homologues of these proteins. Unless the 26S proteasome is inhibited, it is not the rate-limiting step in ubiquitin-mediated proteolysis (39); rather, ubiquitination of proteins is most likely at the ubiquitin-ligase step (43, 51). Thus, increases in ubiquitinated proteins would be more indicative of decreased activity of the 26S proteasome.

The results of the proteasome inhibitor studies further support the hypothesis that myocardial ischemia can result in dysregulation. If proteasome activity has any role in the determination of postischemic recovery, then additional inhibition prior to ischemia should worsen postischemic recovery coincident with increases in ubiquitinated proteins, which was the observed result. The observation of dose-related increases in ubiquitinated proteins in the same 34-, 50-, and 55-kDa bands observed in ischemic hearts alone suggests that these proteins are dysregulated, and suggests a putative role for the proteasome in recovery of postischemic myocardial function.

The inhibitor studies would appear to be at odds with previous studies of a proteasome inhibitor in ischemic myocardium. Two studies (6, 38) have indicated that treatment with the proteasome inhibitor, PS-519 (Millennium Pharmaceuticals, Cambridge, MA, U.S.A.), has protective effects in the ischemic myocardium. Both of these studies (6, 38) used the inhibitor to decrease leukocyte adhesion to endothelial cells, thus limiting the inflammatory response associated with myocardial ischemia. In one of these studies (6), an isolated perfused heart preparation was perfused with leukocyte-supplemented buffer, and in the absence of the leukocytes, no effect of the inhibitor was observed. The ability of proteasome inhibitors to decrease the inflammatory response has been well documented (9) and, besides effects on leukocyte adhesion, has been attributed to inhibition of NFκB nuclear translocation resulting from dysregulation of IκB (7). Whether a proteasome inhibitor has a beneficial (antiinflammatory) or negative (proapoptotic) effect is notoriously dose-related (27), and will be somewhat dependent on degrees of proteasome activity in the different tissues (*e.g.*, leukocyte versus heart). It may well be that after brief ischemia, where little or no proteasome inhibition is present, decreasing leukocyte-mediated inflammation with a peripherally acting inhibitor may be beneficial. However, in the presence of significant proteasome inhibition, an inhibitor may be counterproductive and tilt the cell toward death, similar to what was observed in the current study. Although both of these previous studies (6, 38) determined peripheral leukocyte 20S-proteasome activity, neither measured myocardial 20S- or 26S-proteasome activity or levels of ubiquitin-conjugated proteins. Thus, it is not clear if the beneficial effect had any relation to myocardial proteasome.

In summary, it seems reasonable to conclude that a correlation exists between eventual recovery of postischemic function and levels of oxidized and/or ubiquitinated proteins, to the extent that they may actually be predictive. Although numerous factors, including rates of formation, may affect lev-

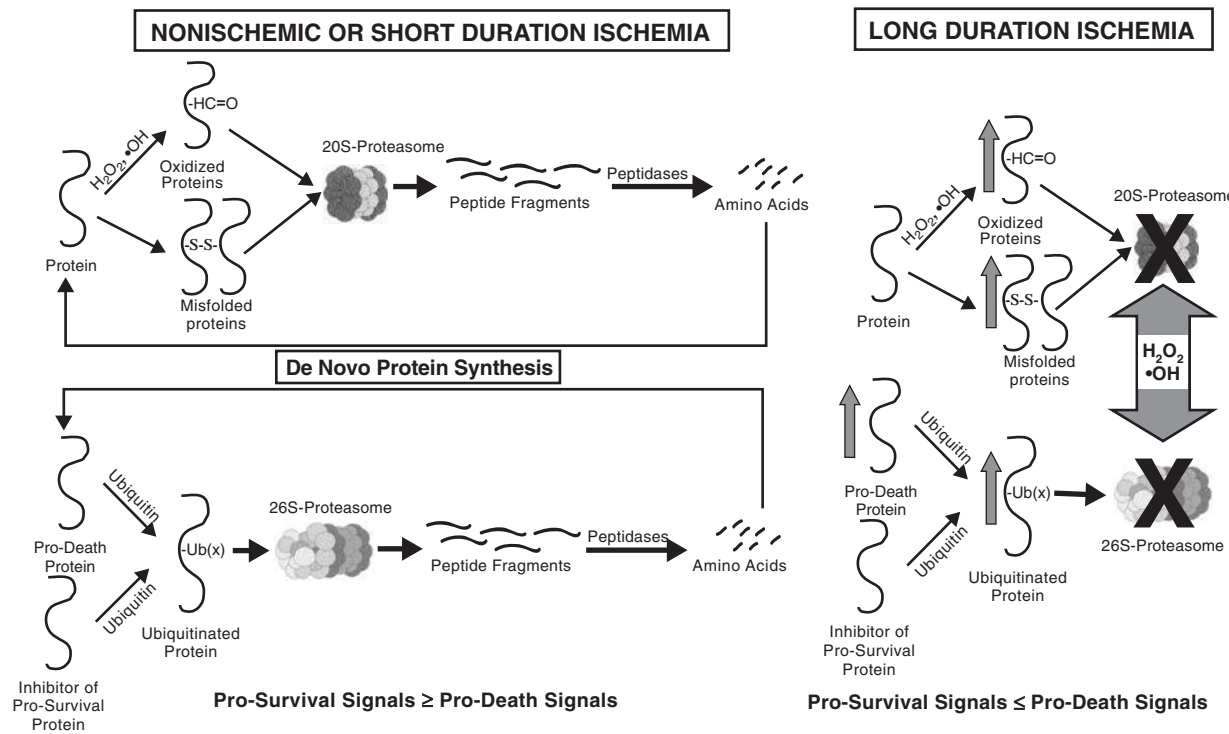

Fig. 7. Scheme illustrating potential roles of 20S and 26S proteasomes in short- and long-duration ischemia. H_2O_2, hydrogen peroxide; •OH, hydroxyl radical.

els of oxidized and ubiquitinated proteins, one major determinant is hydrolysis by the 20S and 26S proteasomes, respectively. Under conditions that foster excessive inhibition of the proteasomes, removal of oxidized proteins by the 20S proteasome would be impaired, thus hindering recovery, and numerous proteins, some of which may be proapoptotic, that are regulated by the 26S proteasome would accumulate, thus pushing the cell toward death (see scheme in Fig. 7). On the other hand, certain treatments, such as preconditioning, may preserve activity of the proteasome and help to convert what would have been cell death signals to cell survival signals as recently suggested by Das and Maulik (8). Although it is obvious that this area requires additional study, the concept that the proteasomes may play significant roles in both myocardial cell death and recovery following an ischemic insult is an important advance that may eventually identify a site amenable to therapeutic intervention.

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ABBREVIATIONS

IP, ischemic preconditioned; KH, Krebs–Henseleit; MG132, Z-leu-leu-leucinal; NFκB, nuclear factor-κB; Nic, Nicorandil; RMANOVA, repeated measures analysis of variance; TNB, 3,3',5,5'-tetramethylbenzidine.

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Address reprint requests to:
Saul R. Powell, Ph.D.
Endocrinology Research Lab—CH049
Long Island Jewish Medical Center
270-05 76th Avenue
New Hyde Park, NY 11042

E-mail: spowell@lij.edu

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