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Original Research

β-Catenin overexpression causes an increase in inflammatory cytokines and NF-κB activation in cardiomyocytes

J. C. Lin[1,2], R-L. Chang[3,4#], Y-F. Chen[5], J-J. Yang[6], R. Baskaran[7], L-C. Chung[8], R-J. Chen[9], C. H. Day[10], V. Vijaya Padma[11], C-Y. Huang[4,7,12*#]

[1] Department of Occupational Therapy, Asia University, Taichung 41354, Taiwan
[2] Department of Internal Medicine, Cheng Ching Hospital, Taichung 40045, Taiwan
[3] College of Chinese Medicine, School of Post-Baccalaureate Chinese Medicine, China Medical University, Taichung City, Taiwan
[4] Graduate Institute of Chinese Medicine, China Medical University, Taichung, Taiwan
[5] Department of Obstetrics and Gynecology, Taichung Veteran's General Hospital, Taichung 40705, Taiwan
[6] Institute of Oral Sciences, College of Oral Medicine, Chung Shan Medical University, Taichung 40201, Taiwan
[7] Graduate Institute of Basic Medical Science, China Medical University, Taichung 40402, Taiwan
[8] Department of Hospital and Health Care Administration, Chia Nan University of Pharmacy & Science, Tainan County 71710 , Taiwan
[9] Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei 11031 ,Taiwan
[10] Department of Nursing, MeiHo University, Pingtung County 91202, Taiwan
[11] Department of Biotechnology, Bharathiar University, Coimbatore-641 046, India
[12] Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan

Correspondence to: cyhuang@mail.cmu.edu.tw

[#] These authors share equal contribution

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Abstract: β-Catenin has been implicated in various developmental and physiological processes. Defective Wnt signaling can result in different cardiac and vascular abnormalities and is activated under pathological conditions such as inflammation and obesity. In this study, roles of β-catenin in inflammation in cardiomyocytes were investigated. 10 samples from hearts of patients with acute infarction and 10 from normal ones were collected in order to access roles of β-catenin in cardiomyocytes. H9c2 cardiomyoblasts and primary neonatal rat cardiomyocytes were transfected with porcine cytomegalovirus (pCMV)-β-catenin plasmid in order to overexpress β-catenin. Protein level of β-catenin protein was increased in human acute infarction tissues compared to ones from normal patients. The transcription factor had increased nuclear localization in cardiomyocytes of the Wistar rats with cardiac hypertension. Furthermore, expression of fibrosis protein markers increased. Protein expression of β-catenin was increased in human acute infarction inflammatory heart tissues and in hearts of inflammatory obesity rats. After pCMV-β-catenin plasmid was transfected in a dose-dependent manner, inflammation protein markers, TNF-α and IL-8, were upregulated in hypertensive neonatal rat cardiomyocytes and H9c2 cardiomyoblasts. In addition, overexpression of β-catenin induced activation and nuclear localization of NF-κB. Therefore, β-catenin is a potential molecular target for treatment of inflammation and fibrosis in cardiomyocytes.

Key words: Acute infarction; H9c2 cardiomyocytes; β-catenin; inflammation; NF-κB.

Introduction

When cardiomyocytes are under mechanical stress stimuli, various hormones are secreted and a number of intra- and intercellular signaling cascades are activated and function in an autocrine or paracrine manner (1). These ligands are capable of altering myocardial ultrastructure, determining hypertrophy and/or apoptosis of cardiac fibroblasts, mesenchymal fibrotic and inflammatory processes and induce cardiac gene expression modifications (1-5).

This series of events is the basis of myocardial remodeling, which is a complex phenomenon of ultrastructural cardiac rearrangement (6). Because of the sensible changes in cardiomyocyte viability, energy metabolism, kinetic and electrical properties and cytoskeleton and extracellular matrix compositions, myocardial remodeling is considered as the key pathogenetic factor of Chronic Heart Failure (CHF) and of its natural history, which is marked by inexorable, progressive cardiac dysfunctions (7-9).

In response to myocardial ischemia (MI) and systemic inflammatory stimuli such as sepsis, cardiomyocytes express pro-inflammatory cytokines, which are able to initiate a local inflammatory response (10-13). Inflammatory response in cardiomyocytes, which involves cytokines, chemokines and subsequently recruited leukocytes (14, 15) and cell surface adhesion molecules (15, 16), leads to decreased cardiomyocyte contractility and may impact tissue repair processes. Analogous to that of dendritic cells, cardiomyocytes have an initial response directed by Toll-like receptors (TLRs) against damaging stimuli (17). Moreover, β-Catenin signaling was recently found to be activated by pressure overload-induced pathological cardiac remodeling in injured arteries and after myocardial infarction (18). β-Catenin signaling is clearly involved in hypertrophic growth (19). However, little is known about Wnt/frizzled/β-catenin pathway in

cardiomyocyte inflammation and fibrosis.

Involvement of β-catenin in Wnt signaling pathway has been extensively studied (20, 21). Cytosolic β-catenin is tightly regulated by a destruction complex consisting of Axin, adenomatous polyposis coli (APC), casein kinase I (CKI) and glycogen synthase kinase-3β (GSK-3β) (22). GSK-3β phosphorylates β-catenin, leading to degradation of the latter. Inhibition of GSK-3β activity by Wnt or other pathways results in stabilization and nuclear localization of β-catenin (23).

β-Catenin, which is the central transcription factor in Wnt signaling pathway, has been implicated in cell-cell communication in a wide variety of developmental and physiological processes. Wnt/β-catenin signaling pathway is required for different aspects of cardiac and vascular development and its defective state can result in different cardiac and vascular abnormalities. In adult heart and blood vessels, its activity is quite low under normal conditions. However, it is reactivated during pathological cardiac remodeling induced by pressure overload in injured arteries and after myocardial infarction (2).

In several types of organs, including, lungs, kidneys and liver, Wnt/β-catenin intracellular signaling was found be involved in expression of fibrosis (18, 19, 24). WNT1 and WNT10B are upregulated in pulmonary fibrosis and in liver cirrhosis (19). In cardiomyocytes, cardiac fibroblasts are regulated in vitro by Wnt/β-catenin pathway (5). Specific combinations of Wnt ligands and Frizzled receptors elicit distinct responses. In vivo, pediatric heart allographs with diastolic dysfunction and severe epicardial fibrosis display nuclear β-catenin accumulation in fibroblasts, suggesting Wnt signaling activation (24).

Toll-like receptor (TLR) family members play important roles in immune responses, especially ones against foreign microorganisms which are potentially pathogenic, by recognizing receptor-specific pathogen -associated molecular patterns (PAMPs), which were commonly found in bacteria, viruses and so on (25, 26). Pathogenic microbe components are sources of their ligands which are often considered as PAMPs. Binding of PAMPs to specific TLRs represents a critical event of innate immunity and provides an immediate response to pathogens in various animal species (27). In addition, TLR2 and TLR4 were found to bind a separate set of ligands, damage-associated molecular patterns (DAMPs), in injured and inflamed tissues (28).

Binding of TLR ligands to TLRs pass down signals to other parts of a cell through at least one of the following adaptor proteins: myeloid differentiation primary response gene 88 (MyD88), toll/interleukin-1-receptor-domain-containing adaptor inducing interferon-β (TRIF), toll/interleukin-1-receptor-domain-containing adaptor protein and TRIF-related adaptor molecule.

TLR2 or TLR4 agonists stimulate MyD88 signaling pathway in antigen-presenting cells such as dendritic cells and macrophages, which leads to subsequent downstream activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (29). This activation leads to the rapid expression of inducible nitric oxide synthase and a wide variety of pro-inflammatory cytokines, chemokines and their receptors, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-6 and IL-8 (30-32).

This particular study aimed to investigate roles of β-catenin in apoptosis, cardiac hypertrophy, inflammation, fibrosis and survival in cardiomyocytes.

Materials and Methods

Cell culture

H9c2 cardiomyoblast cell line was purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in 100-mm culture plates in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% cosmic calf serum (CCS; HyClone, South Logan, UT, USA) in a cell incubator at 37°C. Cell medium was replaced 48 hours after sub-cultivation. H9c2 cells were transfected with β-catenin plasmid in time-dependent or dose-dependent treatments.

Immunohistochemical staining

Samples of tissue biopsies from patients described in the Human Cardiovascular Tissue Microarray section were dried at 58°C overnight. The tissue sections were dewaxed in xylene for 40 minutes and sequentially rehydrated using a graded series of ethanol. Endogenous peroxidase activity was blocked with hydrogen peroxide blocking buffer (3% H_2O_2) for 13 minutes. After the biopsy microarray was rinsed in tap water for 15 minutes, it was microwave treated with pre-warmed citrate buffer (0.01M citric acid (pH6.0)) for 15 minutes and cooled to room temperature (RT) for 30 minutes. Nonspecific binding was blocked with 5% CCS for 1 hour and then the array was incubated with 1:100 diluted primary antibodies against β-catenin (Santa Cruz Biotechnology, Dallas, TX, USA) at RT overnight, which was followed by 1:100 diluted secondary antibodies application (Santa Cruz Biotechnology, Dallas, TX, USA) at RT for 1 hour. Immunoreactivity was visualized with 3,3'-diaminobenzidine substrate (Roche Diagnostics GmbH, Mannheim, Germany) for 5 minutes. After it was washed using 1× phosphate-buffered saline (1×PBS) (GIBCO, Auckland, New Zealand) for 10 minutes, the tissue microarray was then analyzed using microscopy (magnification: 200×). The study procedures conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were reviewed and approved by the Institutional Review Board of the Armed Force Taichung General Hospital, Taichung and the China Medical University, Taichung, Republic of China. Verbal and written informed consents for inclusion in the study were obtained prior to sample collection.

Neonatal rat primary cardiomyocyte culture

Primary cardiomyocytes from Wistar rats with obesity were isolated with Neonatal Rat/Mouse Cardiomyocyte Isolation System Kit (Cellutron Life Technology; Baltimore, MD, USA). Each of 100-mm culture dishes was coated with SureCoat solution (Cellutron Life Technology, Baltimore, MD, USA) for 2 hours in at 37°C. Hearts of the diabetic rats were isolated and incubated in digestion solution at 37°C. Each plate was coated with an extra layer of SureCoat solution for another 2 hours. Then all isolated cells were rested in non-coated cell culture plates for 1 hour, fibroblasts from

the isolated hearts first attached to bottom of each plate and cells which floated on culture medium were primary cardiomyocytes. The cardiomyocytes were finally transferred to another set of culture plates pre-coated with SureCoat solution twice. Finally, ventricular cardiomyocytes were cultured in DMEM containing 10% CCS and subsequently were transfected with β-catenin plasmid in dose- and time-dependent manners 3-4 days later.

Nuclear and cytoplasmic fractionation

H9c2 cardiomyocytes were seed in 100-mm culture dishes containing DMEM with 10% CCS at 80% confluency. Subsequently, the cels were transfected with pCMV-β-catenin plasmid. The transfected cells were washed with 1×PBS and prepared for nuclear protein extraction with a Nuclear/cytosol Fractionation Kit (BioVision Inc., Milpitas, CA, USA). Cells were trypsinized (0.05% trypsin/0.53mM EDTA) and resuspended in 1×PBS. Cells were collected and then centrifuged at 600 ×g for 5 minutes at 4°C. After carefully aspirating supernatants, the cells were resuspended with 200 µl CEB-A Mix containing 1mM DTT and protease inhibitors, vigorously vortexed for 15 seconds and then incubated on ice for 10 minutes at 4°C. Then 11 µl Cytosol Extraction Buffer-B was added to the tube. After it was vigorously vortexed for 15 seconds, incubated on ice for 1 minute and centrifuged at 16,000 ×g for 10 minutes at 4°C, supernatants (cytoplasmic fractions) were carefully aspirated and pellets were resuspended with 100 µl ice-cold Nuclear Extraction Buffer Mix containing 1mM DTT and protease inhibitors and vigorously vortexed for 15 seconds. After vortexing, the suspensions were placed on ice for 10 minutes. This step was repeated for a total of 40 minutes. Finally, the tube was centrifuged at 16,000 ×g for 10 minutes at 4°C. The supernatants (nuclear extracts) were stored in aliquots at -80°C. Lowry assay is used to determine protein concentration in each sample and western blot analysis was conducted in order to obtain results.

Tissue homogenization

Tissue samples from spontaneously hypertensive Wistar Kyoto rats (National Laboratory Animal Center, Taiwan) with hypertension or diabetes were homogenized for protein extraction in a lysis buffer at a concentration of 100 mg tissue/ml buffer. The homogenates were placed on ice for 10 minutes and then centrifuged at 13,000 ×g for 40 minutes. The supernatants were collected and stored at -80°C for further analysis. All protocols were reviewed and approved by the Institutional Review Board and the animal care and use committee of the China Medical University, Taichung, Taiwan.

Transient transfection

Cells at 50% confluency were seeded into 6-cm culture plates containing fresh DMEM with 10% CCS 2 hours before transfection and then pCMV-β-catenin plasmid was transfected into the cells for 24 hours using PureFection™ Nanotechnology-based Transfection Reagent (System Biosciences, Palo Alto, CA, USA) following the manufacturer's protocol. In each experiment, the efficiency of protein overexpression was accessed by western blot analysis.

Western blot analysis

H9c2 cardiomyocytes were seeded in 100-mm culture dishes containing DMEM (10% CCS) to 80% confluency. After transfection with pCMV-β-catenin plasmid at different time points or at different doses, each plate was washed with 3 ml 1×PBS twice and the remaining fluid in each plate was sucked off. Then 100 µl cell lysis buffer (50mM Tris-base (pH7.5), 0.5M NaCl, 1mM EDTA (pH8.0), 1mM β-mercaptoethanol, 1% NP-40, 1% glycerol and protease inhibitor cocktail tablets) per plate was added to lyse the cells. The cells were scraped down and collected in appropriate 1.5-ml microcentrifuge tubes on ice, which were then vortexed once every 10 minutes 3 times and centrifuged for 20 minutes at 12000 rpm, 4°C. The supernatants were transferred to another set of 1.5-ml microcentrifuge tubes. These were the total protein samples. Protein concentrations of the samples were determined by Lowry assay and western blot analysis was conducted.

Statistical analysis

Each sample was analyzed based on results that were repeated at least three times and SigmaPlot 10.0 software and standard t-test was used to analyze each numeric data. In all cases, differences at $p < 0.05$ were regarded as statistically significant, ones at $p < 0.01$ or $p < 0.001$ were considered higher statistical significances.

Results

Expression patterns of β-catenin in human infarct hearts and hearts of inflammatory disease models

Cardiac infarction is associated inflammatory response, in which secretion of inflammatory cytokines is involved (33, 34). Expression of β-catenin protein in human and rat inflammatory were determined by conducting western blot analysis and examining immunohistochemical staining patterns in myocardial infarction specimens from cardiovascular tissue microarray. The results show that protein expression of β-catenin was increased in the acute infarction tissues. Moreover, protein level of the transcription factor was also elevated in the hearts of obese rats (Figure 1). Therefore, β-catenin signaling was elevated by cardiac infarction in humans and inflammation in rats.

Transfection of pCMV-β-catenin plasmid in a dose-dependent manner induced inflammation markers and nuclear localizations of β-catenin and NF-κB

In order to access effects of β-catenin on inflammation and nuclear localizations of NF-κB and β-catenin in cardiomyocytes, nuclear and cytoplasmic fractionation and western blot analysis were conducted. Transfection with the pCMV-β-catenin plasmid in a dose-dependent manners upregulated expression of inflammatory protein markers, TNF-α, p-NF-κB and IL-8, in both neonatal rat cardiomyocytes and H9c2 cardiomyoblast cells (Figure 2). Moreover, at increasing doses of the plasmid, both β-catenin and NF-κB were shown to have increasingly higher nuclear localization levels (Figure 3). Therefore, β-catenin led to increased inflammation and NF-κB nuclear localization.

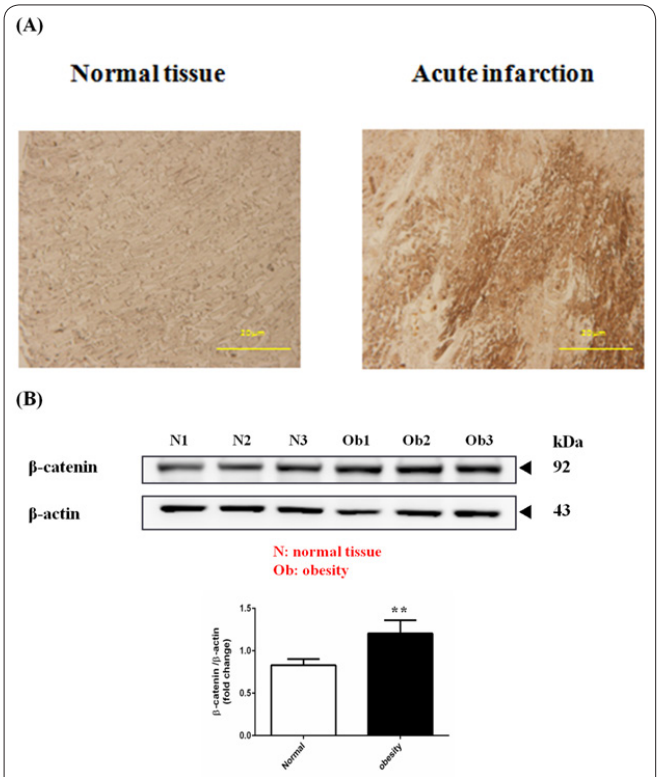

Figure 1. Immunohistochemical analysis for β-catenin in sections from the human cardiovascular tissue with myocardial infarction (MI) disease and hearts of animal metabolic syndrome. (A) Immunohistochemical analysis of β-catenin protein expression (brown color) in human cardiovascular tissue with MI. Panel: a normal tissue (n=10) and an acute infarction (n=10). Final magnifications: 200× (bar, 200μm). (B) Western blot analysis of β-catenin expression in hearts of obesity rats. **p < 0.001 vs normal (increase).

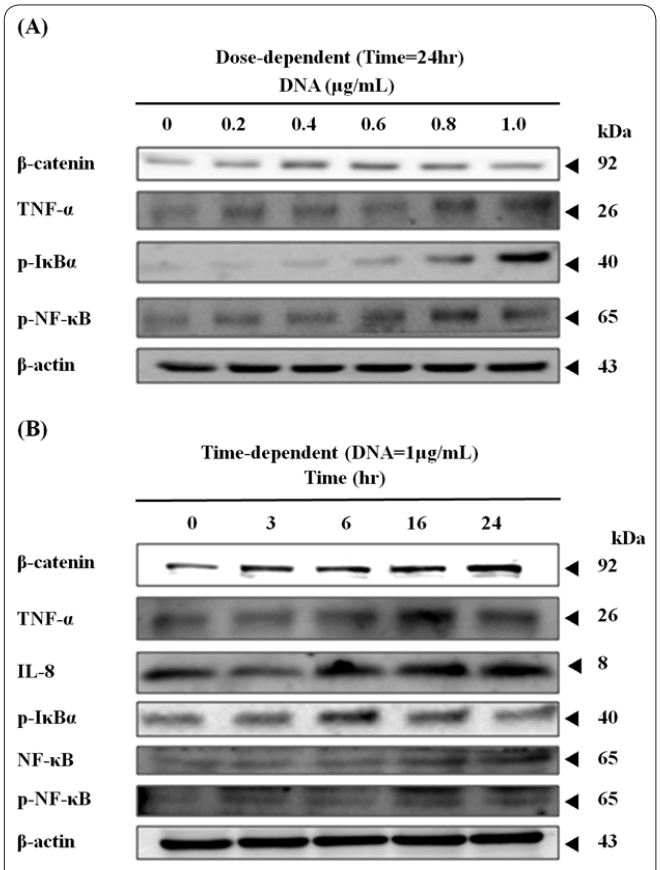

Figure 2. β-Catenin over-expression causes increased inflammatory cytokines. (A) Neonatal Rat Cardiomyocytes were transfected with dose-dependent pCMVβ-catenin plasmid for 24 hours. Western blot analysis of effects of β-catenin on TNF-α, p-IκBα and p-NF-κB in neonatal rat cardiomyocytes in a dose-dependent manner. (B) H9c2 cells were transfected with a 1 μg/ml pCMVβ-catenin plasmid for a time-dependent course. Western blot analysis of effects of β-catenin on inflammatory cytokines in neonatal rat cardiomyocytes in time-dependent manner.

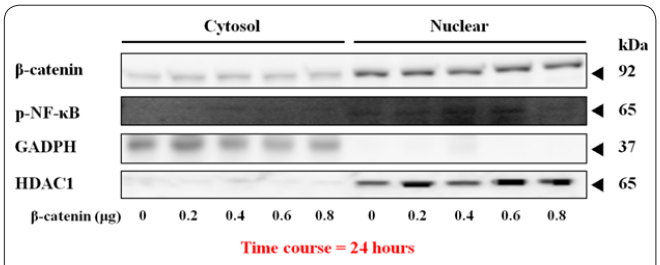

Figure 3. β-Catenin over-expression induced inflammation and NF-κB nuclear translocation. H9c2 cells were transfected with a dose-dependent pCMVβ-catenin plasmid for 24 hours. Investigation of 24-hour β-catenin overexpression on nuclear localizations of β-catenin and NF-κB in H9c2 cardiomyoblasts.

Discussion

Cardiac hypertrophy is a decompensated state with profound changes in gene expression, contractile dysfunction and extracellular remodeling (6, 31, 35). Differential activation of MAPK family members results in specific cardiac morphologic and functional phenotypes (36-38). Cytosolic β-catenin is tightly regulated by a destruction complex consisting of Axin, APC, CKI and GSK-3β (22). Overexpression of β-catenin induced hypertrophic growth in cardiomyocytes (19, 39, 40). In addition, β-catenin can potentially regulate hypertrophic responses following various stimuli; however, there is no clear relationship between β-catenin levels and degree of cardiac hypertrophy.

Evidence from both experimental and clinical trials indicates that inflammatory mediators are important in chronic heart failure pathogenesis and contribute to cardiac remodeling and peripheral vascular disturbances. Several studies have shown increased inflammatory cytokine levels such as TNF-α, IL-1β and IL-6 in blood and circulating leukocytes of HF patients, as well as in ones with failing myocardium. There is strong evidence that these mediators are involved in processes leading to cardiac remodeling such as hypertrophy, fibrosis and apoptosis (41).

In this particular study, protein levels of β-catenin were increased in hearts of obese rats and in those of patients with acute cardiac infarction. Inflammatory protein markers (TNF-α, p-NF-κB and IL-8) were upregulated in neonatal rat cardiomyocytes and H9c2 cardiomyoblasts after transfection with pCMV-β-catenin plasmid in dose- and time-dependent manners. It was previously shown that obesity is associated with white adipose tissue (WAT) macrophage infiltration and increased local concentrations of IL-6 and TNF-α (42, 43). Interestingly, anti-adipogenic actions of IL-6 and TNF-α seemed to mediate, at least in part, through Wnt/β-catenin signaling cascade (30, 44). Numerous experimental studies have also shown that both endogenous β-catenin knock-down and dn*TCF712* over-expression reversed anti-adipogenic effect of TNF-α, indicating that Wnt/β-catenin signaling activation is a prerequisite

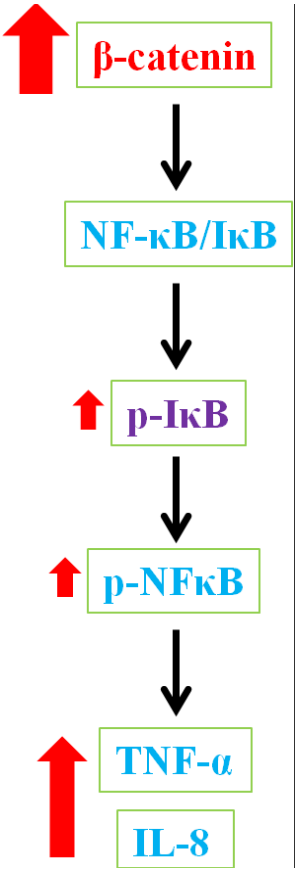

Figure 4. β-Catenin over-expression induced inflammation and activation of NF-κB while reducing survival in cardiomyocytes.

for inhibiting adipogenesis using this cytokine (45).

In conclusion, Wnt/β-catenin signaling cascade is capable of damaging cardiomyocytes and that this study will give a good picture in treatment of cardiovascular disease. Strategies to target β-catenin expression in cardiomyocytes would help in protecting heart cells from myocardial infarction-induced inflammation and expression and activity of NF-κB. β-Catenin protein levels were increased in the hearts of obese rats. Therefore, down-regulation of β-catenin might impair WAT expansion during obesity by hijacking canonical Wnt signaling pathway, thereby decreasing the inflammatory mediators in chronic heart failure pathogenesis (Figure 4) . In conclusion, β-catenin is capable of transcribing a wide range of genes depending on the cell context.

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