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Original Article

Bioactive peptides attenuate cardiac hypertrophy and fibrosis in spontaneously hypertensive rat hearts

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ABSTRACT

Alcalase potato protein hydrolysate (APPH), a nutraceutical food, might an have important role in anti-obesity activity. Recent studies from our lab indicated that APPH treatment had lipolysis stimulating activity and identified was an efficient anti-obesity diet ingredient. In this study we aim to investigate the beneficial effects of pure peptide amino acid sequences (DIKTNKPVIF (DI) and IF) from APPH supplement in the regulation of cardiac hypertrophy and fibrosis on spontaneously hypertensive rats (SHR). We examined hematoxylin and eosin staining, Masson's trichrome staining, echocardiographic parameters, serum parameters, hypertrophy, inflammation and fibrotic marker expression to demonstrate efficacy of bioactive peptides in a SHR model. There was a significant upregulation between SHR and bioactive peptides treated groups in left heart weight (LHW), LHW/WHW, LHW/Tibia, LVIDd, and LVd mass. In addition, the bioactive peptides repress the protein expression of hypertrophy markers (BNP, MYH7), inflammation (TLR-4, p-NFkB, TNF-α, IL-6), and fibrotic markers (uPA, MMP-2, TIMP1, CTGF). In summary, these results indicate that DI and IF bioactive peptides from APPH attenuate cardiac hypertrophy, inflammation and fibrosis in the SHR model.

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1. Introduction

Hypertension, an age related chronic disease affecting millions of people worldwide, is a crucial risk factor for myocardial infarction, heart failure, stroke, and renal damage [1,2]. Among the several angiotensin peptides, Ang-II is the major effector of the RAS system. Ang-II can constrict vascular smooth muscle, promote aldosterone production, stimulate catecholamines release, regulate sodium transport in kidney and remodel cardiovascular organs [3,4]. Synthetic ACE (Angiotensin-converting enzyme) inhibitors, such as captopril, enalapril and lisinopril are effective for reducing blood pressure. However, some undesirable side effects have been reported, including coughing, dizziness, headache, kidney and liver problems [5]. Thus, an ACE inhibitor peptide from food sources might provide a natural way to protect against hypertension with fewer side effects.

ACE inhibitor peptides are produced from various food proteins, including casein, zein, soybean protein, dried salted fish, ovalbumin, fish sauce, and fish water soluble protein. Food consumed daily with the protective peptides with ACE inhibitor activity may effectively maintain blood pressure naturally. Nutraceutical foods include proteins, protein hydrolyzates and peptides obtained from hydrolyzed food protein and from fermented products [6]. Peptides from plant source such as soybean have good antihypertensive, hypocholesterolemic, antiobesity and anticancer activity [7] and soy peptides have been reported to decrease the risk of cardiovascular disease [8–10]. Potato protein hydrolyzate (PPH) was reported to protect against ethanol-induced gastric mucosal damage through its antioxidative activity [11]. Alcalase was reported to hydrolyze soy protein into antioxidative hydrolyzates [12,13]. Previous findings from our lab indicated that APPH (Alcalase potato protein hydrolyzate) attenuate high fat diet-induced hepatic lipid accumulation, apoptosis and fibrosis [14–16]. In addition, we also reported that DIKTNKPVIF, a bioactive peptide, showed anti-hepatosteatosis activity [17]. In this study, we investigated the beneficial effect of bioactive peptides DIKTNKPVIF and IF on cardiac hypertrophy, inflammation and fibrosis in SHR rats. The present findings show clear evidence that the administration of bioactive peptides might ameliorate hypertension-induced cardiac hypertrophy, inflammation and fibrosis in SHR rats. Thus, bioactive peptides can be considered as a possible therapeutic agent to attenuate cardiac hypertrophy and fibrosis.

2. Materials and methods

2.1. Animal procedure

Twelve-week old SHR and WKY rats were procured from Bio-Lasco Co., Ltd., (Taipei, Taiwan). All rats were supplied with a standard diet, tap water and maintained at a constant temperature (22 °C) on a 12-h light/dark cycle. After four week acclimatization period, the animals were divided into 5 groups with 6 animals in each group: SHR (Control), SHR - IF, SHR-DIKTNKPVIF (DI), SHR - Captopril (ACE inhibitor). WKY rats served as the normal control group. The treatment period is for about eight weeks. After treatment, the animals were sacrificed. Finally, the heart tissue and serum were collected and stored at −80 °C for further analysis. All animal experiments were performed in accordance with the IACUC-10525 protocol and with prior approval from the Institutional Animal Care and Use Committee (IACUC), Tunghai University, Taichung, Taiwan.

2.2. Drug treatment

The bioactive peptides are commercially synthesized from DG peptides Co. Ltd., China. The bioactive peptides IF (I-Isoleucine; F- Phenylalanine) (10 mg/kg), DIKTNKPVIF (D-Aspartic acid; I- Isoleucine; K- Lysine; T- Threonine; N- Asparagine; K-Lysine; P- Proline; V- Valine; I- Isoleucine; F- Phenylalanine) (10 mg/kg) and captopril (ACE inhibitor) (5 mg/kg) were given daily to the SHR rats by intragastric administration for 8 weeks. The bioactive peptides are prepared by using PBS. Subsequently, WKY and SHR control groups received PBS by intragastric administration.

2.3. Blood pressure measurement

After treatment, heart rate, systolic blood pressure, medium blood pressure, and diastolic blood pressure were measured by the tail-cuff method using a noninvasive blood pressure measurement system (Softron, BP-2010 series). Before the measurement, the rats were kept in a warm box for 10 min.

2.4. Echocardiography parameters

Echocardiography was performed for all groups before sacrificing. Rats were anesthetized with isoflurane, and echocardiography was performed using 12 MHz linear transducers and 5–8 MHz sector transducer (Vivid 3, General Electric Medical Systems Ultrasound, Tirat Carmel, Israel). Measurements were contrived from M-mode planes and two-dimensional images obtained in the parasternal long and short axes at the level of the papillary muscles after observation of at least six cardiac cycles.

2.5. Measurement of blood serum biochemical parameters

The levels of uric acid, creatine, aspartate transaminase, alanine transaminase and creatine kinase were determined using specific ELISA kits according to the manufacturer's protocol (Sigma–Aldrich, St.Louis, MO, USA).

2.6. Hematoxylin and eosin/Masson's trichrome staining

The rat hearts were removed and fixed in formalin followed by dehydration using alcohol gradient (100%, 95%, and 75%, for 5 min each) and were embedded in paraffin wax. Paraffin-embedded tissue blocks were sectioned into 2 μm-thick slides, and deparaffinized by submersion in xylene, followed by rehydration with alcohol gradient. The slides were stained with hematoxylin and eosin/Masson's trichrome dye. Images of the samples were obtained using a microscope under 400× magnification.

2.7. Tissue protein extraction

The left ventricle tissue was homogenized using tissue lysis buffer with protease inhibitor and phosphatase inhibitor. The homogenates were centrifuged at 12,000 rpm for 30 min and the supernatant was stored at −80 °C.

2.8. Western blotting

Protein samples 40 μg/lane were resolved by 10–15% gradient SDS-PAGE with a constant voltage. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences) for 90 min at 90V. Then, membranes were incubated with blocking solution (3% BSA) for 1 h at RT. After washing with TBST, membranes were incubated with specific primary antibodies overnight at 4 °C. Followed by TBST wash, and horseradish peroxidase labeled secondary antibodies were added and incubated for 1 h at RT. The blots were visualized using a chemiluminescence ECL western blotting reagent (Millipore) in Fujifilm LAS-3000 (GE Healthcare) and intensities were quantified using ImageJ.

2.9. Statistical analysis

Statistical analysis was performed with GraphPad Prism software, version 5.0, California. All data are expressed as Means ± SD. The overall significance of means of multiple groups was assessed by analysis of variance. Statistical significance was considered at the level of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Bioactive peptide attenuate cardiac hypertrophy, inflammation, and fibrosis in the SHR model

The body weight and cardiac characteristics are presented in Table 1. SHR mice had significantly higher body weight ($p < 0.05$), WHW ($p < 0.01$), LHW ($p < 0.001$), WHW/Tibia ($p < 0.01$) and LHW/Tibia ($p < 0.001$) compared to WKY. In addition, we observed that left heart weight was significantly decreased in peptide treated groups compared to SHR ($p < 0.001$). Histopathological analysis using hematoxylin and eosin staining and Masson's trichrome staining revealed that cardiac myocyte area and collagen deposition was higher in SHR compared to the WKY and peptide treated groups (Fig. 1). The echocardiographic parameters are represented in Table 2. A significant difference between WKY and SHR in IVSd ($p < 0.01$), LVIDd ($p < 0.01$), LVIDs ($p < 0.001$), EDV ($p < 0.001$), ESV ($p < 0.001$), SV ($p < 0.05$), LVd mass ($p < 0.001$) and LVs mass ($p < 0.001$) was observed. Subsequently, there was a significant decrease between IF peptide-treated group and SHR in LVIDs ($p < 0.001$), EDV ($p < 0.05$) and ESV ($p < 0.01$). In addition, we found a significant downregulation between DI peptide-treated group and SHR in LVIDs ($p < 0.01$), ESV ($p < 0.01$) and LVd mass ($p < 0.05$). These findings showed that bioactive peptides attenuate the cardiac damage in SHR rats. Serum parameters such as uric acid, creatine, aspartate transaminase, alanine transaminase, and creatine kinase parameters are summarized in Fig. 2A. The uric acid parameter showed significant increase between SHR and WKY groups ($P < 0.05$). The downregulation of serum parameters was found in peptide treated groups compared to the SHR.

3.2. Treatment with bioactive peptides downregulates expression of MAPK and cardiac hypertrophy markers

The protein expression of MAPK (ERK/JNK/p38) was presented in Fig. 2B and C. There was no significant difference between WKY and SHR groups in p-ERK and p-JNK expression. The results showed that IF and DI treatment significantly downregulates p-p38 expression compared to the SHR ($p < 0.01$). In addition, p-p38 expression was significantly higher in SHR compare to the WKY group ($p < 0.01$). The protein expression of hypertrophic markers (BNP, MYH-7) was fivefold increase in SHR compared to the WKY group significantly (Fig. 3). Treatment with IF and DI significantly downregulates BNP and MYH-7 expression compared to the SHR rats ($p < 0.05$).

Protein expression of eccentric hypertrophic markers (Rac-1/p-JAK2/STAT3/IL-6) is summarized in Fig. 3. We found a significant ($p < 0.01$) upregulation between SHR and WKY rats in Rac-1 and STAT3 expression. In addition, there was a significant downregulation between peptide-treated groups compared to the SHR group of Rac-1 protein expression ($p < 0.05$). On the other hand, we found significant downregulation of p-JAK2 expression in DI treated groups compared to the SHR group ($p < 0.05$). These findings show that bioactive peptides attenuate the cardiac hypertrophy in SHR rats.

Table 1 – Body weight and cardiac characteristics of control and treatment groups.

	WKY	SHR	IF	DI	ACE
Body weight (g)	0.287 ± 0.020	0.338 ± 0.021 *	0.344 ± 0.018 **	0.328 ± 0.015	0.335 ± 0.011 *
WHW (g)	0.985 ± 0.09	1.224 ± 0.06 **	1.118 ± 0.107 **	1.086 ± 0.04	1.098 ± 0.09
LHW (g)	0.722 ± 0.03	0.953 ± 0.08 ***	0.779 ± 0.02 ###	0.754 ± 0.03 ###	0.726 ± 0.02 ###
Tibia (mm)	36.2 ± 0.45	35.77 ± 0.13	36.31 ± 0.24	36.29 ± 0.23	37.13 ± 0.10
LHW/WHW	0.734 ± 0.03	0.780 ± 0.07	0.702 ± 0.07 ##	0.695 ± 0.03 #	0.665 ± 0.06 #
WHW/Tibia (100 g/mm)	2.74 ± 0.24	3.43 ± 0.15 **	3.07 ± 0.29 *	3.00 ± 0.10	2.96 ± 0.23
LHW/Tibia (100 g/mm)	2.00 ± 0.09	2.67 ± 0.21 ***	2.14 ± 0.06 ###	2.08 ± 0.09 ###	1.96 ± 0.06 ###

Values are Mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ are compared to WKY; #p < 0.05, ##p < 0.01 and ###p < 0.001 are compared to SHR. WHW – Whole heart weight; LHW – Left heart weight.

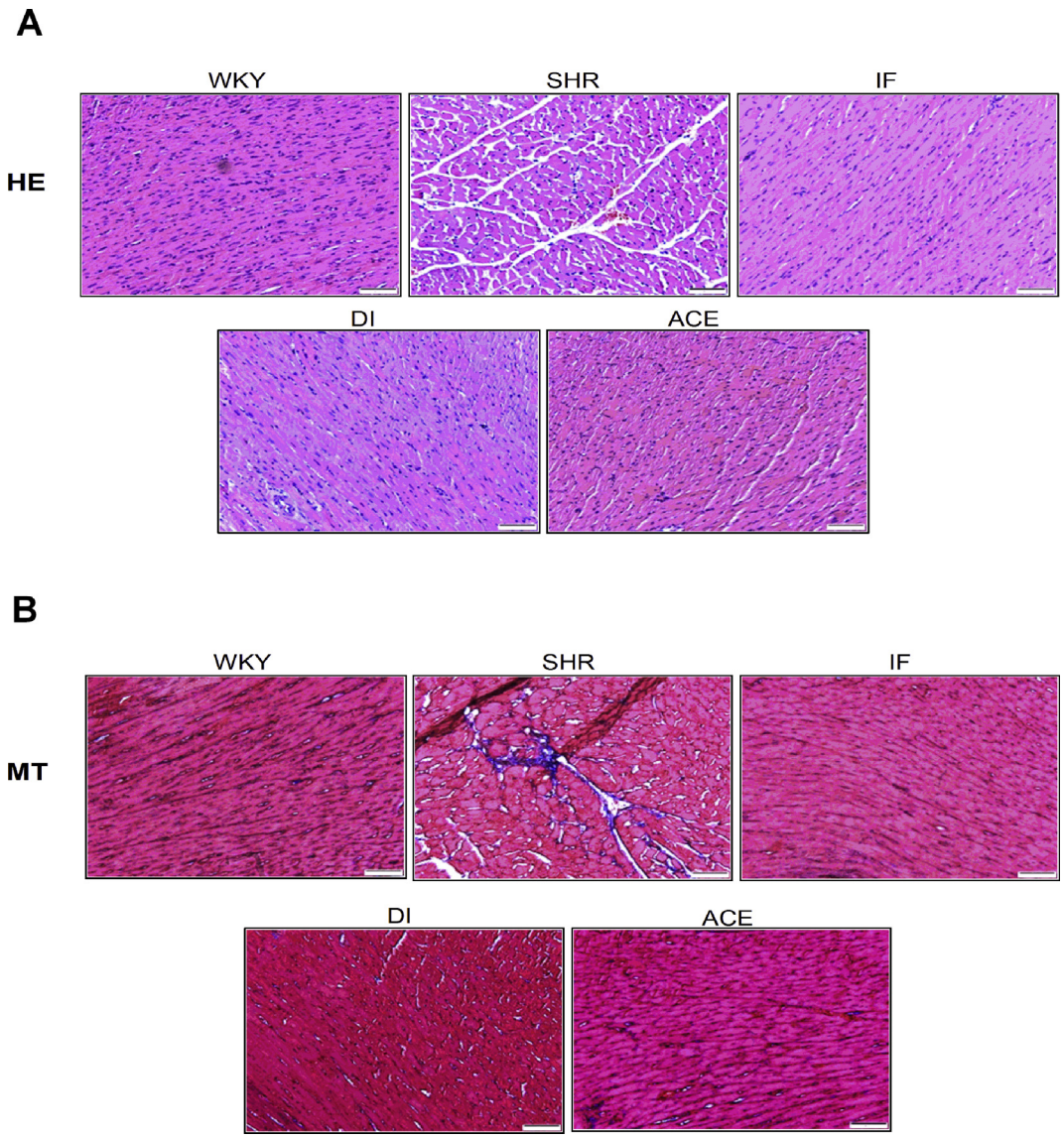

Fig. 1 – Histological observations of control and treatment groups. A. Hematoxylin and eosin staining and B. Masson's trichrome staining.

Table: 2 – Echocardiographic parameters of control and treatment groups.

	WKY	SHR	IF	DI	ACE
IVSd (mm)	0.90 ± 0.11	1.19 ± 0.09 **	1.09 ± 0.07 *	1.10 ± 0.07 *	1.10 ± 0.14
LVIDd (mm)	7.54 ± 0.37	8.68 ± 0.36 **	7.90 ± 0.54	7.94 ± 0.40	8.34 ± 0.57 **
LVPWd (mm)	0.98 ± 0.07	1.26 ± 0.07	1.17 ± 0.11	1.01 ± 0.11	1.04 ± 0.09
IVSs (mm)	2.20 ± 0.20	2.47 ± 0.18	2.29 ± 0.27	2.29 ± 0.21	2.31 ± 0.25
LVIDs (mm)	4.37 ± 0.39	5.67 ± 0.28 ***	4.73 ± 0.49 ###	4.72 ± 0.25 ##	4.80 ± 0.32 ##
LVPWs (mm)	2.04 ± 0.16	2.33 ± 0.06	2.20 ± 0.29	2.15 ± 0.29	2.26 ± 0.21
EDV (Teich)	0.96 ± 0.13	1.49 ± 0.10 ***	1.17 ± 0.25 #	1.18 ± 0.15	1.27 ± 0.22 **
ESV (Teich)	0.21 ± 0.05	0.42 ± 0.07 ***	0.26 ± 0.07 ##	0.26 ± 0.04 ##	0.27 ± 0.05 #
SV (Teich)	0.75 ± 0.09	1.04 ± 0.08 *	0.90 ± 0.19	0.91 ± 0.12	1 ± 0.18 **
LVd Mass (ASE)	0.94 ± 0.02	1.21 ± 0.05 ***	1.11 ± 0.10 **	1.06 ± 0.06 #	1.08 ± 0.08 *
LVs Mass (ASE)	1.06 ± 0.06	1.36 ± 0.07 ***	1.20 ± 0.15 *	1.18 ± 0.07	1.20 ± 0.11

Values are Mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001 are compared to WKY; #p < 0.05, ##p < 0.01 and ###p < 0.001 are compared to SHR. IVSd - Interventricular septal thickness at end-diastole; LVIDd - Left ventricular internal dimension at end-diastole; LVPWd - Left ventricular posterior wall thickness at end-diastole; IVSs - Interventricular septal thickness at end-systole; LVIDs - Left ventricular internal dimension at end-systole; LVPWs - Left ventricular posterior wall thickness at end-systole; EDV - End-diastolic volume; ESV -End-systolic volume; SV – Stroke Volume; LVd Mass - Left ventricular end diastole mass; LVs Mass – Left ventricular end systole mass.

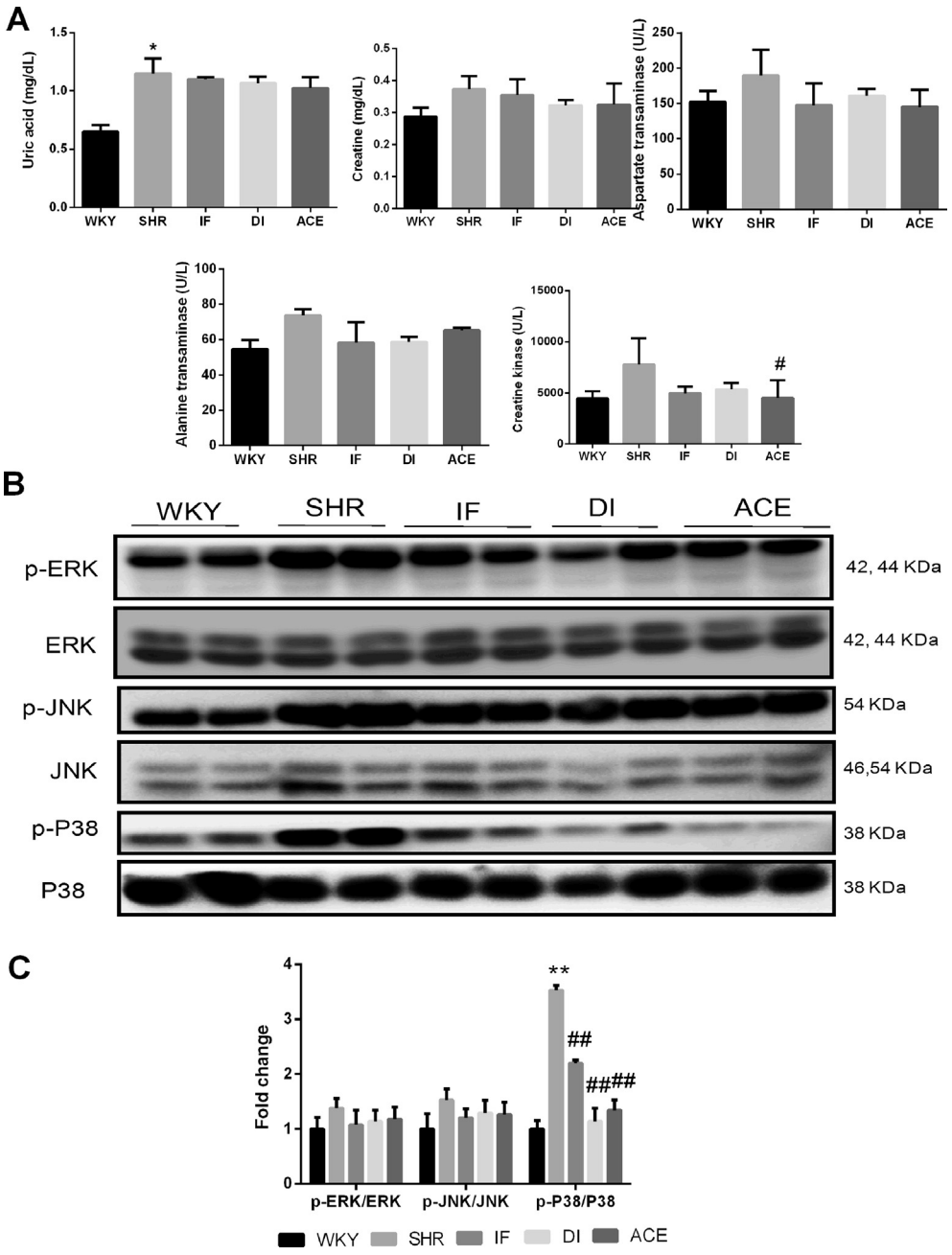

Fig. 2 – Serum parameters of control and treatment groups and protein expression of MAPK for all five groups. A. Serum parameters of uric acid, creatine, aspartate transaminase, alanine transaminase and creatine kinase. B. Protein expression of p-ERK/ERK, p-JNK/JNK and p-p38/p38 and C. The expression levels of MAPK proteins are plotted on the bar graph. *-p<0.05 compared to the WKY; **-p<0.01 compared to the WKY; #-p<0.05 compared to the SHR; ##-p<0.01 compared to the SHR.

3.3. *Bioactive peptides repress the expression of upstream signaling in SHR*

Next, we determined the upstream signaling in regulation of cardiac hypertrophy and fibrosis. The protein expression of upstream pathway and transcriptional factors markers are upregulated in SHR compared to the control and peptide treated groups (Fig. 4). There was a significant increase in AT1R ($p < 0.01$), IGF-IIR ($p < 0.05$), p-GATA4 ($p < 0.01$) and p-PKC ($p < 0.05$) in SHR compared to the WKY groups. However, bioactive peptides showed a protective effect by down-regulating AT1R ($p < 0.05$), p-GATA4 ($p < 0.05$) and p-PKC ($p < 0.05$) expression compared to the SHR groups.

3.4. *Protective effect of IF and DI in cardiac fibrosis and inflammation in SHR*

The protein expression of fibrosis (uPA/MMP-2/TIMP1/CTGF) and inflammation (TLR4/pNFkBp65/TNF-α) is presented in Fig. 5. The fibrotic and inflammatory markers were

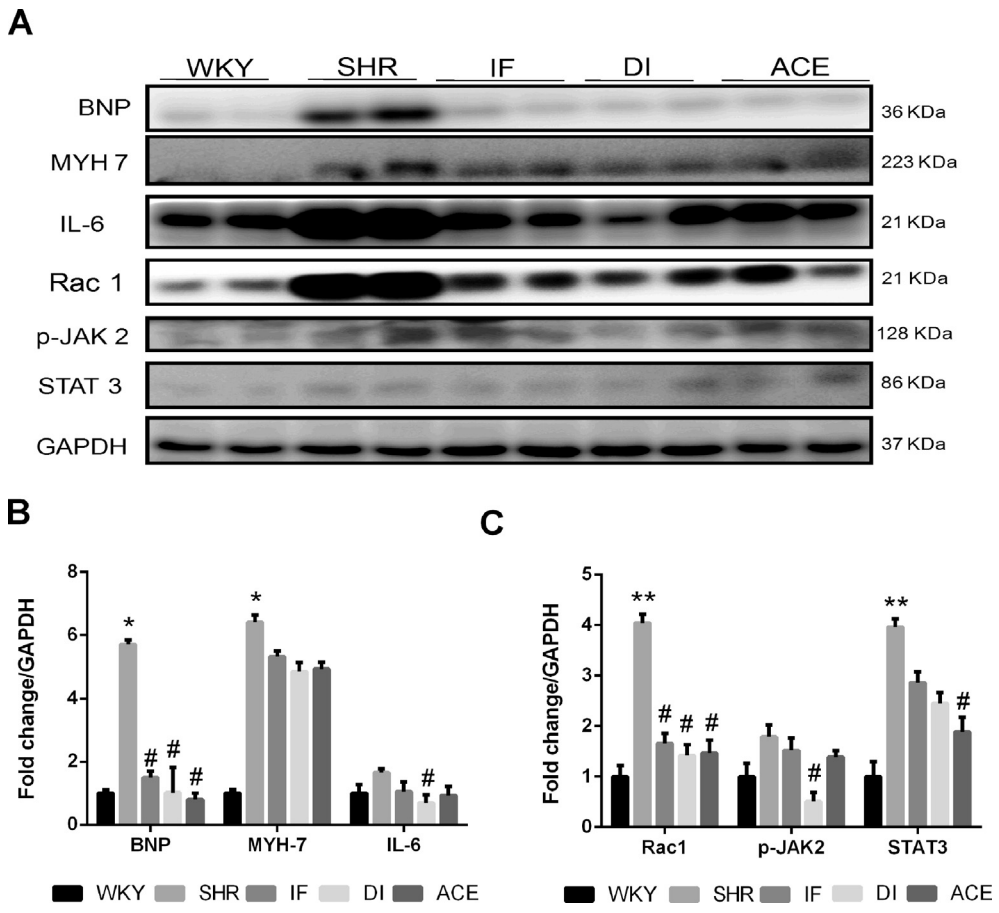

Fig. 3 – Protein expression of hypertrophy and eccentric hypertrophy markers. A. The hypertrophy marker expressions of BNP, MYH-7 and the eccentric hypertrophy markers expression of Rac-1, p-JAK2, STAT3 and IL-6. B. The protein expression levels of BNP, MYH-7 and IL-6 were plotted on the bar graph and C. The upregulation of Rac-1, p-JAK2 and STAT3 expressions in SHR and downregulation of protein expression in bioactive peptide treated groups. *-p<0.05 compared to the WKY; **-p<0.01 compared to the WKY; #-p<0.05 compared to the SHR.

upregulated in SHR rats compared to the control and peptide treated groups. We found a significant downregulation in TIMP1 ($p < 0.05$), uPA ($p < 0.01$), TLR4 ($p < 0.05$) and p-NFkBp65 ($p < 0.05$) expression in peptide treated groups compared to the SHR group. These observations showed that IF bioactive peptides attenuate cardiac fibrosis and inflammation.

4. Discussion

Hypertension is one of the main mediators of cardiovascular diseases [2]. Pressure overload exerts mechanical stress in the ventricles and triggers cardiac hypertrophy and fibrosis [18]. Bioactive peptides were recently identified as significant contributors in protecting against cardiac disease progression. In this study, we found that SHR group showed structural changes in heart weight along with heart weight-body weight ratio compared to WKY group. Further, pathological changes in the heart of SHR show increased myocyte area and collagen accumulation compared to WKY rats. The present findings are consistent with Lin et al. [19] on the structural and pathological changes in SHR. In addition to cardiac dysfunction, we noticed a significant amount of increase in hepatic serum aminotransferases and renal markers such as serum uric acid and creatine levels compared to WKY rats. Right ventricular dysfunction and low cardiac output lead to congestion and hepatic necrosis [20]. Studies show evidence that heart failure leads to chronic kidney disease and cause decline in renal function [21,22]. Treatment with bioactive peptides, IF and DI largely improved the cardiac function along with protective roles against renal and hepatic dysfunction as that of control rats. We have previously reported that bioactive peptide from soy (VHVV) and potato protein hydrolyzate DIKTNKPVIF peptide ameliorates high-fat diet-induced hepatosteatosis development respectively [17,23].

Prolonged cellular adaption leads to stress-induced cardiac remodeling (physiological and pathological hypertrophy) and causes irreversible functional deterioration leading to heart failure [24]. In this study, we found increased levels of hypertrophy markers - brain (B-type) natriuretic peptide (BNP) in SHR compared to normotensive rats. Importantly, the occurrence of pathological hypertrophy in SHR rats was significantly increased. Inflammatory mediators such as TNF-α and IL-6 are known to regulate hypertrophy [25]. Our findings

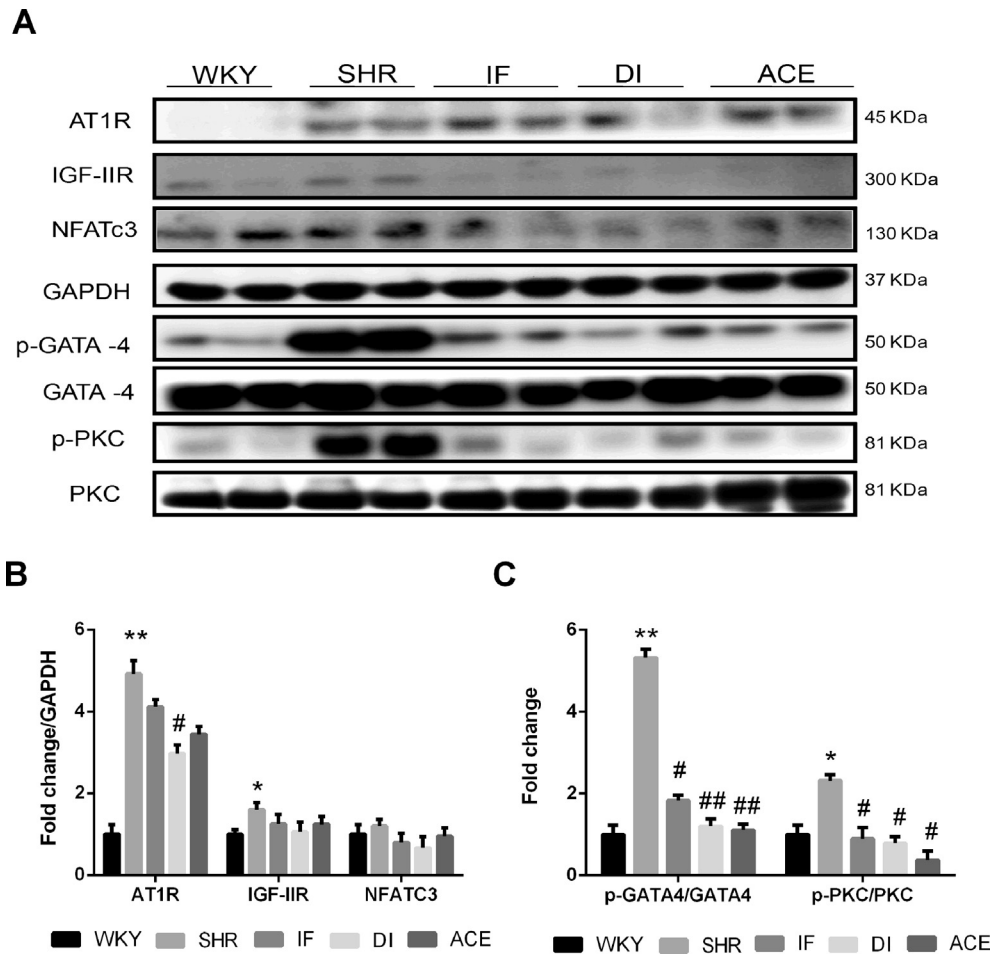

Fig. 4 – The protein expression of upstream signaling and transcriptional factors in control and treatment groups. A. The protein expression of upstream signaling markers AT1R and IGF-IIR and transcriptional factors NFATc3, p-GATA4 and p-PKC and B, C. The upregulation of protein expression levels in SHR and downregulation of expression levels in bioactive peptide treated groups. *-p<0.05 compared to the WKY; **-p<0.01 compared to the WKY; #-p<0.05 compared to the SHR; ##-p<0.01 compared to the SHR.

show that SHR rats had significantly increased TNF-α and IL-6 protein levels. TNF-α is identified to regulate hypertrophic growth response in cardiac myocytes [26]. IL-6 along with gp130 activate the JAK2-STAT3 pathway causing cardiac myocyte hypertrophy [27–29]. Thus, observed upregulation of JAK2 and STAT3 in the SHR groups show evidence that inflammatory cytokines activation play a significant role in eliciting pathological hypertrophy. Rac-1 is a key mediator in Ang-II induced STAT3 regulation via JAK2 activation [30]. We found that SHR rats showed a significant increase in Rac-1 expression. Rac-1 transgenic mice showed atrial dilatation and promoted the development of structural atrial changes [31]. Here, we demonstrate key findings that bioactive peptides (IF and DI) significantly decrease eccentric hypertrophy in SHR through Rac-1/JAK2 pathway. Previously, pharmacologic attenuation of JAK2 and its protection against LV remodeling in pressure overload has been reported [32]. The statin, simvastatin protected against cardiac hypertrophic and fibrotic changes by targeting Rac-1 and Ang-II/JAK/STAT signaling [33]. Thus, our study shows that bioactive peptides play a potent role in protection against the pathological hypertrophy response in SHR. IGF-IIR activation regulates pathological hypertrophy through Ca^{2+} release and protein kinase C/NFATc3 signaling. Our lab has previously demonstrated that, IGF-IIR regulates hypertrophic mechanisms through p38/NFATc3/GATA4 pathway [34].

The systemic inflammation was associated with cardiac fibrosis and diastolic dysfunction [35]. Increased thickness of ECM under exuberated hypertrophy and cardiac fibrosis causes irreversible heart failure. Multiple signaling mechanisms culminate together in oschestring myocardial fibrosis and atrial fibrillation including MAPKs [36,37]. In the present study, we identified significant upregulation of TLR4 and NF-kB activation in SHR rats. TLR4 is an important receptor in mediating fibrosis by regulating inflammatory cytokine production through NF-kB activation [38,39]. Previous studies report that, TLR4 in the regulation of hypertension, inflammation and I/R injury, leading to cardiac failure [38,40]. The absence of TLR4 significantly reduced inflammation and cardiac I/R injury [41,42]. The bioactive peptide - DI, significantly reduced fibrotic changes with a decline in uPA mediated signaling pathways and thereby protected SHR-induced fibrosis. Furthermore blockade of uPA- or MMP-signaling prevents pressure overload-induced LV fibrosis in mice [43].

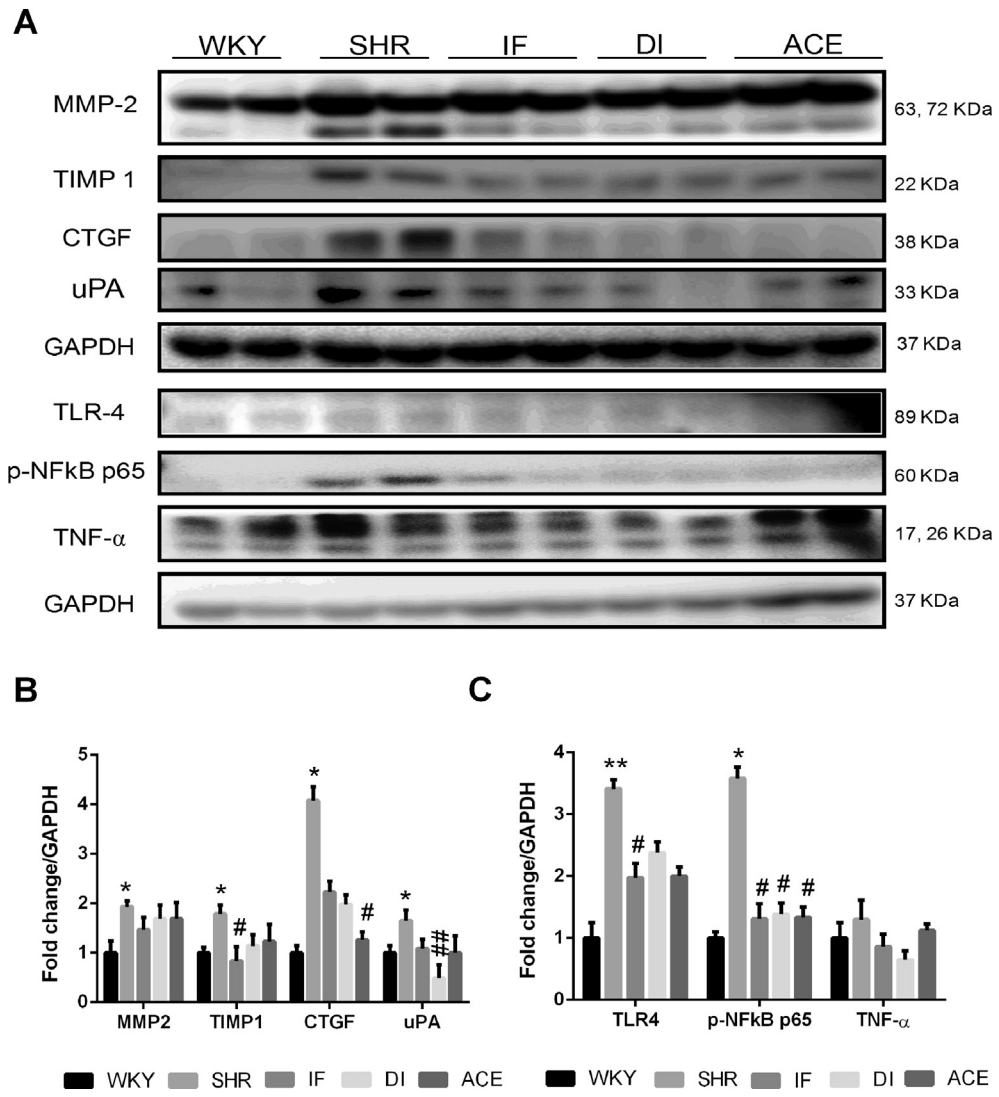

Fig. 5 – Protein expression of cardiac fibrosis and inflammation markers in control and treatment groups. A. The cardiac fibrotic protein expression of MMP-2, TIMP-1, CTGF, uPA and inflammation markers TLR-4, p-NFkBp65 and TNF-α and B, C. Downregulation of cardiac fibrosis and inflammation markers in bioactive peptide treated groups. *-p<0.05 compared to the WKY; **-p<0.01 compared to the WKY; #-p<0.05 compared to the SHR; ##-p<0.01 compared to the SHR.

The present findings showed clear evidence that bioactive peptides IF and DI may have therapeutic benefit in protecting against cardiac inflammation, hypertrophy, and fibrosis under hypertensive conditions.

Declaration of Competing Interest

Authors declared no conflicts of interest.

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