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Expression of Notch-1 and nuclear factor-κB signal pathway in myocardial cells of coronary heart disease rats

JIAHUI ZHENG[1*], SHUFENG ZHANG[2*] and TINGTING WANG[2]

Departments of [1]Cardiology and [2]Preventive Medicine, Weifang People's Hospital, Weifang, Shandong 261041, P.R. China

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Abstract. Expression changes of Notch-1 and nuclear factor-κB (NF-κB) in cardiomyocytes from coronary heart disease (CHD) rats were investigated. The CHD model established by high-fat diet in 48 clean SD rats was set as the observation group, and another 48 healthy rats routinely fed were the control group. Rats were sacrificed on the 1st, 3rd, 7th, and 14th days after successful modeling. The rat myocardial cells were harvested to examine the changes of Notch-1 and NF-κB using immunoblot (western blot analysis) and TUNEL assay. Cardiomyocyte apoptotic rate, by Pearson's correlation test was used to analyze the correlation between Notch-1 and NF-κB. With the prolongation of the course of CHD in rats, the expression levels of Notch-1 and NF-κB proteins gradually increased, and the expression in the observation group was significantly higher than that in the control group (P<0.01). On the seventh day, the expression levels of Notch-1 and NF-κB protein in the rats in the observation group showed significant difference from those on day 1 and day 3 (P<0.05), and they were significantly different on the 7th and 14th days (P<0.05). There was no correlation between the expression level of the two proteins with the age and sex of the rats. Pearson's correlation analysis showed that Notch-1 was positively correlated with NF-κB protein (r=0.745, P=0.005). The results of myocardial apoptosis test showed that with the prolongation of the CHD course in the rats of the observation group, the cardiomyocyte apoptosis rate did not differ from the control group. The expression of Notch-1 and NF-κB protein is increased in cardiomyocytes of CHD rats. Notch-1 and NF-κB participate in the occurrence and development of CHD.

Correspondence to: Dr Tingting Wang, Department of Preventive Medicine, Weifang People's Hospital, 151 Guangwen Street, Weifang, Shandong 261041, P.R. China
E-mail: t8c2rf@163.com

*Contributed equally

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Introduction

Coronary heart disease (CHD), is considered to be one of the diseases that have the greatest threat to human health in recent decades. According to surveys, the total CHD patients in developed countries are over 750,000. There are more than 450,000 patients with secondary CHD (1). Other studies have shown (2) that in 2004 there were approximately 400,000 people who died of cardiovascular disease globally. The incidence of CHD in China is relatively low, but with the development of the aging population, the incidence rate is also rising year by year (3). This not only threatens the patient's prognosis and lifespan, but also has an impact on the social and economic benefits such as labor loss and brings a heavy burden on society and the family. CHD is due to changes in coronary function, leading to stenosis or occlusion of the lumen, causing myocardial ischemia and hypoxia in patients (4). The pathogenesis of CHD is not yet clear. At present, most scholars believe that CHD is caused by a combination of multiple genes and is associated with environmental and genetic factors (5).

The Notch signaling pathway serves as a highly-conserved cell-to-cell communication pathway. Notch was first discovered when a mutant allele caused an incision in the wing of the fly (6). Studies have shown that (7) Notch-1 signaling pathway plays a key role in the physiology, pathology, occurrence and development of cardiovascular system. In recent years, with in-depth study, it was found that Notch signaling pathway plays a regulatory role in the course of CHD (8). Nuclear factor-κB (NF-κB) is a protein complex that regulates transcription of DNA, and NF-κB is expressed in all animal cells (9). In recent years, studies have shown that (10) NF-κB-p65 signaling pathway also plays a regulatory role in the CHD process. However, Notch-1 signaling is associated with NF-κB-p65 signaling pathway at multiple levels. For example, NF-κB-p65 signaling pathway is involved in the development of rheumatoid arthritis. The activation of Notch-1 signaling pathway is closely related to the occurrence and development of tumors (11,12). We speculate that Notch-1 and NF-κB-p65 may be involved in the pathogenesis of CHD.

Through this study, the changes of Notch-1 and NF-κB pathways in the pathogenesis of CHD were explored, in order to provide a theoretical basis for clinical prevention and control of CHD.

Materials and methods

Animal sources. A total of 96 wistar rats were used in this study, including 48 male and 48 female rats. The weight range was 160-210 g, the average weight was 185±25 g, age 8-12 weeks, and the average age 10.51±1.25 weeks, provided by Daping Medical Laboratory Animal Center, Third Military Medical University, license no. SCXK (Yu) 2012-0005.

Experimental materials. Tissue protein lysate, BCA protein quantification kit (Biotime Biotechnology Institute, Shanghai, China), TUNEL apoptosis detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), rabbit anti-mouse Notch-1 polyclonal antibody and NF-κB-p65 polyclonal antibody (cat. nos. 4147 and 4764; both from Cell Signaling Technology, Inc., Danvers, MA, USA), horseradish peroxidase labeled goat anti-rabbit IgG (cat. no. 31460; Zymed; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and GAPDH internal reference was provided by Cell Signaling Technology, Inc. Isoproterenol hydrochloride was purchased from Shanghai Shifeng Biotechnology Co., Ltd. (Shanghai, China). The study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China).

Animal model construction. A total of 96 rats were randomly divided into a control and an observation group according to random method. The rats of the control group were 25 males and 23 females, and the age was 10.05±1.62 weeks. There were 23 males and 25 females in the observation group, aged 10.32±1.32 weeks, there was no statistically significant difference in sex and age between the two groups, and there was comparability. In the week before modeling, the rats of the two groups were fed with common feed, and had free access to drinking water with a 12 h light/dark cycle to adapt to the environment. After one week, the rats in the observation group were fed with high-fat diet for 6 weeks by the laboratory (propyl thioxymidine 0.2%, sodium taurocholate 0.5%, cholesterol 2%, lard 10%, and basal diet 87.3%). After 6 weeks, the rats were injected with isoproterenol hydrochloride (5 mg/kg) continuously for 3 days, once daily. After 3 days of injection, 10% chloral hydrate (0.3 ml/100 g) was used for intraperitoneal anesthesia and fixed. The ST segment changes were observed using an electrocardiograph and the ST segment elevation ≥0.1 mv in the rat electrocardiogram suggested that the modeling was successful. The control group was fed with conventional feed for 6 weeks.

Tissue collection. Rats were sacrificed by cervical dislocation on the 1st, 3rd, 7th, and 14th days after successful modeling. Each time, 12 rats were deprived of their hearts. The infarcted and non-infarcted areas were separated and some of them were subjected to follow-up experiments. The rest of the tissue was stored at 80˚C until use.

Detection of Notch-1 and NF-κB proteins in rat cardiomyocytes by western blot analysis. The collected tissues were lysed using the cell lysate RIPA, and the total protein was lysed. The protein concentration was measured using a BCA protein quantitation kit, electrophoresis separation was performed using a 12% SDS-PAGE gel, and electrotransfer was performed at a constant voltage. The membrane was sealed in 5% skim milk in TBS buffer and protected from light at room temperature for 1 h. Primary antibody (dilution, 1,000 both for rabbit anti-mouse Notch-1 polyclonal antibody and NF-κB-p65 polyclonal antibody) was plated overnight at 4˚C. Washing with PBS followed by labeling with horseradish peroxidase goat anti-rabbit IgG secondary antibody, incubated at 4˚C on a rocking shaker for one hour, shaking, and visualization. GAPDH was used as an internal control in this study. Gray scale of the protein bands was measured using the Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Product relative expression = grayscale of target protein / grayscale of internal control bands.

Apoptosis. Apoptosis of rat cardiomyocytes was detected using the TUNEL Cell Apoptosis Detection kit. The experimental method was performed in strict accordance with the manufacturer's instructions. After staining, observations were performed with an optical microscope (Olympus, Tokyo, Japan). After nuclear myocardial cell nuclear staining, the normal myocardial cell nuclear staining was blue, and the apoptotic cardiomyocyte nuclear staining was yellow or brown-yellow. Each field was randomly taken from 5 fields to observe and count the apoptotic cells in the visual field. Apoptosis rate = total apoptotic number / total cell number x100%.

Statistical analysis. In this study, we used SPSS20.0 software (IBM Corp., Armonk, NY, USA) to perform statistical analysis on all collected data, GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to create images. Enumeration data are expressed as rate (%), examined using Chi-square test, and measurement data used for the analysis were expressed as mean ± standard deviation (SD). The t-test was used for analysis between the two groups. Multiple groups were compared using repeated measures analysis of variance (ANOVA) and the post hoc test used was Fisher's test. Pearson's analysis was used to analyze protein expression correlations. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rat modeling. We established the two groups of rat models in 6 weeks. The results showed that the rats in the observation group successfully established the CHD model in 6 weeks. During the modeling period, the two groups of model rats did not die.

The expression of Notch-1 and NF-κB-p65 protein in rat cardiomyocytes. After successfully establishing the rat model, rats were sacrificed on the 1st, 3rd, 7th, and 14th day. The rat cardiomyocytes were used to detect the expression of Notch-1 and NF-κB-p65 protein. The expression of Notch-1 and NF-κB-p65 protein was significantly higher in the observation than in the control group ($P<0.05$). Repeated measurement analysis of variance revealed that the Notch-1 and NF-κB-p65 proteins were expressed in the control group and there was no statistical difference ($P>0.05$), but the expression of Notch-1 and NF-κB-p65 protein in the observation group was statistically different ($P<0.05$). There was a statistically

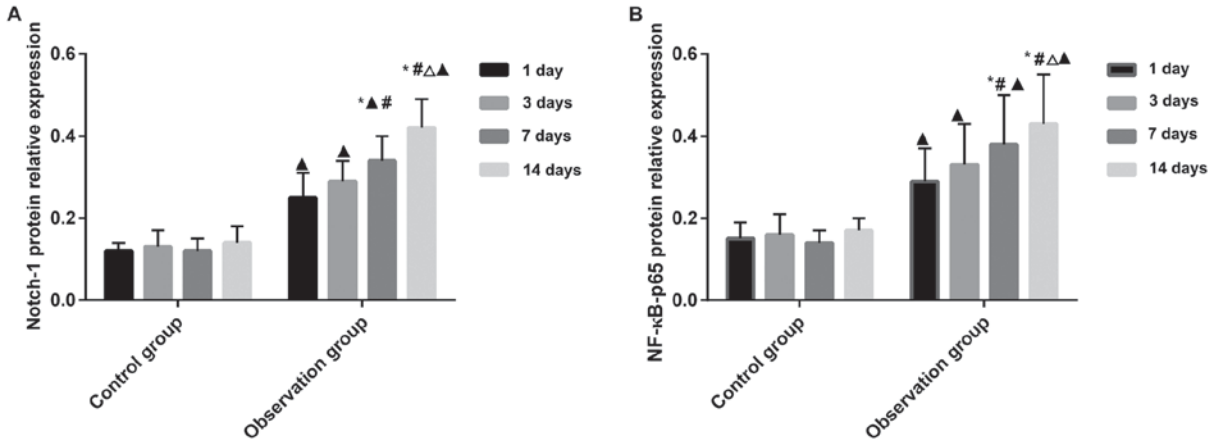

Figure 1. (A) The relative expression of Notch-1 protein in the control and observation group. *P<0.05, there is a difference in the expression of Notch-1 protein compared to the 1 day observation group; #P<0.05, there is a difference in Notch-1 protein expression compared to the 3 day observation group; △P<0.05, there was a difference in the expression of Notch-1 protein compared to the 14 day observation group. ▲P<0.05, indicates that there is a difference compared with the control group at the same time. (B) The relative expression of NF-κB1 protein in the control and observation group. *P<0.05, there is a difference in the expression of NF-κB1 protein compared to the 1 day observation group; #P<0.05, there is a difference in NF-κB1 protein expression compared to the 3 day observation group; △P<0.05, indicates that there was a difference in the expression of NF-κB1 protein compared to the 14 day observation group; ▲P<0.05, indicates that there is a difference compared with the control group at the same time. NF-κB, nuclear factor-κB.

Table I. The expression of Notch-1 protein in two groups of rats.

Items	1 day (n=12)	3 day (n=12)	7 day (n=12)	14 day (n=12)	F-value	P-value
Control	0.12±0.02	0.13±0.04	0.12±0.03	0.14±0.04	0.978	0.412
Observation	0.25±0.06	0.29±0.05	0.34±0.06[a-c]	0.42±0.07[a,b]	17.644	0.001
t value	7.120	8.656	11.361	12.031		
P-value	0.001	0.001	0.001	0.001		

[a]P<0.05, there is a difference in the expression of Notch-1 protein compared to the 1 day observation group;[b]P<0.05, there is a difference in Notch-1 protein expression compared to the 3 days observation group; [c]P<0.05, there was a difference in the expression of Notch-1 protein compared to the 14 days observation group.

Table II. The expression of NF-κB-p65 protein in two groups of rats.

Items	1 day (n=12)	3 day (n=12)	7 day (n=12)	14 day (n=12)	F-value	P-value
Control	0.15±0.04	0.16±0.05	0.15±0.03	0.17±0.03	0.746	0.531
Observation	0.29±0.08	0.33±0.10	0.38±0.12[a,b]	0.43±0.12[a,b]	3.920	0.015
t value	5.422	5.267	6.411	7.281		
P-value	0.001	0.001	0.001	0.001		

[a]P<0.05, there is a difference in the expression of NF-κB-p65 protein compared to the 1 day observation group; [b]P<0.05, there is a difference in NF-κB-p65 protein expression compared to the 3 days observation group. NF-κB, nuclear factor-κB.

significant difference between in the expression of Notch-1 and NF-κB-p65 protein in the observation group between 7 days, 14 days and 1 day, 3 days of modeling (P<0.05), and the expression of Notch-1 protein in the observation group at day 7 was different from that at the 14 day (P<0.05) (Fig. 1; Tables I and II).

Relationship between expression of Notch-1, NF-κB-p65 protein and sex and age of rats. We observed the expression of Notch-1 and NF-κB-p65 protein in the observation group at the 14th day after successful modeling. There was no statistical difference between the expression of Notch-1 and NF-κB-p65 protein and the sex and age of the rats (P>0.05) (Table III).

Table III. Relationship between expression of Notch-1, NF-κB-p65 protein and sex, age in rats (n, %).

Groups	Notch-1 protein expression			NF-κB-p65 protein expression		
	Low expression (n=6)	High expression (n=6)	P-value	Low expression (n=6)	High expression (n=6)	P-value
Sex						
Male (n=6)	3 (50)	3 (50)	>0.05	3 (60)	3 (42.86)	>0.05
Female (n=6)	3 (50)	3 (50)		2 (40)	4 (57.14)	
Age						
>10 weeks (n=5)	3 (50)	2 (40)	>0.05	2 (40)	3 (42.86)	>0.05
≤10 weeks (n=7)	3 (50)	4 (60)		3 (60)	4 (57.14)	

NF-κB, nuclear factor-κB.

Table IV. Cardiomyocyte apoptosis expression in rats.

Items	1 day (n=12)	3 day (n=12)	7 day (n=12)	14 day (n=12)	F-value	P-value
Control	4.05±0.84	3.94±0.79	3.98±0.80	4.24±0.91	0.304	0.823
Observation	4.25±0.92	4.21±0.95	3.84±0.81	3.75±0.85	0.993	0.405
t value	0.556	0.806	0.426	1.363		
P-value	0.584	0.429	0.674	0.186		

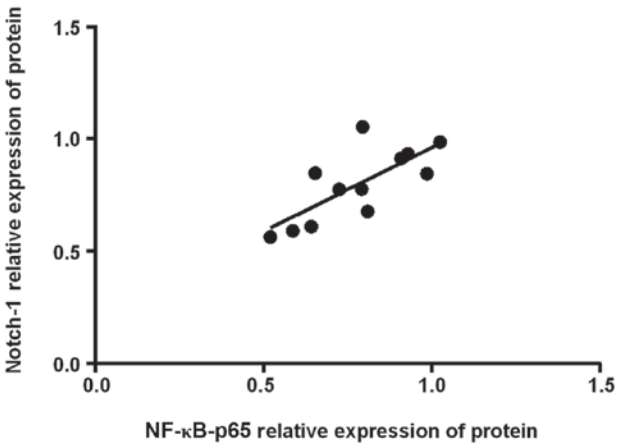

Figure 2. The correlation between the relative expression of Notch-1 protein and the relative expression of NF-κB1 protein in the 14th day of the control and observation group. Pearson's correlation test showed that the relative expression of Notch-1 protein was positively correlated with the relative expression of NF-κB1 protein on the 14th day (P<0.05). NF-κB, nuclear factor-κB.

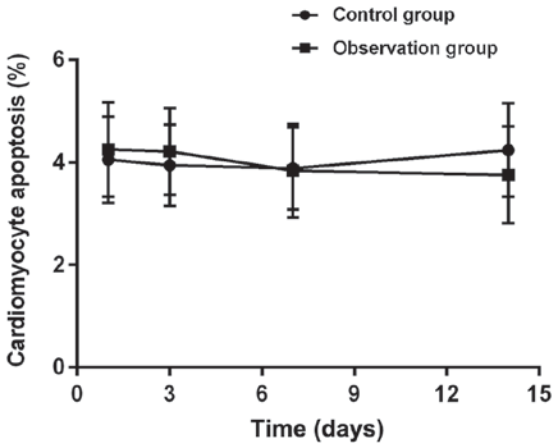

Figure 3. Comparison of cardiomyocyte apoptotic rate between control and observation group. After rat model was successfully established, there was no difference in myocardial apoptosis between the observation and the control group (P>0.05). The analysis of variance showed no difference in myocardial cell apoptosis between the control and the observation group (P>0.05).

Correlation analysis between Notch-1 and NF-κB-p65 protein expression. Pearson's correlation analysis of Notch-1 and NF-κB protein in rats in 14 days showed that Notch-1 was positively correlated with NF-κB-p65 protein expression (r=0.745, P=0.05) (Fig. 2).

Apoptosis. The TUNEL assay was used to detect apoptosis of the two groups of rats according to the time period. The results showed that after the rats were successfully modeled, there was no statistical difference between the observation group and the control group (P<0.05), repeated measurement analysis of variance found that there was no statistical difference in myocardial cell apoptosis between the control and the observation group (P>0.05) (Fig. 3; Table IV).

Discussion

The total number of cardiovascular patients in China is as high as 290 million, and the death rate is >30%. The number of deaths due to cardiovascular and cerebrovascular diseases is

second only to malignant tumors. There are also data showing that the age of onset is tending to be younger (13). CHD is the most common disease in cardiovascular and cerebrovascular diseases. CHD is caused by atherosclerosis of the coronary arteries causing obstruction or stenosis of the coronary arteries, which leads to myocardial ischemia and hypoxia. It is common in clinical, if the treatment is not timely, it may lead to death or disability of the patient or cause serious effects on the quality of life (14).

On the occurrence and development process of CHD, there are various opinions from various angles, including thrombosis, cloning of smooth muscle cells, lipid infiltration, and endothelial injury response (15). At present, most scholars still believe that the endothelium injury response is the main cause of CHD, and the theory of endothelial injury response is that the coronary endothelium or endometrial injury causes inflammation forming atherosclerosis (16).

Notch/NF-κB signaling pathway has been fully studied as a classical signaling pathway. Compared with non-classical pathways, the crosstalk between Notch/NF-κB signaling pathways is relatively simple (17), and studies have shown (18) that Notch signaling pathway is linked to various signals, but their crosstalk relationship is more complex and requires further studies. In the past decade, a large number of studies have shown that (19) Notch and NF-κB are interrelated in the life cycle and the cancer process. Notch signaling pathway is composed of Notch receptor and Notch ligand and CSL (CBF1/RBP-J/SU(H)/Lag-1). It mainly exists in mammals in four forms (Notch1-4) (20). Studies have shown that (21) Notch-1 regulates the development and function of inflammatory cells, and acts on T cells in the thymus to mediate its activation, proliferation, and secretion of cytokines. NF-κB signaling is an important signaling pathway for multicellular animals to determine cell fate. As an important transcription factor, NF-κB is widely present in eukaryotic cells. After activation by multiple stimuli, NF-κB transcription factors directly regulate target genes, among which NF-κB plays an important role in immune and inflammatory responses. NF-κB is also a very important inflammatory factor, and may be responsible for regulating a large number of inflammatory factors (IL-1, IL-2, TNF-α) and death-related factors (Bcl-2, Bax, Fas) (22), and NF-κB-p65 is one of the important subgroups of the NF-κB pathway, and its expression changes can well reflect the conditions of the pathway (23).

In this study, we successfully established a rat model of CHD and detected the expression of Notch-1 and NF-κB-p65 protein in rat cardiomyocytes. The results showed that the Notch-1 and NF-κB-p65 protein expression in the observation group 1 day after successful modeling was significantly higher than that of the control group, and the expression was significantly higher at the subsequent time. However, the expression of Notch-1 and NF-κB-p65 protein was found to be different on day 7 after the modeling success compared to day 1 and day 3. This may suggest that the Notch and NF-κB pathways were activated and involved in the development of CHD one week after rat modelling. In the study of Jin *et al* (24), the expression of Notch-1 receptor protein and its soluble intracellular fragment NICD1 protein in the rat model of myocardial infarction was significantly increased, and the downstream target gene Hes1 was found to increase. In addition, the NF-κB pathway in the myocardial infarction group was activated in the first week, which was significantly higher than that in the unmodeled control group. Myocardial infarction is the basis of CHD, a variety of incentives lead to coronary atherosclerotic plaque rupture, causing platelet aggregation in the rupture and the formation of thrombosis, which is the main cause of death in patients with CHD. The above literature and the results of this study suggest that Notch-1 and NF-κB-p65 signaling pathway may be involved in the development of CHD. We also examined the correlation between the two proteins and whether they differed in sex and age. We found that the expression of Notch-1 and NF-κB-p65 protein was positively correlated. In the study of Li *et al* (25), increasing the activity of Notch-1 can upregulate the expression of NF-κB target genes, and we have better proved the relationship between them by Pearson correlation test. Moreover, we analyzed the expression of Notch-1 and NF-κB-p65 proteins with sex, and age, but we have few specimens, which cannot explain its accuracy. Studies have shown that (26) the inhibition of Notch-1 signaling pathway by pcDNA3.1-Myc-His plasmid and RNA interference can inhibit the expression of Bcl-2 and Bax proteins and inhibit cardiomyocyte apoptosis, and our study found that there was no difference in apoptosis of murine cardiomyocytes between the two groups, which may be due to the fact that we did not modulate them resulting in insignificant differences. Through the above we initially demonstrated the relationship between Notch-1 and NF-κB signaling pathway in the development of CHD, but failed to further detect its target genes, and we have fewer samples in this study, which cannot explain the accuracy of the results. We hope to increase our testing projects in future research, conduct more in-depth research, and obtain more accurate results to support our experimental conclusions.

In conclusion, the expression levels of Notch-1 and NF-κB proteins were increased in CHD rat cardiomyocytes, and Notch-1 was positively correlated with NF-κB protein expression, they are involved in the development of CHD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and SZ were responsible for animal model construction. JZ, SZ and TW were mainly devoted to western blot analysis and TUNEL assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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