ALTERATIONS IN PROTEIN P53 EXPRESSION DURING
THE DEVELOPMENT OF PRESSURE OVERLOAD-INDUCED
LEFT VENTRICULAR HYPERTROPHY IN RATS

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ABSTRACT

The relation between myocardial structural and functional changes and p53 proteins during the development of left ventricular hypertrophy (LVH) has not yet been reported. The purpose of this study was to determine whether p53 protein expression is altered during development of LVH, induced by pressure overload.

LVH was induced in 80 male Wistar adult rats by abdominal aortic banding (AAB) and was monitored at the 10th, 15th, 20th, 25th, 35th and 45th post-operation days by echocardiography and validated by postmortem examination. Sham operated (SO) rats (n=60) went the same operation without banding. The expression of p53 protein in the nuclear and cytoplasmic extracts from left ventricular tissue from AAB and SO rats were analyzed by immunoblotting at each of the pointed days.

Relative to SO, echo-left ventricular mass-to-tibia length (LVM/TL) ratio in AAB increased progressively on the 10th and the 15th day. On the 20th day we observed a short lasting regression of LVH, followed by a new marked increase on the 25th and 35th day; then there was presented a plateau. Relative to SO the changes in LV%FS (77% ± 2.2 %) in AAB rats remained at the same level throughout the studied period without significant differences in body weight or tibia length (TL). Results from echocardiography were validated by necropsy. Echo LVM/TL correlated significantly with actual heart weight-to-tibia length ratio (r=0.69, P< 0.0001, n=140).

The level of p53 protein expression in the nuclear extracts from LV tissues obtained from AAB rats increased significantly by 77%, 78%, 35% and 63% at day 10th, 20th, 35th and 45th, respectively compared with SO, and returned to SO levels by day 15th and 25th post-operation. The highest level of p53 protein in the nuclear extracts from LV tissues obtained from AAB rats was determined on 20th day concomitant with significant reduction of the actual heart mass in the group with AAB rats, compared with the same groups on the other days. In the cytoplasm the expression of p53 was always higher in the SO, compared with AAB groups. Furthermore, the level of p53 protein in the nuclear extracts from AAB rats significantly correlated with the actual heart weight during the studied period (r= -0.59, P< 0.0001, n=60).

Following the development of LVH over a relatively long period of time and providing the changes in short intervals, it was found a short lasting regression during ongoing pressure overload concomitant with significantly increased expression of p53 protein in the nuclear extracts from AAB rats.

This study adds new important concepts for the time dependency of LVH and suggests that p53 protein may modulate the adaptive growth of pressure overload induced LVH.

Keywords: protein p53, left ventricular hypertrophy, rats

Introduction

Left ventricular hypertrophy (LVH) is an adaptive response to increased hemodynamic load that myocardium undergoes during postnatal maturation, characterized by the enlargement of individual cardiomyocytes, without cell division. Although this process is initially compensatory, findings from the Framingham studies have provided evidence that the presence of LVH is an independent risk factor for the development of heart failure (15). Aspects of developmental hypertrophy are reemployed in the adult heart in response to diverse pathophysiologic stimuli such as hypertension, ischemic heart disease, valvular insufficiency, and cardiomyopathy (20). Previous studies have demonstrated that LVH is not a sole adaptive response to an increased workload because other biological changes play an important role in this process (9, 10) such as alterations in gene expression, including induction of the expression of immediate early genes (e.g., c-myc and c-fos), embryonic genes [e.g., atrial natriuretic factor (ANF)], and other genes (e.g., transforming growth factor-p) that regulate cell growth and differentiation. However, the molecules that mediate the growth of cardiomyocytes during LVH remain unknown.

Observations have suggested that cell cycle control molecules, such as tumor suppressor proteins (8, 25, 28) may be implicated in this process. Regulation of the cell cycle in the cardiac muscle is unique. Most adult cardiomyocytes are highly differentiated and little DNA synthesis or cell proliferation occurs in myocardium. But upon hormonal or mechanical stimulation significant DNA synthesis can be elicited and progression of cardiomyocytes into S phase can occur (5). It is known, however, that regulation of cell growth and differentiation involves stimulatory and inhibitory pathways of cell cycle progression (21). Several lines of evidence suggest...
that p53 is a critical component of an inhibitory pathway that negatively regulates progression of the cell cycle (24) and prevents the cells from entering S phase. p53 plays multiple roles in cells. A wide range of stress factors can activate p53 to elicit adaptive responses that include, but that are by no means restricted to, growth arrest and apoptosis. These properties are profoundly influenced by cell type and tissue-specific modifiers, which are of great importance but remain poorly understood (12).

Evidence in vitro suggests a functional association between cardiomyocyte stretch and p53 activation (17). In several in vivo injury models (ie, myocardial infarction (22), reperfusion injury (30), pacing-induced heart failure (3), and isoproterenol-induced hypertrophy (18) cardiac p53 expression is known to increase. Nevertheless, our current knowledge of the expression of protein p53 during the development of LVH induced by pressure overload remains unclear.

With the use of abdominal aortic banding (AAB) - a simple technique for inducing LVH in rats, the aim of this study was to examine by immunoblotting the effect of pressure overload on the expression of protein p53 in left ventricular (LV) tissues. Our data suggest that protein p53 may be involved in the adaptive growth of LV tissue during pressure overload induced LVH in rats.

Materials and Methods

Animals

Experiments were performed with male Wistar rats, 200±10 g body weight and 9-10 weeks old at the beginning of the experiment. All procedures were performed in accordance with the Ethical Committee Note of the Medical University Plovdiv (Bulgaria) for care and use of laboratory animals.

Animal model

Surgical Induction of Pressure Overload

Pressure overload was produced by AAB subdiaphragmatically as previously described (27). Briefly, under anesthesia with pentobarbital sodium (65mg/kg ip) intraperitoneally and a midline abdominal laparotomy, a nearly 1 cm segment of abdominal aorta was separated free just bellow the diaphragma and a needle, having 0.95 mm external diameter was placed on the top of the aorta. A 2-0 surgical silk was tightly banded around the needle and aorta providing a uniform degree of constriction. The needle was then carefully withdrawn from the ligature so that the diameter of the constriction approximated that of the needle. Muscles and skin were closed layer by layer with 3-0 silk suture. For sham operated (SO) animals the identical procedure was performed except AAB. Postoperatively, all animals were fed commercial rat chow and had free access to water. On the final day of the study each rat with AAB was examined to verify the constriction.

Echocardiographic study

After sedation with pentobarbital sodium (65mg/kg) injected intraperitoneally, rats were secured in a supine position, shaved at the precordium, and transthoracic echocardiography was performed by using Sono Site 180 (SonoSite Inc., USA) equipped with a 10 MHz linear transducer.

The heart was imaged in the 2-D mode in the parasternal long-axis view. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricular at the level of the papillary muscles. Left ventricular anterior wall thickness (AWT), posterior wall thickness (PWT), left ventricular end-diastolic diameter (LVEDD), and left ventricular end-systolic diameter (LVESD) were measured. All measurements were done from leading edge to leading edge according to the American Society of Echocardiography guidelines (26). Three representative cardiac cycles were analyzed and averaged for each measurement. Left ventricular mass (LVM) was calculated by use of the following formula, assuming a spherical left ventricular geometry and validated in rats (19): LVM (g) = 1.04 x [(LVEDD+ PWT+ AWT)3-LVEDD3], where 1.04 is the specific gravity of myocardium. The percentage of LV fractional shortening (LV%FS) was calculated as [(LVEDD-LVESD)/LVEDD] x 100.

Hemodynamic Measurements

Mean blood pressure (MBP) was measured in anesthetized (pentobarbital sodium - 65mg/kg ip) rats (n=5 from each group and at each time point). For this purpose the left carotid artery was cannulated with a fluid-filled polyethylene (PE-50) catheter connected to a pressure transducer (Experimetria, Ltd, Hungary) in-line to Cardiostar CO-100 polygraph (Cardiostar CO-100, Experimetria MM, Ltd., Budapest, Hungary).

Cytoplasmic and nuclear protein extraction.

Cytoplasmic and nuclear extracts were prepared from snap-frozen LV tissue (14). The LV tissue (100 mg) was homogenized, using a DIAx900 P/N595 homogenizer in 300µl an ice-cold phosphate-buffered saline (PBS) containing 5µl protease inhibitor cocktail (AEBSF 104mM, Aprotinin 0.08mM, Leupeptin 2mM, Bestatin 4mM, Pepstatin A 1.5mM, E-64 1.4mM - Sigma). Homogenate was centrifuged for 10 min at 12000g at 4°C. The supernatant was kept as cytoplasmic extract and stored at -80°C. For preparation of nuclear extracts, pelleted nuclei were resuspended in PBS, centrifuged, and further lysed for 15 min on ice in PBS and Triton X-100 added to a final concentration of 0.5% in the presence of a cocktail of protease inhibitors as described above. After centrifugation at 12000g at 4°C for 10 min the supernatant was transferred to a new tube as nuclear extract and stored at -80°C until needed. Protein concentrations in each sample were determined according to the method of Bradford (4) using bovine serum albumin (BSA) as a standard.

Immunoblotting

Samples (45 µg of protein) were heat-denatured in Laemmli sample buffer (10% glycerol, 5% b-mercaptoethanol, 2% sodium dodecyl sulphate (SDS), 62.5mM Tris (pH 6.8), 0.05% bromphenol blue, final concentrations) and after centrifugation
Experimental protocol

After the initial assessment by using echocardiography rats were randomly divided into 2 groups and subjected to AAB or sham operation.

In 10-16 AAB- rats and 10 SO- rats transthoracic echocardiography was performed at 10th day, 15th day, 20th day, 25th day, 35th day and 45th day after surgery and on the appointed day they were euthanized.

Body weight (BW) was recorded for each rat, both on the day of operation and on the day of death. On the last day, after all measurements were completed, each animal chest was opened through a left thoracotomy, the pericardium was exposed, and the heart was quickly removed, immersed in ice-cold PBS and weighed. Left ventricular free wall was separated and each sample of LV tissue was used for protein preparation. Left ventricular tissue was frozen immediately in liquid nitrogen and stored at -70°C until required for biochemical analysis. The length of the tibia was measured. The right tibia was dissected, and its length from the condyles to the tip of the medial malleolus was measured with a micrometer caliper by the method of Yin et al. (29).

Echocardiographic measurements

In vivo 2D guided M-mode echocardiograms were obtained on the 10th, 15th, 20th, 25th, 35th and 45th day after operation in all rats. Echo-LVM was calculated and results were expressed as the ratio of echo LVM-to-TL. In relation to SO, echo LVM-to-TL ratio in AAB rats increased significantly (e.g., by 24%)

TABLE 1

| Day   | BW g   | TL mm | MBP mmHg | HR beats/min |
|-------|--------|-------|-----------|--------------|
|       | SO     | AAB   | SO        | AAB          | SO  | AAB | SO  | AAB |
| 10th d| 197±9  | 197±18| 34.9±0.6  | 34.7±1.4     | 111±3| 165±5* | 403±15| 372±16|
| 15th d| 211±9  | 219±11| 35.8±0.7  | 35.9±0.8     | 112±3| 167±6* | 410±11| 399±17|
| 20th d| 223±9  | 228±15| 36.5±0.7  | 36.4±0.6     | 113±3| 166±4* | 409±8 | 409±13|
| 25th d| 244±9  | 253±30| 36.8±0.4  | 36.7±1.0     | 113±5| 164±2* | 410±7 | 405±17|
| 35th d| 260±7  | 283±19| 37.5±0.5  | 38.1±0.8     | 113±2| 165±3* | 410±7 | 406±17|
| 45th d| 290±14 | 301±20| 38.4±0.5  | 38.5±0.8     | 114±4| 168±4* | 409±8 | 402±16|

Data are means±SD; # - P<0.05 AAB vs. respective SO. d-day.

Statistical analysis

Values are expressed as means ± standard deviation. Serial echocardiographic studies were tested by repeated measures ANOVA followed by Fisher’s protected least significant difference test. Results obtained in postmortem examination, in echocardiographic studies and protein levels were analyzed with analysis of variance of the multivariate profiles for dependent groups (MANOVA), followed by the Duncan post hoc test to assess differences between groups (AAB and SO), as well as the effect of aortic banding in time. A value of P<0.05 was considered statistically significant. The relation between variables of echo-LVM, actual HW and protein levels were examined by regression analysis.

Results and Discussion

Mortality

A total of 155 rats were initially enrolled in this study – 95 underwent AAB and 60 rats - SO. Upon surgery, the death rate for AAB was nearly 16% - mainly in the first 12 hours after the operation and only two died on the third day. There were no fatalities in SO.

Hemodynamic measurements

The time course for alterations in MBP and heart rate after 10, 15, 20, 25, 35 and 45 days of pressure overload is presented in Table 1. Relative to SO MBP was increased by 53 mmHg in AAB rats at each time point after pressure overload. No significant difference was observed in heart rate (HR) between AAB and SO rats and within each group across time. Body weight (BW) and tibia length (TL) were analyzed across groups and in time. There was significant increase in BW and TL with time in rats from both groups, with no significant difference between the groups at each time point (Table 1).

Echocardiographic measurements

After the initial assessment by using echocardiography rats were randomly divided into 2 groups and subjected to AAB or sham operation.

In 10-16 AAB- rats and 10 SO- rats transthoracic echocardiography was performed at 10th day, 15th day, 20th day, 25th day, 35th day and 45th day after surgery and on the appointed day they were euthanized.

Body weight (BW) was recorded for each rat, both on the day of operation and on the day of death. On the last day, after all measurements were completed, each animal chest was opened through a left thoracotomy, the pericardium was exposed, and the heart was quickly removed, immersed in ice-cold PBS and weighed. Left ventricular free wall was separated and each sample of LV tissue was used for protein preparation. Left ventricular tissue was frozen immediately in liquid nitrogen and stored at -70°C until required for biochemical analysis. The length of the tibia was measured. The right tibia was dissected, and its length from the condyles to the tip of the medial malleolus was measured with a micrometer caliper by the method of Yin et al. (29).

Measurements of body weight (BW), tibia length (TL), mean blood pressure (MBP), heart rate (HR) in sham-operated (SO) and abdominal aortic banded (AAB) rats at each time point after surgery.
at day 10, 51% at day 15, 21% at day 20, 45% at day 25, 69% at day 35 and 70% at day 45 (Fig. 1A). Interestingly, a short-lasting regression of LVH at day 20 was observed. On this exact day echo LVM-to-TL ratio was significantly lesser in respect to the other time points in AAB group (e.g. by 16% to 10\textsuperscript{th} day, by 27% to 15\textsuperscript{th} day, by 28% to 25\textsuperscript{th} day, by 49% to both 35\textsuperscript{th} and 45\textsuperscript{th} days).

All echocardiographic parameters remained with no significant changes in SO during the studied period. Echocardiographic data showed that hearts of the rats subjected to AAB maintained normal LV chamber size and systolic function. Relative to SO the changes in LV%FS (77% ± 2.2%) in AAB rats were not statistically significant and remained at the same level throughout the studied period (Fig. 1C).

**Necropsy validation**

The time dependency of LVH and the short lasting regression of the hypertrophy on the 20\textsuperscript{th} day were verified by necropsy at each time point. Actual heart weight (HW) was measured at each time point and results expressed as the ratio of HW-to-TL (Fig. 1B). They supported the observed by echocardiographic study changes in the development of LVH. Furthermore, echo LVM-to-TL ratio correlated significantly with HW-to-TL ratio ($r=0.69; P<0.0001$) (Fig. 1D).

### Changes in p53 protein expression after AAB and SO operation

To determine the expression of p53 protein during the development of LVH, nuclear and cytoplasmic extracts from LV tissue of AAB and SO rats were analyzed by immunoblotting. We measured the level of p53 protein in cytoplasmic and nuclear extracts of LV tissues from AAB and SO rats at days 10\textsuperscript{th}, 15\textsuperscript{th}, 20\textsuperscript{th}, 25\textsuperscript{th}, 35\textsuperscript{th} and 45\textsuperscript{th} after operation. Equal protein loading in each experiment was confirmed by Coomassie blue staining of the gels and by reprobing each membrane with a mouse monoclonal antibody to α-actin.

Each experiment was repeated twice to confirm the reproducibility of the results. A representative experiment is shown in Fig. 2. LV tissue obtained from SO rats showed a constant level of p53 protein in the nuclear and cytoplasmic extracts throughout the day 10-45 postoperative period. Relative to the nuclear extracts from the LV tissue obtained from SO rats at each time point, the protein levels of p53 detected in the cytoplasmic extracts from the same samples were significantly higher.

The level of p53 protein in the cytoplasmic extracts from AAB rats was higher on 15\textsuperscript{th} and 25\textsuperscript{th} days (0.39±0.05 and 0.38±0.09 respectively) compared with AAB rats on the other days (10\textsuperscript{th} -0.33±0.08, 20\textsuperscript{th} -0.34±0.09, 35\textsuperscript{th} - 0.34±0.08 and 45\textsuperscript{th}.

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**Fig. 1.** Time-course changes in (A) echocardiographically derived left ventricular mass ventricular –to-tibia length ratio [echo-LVM/TL], (B) actual heart weight to tibia length ratio [HW/TL], (C) percentage of LV fractional shortening (LV%FS) assessed by echocardiography of 10-16 abdominal aortic banding rats (AAB) and 10 sham-operated (SO). (D) Correlation between actual heart weight-to-tibia length ratio [HW/TL] and echo-LV mass-to tibia length ratio [echo-LVM/TL]; $r =0.69, P<0.0001; n=140$. d - day; Data are means±SD; a - P<0.05 vs. 10 day AAB; b - P<0.05 vs. 15 day AAB; c - P<0.05 vs. 20 day AAB; d - P<0.05 vs 25 day AAB; e - P<0.05 vs 35 day AAB; f - P<0.05 vs 45 day AAB; # - P<0.05 vs respective SO.
-0.17±0.09), such that no significant differences was observed between the cytoplasmic levels of p53 protein in AAB and SO rats at 15th and 25th days (Fig. 3A).

Interestingly, the level of p53 protein in the nuclear extracts from LV tissues obtained from AAB rats increased significantly by 77%, 78%, 35% and 63% at day 10th, 20th, 35th and 45th respectively, compared with SO, and returned to SO levels by day 15th and 25th post operation (Fig. 3B). However, the highest level of p53 protein in the nuclear extracts from LV tissues obtained from AAB rats was noted on the 20th day concomitant with significant reduction of the actual heart mass in the group with AAB rats, compared with the same groups on the other days. As shown in Fig. 3C the level of p53 protein in the nuclear extracts from AAB rats significantly correlates with the actual heart weight for the studied period (r= -0.59, P< 0.0001).

Figure 2 also shows that the changes in p53 protein level in AAB rats was not due to variations in the amount of protein loaded as each membrane was reprobed with an antibody to α actin and showed a band at 42 kDa, for which the level altered very little in samples from both AAB and SO groups throughout the studied period.

The changes in the expression of p53 protein from nuclear and cytoplasmic extracts from LV tissue of AAB and SO rats at each time point were quantified by measuring the optical densities of the bands obtained from 9-13 separate hearts with two determinations per tissue sample. p53 protein expression was normalized individually to the level of expression of the total cytoplasmic and nuclear protein for each sample respectively (Fig. 3A and B).

In this study we report the subcellular expression of one of the main modulator of the cell cycle protein p53 during the development of LVH. Recent studies have proved that despite the diminishing ability of the cardiac myocyte to undergo cell division, in some instances, cardiac myocyte nuclear division can be re-initiated in the adult rat heart (2, 5). Our evaluation of myocardial expression of protein p53 has been somewhat biased, as we have assumed that this protein exists primarily to regulate the cell cycle.

The potential role of tumor suppressor gene product expression in myocardial development and pathology is discussed by Kim and colleagues (16). These authors demonstrated that in isoproterenol-induced hypertrophy in mice they did not find significantly induced tumor suppressor gene transcription. But they noticed that the study utilized total heart preparations and also they did not study the expression of these proteins for a long period after induction of LVH. Recent observations have underscored the importance of p53 in regulating cardiomyocyte cell cycle activity in response to expression of DNA tumor virus oncoproteins (22). The profound roles of p53 in DNA tumor virus-induced cardiomyocyte proliferation raised the possibility that this protein might also exert a cell cycle regulatory role in the adult heart. In this study we have shown by immunoblotting that the expression of protein p53 alters dramatically, but transiently during the development of pressure overload induced LVH in rats.

That LVH had indeed occurred in our model was demonstrated by a 70% increase in echo LVM-to-TL ratio in AAB rats compared with SO control animals by 45 days after operation (Fig. 1). Previous investigators demonstrated that
pressure overload is initially characterized by the development of concentric LVH with compensated left ventricular contractile performance (11, 19). Our results demonstrate that hypertrophy of the left ventricle within the whole period of 45 days after AAB compensates to maintain baseline cardiac function. Despite the increased blood pressure in AAB rats, the LV%FS in AAB groups was not different from that in SO groups, which is most likely due to the greater wall thickness of the AAB groups in the presence of similar LVEDD.

In our model the magnitude of the pressure overload was sufficient to produce significant LVH within 10 day time frame. However, we report a further increase of echo LVM on the 15th day after AAB. Interestingly, on 20th day after operation there was a short lasting regression in LVM, followed by a new progression on 25th and 35th days. Above all, one may ask whether our finding is simply an artifact. The significant correlation between echo-LVM-to-TL ratio and actual HW-to-TL ratio as well as the consistence of echocardiographic findings with postmortem measurements exclude this possibility. Therefore, by assessing the changes in left ventricular structure and function in rats in short intervals of time for a period of 45 days we suppose that during the progression of compensated LVH there is a short-lasting regression of LVH, found on the 20th day in this model. Our findings were comparable to those of Hill et al. (13). They followed the development of cardiac hypertrophy in mice 5 weeks after thoracic aortic banding. On the other hand, Akers et al. (1) assessed the changes in rats but only in long intervals of time - on the 3, 10, 30, 60 days after banding. We assume that the other investigators (1, 6) have not encountered the short-lasting regression of LVH because of the long intervals of time at which they study the development of LVH.

Our results suggest that protein p53 may take part in the hypertrophic growth, induced by pressure overload. We observed a fluctuation of the level of protein p53 in the nucleus of LV tissue during the development of LVH. The increased level of protein p53 in LV tissue was only seen during a relatively short period in the nucleus in our model of LVH. The increased level of protein p53 in LV tissue was not different from that of SO rats. Similar decrease of this protein was observed in LV tissue five days after the period of upregulation during hypertrophy remains unknown at the present time. However, it is possible that the activity of certain cardiac p53-activating signals may be involved which can lead within a relatively short time to a marked elevation in p53 protein, or inactivation of the proteins that control its degradation (23). Furthermore, Wang et al. (28) detected increased expression of p53 induced by IGF I in the continuously proliferating H9C2 cells and in the primary cardiomyocytes that do not replicate one week after isolation. They suggest that protein p53 is involved in one of the fundamental signaling machinery in cardiac muscle cells. According to Anversa et al. (2) adult cardiomyocytes are not terminally differentiated. In light of these hypotheses, a possible explanation of our finding is that adult ventricular cardiomyocytes, under the certain circumstances during the development of LVH, appear to re-enter the cell cycle and proliferate (7). The short-lasting regression of LVH on 20th day in our model may be a result of the small size of the new cardiomyocytes immediately after the mitosis. In this aspect the increased expression of protein p53 in the nucleus on this day is explicable as a cell cycle checkpoint determinant. Protein p53 is a critical component of an inhibitory pathway that negatively regulates progression of the cell cycle. The inhibitory pathway mediated by p53 prevents the cells from entering S phase and thus may divert the cells toward differentiation or apoptosis pathways (8).

Conclusions

The observation of the increased expression of p53 protein and its correlation with the short lasting regression during the development of LVH suggests that p53 protein may modulate the adaptive growth of pressure overload induced LVH. It may also provide us with an opportunity to understand more clearly the mechanisms of this process and may eventually lead to successful strategies for the treatment of this process.
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