

# Molecular Mechanisms of Age-Related Cardiac Hypertrophy in the F344XBN Rat Model

Nandini DPK Manne<sup>1,2</sup>, Sunil K Kakarla<sup>1,2</sup>, Ravikumar Arvapalli<sup>2</sup>, Kevin M Rice<sup>2</sup> and Eric R Blough<sup>1,2,3\*</sup>

<sup>1</sup>Department of Pharmacology, Physiology and Toxicology, Marshall University, Joan C. Edwards School of Medicine, Huntington, WV, United States

<sup>2</sup>Center for Diagnostic Nanosystems, Marshall University, Huntington, WV, United States

<sup>3</sup>Department of Pharmaceutical Sciences and Research, Marshall University, Huntington, WV, United States

\*Corresponding author: Eric Blough, Center for Diagnostic Nanosystems, Department of Pharmaceutical Science and Research, Marshall University, Huntington, WV, USA- 25755-1090, Tel: 304-696-2708; E-mail: blough@marshall.edu

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## Abstract

**Background:** The aging heart undergoes well characterized changes in cardiac structure and function. Recent studies suggest that age-related increase in oxidative stress is associated with increased cardiomyocyte apoptosis and possible compensatory hypertrophy of the remaining cardiomyocytes. However, the underlying molecular mechanisms remain obscure. The purpose of this study is to examine the possible molecular mechanisms underlying the age-related hypertrophy seen in male F344XBN rat hearts.

**Methods and results:** Male F344XBN rats aged 6, 27, 30, 33, and 36-months were divided into five groups and were used to examine the molecular mechanisms underlying age-associated cardiac hypertrophy through immunohistochemical staining and immunoblotting. Compared to 6-month animals, the heart to body weight ratio remained unchanged in the 27 and 30-month rats, while it was significantly increased by  $27 \pm 5\%$  and  $38 \pm 7\%$  in 33 and 36-month old rats, respectively ( $P < 0.05$ ). Consistent with these data, semi-quantitative morphological analysis suggested that the average cardiomyocyte fiber cross sectional area was five- and nine fold higher in the 33 and 36-month old animals, respectively ( $P < 0.05$ ). Increases in cardiomyocyte size were accompanied by the hyperphosphorylation of several different signalling molecules involved in the ERK 1/2- Akt signalling pathway ( $P < 0.05$ ).

**Conclusion:** Taken together, these data suggest that age related cardiomyocyte hypertrophy in the F344XBN rat heart is associated with the alterations in ERK1/2 and Akt signalling.

**Keywords:** Aging; Myocardial hypertrophy; Protein translational signalling; ERK 1/2; Akt

## Introduction

Cardiovascular disease remains the leading cause of death in the elderly population and it is estimated that by 2035, nearly one in four individuals in the United States will be sixty-five years of age or older [1]. The aged heart undergoes well characterized structural changes leading to a diminished functional and adaptive reserve capacity, an increased susceptibility to incur damage and a limited practical ability for repair/regeneration [2]. Cellular and molecular mechanisms that have been implicated in age-associated changes in myocardial structure and function in humans have been studied largely in rodents. The Fischer 344/Brown Norway F1 (F344/BNF1) rat is recommended for age-related studies by the National Institutes on Aging because this hybrid rat lives longer and has a lower rate of pathological conditions than inbred rats [3]. According to the probability of aging curves generated by National Institute on Aging, the aged 30 to 36-month rats correspond roughly to humans in their sixth and eighth decades of life, respectively [4]. It is thought that aging in the F344XBN rats, similar to that in humans, is associated with increased left ventricular hypertrophy, fibrosis, and diastolic dysfunction [4]. The mechanism(s) responsible for these changes have yet to be elucidated, however recent data has suggested that these changes in cardiac structure and function

are associated with increases in heart weight, cardiomyocyte apoptosis and elevations in oxidative-nitrosative stress [5,6].

The increased heart size (hypertrophy) seen with aging and in response to other types of physiologic stimuli is initially beneficial as it functions as a compensatory mechanism to maintain cardiac function. Nonetheless, if allowed to proceed unchecked, the hypertrophic response can cause cardiac arrhythmias, contractile dysfunction, cardiac failure, and death [7]. The factors thought to regulate cardiac hypertrophy are not well understood although recent data has established that increased protein synthesis is likely a key requirement for the cardiac growth [8]. Previous studies have shown that the extracellular signal-regulated kinase 1/2 (ERK1/2) plays a central regulatory role in promoting cardiac hypertrophy through Ras/Raf/MEK/ERK signalling cascade both in vivo and in vitro [9,10]. Similarly, other work has demonstrated that the protein kinase B or Akt, a serine threonine kinase, and its downstream substrate, the mammalian target of rapamycin (mTOR), appear to be involved in regulating the cardiac hypertrophy produced by numerous stimuli [11,12]. Whether these signalling cascades function to regulate the age-associated hypertrophy seen in the male F344XBN rat, has to our knowledge, not been investigated.

## Methods

### Animals

Animal care and procedures were conducted in accordance with the Animal Use Review Board of Marshall University using the criteria outlined by the American Association of Laboratory Animal Care (AALAC) as proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279). Young (6 months, n=6), adult (27 months, n=6), aged (30-months, n=6), and very aged (33- and 36-months, n=6/group) male F344XBN rats were obtained from the National Institute on Aging. Rats were housed two per cage in an AALAC-approved vivarium. Housing conditions consisted of a 12 hour: 12 hour dark-light cycle with temperature maintained at  $22 \pm 2^\circ\text{C}$ . Animals were provided food and water ad libitum. Rats were allowed to acclimate to the housing facilities for at least two weeks before experimentation began.

### Tissue collection

Rats were anesthetized with a cocktail mixture of ketamine-xylazine (4:1) (50 mg/kg, I/P) and supplemented as necessary for reflexive response before sacrifice. A midline laparotomy was performed and the heart was removed and rinsed with in Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with 5%  $\text{CO}_2$ /95%  $\text{O}_2$  maintained at  $37^\circ\text{C}$  as described previously [13]. Isolated hearts were removed of connective tissue; dried, and snap frozen in liquid nitrogen immediately and stored at  $-80^\circ\text{C}$  until further analysis.

### Tissue homogenization and determination of protein concentration

Cardiac tissue was homogenized in a tissue protein extraction reagent (T-PER) (10 mL/g tissue; Rockford, IL, USA) with protease inhibitors (P8340, Sigma-Aldrich, Inc., St. Louis, MO, USA) and phosphatase inhibitors (P5726, Sigma-Aldrich, Inc., St. Louis, MO, USA). Samples were incubated on ice for 30 min and centrifuged at 12,000 g for 5 min at  $4^\circ\text{C}$  to collect the supernatant. Concentration of protein in the homogenates was determined via the Bradford method (Fisher Scientific, Rockford, IL, USA), normalized with T-PER and then boiled in a 2X Laemmli sample buffer (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 5 min at  $95^\circ\text{C}$ .

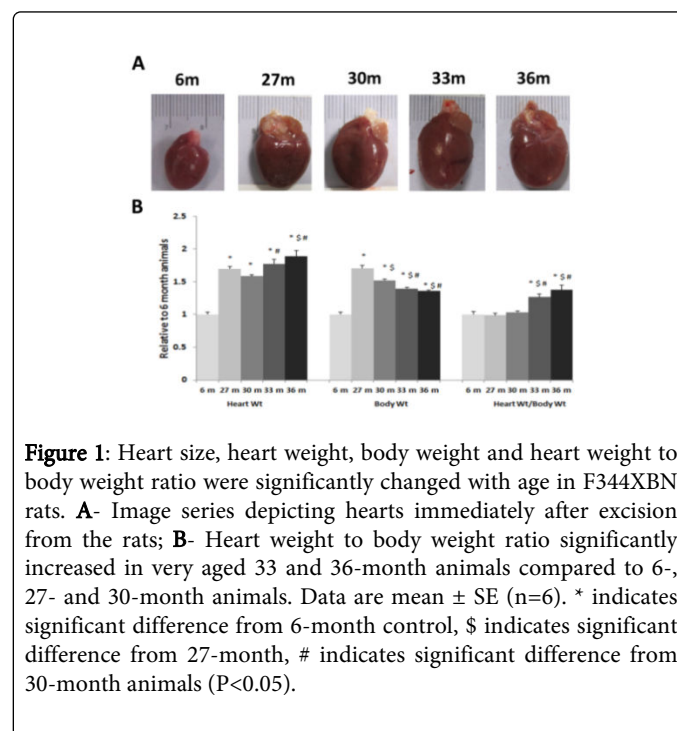
### SDS-PAGE and Immunoblotting

Fifty micrograms of total protein from each sample was separated on a 10% PAGEr Gold Precast gel (Lonza, Rockland, ME, USA) and then transferred onto a nitrocellulose membrane as described previously [14,15]. Ponceau S was used to stain the membranes to verify equal loading of protein across different lanes. Membranes were blocked for 1 hour at room temperature in blocking buffer (5% non-fat dry milk in TBS-T) (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20), pH 7.6), washed with TBS-T thrice for 5 min each at room temperature and probed with antibodies for the detection of Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), GSK-3 $\beta$ , p-GSK-3 $\beta$  (Ser 9), PTEN, p-PTEN (Ser380/Thr382/383), ERK 1/2, p-ERK 1/2 (Thr202/Tyr204), ERK 5 and p-ERK 5 (Thr218/Tyr220) (from Cell Signalling Technology, Inc., Beverly, MA, USA). Membranes were incubated overnight at  $4^\circ\text{C}$  in primary antibody buffer (5% BSA in TBS-T, pH 7.6) and washed thrice with TBS-T for 5 min each. After washing, membranes were incubated with HRP- conjugated secondary antibody (anti-rabbit or anti-mouse, Cell Signalling Technology, Inc., Danvers,

MA, USA) in blocking buffer for 1 hour at room temperature. Membranes were washed again as done previously in TBS-T. Membranes were then incubated with ECL reagent to visualize the protein bands (Amersham ECL Western Blotting reagent RPN 2106, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Protein bands of interest were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA).

### Immunohistochemistry

Briefly, tissue sections (8  $\mu\text{m}$ ) were washed thrice with phosphate-buffered saline (PBS) containing 0.05% Tween-20, pH 7.4. Sections were then incubated in blocking solution (5% BSA in PBS-T) for 30 min followed by incubation with specific antisera (1:100 anti-dystrophin, #NBPI-45532, Novus Biologicals, Littleton, CO) diluted in 3% BSA in PSB-T (antibody dilution of 1:100) for 1 h at  $24^\circ\text{C}$ . Sections were washed thrice again with PBS-T and incubated with FITC labeled anti-rabbit IgG (1:200) for 30 min at  $24^\circ\text{C}$  in a humidified chamber. Experiments performed in parallel omitting either the primary or the secondary antibodies were used as controls to confirm specificity and background immunoreactivity. DAPI was used to stain nuclei and used in the secondary antibody solution at a concentration of 1.5  $\mu\text{g}/\text{ml}$ . Slides were again washed with PBS, mounted and were visualized by epifluorescence using an Olympus fluorescence microscope (Melville, NY, USA). Images were captured digitally using a CCD camera and three random areas were selected from each slide and the total number of cross sectional area of all muscle fibers in these fields were counted using Image J software.



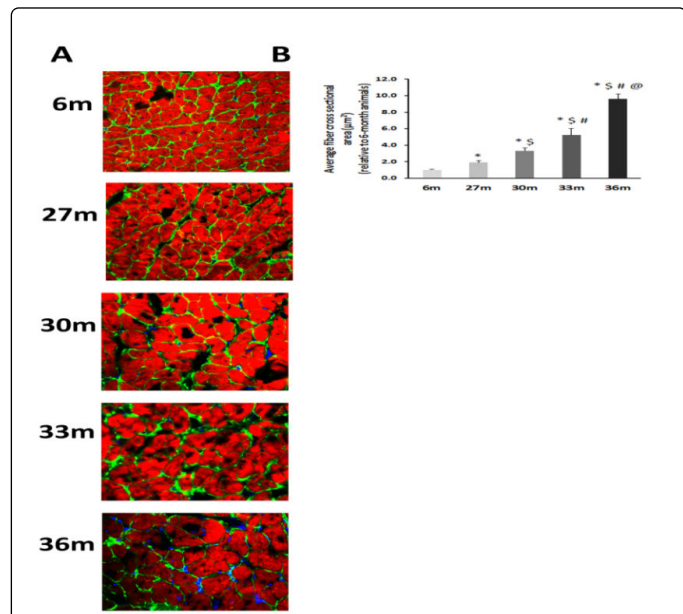
**Figure 1:** Heart size, heart weight, body weight and heart weight to body weight ratio were significantly changed with age in F344XBN rats. **A-** Image series depicting hearts immediately after excision from the rats; **B-** Heart weight to body weight ratio significantly increased in very aged 33 and 36-month animals compared to 6-, 27- and 30-month animals. Data are mean  $\pm$  SE (n=6). \* indicates significant difference from 6-month control, \$ indicates significant difference from 27-month, # indicates significant difference from 30-month animals ( $P < 0.05$ ).

### Data analysis

Results are presented as mean  $\pm$  standard error of mean (SEM). Sigmaplot 12.1 statistical program was used to evaluate the statistical significance. A one-way analysis of variance (ANOVA) or ANOVA on ranks was performed for comparisons while the Student-Newman-

Keuls post hoc test was used to determine the differences across groups. The level of significance accepted a priori was  $P < 0.05$ .

## Results



**Figure 2:** Aging in F344XBN rats is associated with cardiomyocyte hypertrophy. A- Immunofluorescence labeling of dystrophin (FITC-green), actin staining (rhodamine phalloidin-red) and nuclei (DAPI-blue) in 6-, 27-, 30-, 33- and 36-month F344XBN rat heart cross sections. Magnification 400x. B- Average muscle fiber cross sectional area ( $\mu\text{m}^2/\text{fiber}$ ). Number of fibers measured for 6-, 27-, 30-, 33- and 36-month rats was 640, 423, 311, 235 and 190 respectively. Data are mean  $\pm$  SE (n=3). \* indicates significant difference from 6-month group, § indicates significant difference from 27-month group, # indicates significant difference from 30-month group and @ indicates significant difference from 33-month group ( $P < 0.05$ ).

### Aging in the F344XBN rats is associated with cardiac hypertrophy

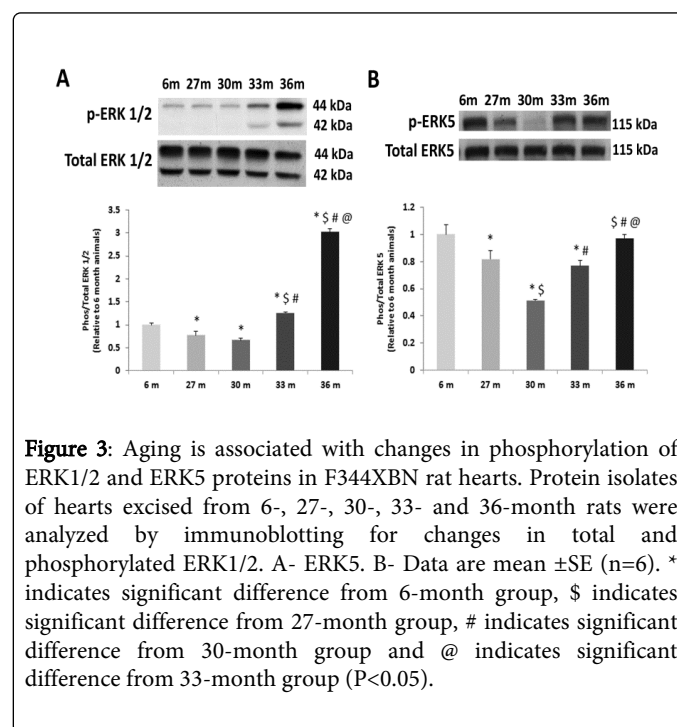
Heart size was visibly larger at 27 and 30-month and appeared to undergo a second phase of hypertrophy in 33 and 36-month animals (Figures 1A,B). Compared to hearts obtained from the 6-month old animals, cardiac mass was increased by  $70 \pm 4\%$ ,  $59 \pm 2\%$ ,  $78 \pm 6\%$  and  $89 \pm 9\%$  at 27, 30, 33 and 36-months (Figure 1B,  $P < 0.05$ ). Similarly, the hearts from the 36-month old animals was also 11% and 19% higher than that observed in the 27 and 30-month old animals (Figure 1B,  $P < 0.05$ ). Compared to 6-month animals, the body weight was significantly increased by  $71 \pm 4\%$  at 27-months and then gradually decreased with age in 30, 33 and 36-month animals but remained significantly higher when compared to young 6-month animals (Figure 1B,  $P < 0.05$ ). Evaluation of the heart weight to body weight ratio demonstrated that the ratio remained unchanged between 6, 27 and 30-month animals, while it was  $27 \pm 5\%$  and  $38 \pm 7\%$  higher in the 33 and 36-month animals compared to 6-month animals (Figure 1B,  $P < 0.05$ ).

To investigate whether increases in heart mass were associated with larger cardiac myocytes, we next measured the muscle fiber Cross Sectional Area (CSA). Because the myofibres in different areas of the heart are orientated in different directions we imaged only those areas of the left ventricle where all the muscle fibers were orientated in a transverse direction.

This task was accomplished by using immunofluorescence after labelling for dystrophin on tissue cross sections obtained from 6, 27, 30, 33 and 36-month rat hearts. Compared to that observed in the 6-month animals, our analysis indicated that the average muscle fiber CSA was  $90.1 \pm 23\%$ ,  $231.8 \pm 36\%$ ,  $421.6 \pm 80\%$ , and  $860 \pm 63\%$  higher in the 27, 30, 33 and 36-month rat hearts, respectively (Figure 2,  $P < 0.05$ ).

### Aging associated cardiac hypertrophy in F344XBN rats is associated with increase in the activation of the ERK signalling cascade

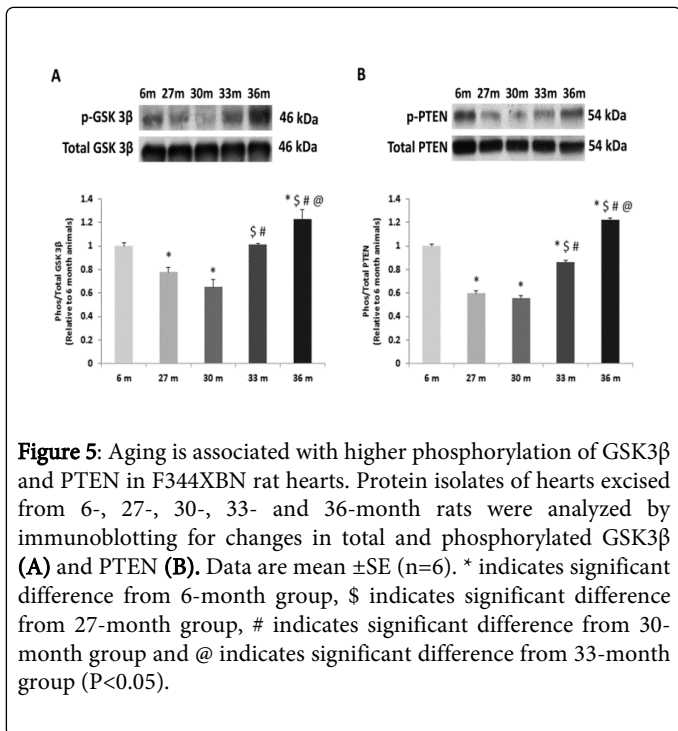
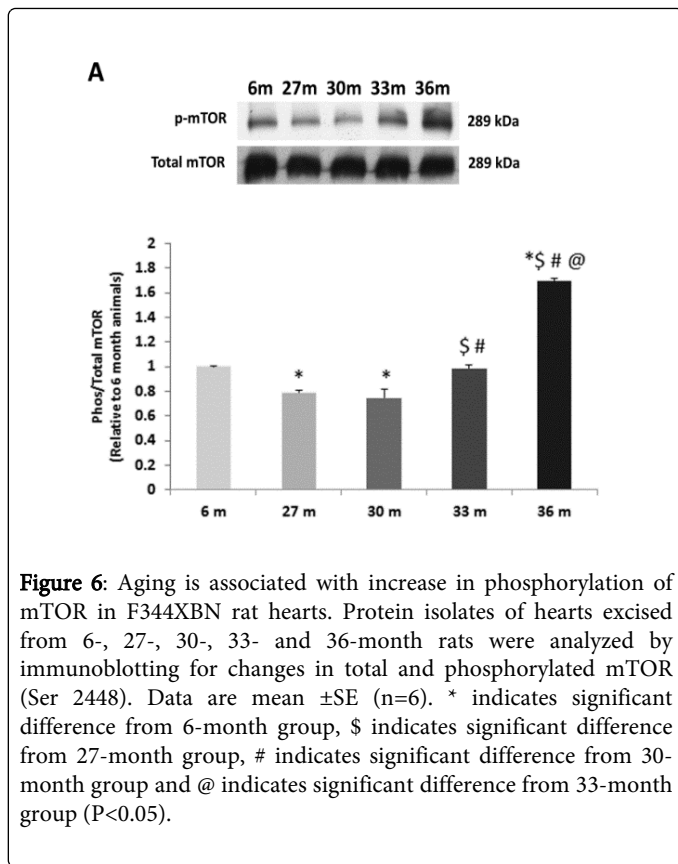
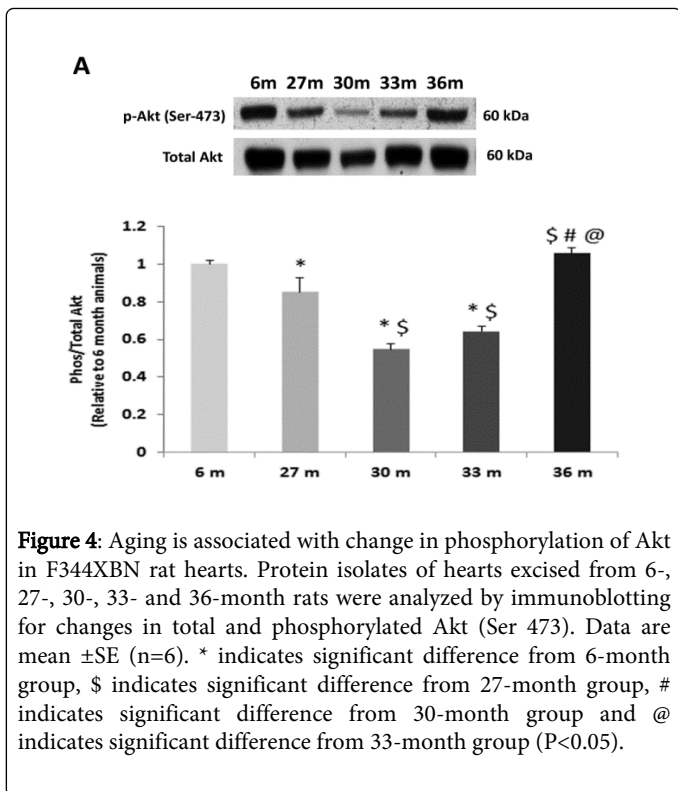
Compared to that observed in the hearts obtained from 6-month animals, the phosphorylated to total levels of ERK1/2 was significantly decreased in 27 and 30-month hearts by  $23 \pm 9\%$ ,  $33 \pm 5\%$  and increased in 33 and 36-month aged hearts by  $26 \pm 2\%$  and  $202 \pm 7\%$  respectively (Figure 3A,  $P < 0.05$ ). Similarly phosphorylated to total levels of ERK5 exhibited a bimodal signaling response with aging demonstrating a significant decrease of  $18 \pm 6\%$  and  $49 \pm 1\%$  with age at 27 and 30-month, when compared to 6 month levels, before returning to 6-month levels (Figure 3B,  $P < 0.05$ ).



**Figure 3:** Aging is associated with changes in phosphorylation of ERK1/2 and ERK5 proteins in F344XBN rat hearts. Protein isolates of hearts excised from 6-, 27-, 30-, 33- and 36-month rats were analyzed by immunoblotting for changes in total and phosphorylated ERK1/2. A- ERK5. B- Data are mean  $\pm$  SE (n=6). \* indicates significant difference from 6-month group, § indicates significant difference from 27-month group, # indicates significant difference from 30-month group and @ indicates significant difference from 33-month group ( $P < 0.05$ ).

### PI3K/Akt signaling in age associated cardiac hypertrophy in the F344XBN rat

Compared to 6-month animals, phosphorylated to total levels of Akt at Ser 473 was significantly decreased by  $15 \pm 8\%$ ,  $45 \pm 3\%$  at 27- and 30-month, respectively, before returning to 6-month levels at 36-month (Figure 4,  $P < 0.05$ ).



Consistent with our Akt findings, the phosphorylated to total levels of glycogen synthase kinase-3β (GSK-3β) was 22 ± 4% and 35 ± 6% lower in 27- and 30-month old animals, before increasing to a level 23 ± 8% higher than that seen in the 6-month animals at 36-months (Figure 5A, P<0.05).

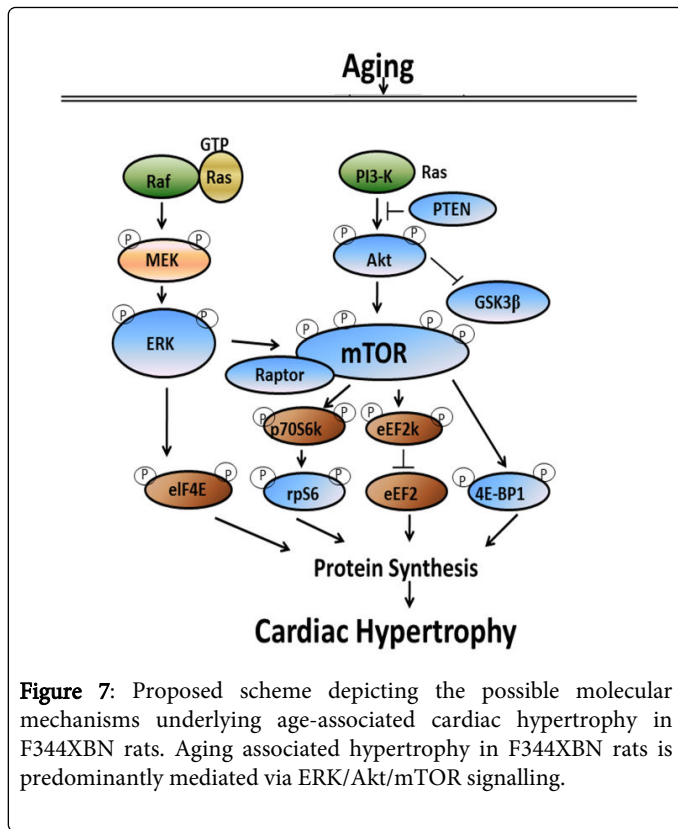


Figure 7: Proposed scheme depicting the possible molecular mechanisms underlying age-associated cardiac hypertrophy in F344XBN rats. Aging associated hypertrophy in F344XBN rats is predominantly mediated via ERK/Akt/mTOR signalling.



Compared to 6-month animals, the phosphorylated to total levels of Phosphatase and tensin homolog (PTEN) significantly decreased with age in 27- and 30-month hearts by  $40 \pm 2\%$  and  $45 \pm 2\%$ , respectively, before a gradual increase to a level of  $22 \pm 1\%$  higher than that seen in the 6-month animals at 36-months (Figure 5B,  $P < 0.05$ ).

Similarly, phosphorylated to total levels of mTOR significantly decreased with age in 27- and 30-month hearts by  $21 \pm 2\%$  and  $26 \pm 7\%$  when compared to 6-month animals, before increasing further by  $69 \pm 3\%$  in the hearts of 36-month old animals (Figure 6,  $P < 0.05$ ).

## Discussion

In spite of advances in treatment and early diagnosis, cardiovascular disease still remains the leading cause of death in the elderly. Studies in both humans and animal models indicate that aging is associated with alterations in cardiac structure and function including myocyte dropout and a compensatory hypertrophy of the remaining myocytes [4,16,17]. Similar to that seen in humans, previous work using the aging F344XBN rat model, has demonstrated aging in these rats is associated with increases in cardiac hypertrophy, oxidative-nitrosative stress, and apoptosis [6,18]. Here we examine the signalling processes that may be associated with age-related cardiomyocytes hypertrophy.

The increase in heart size and weight seen at 27 and 30-months did not illicit a change in heart weight to body weight ratios even in the presence of a slight decrease in body weight at 30-months of age. The stark change in heart weight, heart size, and heart weight to body weight ratio at 33- and 36-months of age was even more pronounced by the significant decrease in body weight (Figure 1). These findings suggest that the heart seems to grow in a parallel fashion with body weight until 30-months of age before undergoing hypertrophy. To investigate whether the increase in heart weight were due to cardiomyocyte hypertrophy, increase in fibrosis, or some other alteration, we next measured the cardiomyocytes cross sectional area. Our data showed that the cardiomyocytes fiber cross sectional area tended to increase rapidly from 30–36 months (Figure 2). The factors regulating cardiac hypertrophy are not fully understood however, several reports have demonstrated a role for the Raf/MEK/ERK signalling pathway [19–24]. Using a transgenic mice model, Sanna and colleagues demonstrated that the MEK1–ERK1/2 pathway induces cardiac hypertrophy *in vivo*, at least in part, by enhancing the transcriptional activity of NFAT [25]. The data of the present study support this contention as the phosphorylated (activation) to total levels of ERK1/2 was robustly increased in the 36-month rat hearts (Figure 3A).

In addition to the mitogen activated protein kinases, it is well accepted that the activation of the PI3K/Akt signalling pathway also participates in the development of myocardial hypertrophy [26,27]. Indeed, PI3K/Akt signalling has been shown to be activated in response to pressure overload in the rodent heart and following the incubation of cultured cardiomyocytes with growth factors [28]. PI3K inhibitors have also been shown to block ligand-induced cardiac hypertrophy [29] while the over-expression of a constitutively active Akt (caAkt) in the heart has been reported to increase heart weight and cell size [30]. Our data are in agreement with these findings as we observed modest increase in Akt phosphorylation at Ser 473 in the 36-month old animals (Figure 4). The GSK3 $\beta$  is a downstream substrate of Akt that is inhibited by Akt-mediated phosphorylation. GSK3 $\beta$  functions to negatively regulate the activity of GATA4,  $\beta$ -catenin, c-Myc and NFAT [31] that in turn attenuates the hypertrophy response.

In agreement with this data, we note an increased phosphorylated to total levels of GSK3 $\beta$  in the hypertrophied hearts of the 36-month old F344XBN rat (Figure 5A). The PTEN has been shown to function as a negative modulator of PI3K/Akt/mTOR pathway that is inactivated upon phosphorylation [32]. Consistent with our previous data, changes in the phosphorylated to total levels of PTEN appeared to mirror that observed for GSK-3 $\beta$  and were found to be significantly increased in the very aged 36-month animals (5B). Taken together, our data suggests that the PI3K/Akt pathway may be involved in regulating age-associated cardiac hypertrophy in F344XBN rats.

Like Akt, a growing body of evidence suggests that mTOR may play a key role in regulating cell growth and protein synthesis [12]. Rapamycin, an inhibitor of mTOR activity has been shown to attenuate pressure overload cardiac hypertrophy induced by aortic banding [33]. Here we demonstrate a large increase in the amount of phosphorylated to total mTOR in the very aged 36-month animals (Figure 6). Given that recent studies have suggested a cross talk between Ras/ERK signalling and mTOR during cardiac hypertrophy [24], it is possible that the increased phosphorylation of mTOR we see with aging may be related to increase ERK1/2 signalling. Future studies designed to directly investigate this linkage may be useful for increasing our understanding of the molecular mechanisms involved in age-related cardiac hypertrophy.

In summary, the findings from this study suggest that aging in F344XBN rats is associated with cardiac hypertrophy that appears to involve the activation of the ERK1/2 and Akt signaling pathways (Figure 7). Given the high prevalence of age-associated heart disease worldwide and the ever growing expansion of the elderly population additional mechanistic studies examining these pathways is likely needed to better understand the molecular events underlying age-related cardiac hypertrophy.

## Acknowledgements

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