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Original Research Communication

Association of Increased Ubiquitinated Proteins with Cardiac Apoptosis

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ABSTRACT

Intracellular proteases play an important role in the regulation of apoptosis. A study was performed to determine whether inhibition of the cardiac ATP-dependent ubiquitin 26S protease complex affects cardiomyocyte apoptosis. Isolated rat hearts were perfused for up to 80 min with Krebs-Henseleit buffer ± the 26S-proteasome inhibitor, MG132 (Z-leu-leu-leucinal). TUNEL-staining of hearts perfused with 25 μM MG132 for 50 min revealed a significant increase ($p < 0.05$) in the apoptotic index from 1.1% to 15.5% when compared with control hearts perfused with buffer only. Histology of adjacent myocardial sections revealed no signs of necrotic or late apoptotic (nuclear condensation) changes, indicating that the TUNEL-positive nuclei were in the early stages of apoptosis. This early stage of apoptosis was associated with a significant ($p < 0.05$) reduction in cardiac function. There was a 63% decrease in the rate · pressure product in hearts perfused with 25 μM MG132 as compared with a 35% decrease in control hearts over the 80-min perfusion period. Soluble ubiquitin-conjugated proteins, as detected by probing with a specific antibody to ubiquitin, were increased in MG132-treated hearts. In hearts perfused with 50 μM MG132, a greater accumulation of ubiquinated proteins was observed accompanied by a more rapid and greater reduction in hemodynamic function. These observations indicate that prolonged inhibition of the ubiquitin-26S-proteasome results in cardiomyocyte apoptosis accompanied by increased ubiquinated proteins, thus suggesting that accumulation of these abnormal proteins may act as a signal to activate the cell death program. Antiox. Redox Signal. 2, 103–112.

INTRODUCTION

INTRACELLULAR PROTEASES play an important role in the regulation of cell functions and degradation of proteins. Protein degradation occurs by two basic processes: (i) lysosomal, involving bulk turnover; and (ii) nonlysosomal, responsible for the selective turnover of intracellular proteins under basal metabolic conditions (Ciechanover, 1994). The major nonlysosomal protease for most soluble intracellular proteins is an ATP-dependent, macromolecular complex, the 26S protease (proteasome). This contains two large subunits (100 kDa and 110 kDa), 10 smaller subunits (40 and 62 kDa), as well as several subunits of 21 and 34 kDa (Hough *et al.*, 1987; Hershko and Ciechanover, 1992; Coux *et al.*, 1996). Within the complex is a 20S proteasome (CF-3), which is the catalytic core, and two regulatory subunits (CF-1 and CF-2) (Hough *et al.*, 1988). The 26S protease complex degrades proteins that have been con-

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jugated with ubiquitin, a highly conserved 76-amino-acid polypeptide, that can be covalently linked to protein substrates by an ATP-dependent reaction. Ubiquitination involves coordinated function of a conjugating system that includes a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Ciechanover and colleagues (Ciechanover *et al.*, 1980; Hershko *et al.*, 1980) were the first in 1980 to propose that ubiquitination commits proteins to degradation. Protein ubiquitination has been implicated in a variety of cellular processes such as cell cycle progression through selective degradation of cyclins (Hershko, 1997), antigen presentation (Gaczynska *et al.*, 1993), activation of transcription factors (Palombella *et al.*, 1994), and amino acid metabolism (Gross-Mesilaty *et al.*, 1997).

Considering the importance of the 26S protease complex in regulation of basal cellular metabolism, it seems reasonable to suggest that inhibition could have serious consequences for normal cellular function. In the present study, the effects of proteasome inhibition on myocardial function and cell death were examined. Z-leu-leu-leucinal (MG132), a peptidyl aldehyde analogue, is a potent, reversible inhibitor of the 26S protease complex that inactivates multiple catalytic sites by forming a transient, covalent hemiacetal with the catalytic amino-terminal threonine hydroxyl (Rock *et al.*, 1994). These compounds do not show complete specificity for the proteasome as they may inhibit cellular thiol proteases as well (Rock *et al.*, 1994; Coux *et al.*, 1996). Using the isolated perfused heart preparation, we demonstrate that inhibition of the 26S protease complex has deleterious effects on cardiac hemodynamic function that are simultaneous with increased levels of ubiquitinated proteins and apoptotic nuclei.

MATERIALS AND METHODS

Animals and perfused heart preparation

All studies were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (275–300 g) were obtained from Charles River Laboratory, Inc. (Wilmington, MA), Taconic Farms (Germantown, NY), or Hilltop Farms (Scottsdale, PA) and were allowed at least 3 days of in-house acclimatization prior to experimental use. During this time, all animals were fed *ad libitum* with Purina lab chow (Ralston Purina Co., St. Louis, Mo.) and water. Rats were injected with sodium heparin (500 units, i.p.) 30 min before being anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Hearts were removed rapidly and placed in ice-cold heparinized saline. The hearts were then perfused orthogradely through the coronary arteries (Langendorff, 1895) as previously described (Powell *et al.*, 1994a) at a constant pressure of 95 cm H_2O. The perfusate was a modified Krebs-Henseleit buffer consisting of (m*M*): 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 calcium chloride. All concentrated solutions, with the exception of the magnesium sulfate, were treated with iminodiacetic acid chelating resin beads, 50–100 mesh (Chelex 100® (Bio-Rad, Hercules CA)) obtained from Sigma Chemical Co., St. Louis, MO) as previously described (Powell and Wapnir, 1994b). MG132 (Z-leu-leu-leucinal) (Peptides International, Louisville, KY) was added to the perfusate dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the Krebs-Henseleit buffer (control and treatment groups) was 0.25% and in preliminary experiments had no effect on hemodynamic function (data not shown). All coronary effluents were collected and recirculated one time.

Treatment protocols

Apoptosis and Histology Studies: Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 for up to 50 min and then perfusion-fixed with 10% buffered formalin and analyzed for histology or the presence of apoptotic nuclei.

Hemodynamic Function Studies: Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 or 50 μM MG132 for up to 80 min. Hemodynamic function was determined as the rate · pressure product or first derivative of the pressure curve.

Ubiquitin-Conjugated Protein Studies: Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 for 50 min or 50 μM MG132 for 20 min and then analyzed for ubiquitin-conjugated proteins. Because of the severe effects of 50 μM MG132 on hemodynamic function perfusion was stopped after 20 min.

Assessment of cardiac function

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 mmHg, as previously described (Powell *et al.*, 1994a). The rate · pressure was calculated as the heart rate multiplied by the developed systolic pressure and is expressed as mmHg/min. Coronary flow was determined by a timed collection of coronary effluent (data not shown). Heart rate was calculated from the R-to-R interval of the electrocardiogram (data not shown). Myocardial contractility was estimated by calculation of the first derivative or rate of rise of the pressure curve ($+dP/dt_{max}$) and expressed as mmHg/sec. Hearts were excluded from the study if they failed to maintain a developed systolic pressure of at least 70 mmHg, or a heart rate of at least 220 beats per minute during the 10-min pretreatment equilibration period. Furthermore, hearts were excluded if a persistent arrhythmia was present during the equilibration period.

Assessment of ubiquitin-conjugated proteins

Ubiquitin-conjugated proteins were assessed using the method described by Baracos and coworkers (Baracos *et al.*, 1995). Cardiac tissue was homogenized in HEPES suspension buffer at 4°C containing a cocktail of protease inhibitors (leupeptin, 5 μg/ml; aprotinin, 5 μg/ml; pepstatin, 7 μg/ml; and phenylmethylsulfonyl fluoride, 40 μg/ml) and then centrifuged at 10,000 × g to obtain the soluble fraction. Cellular proteins (5 μg) were separated on a 4–20% gradient gel (Ready Gel®, Bio-Rad Laboratories, Hercules, CA) using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques, transferred onto a PVDF membrane, and blocked for 2 hr with high-salt Tris–buffered saline (TBS) (pH 7.4) containing 25 mg/ml bovine serum albumin (BSA) and 5% nonfat dry milk. The membrane was then incubated with the appropriate polyclonal antibody to ubiquitin (Sigma, St. Louis, MO), washed, and then incubated with horseradish peroxidase-conjugated secondary antibody (to rabbit) (Vectastain®, Vector Labs, Inc., Burlingame, CA) and developed with 3,3′,5,5′-tetramthylbenzidine substrate (Vector Labs, Inc., Burlingame, CA). Relative quantitation of protein bands was performed using computer-assisted densitometry (SigmaScan, Jandel Scientific, Chicago, IL).

Preparation of cardiac tissue for apoptosis and histology

At the appropriate time-point, hearts were perfusion-fixed with 10 ml of phosphate-buffered 10% formalin (Baxter Labs.). The apex was then dissected off and sectioned into two full-thickness blocks (3 mm × 2 mm). One block was allowed to sit in 10% formalin at 4°C for no more than 48 hr and then paraffin-embedded and sectioned into thin slices for use in the terminal transferase-dUTP-nick end labeling (TUNEL) assay. The other block was chopped into pieces no larger than 1 mm in diameter and prepared for light microscopy as previously described (Powell *et al.*, 1994a). One micrometer plastic sections of tissue were cut and stained with toluidine blue and examined by light microscopy.

TUNEL assay for detection of apoptosis and analysis

Tissue sections (4–5 μm) were mounted onto slides pretreated with 3-aminopropylethoxysilane (Digene Diagnostics, Inc., Beltsville, MD), baked for 30 min at 60°C, and washed twice in fresh xylenes for 5 min each to remove paraffin. The slides were rehydrated through a series of graded alcohols and then washed in distilled water for 3 min. TUNEL assays were

performed as previously described (Mantell *et al.*, 1997) using the method of Tornusciolo *et al.* (1995). All reagents for the TUNEL assay were obtained from Boehringer Mannheim (Indianapolis, IN). Tissue sections were counterstained with 2 μg/ml 4',6-diamidine-2-phenylindole-dihydrochloride (DAPI) for 10 min at room temperature. To quantify the extent of apoptosis, tissue sections were illuminated with UV light to detect either TUNEL-positive nuclei (590 nm) or total, DAPI-stained nuclei (420 nm). Images were captured with a CCD video camera. Uniform camera control settings were used for image capture, and image thresholding was identical for all images. The captured images were analyzed on a personal computer using the Image 1 system (Universal Imaging, West Chester, PA). Thirty random fields were analyzed on each of two tissue sections from each heart. The apoptotic index was calculated as the percent of TUNEL-positive apoptotic nuclei divided by the DAPI-staining nuclei.

Statistical analysis

Analysis of differences between multiple groups was with a repeated measures analysis of variance (RMANOVA) in which the within factor was time. Differences between two individual groups were analyzed with an independent Student *t*-test for independent variables. In all cases, results were considered to be significant at the $p < 0.05$ level. All statistics were performed with the SigmaStat (Jandel Scientific, Chicago, IL) statistical analysis package.

RESULTS

The effect of proteasome inhibition on cardiomyocyte apoptosis

To determine if inhibition of the ubiquitin 26S protease complex affects cardiomyocyte apoptosis, hearts were perfused with buffer containing 25 μM MG132 for up to 50 min. As depicted in Fig. 1, after 20 min of perfusion with 25 μM MG132, the number of TUNEL-positive nuclei had only increased approximately two-fold, which was not significantly different from control. However, after 50 min

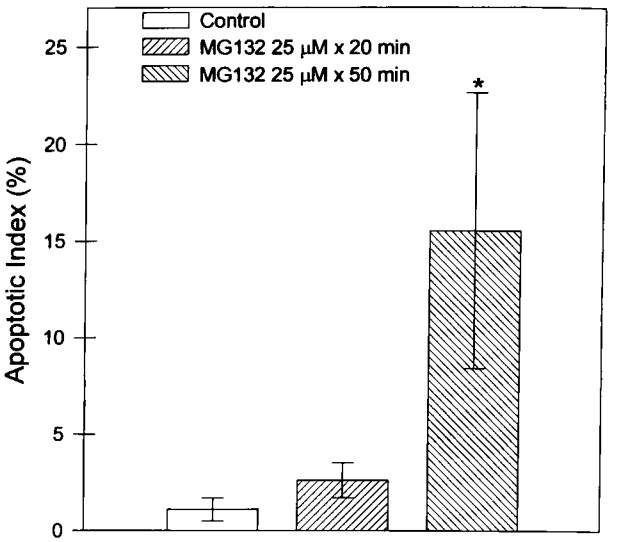

FIG. 1. The effect of MG132 on cardiomyocyte apoptosis. Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 for up to 50 min and then perfusion fixed with 10% formalin and analyzed for apoptotic nuclei using the TUNEL assay. The values are expressed as the apoptotic index, which is the percent of nuclei staining positively versus the total number of nuclei. The values represent the mean ± SEM of 8 or 9 sections from three hearts from each group as described in the METHODS section. *$p < 0.05$ when compared with control (*t*-test).

of perfusion, the number of TUNEL positive nuclei was significantly ($p < 0.05$, *t*-test) increased 15-fold, thus suggesting that initiation of apoptosis was time dependent. The increase in TUNEL staining is apparent in the photomicrographs in Fig. 2, A and B. Several toluidine blue-stained sections from each of three hearts from control and treated groups were examined for morphologic evidence of nuclear chromatin alterations and fragmentation. As shown in Fig. 2, C and D, there was no evidence of these late apoptotic changes. The histology of cardiac myocytes, perfused with or without MG132, appeared normal, suggesting that the TUNEL-positive nuclei were in the early stages of apoptosis.

The effect of proteasome inhibition on cardiac function

To determine if proteasome inhibition was associated with changes in cardiac function, hearts were perfused with buffer containing 25 or 50 μM MG132 for up to 80 min. As depicted in Fig. 3, perfusion with MG132 had a delete-

rious effect on cardiac function as determined by the rate · pressure product. Hearts perfused with 25 μM MG132 experienced a 63% decrease in function as compared with control hearts, which experienced a 35% decrease over the same time period ($p < 0.05$, RMANOVA). The decrease in function was so severe (−70% within 20 min) in hearts treated with 50 μM MG132 that the perfusion was terminated after 50 min, at which point the rate · pressure product was decreased by 77%. Much of the decrease in the rate · pressure product reflects a negative effect on myocardial chronotropy. To determine if myocardial inotropy was affected, the rate of rise of the pressure curve ($+dP/dt_{max}$) was calculated as an indication of contractility. As illustrated by Fig. 4, despite an initial increase after 10 min of perfusion, contractility decreased at a relatively consistent rate, so that by 80 min a 32% decrease was apparent. In hearts perfused with 50 μM MG132, an immediate decrease in contractility was observed with a 44% decrease within 20 min. This was in contrast to the control hearts, which remained relatively stable through 60 min and by 80 min had experienced only a 10% decrease ($p < 0.05$, RMANOVA). Decreases in myocardial inotropy and chronotropy indicate that proteasome inhibition has a deleterious effect on cardiac function in general.

The effect of proteasome inhibition on cardiomyocyte soluble ubiquitin-conjugated proteins

To determine whether inhibition of the proteasome resulted in accumulation of ubiquitinated proteins, myocardial-soluble proteins

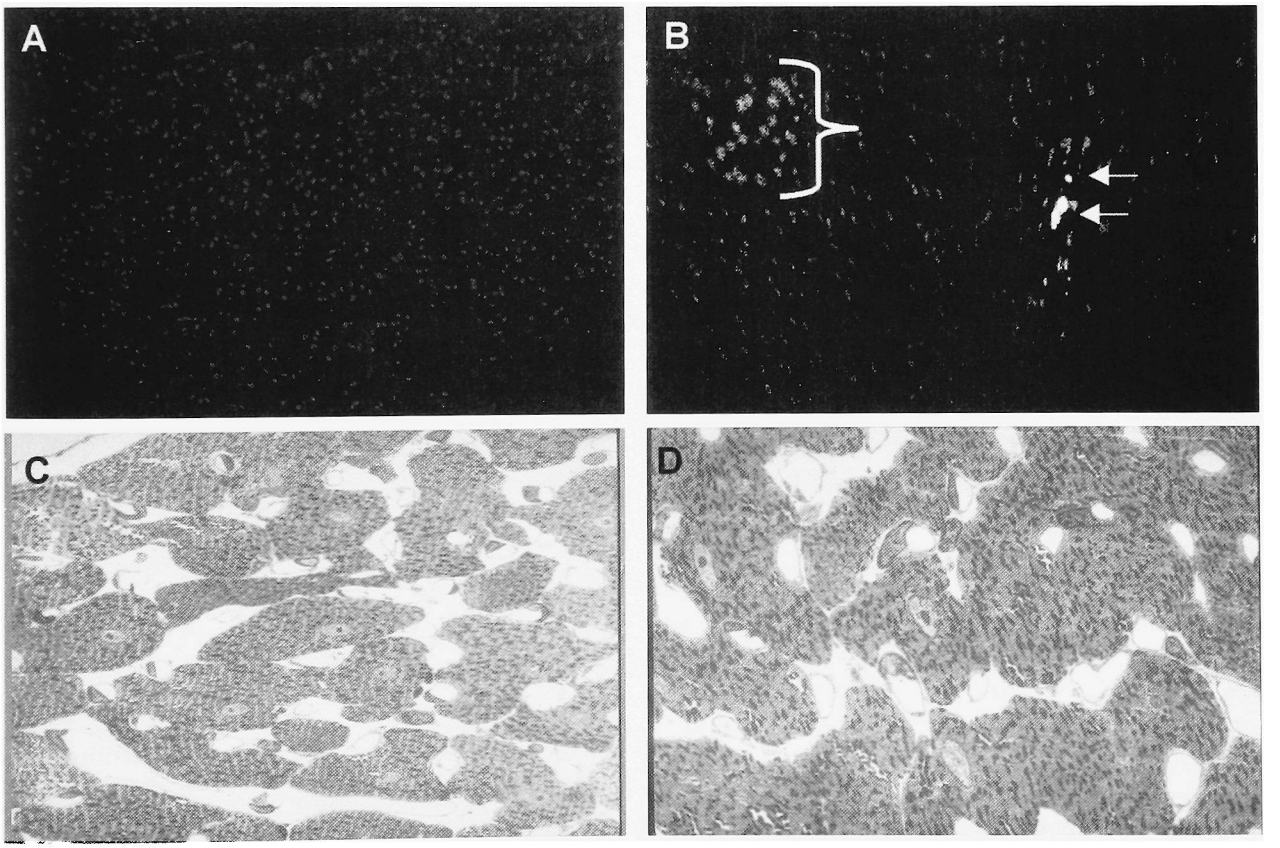

FIG. 2. Representative photomicrographs illustrating the effect of MG132 on cardiomyocyte TUNEL-staining (Panels A and B) and histology (Panels C and D). Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 for 50 min and then perfusion fixed with 10% formalin and analyzed for either apoptosis or histology. The top panels show representative results of TUNEL-staining in control (**Panel A**) and MG132-treated (**Panel B**) hearts (magnification, ×200). These figures were converted to gray scale and adjusted for brightness and contrast. TUNEL-positive nuclei appear as white dots (see bracket and arrow). The bottom panels show the representative histology in control (**Panel C**) and MG132-treated (**Panel D**) hearts (magnification, ×500).

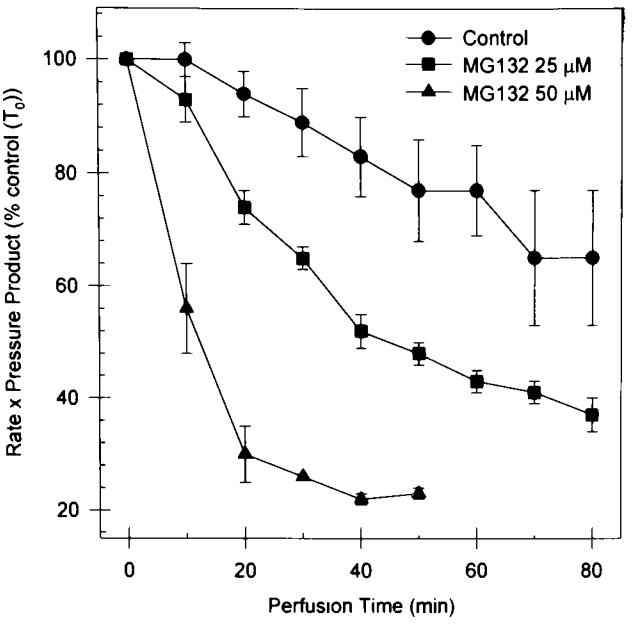

FIG. 3. The effect of MG132 on hemodynamic function of the isolated perfused heart. Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 or 50 μM MG132 for up to 80 min. Hemodynamic function was determined as the rate pressure product and then expressed as percent of control setting T_0 = 100% (Control T_0 = 32,963 ± 2,439 mmHg/min; 25 μM MG132 T_0 = 40,675 ± 3,437 mmHg/min; 50 μM MG132 T_0 = 32,613 ± 2,143 mmHg/min). Perfusion of hearts with 50 μM MG132 was terminated after 50 min because of the extreme effects on hemodynamic function. The values represent the mean ± SEM of six determinations.

were separated using electrophoresis and then probed with a specific anti-ubiquitin antibody. As depicted in Fig. 5, many proteins in the control hearts had significant reactivity with the antibody, indicating that these proteins were already ubiquitin-conjugated. In the presence of MG132, ubiquitin-conjugated proteins are increased. Because the increase was most apparent at molecular weights >80 kDa, we quantitated three proteins (78 kDa, 88 kDa, and 114 kDa) using computer-assisted densitometry. As shown in Table 1, perfusion of hearts with 25 μM MG132 resulted in an increase in ubiquitination of all three proteins. The increase was greater in the presence of 50 μM MG132 in the 88-kDa and 114-kDa proteins. These findings suggest that during normal cellular metabolism, certain myocardial proteins undergo ubiquitination and then probably degradation. In the presence of a 26S protease inhibitor, degradation is inhibited, resulting in an accumulation of these marked proteins.

DISCUSSION

The results of this study demonstrate that inhibition of the 26S protease has deleterious effects on normal myocardial function. We observed decreased myocardial function in the face of the initiation of increased cellular apoptosis and accumulation of ubiquitinated proteins. This observation suggests that the 26S protease and protein degradation play an important role in normal metabolic function of the cardiomyocyte. Intracellular protein degradation is a highly selective process that has important roles in the modulation of the levels of specific proteins and removal of damaged proteins. Rates of degradation of specific proteins vary according to their function. For example, regulatory proteins would be expected to have high turnover rates so that their levels can change rapidly to response to environmental alterations. Interference with these processes could be expected to have serious conse-

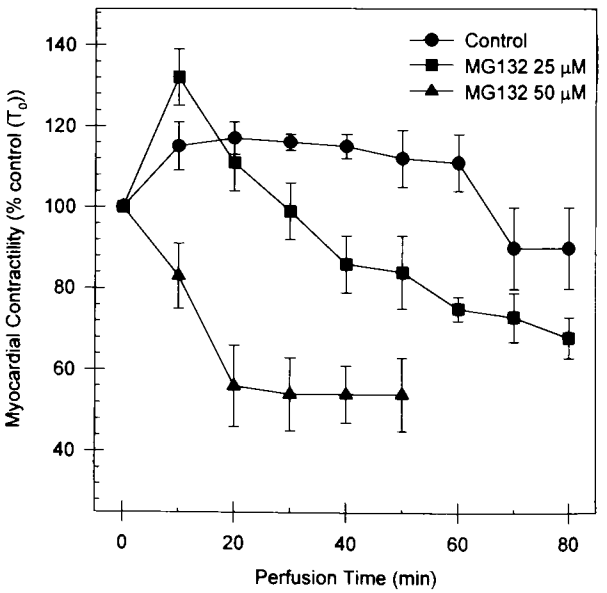

FIG. 4. The effect of MG132 on the contractility of the isolated perfused heart. Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 or 50 μM MG132 for up to 80 min. Myocardial contractility was determined as the first derivative of the pressure curve and then expressed as percent of control setting T_0 = 100% (Control T_0 = 3,500 ± 329 mmHg/sec; 25 μM MG132 T_0 = 4,333 ± 352 mmHg/sec; 50 μM MG132 T_0 = 3,667 ± 144 mmHg/sec). Perfusion of hearts with 60 μM MG132 was terminated after 50 min because of the extreme effects on hemodynamic function. The values represent the mean ± SEM of six determinations.

TABLE 1. THE EFFECT OF MG132 ON ACCUMULATION OF CARDIOMYOCYTE UBIQUITIN-CONJUGATED PROTEINS

	25 μM			50 μM		
	KH	MG132	Δ	KH	MG132	Δ
114 kDa	112	138	26	72	109	37
88 kDa	80	92	12	82	59	23
78 kDa	106	125	20	66	86	20

Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 for 50 min or 50 μM MG132 for 20 min and then analyzed for ubiquitin-conjugated proteins. Because of the severe effects of 50 μM MG132 on hemodynamic function (see Fig. 2) perfusion was stopped after 20 min. The values represent the arithmetic mean of two to three determinations and are expressed as shades of gray.

quences on cellular function and may, in fact, induce cell death or apoptosis.

The involvement of the ubiquitin-proteasome in apoptosis can be inferred from the numerous studies that describe a variety of effects of inhibition on activation of cell death factors and programs. One of the determinants appears to be related to cell cycle status. Actively proliferating cells appear to be more sensitive to the pro-apoptotic consequences of proteasome inhibition. Studies evaluating cell-permeant peptidyl aldehyde inhibitors have demonstrated stimulation of apoptosis in human T-cell leukemia MOLT-4 and mouse lymphocytic leukemia L5178Y cell lines (Shinohara *et al.*, 1996); the murine T-cell lymphoma RVC cell line; and human leukemic HL60 cells (Drexler, 1997). Lactacystin, a nonpeptidyl aldehyde inhibitor that covalently binds to the amino-terminus of the proteasome catalytic subunit (Craiu *et al.*, 1997), induces apoptosis in Ewing's sarcoma cells (Imajoh-Ohmi *et al.*, 1995) and human monoblast U937 cells (Imajoh-Ohmi *et al.*, 1995). In addition, lactacystin sensitizes chemo- and radioresistant human chronic lymphocytic leukemia lymphocytes to tumor necrosis factor-α (TNFα)-initiated apoptosis (Delic *et al.*, 1998). By contrast, proteasome inhibition seems to protect differentiated, nondividing cells from agents that induce apoptosis. For example, several of the peptidyl aldehyde analogues, as well as lactacystin, antagonize thymocyte apoptosis induced by dexamethasone or etoposide (Beyette *et al.*, 1998; Hirsch *et al.*, 1998; Stefanelli *et al.*, 1998). Inhibitors of proteasome function promote neuron survival in rat sympathetic neurons induced to undergo apoptosis following nerve growth factor withdrawal (Sadoul *et al.*, 1996).

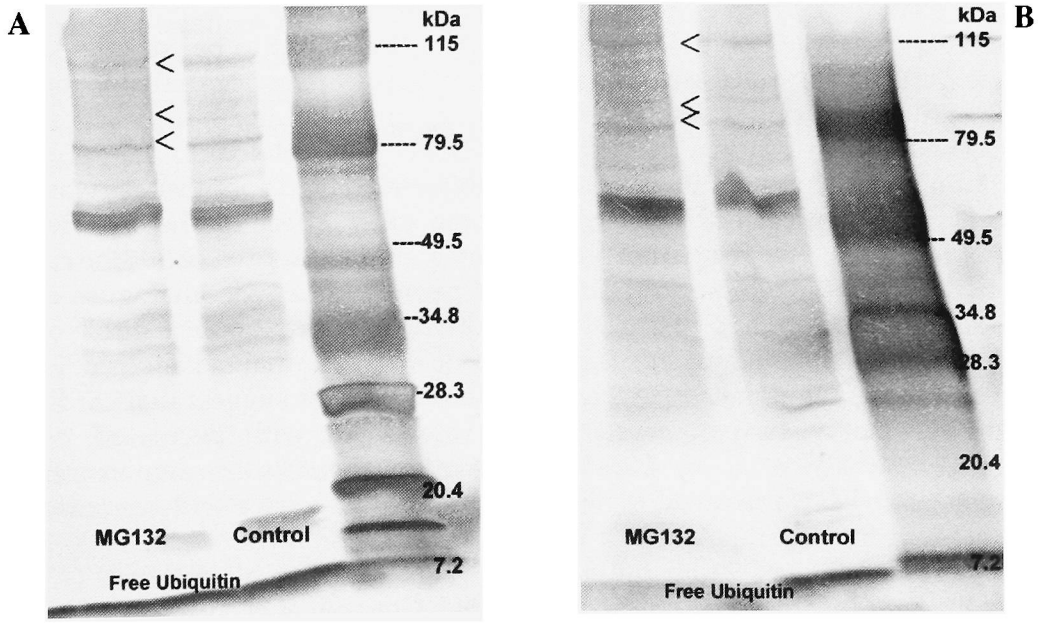

FIG. 5. Representative immunoblots illustrating the effect of MG132 on cardiomyocyte ubiquitin-conjugated proteins. Isolated hearts were perfused with Krebs-Henseleit buffer ± 25 μM MG132 (**A**) for 50 min or 50 μM MG132 (**B**) for 20 min and then analyzed for ubiquitin-conjugated proteins.

In this regard, our results contrast with prior studies in nonmitotic cells. In the present study, inhibition of proteasome function in the cardiomyocyte, a terminally differentiated cell, induces apoptosis. The cardiomyocytes appear to be in the early stages of apoptosis exhibiting only increased TUNEL staining and no nuclear morphological changes. However, our studies were short term; and, if it were possible to keep the isolated heart preparation functional for hours, morphological changes including DNA fragmentation might be observed. It may be relevant that prior studies have been in quiescent cell lines, whereas the current studies were the first to use a functioning isolated organ preparation.

In this study, we have demonstrated an increase in ubiquitinated proteins, particularly those in the >80-kDa size range. This observation is consistent with the accumulation of ubiquitinated proteins in the >75-kDa range in apoptotic Ewing's sarcoma cells exposed to either lactacystin or ionizing radiation (Soldatenkov and Dritschilo, 1997). The possible link between ubiquitination of proteins and cell death was first suggested by studies in the tobacco hawkmoth *Manduca sexta*, in which it was observed that polyubiquitin expression was increased in parallel with an increase in ubiquitinated proteins during muscle atrophy (Schwartz *et al.*, 1990). In mammalian systems, tibialis muscle from dystrophin-deficient mice had increased expression of polyubiquitin and increased ubiquitinated proteins associated with apoptotic nuclear changes. During muscle loss associated with starvation, an increase in polyubiquitin expression and ubiquitinated proteins has been demonstrated (Medina *et al.*, 1995; Wing *et al.*, 1995). A recent study indicates upregulation of the ubiquitin gene in stunned porcine myocardium (Sharma *et al.*, 1996), which is consistent with an increase in ubiquitinated proteins in ischemic myocardium from rat heart that we have observed (unpublished observation).

Other investigators have attempted to define the pathways between ubiquitination of a protein and activation of the cell death program. Considering the multiple systems affected by the proteasome, it is not surprising that numerous pathways have been described linking the proteasome, ubiquitination, and apoptosis. For example, the activation of the stress kinase, c-Jun N-terminal kinase (JNK), has been implicated in apoptosis of human lymphoid tumor cells incubated with MG132 and other peptidyl aldehyde analogues (Meriin *et al.*, 1998). The possible involvement of the apoptotic cascade can be inferred from studies demonstrating that incubation of U937 monoblasts with lactacystin and peptidyl aldehyde analogues increased poly(ADP-ribose) polymerase (PARP) cleavage and CPP32 (ICE)-like activity in the presence of TNFα (Fujita *et al.*, 1996). Other studies have demonstrated that proteasome inhibitors by themselves can induce PARP cleavage in U937 cells (Meriin *et al.*, 1998) and Ewing's sarcoma cells (Soldatenkov and Dritschilo, 1997). In those cell lines in which apoptosis is decreased by proteasome inhibition, PARP cleavage and CPP32-like activity tend to be decreased (Grimm *et al.*, 1996; Sadoul *et al.*, 1996; Stefanelli *et al.*, 1998). In addition, the cell survival protein, Bcl-2, decreases proteasome inhibitor-mediated apoptosis in Ewing's sarcoma cells (Soldatenkov and Dritschilo, 1997). It has been suggested that proteasomes act upstream of the caspase apoptotic cascade (Orlowski, 1999), although it is still possible that apoptosis is mediated via caspase-independent pathways. Yet, despite the association between accumulation of ubiquitinated proteins and apoptosis, there is no definitive proof that these proteins execute the cell death program.

The final point to be considered is whether the decrease in myocardial function, which was quite dramatic in the presence of 50 μM MG132, was related to the proteasome inhibitor-mediated apoptosis, or was the result of some other effect of the inhibitor. Unfortunately, little is known about the cardiac effects of peptidyl aldehyde analogues and virtually nothing is known about the role of the proteasome in myocardial metabolism. However, numerous studies suggest that cardiac apoptosis is associated with decreased hemodynamic function. These studies demonstrate changes in apoptosis associated with myocardial ischemia and reperfusion (Liu *et al.*, 1998; Maulik *et al.*, 1998), congestive heart failure (Anversa *et al.*, 1997; Goussev *et al.*, 1998), and anthracycline-induced cardiomyopathy (Zhang *et al.*, 1996; Sawyer *et al.*, 1999). Although we cannot rule out the possibility that diminished cardiac function is related to some other effect of the

inhibitor, it seems reasonable to suggest that at least part of the diminished function is related to the increased apoptosis.

In summary, we have demonstrated that the proteasome inhibitor, MG132, can be detrimental to cardiac function. Changes in cardiac function were associated with the accumulation of ubiquitinated proteins and initiation of apoptosis. The precise path from proteasome inhibition to activation of the apoptotic cascade is not clear at this time but may involve caspase processing.

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ABBREVIATIONS

BSA, Bovine serum albumin; DAPI, 4',6-diamidine-2-phenylindole-dihydrochloride; DMSO, dimethylsulfoxide; JNK, c-Jun N-terminal kinase; MG132, Z-leu-leu-leucinal; PARP, poly(ADP-ribose) polymerase; RMANOVA, repeated measures analysis of variance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNFα, tumor necrosis factor-α; TUNEL, terminal transferase-dUTP-nick end labeling.

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