Proteasome inhibition promotes regression of left ventricular hypertrophy

William E. Stansfield,1 Ru-Hang Tang,1 Nancy C. Moss,1 Albert S. Baldwin,2 Monte S. Willis,3 and Craig H. Selzman1

1Division of Cardiothoracic Surgery, Department of Surgery, 2Lineberger Comprehensive Cancer Research Center, and 3Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Submitted 14 February 2007; accepted in final form 10 November 2007


METHODS

Animal model. Male C57Bl-6 mice were purchased from Charles River Laboratories (Wilmington, MA) and boarded in an animal facility maintained by the University of North Carolina Department of Laboratory Animal Medicine. Housing conditions were standardized, including 12-h:12-h day-night cycles and unrestricted access to Isopro 3000-irradiated mouse chow and water. All procedures were designed within the guidelines set forth by the Animal Welfare Act and the Animal model. Male C57Bl-6 mice were purchased from Charles River Laboratories (Wilmington, MA) and boarded in an animal facility maintained by the University of North Carolina Department of Laboratory Animal Medicine. Housing conditions were standardized, including 12-h:12-h day-night cycles and unrestricted access to Isopro 3000-irradiated mouse chow and water. All procedures were designed within the guidelines set forth by the Animal Welfare Act and the.

Presently, the vast majority of research and clinical intervention for LVH is focused on interrupting the molecular pathways that give rise to LVH. Few studies have specifically examined the process of LVH regression. Several potentially reversible models of LVH exist, including anemia, aortocaval shunting, and adrenergic overdrive using isoproterenol (Iso). In 2000, a group using this latter model demonstrated that withdrawal of previously administered Iso from mice induced a distinct profile of gene expression (4). By observing that animals undergoing physiological regression are fundamentally different from those undergoing progression, we found that this study reinforced the importance of studying the phenomenon of regression explicitly.

Numerous putative regulators of LVH are reported. Among these is the ubiquitous nuclear transcription factor, nuclear factor-kB (NF-kB). Under basal conditions, NF-kB rests dormant in the cytoplasm as a heterodimer composed of p65 and p50, and an associated inhibitory protein, inhibitory kB (I kB). When activation signals converge, IkB kinase becomes activated. Phosphorylation of IkB leads to subsequent ubiquitination and degradation by the 20S proteasome. This liberates NF-kB for nuclear translocation, where it initiates a host of transcription programs. In 2001, NF-kB inhibition appears to mitigate the progression of LVH induced by previously established hypertrophic stimuli including myotrophin and aortic banding (9, 11). To date, the relationship between NF-kB activation/deactivation and physiological regression remains unknown.

In the present report, we employ the pharmacological model of hypertrophy and regression by Iso administration and withdrawal. We demonstrate that inhibition of NF-kB by proteasome inhibition is effective in preventing the development of hypertrophy in mice. More importantly, we show that proteasome inhibition is capable of inducing regression even in the face of an ongoing hypertrophic stimulus.

LEFT VENTRICULAR (LV) hypertrophy (LVH) is a quiet epidemic in North America. It is widespread, affecting more than 16% of the US adult population. It is progressive, and its prevalence increases with age, affecting more than one-third of individuals over the age of 70 (8). As an independent risk factor for cardiovascular morbidity and mortality, it carries a worse prognosis than three-vessel coronary artery disease (16). Worse still, LVH is not well treated. Even when patients with hypertension are treated with maximal medical therapy, nearly 50% of these patients have continued increases in LV mass by echocardiogram (7). In all cases, regression of hypertrophy is associated with increased survival, whereas patients who fail to regress are marked by increased cardiac morbidity and mortality (6, 7).


The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
National Institutes of Health’s (NIH) Guide for the Care and Use of Laboratory Animals. Before the initiation of the experiments, all procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Experimental design. Experiments were broadly divided into two classes: LVH progression (week 1; Fig. 1A) and LVH regression (week 2; Fig. 2A). Six mice were used for each individual group. To establish our model of LVH progression, we first treated mice with a hypertrophic agent, Iso, for 1 wk. These mice were compared with control animals treated with vehicle alone, animals treated with the inhibitor compound PS-519, and animals treated with both Iso and PS-519. In the regression class of experiments, we extended the experiment to a second week. Again, there were animals that received only vehicle and the inhibitor PS-519. The remaining groups were all allowed to develop hypertrophy over the course of the first week. During the second week, one group received continued hypertrophic stimulation, and another received only vehicle. The third group was treated with both Iso and PS-519. In the regression class of experiments, we extended the experiment to a second week. Again, there were animals that received only vehicle and the inhibitor PS-519. The remaining groups were all allowed to develop hypertrophy over the course of the first week. During the second week, one group received continued hypertrophic stimulation, and another received only vehicle. The third group received both Iso and the inhibitor PS-519 during the second week.

PS-519. In the regression class of experiments, we extended the experiment to a second week. Again, there were animals that received only vehicle and the inhibitor PS-519. The remaining groups were all allowed to develop hypertrophy over the course of the first week. During the second week, one group received continued hypertrophic stimulation, and another received only vehicle. The third group received both Iso and the inhibitor PS-519 during the second week.

Iso-induced hypertrophy and regression. LVH was induced in age-matched mice, weighing ~25 g, by administering 100 mg/kg of Iso (Sigma-Aldrich, St. Louis, MO) in saline vehicle subcutaneously on a daily basis for 7 days. Pharmacological regression was achieved by discontinuing the Iso while continuing to administer saline vehicle for the subsequent 7 days.

Proteasome inhibition. Proteasome inhibition was performed by the administration of the experimental compound PS-519 (generous gift of J. Adams, ProScript, Cambridge, MA). PS-519 is known to inhibit proteasome-mediated degradation of the inhibitory protein IκB. This prevents NF-κB activation and subsequent nuclear translocation. The dose of 1 mg·kg⁻¹·day⁻¹, administered intraperitoneally, has been previously shown to result in a rapid and stable decrease in chymotryptic proteasome activity to ~80–85% of baseline in the mouse model (17). The vehicle used was a 1:1 mix of propylene glycol and normal saline.

Histological analysis. Mice were euthanized by cervical dislocation. Sternotomy exposed the heart. The apex of the left ventricle was cannulated with a 22-gauge needle. The great vessels were then incised, and perfusion was initiated with 10 ml of PBS, followed by 10 ml of paraformaldehyde solution. Successful perfusion was evaluated by ready flow of the infusion agents and blanching of the heart. Hearts were then excised, weighed, and placed in excess volume of paraformaldehyde for 24 h. After fixation, hearts were bisected transversely at the level of the papillary muscles, embedded in paraffin block, and stained with periodic acid Schiff (PAS). Cardiomyocyte cross-sectional areas were determined by collecting images at ×20 magnification from representative heart slices in each group. ImageJ (v.1.38) software was used to outline individual cardiomyocytes that were averaged. For each experimental category, 50–300 cardiomyocytes were sampled. Transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using a standard TUNEL kit assay per manufacturer’s instructions (In Vitrogen, Carlsbad, CA). Staining incorporated propidium iodide and Invitrogen Gold-Antifade reagent. Results were recorded using a fluorescence confocal microscope, and analysis was completed using ImageJ, NIH image processing software.

Transthoracic echocardiography. Mice were placed under general anesthesia using 1% to 2% inhaled isoflurane through a non-rebreather mask. They were positioned on a thermostatically regulated mouse pad with echocardiogram limb leads. Throughout the experiment, the depth of anesthesia was kept to a minimum, with heart rates typically maintained between 480 and 520 beats/min. Body temperature was also maintained between 36° and 38°C. A depilatory agent (Nair) was applied to the back, and mice were allowed to recover for several hours before being placed in a backdrop hatch prior to surgery. A warm blanket was placed under the mouse as it was placed in the back hatch. Mice were allowed to rest for 15 minutes, and then were removed from the hatch and placed in a neutral position. The anesthesia using 1% to 2% inhaled isoflurane through a non-rebreather mask. They were positioned on a thermostatically regulated mouse pad with echocardiogram limb leads. Throughout the experiment, the depth of anesthesia was kept to a minimum, with heart rates typically maintained between 480 and 520 beats/min. Body temperature was also maintained between 36° and 38°C. A depilatory agent (Nair) was applied to the back, and mice were allowed to recover for several hours before being placed in a backdrop hatch prior to surgery. A warm blanket was placed under the mouse as it was placed in the back hatch. Mice were allowed to rest for 15 minutes, and then were removed from the hatch and placed in a neutral position.

Fig. 1. Proteasome inhibition prevents development of left ventricular hypertrophy (LVH). A: experimental design. During week 1, vehicle-treated animals were compared with animals treated with PS-519 (PS), isoproterenol (Iso), and Iso and PS-519 cotreatment. B: after 7 days, hearts were sectioned transversely at the midpapillary level, stained with periodic acid Schiff (PAS) and viewed at ×40 (bar = 0.1 μm). Measurement of cardiomyocyte area is depicted in parenthesis for each group (*P < 0.01 vs. control; †P < 0.01 vs. Iso). C: tabular representation of heart weight-to-body weight ratios (HW/BW) and raw echocardiographic data focusing on left ventricular posterior wall (LVPW) thickness during diastole at baseline and after 1 wk (*P < 0.01: vs. vehicle; †P < 0.001 vs. Iso). D: real-time PCR for evaluating gene expression relative to 18s RNA expression in ventricular apices after 1 wk of treatment (*P < .004 vs. vehicle; **P < 0.04 vs. Iso), β-MHC, β-myosin heavy chain; BNP, brain natriuretic peptide; SM, smooth muscle.

AJP-Heart Circ Physiol • VOL 294 • FEBRUARY 2008 • www.ajpheart.org
used to clean the chest of hair. Aquasonics 100 ultrasound gel (Parker, Fairfield, NJ) warmed to 37°C was applied to the chest, and the mice were tilted into the right lateral decubitus position. Echocardiograms were obtained at the level of the papillary muscle to evaluate ventricular dimensions during diastole. The interpretation was completed using a leading edge-to-leading edge methodology, according to the guidelines set forth by the American Society of Echocardiographers (13). To facilitate the comparison between animals, values were expressed as a percent change in wall thickness for each animal.

Molecular analyses. After cervical dislocation, sternotomy was performed and hearts were excised while still beating. Hearts were cold rinsed with phosphate-buffered solution (PBS) and blotted dry, and then vehicle only or continued Iso with the addition of PS-519 during week 2. A: after 14 days, hearts were sectioned transversely at the midpapillary level, stained with PAS, and viewed at ×40 (bar = 0.1 μm). Measurement of cardiomyocyte area is depicted in parenthesis for each group (*P < 0.01 vs. control; †P < 0.01 vs. Iso, days 1–14). C: tabular representation of HW/BW ratios and raw echocardiographic data focusing on LVPW thickness. Percent change in LVPW depicts the change as measured in individual animals between the end of the first week and the end of the second week (*P < 0.01 vs. vehicle and continuous Iso).

Fig. 2. Proteasome inhibition and LVH regression. A: experimental design. In the 2-wk experiments, animals received continued vehicle, continued PS-519, or continued Iso. To evaluate regression, other animals received Iso during week 1, and then vehicle only or continued Iso with the addition of PS-519 during week 2. B: after 14 days, hearts were sectioned transversely at the midpapillary level, stained with PAS, and viewed at ×40 (bar = 0.1 μm). Measurement of cardiomyocyte area is depicted in parenthesis for each group (*P < 0.01 vs. control; †P < 0.01 vs. Iso, days 1–14). C: tabular representation of HW/BW ratios and raw echocardiographic data focusing on LVPW thickness. Percent change in LVPW depicts the change as measured in individual animals between the end of the first week and the end of the second week (*P < 0.01 vs. vehicle and continuous Iso). D: real-time PCR for evaluating gene expression relative to 18s rRNA expression in ventricular apexes after 2 wk of treatment and depicted as fold change between weeks 1 and 2 (*P < 0.05 vs. vehicle; **P < 0.05 vs. continuous Iso).
Western blot analysis. Nuclear and cytosolic protein fractions were first denatured in loading buffer. Each sample (30 μg) was then loaded into alternating lanes for gel electrophoresis. Membrane transfer was performed overnight, and rabbit anti-mouse p65 antibody (gift of A. S. Baldwin) was used to probe for p65. Tubulin was used as the loading control.

Statistical methods. All data are presented as means ± SE. Comparisons between groups were done using analysis of variance (ANOVA) with Bonferroni-Dunn post hoc analysis or t-test as appropriate. Statistical significance was accepted within the 95% confidence interval. For real-time PCR data, expression fold changes were log transformed before statistical comparison. All analysis was performed using the statistical package Prism 4 (GraphPad, San Diego, CA).

RESULTS

PS-519 prevents the development of LVH.Iso-treated mice had no obvious ill effects and experienced a 95% survival rate during the experimental time course. After 1 wk, mice demonstrated increased heart weight-to-body-weight ratios, cardiomyocyte size on histology, and increased LV wall thickness. Molecular phenotyping for fetal gene expression demonstrated increases in expression of BNP and β-MHC (P < 0.05) and an upward trend in SM α1-actin (Fig. 1). Given alone, PS-519 had no affect on the parameters of LVH. When administered concomitantly, PS-519 appeared to prevent Iso-induced increases in heart weight-to-body weight ratios, cardiomyocyte size and nuclear density, LV wall thickness, and fetal gene expression. There were no differences between PS-519 co-treated and control animals (Fig. 1).

PS-519 induces physiological regression. To determine the role of proteasome inhibition on reversing existing LVH, we first sought to validate the reversibility of the Iso model. As demonstrated in Fig. 2, mice treated for 7 days of Iso were then given a second 7-day course of either vehicle or Iso. When compared with mice receiving continuous Iso during the 2-wk period, mice that had a withdrawal of Iso had substantial reductions in heart weight-to-body weight ratios, cardiomyocyte size and nuclear density. Echocardiography demonstrated a 12% decrease in LV wall thickness over the course of the second week, essentially normalizing the wall thickness. Interestingly, hypertrophic gene expression changed little during the second week. In fact, withdrawal of Iso did not effect β-MHC expression, and it remained elevated throughout the 14-day period (Fig. 2D).

We next sought to determine the influence of proteasome inhibition on LVH provoked with continuous Iso stimulation. Animals that had already developed cardiac hypertrophy after 1 wk of Iso were treated with an additional week of Iso and then cotreated with PS-519. PAS-stained cross sections 1 wk later (2 wk total experiment) showed moderate, but significant, reductions in cardiomyocyte size and nuclear density that roughly compared with specimens from the Iso withdrawal group (Fig. 2B). Heart weight-to-body weight ratio similarly returned to normal, and LV posterior wall, depicted as the fold change in the second experimental week, declined even further than in the Iso-withdrawal group (Fig. 2C). We next focused on the changes in gene expression that occur specifically during the regression period (week 2). When administered during the second week concomitantly with Iso, PS-519 essentially normalized β-MHC expression and reduced SM α1-actin to the level of control (Fig. 2D).

LVH regression is associated with deactivation of NF-κB activation. Although previously known to inhibit NF-κB activation, we next sought to determine the effect of PS-519 on this mechanism within the context of our model. Western blot analyses of nuclear and cytoplasmic protein extracts from LV apaxes were normalized with a β-tubulin loading control. During the development phase (Fig. 3A), 1 wk of Iso increased p65 nuclear translocation that was abrogated with PS-519 cotreatment. During the regression phase, mice that received 2 consecutive wk of Iso maintained elevated nuclear levels of p65 (albeit less than the Iso week 1 seen in Fig. 3A) that returned to near control with Iso withdrawal or with concomitant administration of PS-519 during the second week (Fig. 3B).

DISCUSSION

In this report, we demonstrate the influence of NF-κB inhibition on both the development and regression of structural, functional, and genotypic LVH. We show that NF-κB is required for progression of LVH and that proteasome-mediated inhibition of NF-κB activation effectively impedes this progression. Most importantly, we reveal that, despite ongoing stimulation, NF-κB inhibition can promote myocardial structural and molecular regression of LVH.

Several groups have previously demonstrated that inhibition of NF-κB may attenuate the development of hypertrophy both in vitro and in vivo. Purcell and colleagues (11) cotreated rat neonatal cardiomyocytes exposed to hypertrophic agonists with NF-κB inhibitors and demonstrated that the cells which had failed to enlarge had less expression of atrial natriuretic peptide and lower levels of activated NF-κB. Subsequently, Li and colleagues (9), in an in vivo model of pressure overload, demonstrated that cardiac transfection of an IκB superrepressor significantly reduced the development of LVH relative to banded controls. In the first part of our experiment, we have similarly demonstrated that proteasome-mediated inhibition of NF-κB prevents the development of hypertrophy in vivo. Our model is different in that it employs adrenergic overdrive instead of pressure overload, and we did not control for blood pressure. However, another group has already demonstrated...
that NF-κB inhibition slows the development of hypertrophy in hypertensive rats in a manner that is independent of blood pressure (5). Taken together, our results further support the importance of NF-κB as an important intermediate in the development of cardiac hypertrophy.

One week following adrenergic stimulation, mouse hearts expressed the expected hypertrophic gene pattern including upregulation of β-MHC, BNP, and SM α-1-actin. The dramatic increase in early BNP expression compared with the other genes following hypertrophic stimulus is well recognized (10). During the second week of stimulation, β-MHC rose as the more dominant genetic marker of LVH, whereas BNP fell to near normal levels. This feature is also supported by previous studies (14). However, we were surprised that β-MHC expression remained elevated despite cessation of adrenergic stimulation. We believe that this model system demonstrates two distinct events. We and others have observed that BNP often reflects the acute changes associated with sudden increases in pressure. As such, it is quite possible, after 1 wk of stimulation, that BNP levels do not necessarily increase more (Fig. 2D). Conversely, β-MHC is a structural protein that has a longer expression period, and thus might stay elevated longer after withdrawal of a stimulus (Iso), but can actively be shut down when provided a regressive agent (PS-519). Nevertheless, these findings suggest that proteasome inhibition can both potently interfere with specific hypertrophic gene expression programs and promote physiological changes associated with both LVH development and regression.

PS-519 is a proteasome inhibitor that is based on the naturally occurring compound lactacystin and permanently interrupts the chymotryptic activity of the 20S proteasome. This blocks degradation of IκB, effectively preventing nuclear translocation and activation of NF-κB. Because so many intracellular signaling pathways are at least partially regulated by proteasome activity, certain inhibitors have already found their way to clinical application. PS-341, also known as Bortezomib (Velcade), is a functionally similar but structurally different compound than our inhibitor. It is currently in clinical use for the treatment of multiple myeloma and advanced solid tumors (3). It acts by partially inhibiting NF-κB in such a way that the cell is more sensitive to environmental and oxidative stresses, and apoptosis is induced more readily. Alternatively, PS-519 inhibits activation of NF-κB in a manner that confers resistance to ischemic injury, as previously demonstrated in a swine left anterior descending coronary artery occlusion model (12). Indeed, in our series of experiments, we were unable to detect any differences in apoptosis associated with adrenergic overdrive or proteasome inhibition as measured by TUNEL staining or real-time PCR expression of Bax-to-Bcl2 ratios. PS-519 has also undergone phase-1 clinical trials for use in the treatment of acute stroke, where it has similarly been shown to attenuate ischemia-reperfusion injury (15). The current report is, to the best of our knowledge, the first to demonstrate the influence of proteasome inhibition on both the development and regression of LVH.

We acknowledge that there are several limitations in applying this study directly to our mechanistic understanding of LVH in humans. First, we cannot confirm the specificity of PS-519-afforded proteasome inhibition. There may be confounding effects in addition to inhibiting NF-κB activation. This compound was selected for its known efficacy in NF-κB inhibition and for its relative clinical accessibility, including documented safe use in humans in the acute setting (15). Another potential limitation is the adrenergic overdrive model we selected for the development of hypertrophy. This model may be less clinically relevant than other models (e.g., aortic banding and renin hypersecretion). However, our priority in this study was to examine the possibility of both spontaneous and imposed LVH regression. Furthermore, from the current literature, it is unknown whether the pathways of hypertrophy progression determine or influence the pathways available for physiological regression.

In conclusion, NF-κB is essential for the development of LVH in an adrenergic overdrive model of LVH, and its inhibition can promote LVH regression. In humans, it is nearly impossible to block the myriad of pathways that lead to LVH. Thus the effect of PS-519 inducing LVH regression, despite well-formed LVH and ongoing stimulus, becomes very compelling. The possibility that pharmaceuticals, such as proteasome inhibitors, can induce regression of LVH, and not merely delay its progression, offers hope for many of the patients currently living with LVH.

ACKNOWLEDGMENTS

We appreciate the editorial assistance of Margaret Alford Cloud.

GRANTS

This work is supported in part by grants from the American College of Surgeons and the Thoracic Surgery Foundation for Research and Education.

REFERENCES

10. Magga J, Marttila M, Mantymaa P, Vuolteenaho O, Ruskoaho H. Brain natriuretic peptide in plasma, atria, and ventricles of vasopressin-


