

## Original Article

# Expression of ryanodine receptor 3 during the atherosclerotic plaque formation of apolipoprotein E gene-deficient (ApoE<sup>-/-</sup>) mice

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**Abstract:** Background: To analyze the expression of ryanodine receptor 3 (RYR3) during the atherosclerotic plaque formation of apolipoprotein E knockout (ApoE<sup>-/-</sup>) mice. Methods: Male ApoE<sup>-/-</sup> and wild-type C57BL/6J mice aged 7-8 weeks old were selected, fed high-fat diet for 20, 27 and 33 weeks respectively and killed. Serial aortic sections were prepared and subjected to HE and Masson staining to determine the percentage of plaque area to lumen area and the percentage of collagen area to plaque area. Lipid content in the plaque was detected by oil red O staining. The percentage of smooth muscle cells with positive RYR3 expression was detected by immunohistochemical staining. Dynamic expression of RYR3 in the formation of vulnerable plaque was analyzed with Western blot. Results: Compared with C57BL/6J mice at the same age, aortic RYR3 expression increased in ApoE<sup>-/-</sup> mice dynamically as they grew older (P<0.05). Conclusion: RYR3 was involved in the pathological process of atherosclerotic plaque formation, during which its expression was up-regulated.

**Keywords:** RYR3, atherosclerotic plaque, apolipoprotein E, knockout

## Introduction

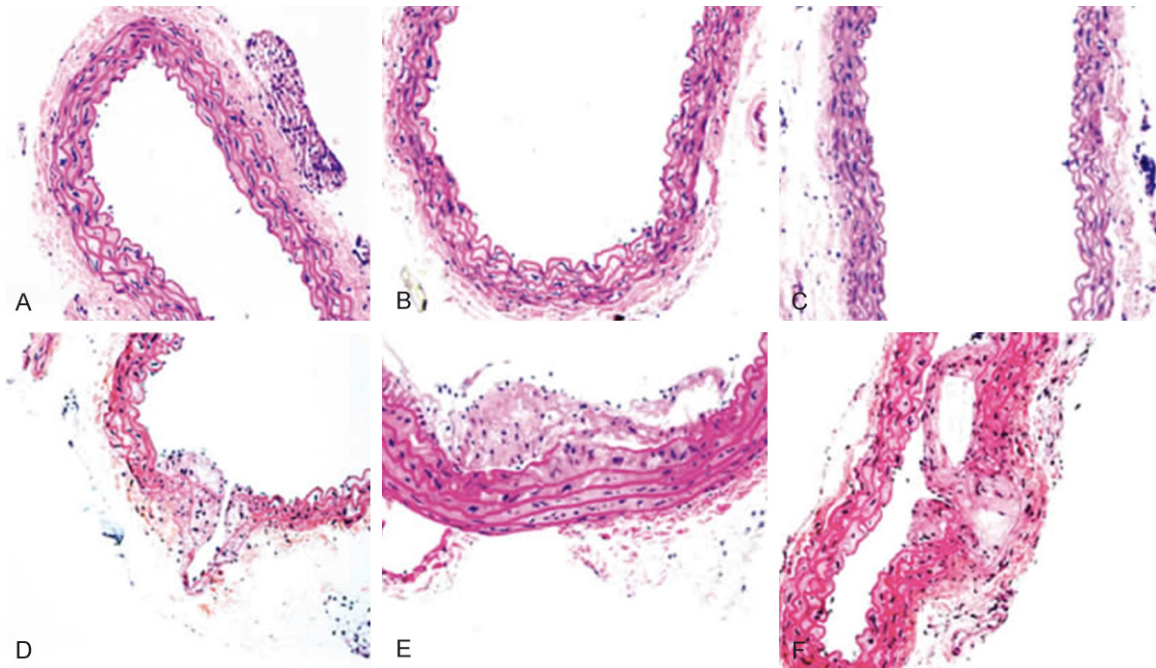
Atherosclerosis, as an initiating factor of coronary heart disease, results from multiple risk factors based on vascular endothelial injury, mainly including vascular remodeling and formation of vulnerable plaques. Proliferation and apoptosis of vascular smooth muscle cells (VSMCs) predominantly control the onset and progression of atherosclerotic plaques. Calcium ions participate in cell proliferation and apoptosis, and maintenance of calcium homeostasis is regulated by ion pump. Opening and activation of cell ion channels are closely associated with atherosclerotic vascular disease [1, 2]. Prior to atherosclerotic plaque formation, baseline Ca<sup>2+</sup> level increases in the VSMCs of apolipoprotein E gene-deficient (ApoE<sup>-/-</sup>) mice [3], accompanied by obviously increased content of inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) and Ca<sup>2+</sup> release. However, the content of ryanodine receptor (RYR) barely changes. Meanwhile,

changes in the dynamic expression of this protein during atherosclerotic plaque formation and the relationship between them remain unclear. In this study, we established an apolipoprotein E gene-deficient (ApoE<sup>-/-</sup>) mouse model of atherosclerosis to observe the RYR3 expression change in aortic VSMCs, and to clarify its relationship with atherosclerotic plaque.

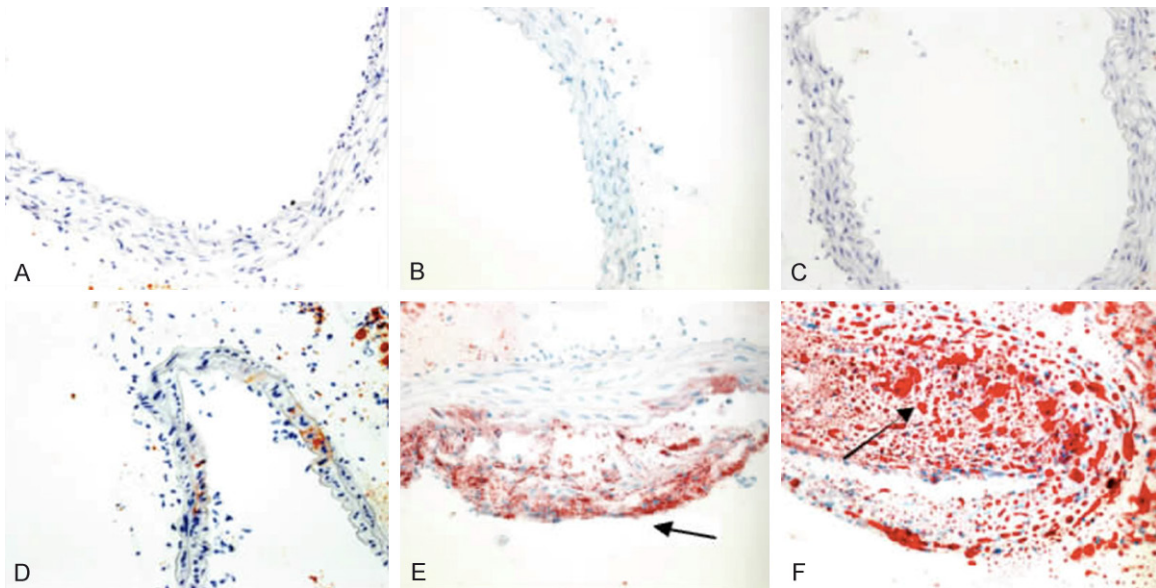
## Materials and methods

### Animals and reagents

Forty-five male ApoE<sup>-/-</sup> (Changzhou Cavens Lab Animal Co., Ltd.) and another 45 wild-type C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.) aged 7-8 weeks old weighing 19-21 g were used. Under Grade 2 feeding condition, they were kept at 22-24°C and humidity of 50% in a 12 h light/12 h dark cycle. RYR3 primary antibody was purchased from Abcom. β-Actin primary antibody, two-step



**Figure 1.** Aortic pathological changes (HE staining,  $\times 200$ ). A. 20-week-old control group; B. 27-week-old control group; C. 33-week-old control group; D. 20-week-old model group; E. 27-week-old model group; F. 33-week-old model group.

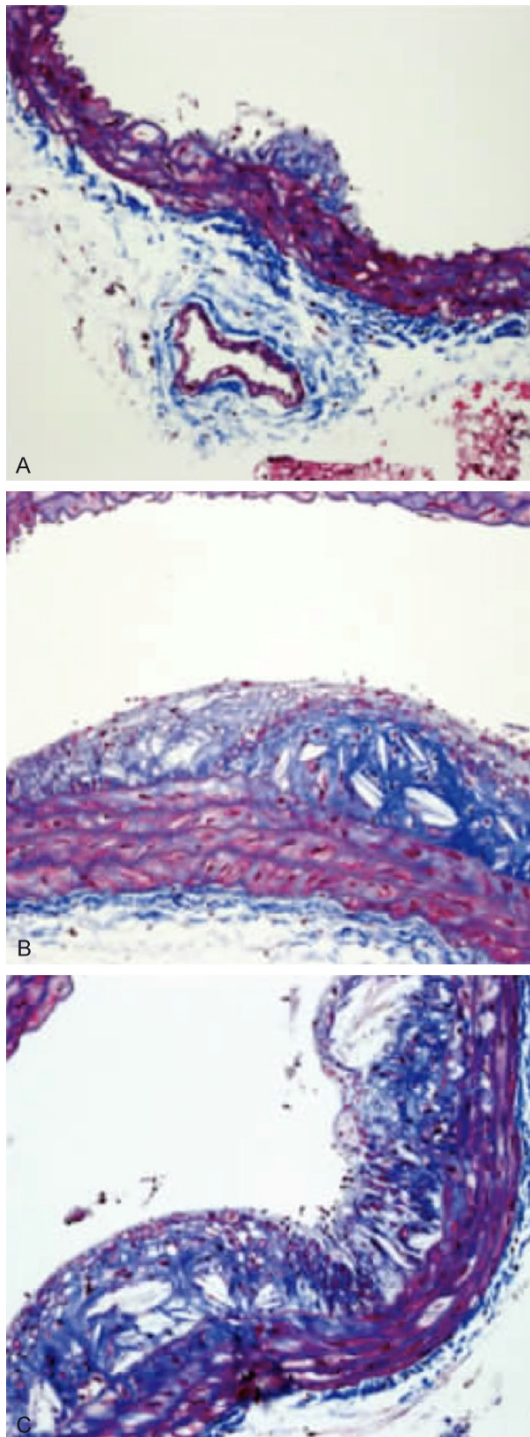


**Figure 2.** Oil red O staining results ( $\times 200$ ). A. 20-week-old control group; B. 27-week-old control group; C. 33-week-old control group; D. 20-week-old model group; E. 27-week-old model group; F. 33-week-old model group.

immunohistochemical detection kit (goat anti-rabbit IgG antibody-HRP polymer) and concentrated DAB color development kit were bought from Life Technologies (USA).

## Animal modeling and grouping

The ApoE<sup>-/-</sup> and wild-type mice were used as model and control groups respectively, and fed



**Figure 3.** Masson staining results (×200). A: 20-week-old model group; B: 27-week-old model group; C: 33-week-old model group.

high-fat diet (basal feed: 78.85%, fat: 21%, cholesterol: 0.15%) until they grew to 20, 27 and 33 weeks old. Random 15 mice from each group were killed at indicated time point, with water deprived in the last night.

## *Morphological detection of pathological tissues*

The heart was infused with heparin/normal saline, and the aortic root was taken out under sterile conditions, fixed in 10% formaldehyde, routinely paraffin-embedded and serially sectioned on the cross section. According to the method of Suzuki et al. [4], four same sections of the aortic root from each mouse: 1) circular-shaped, nearest proximal cross section of the ascending aorta; 2) aortic valve-attachment site, with coronary ostium; 3) starting cross section of the aortic valve; 4) complete exposure and convergence of the aortic valve. Ratio of plaque area/lumen area was determined by HE staining, and that of collagen area/plaque area was detected by Masson staining.

The heart and aorta were rinsed with heparin/normal saline, fixed in 4% paraformaldehyde, dehydrated in 20% sucrose solution, embedded with OCT, and snap-frozen in liquid nitrogen. The aortic root was prepared into serial frozen sections and subjected to oil red O staining to analyze the ratio of lipid area/plaque area.

## *Immunohistochemical staining*

Paraffin sections were deparaffinized, shaken and washed with distilled water three times (5 min each time), antigen-repaired with citrate buffer (pH=6.0) by microwave at 92-98°C, cooled naturally, washed with PBS three times, reacted with 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 15 min, and washed three times with PBS (3 min each time). Then the sections were blocked with goat serum for 1 h at room temperature, incubated with 1:500 diluted rabbit anti-mouse RYR3 primary antibody at 4°C for 24 h, washed with PBS three times, incubated with HRP-labeled goat anti-rabbit IgG antibody at room temperature for 30 min, observed under a microscope after dropping DAB chromogenic reagent, and washed three times with PBS (3 min each time). After 3 min of double staining with hematoxylin, the sections were washed with distilled water for 1 min and differentiated for 2 s. After the sections changed back to blue in tap water within 5 min, they were dehydrated, sealed after becoming transparent and observed under the microscope. Known positive sections were used as positive control, and PBS buffer was used as negative control. Qu-



**Table 1.** Aortic pathological changes ( $\bar{x} \pm s$ , n=5)

Group	Age (week)	n	Plaque area/ lumen area	Lipid area/ plaque area	Collagen area/ plaque area
Control	20	5	-	-	-
	27	5	-	-	-
	33	5	-	-	-
Model	20	5	0.097 $\pm$ 0.031	0.155 $\pm$ 0.024	0.572 $\pm$ 0.034
	27	5	0.214 $\pm$ 0.041 <sup>b</sup>	0.259 $\pm$ 0.031 <sup>b</sup>	0.459 $\pm$ 0.071 <sup>a</sup>
	33	5	0.371 $\pm$ 0.071 <sup>b,c</sup>	0.371 $\pm$ 0.051 <sup>b,c</sup>	0.351 $\pm$ 0.033 <sup>b,c</sup>

<sup>a</sup>: P<0.05, <sup>b</sup>: P<0.01, compared with the 20-week-old model group, <sup>c</sup>: P<0.05, compared with the 27-week-old model group.

antitative analysis: Image analysis was performed under the microscope using a Leica Quantimet 5501W computer at  $\times 200$  magnification to determine the area of positive RYR3 expression in VSMCs.

#### Western blot

RIPA protein extraction buffer was pre-cooled and added protease inhibitor (this inhibitor should be added simultaneously to phosphorylate proteins). Before protein extraction, 0.5-1 mL of PMSF stock solution was added (final concentration: 1 mmol/L). The tissues were weighed (weight: volume of lysate =1:9) and put in the globular portion of a 1-2 mL homogenizer, from which the adventitia was gently peeled off with a toothpick. The residual tissues were smashed with a clean ophthalmic scissor, homogenized at 15000 r/min in Fluka electric tissue homogenizer, put on ice, ground for a while after several minutes and placed on ice again. This procedure was repeated several times to make the tissue pieces as small as possible. After 30 min of lysis, the lysate was transferred into a 1.5 mL centrifuge tube by using a pipette and centrifuged at 12000 r/min and 4°C for 5 min, from which the supernatant (i.e. protein) was collected, subpackaged into 0.5 mL centrifuge tubes and stored at -20°C or -70°C.

The protein concentration was detected with the BCA method. Protein was denatured by boiling for 3-5 min, separated by SDS-PAGE, and transferred to a membrane that was rinsed with 1 $\times$ TBST for 1-2 min and blocked in 5% skimmed milk dissolved in 1 $\times$ TBST for 60 min at room temperature on a slow-speed shaker. Subsequently, the membrane was incubated overnight with TBST-diluted (1:1000) primary antibody at 4°C, washed three times with 1 $\times$  TBST on the shaker (5-10 min each time), incu-

bated with TBST-diluted (1:3000) secondary antibody at room temperature for 60 min, and washed five times with TBST (3 min each time). After ECL reaction, the membrane was color-developed and fixed. Optical density was measured with Quantity One software. Expression level of target protein in tissues was represented by ratio of signal intensity of target band to that of internal reference band.

#### Detection of blood lipid levels

Twelve hours after water and food deprivation, the mice were anesthetized, and blood samples collected from the orbital venous plexus and centrifuged at 3000 r/min for 10 min to separate the serum for detection of blood lipid levels. The levels of total cholesterol (TC) and triglyceride (TG) were determined by using the oxidase method, and those of high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL) were detected with the direct method.

#### Statistical analysis

All data were analyzed by SPSS17.0. The categorical data were expressed as  $\bar{x} \pm s$ . Means of mice at different ages within the same group were compared by one-way ANOVA, and pairwise comparisons were performed with the Bonferroni's method. Results of model and control groups at the same time point were compared by using independent samples t-test. P<0.05 was considered statistically significant.

## Results

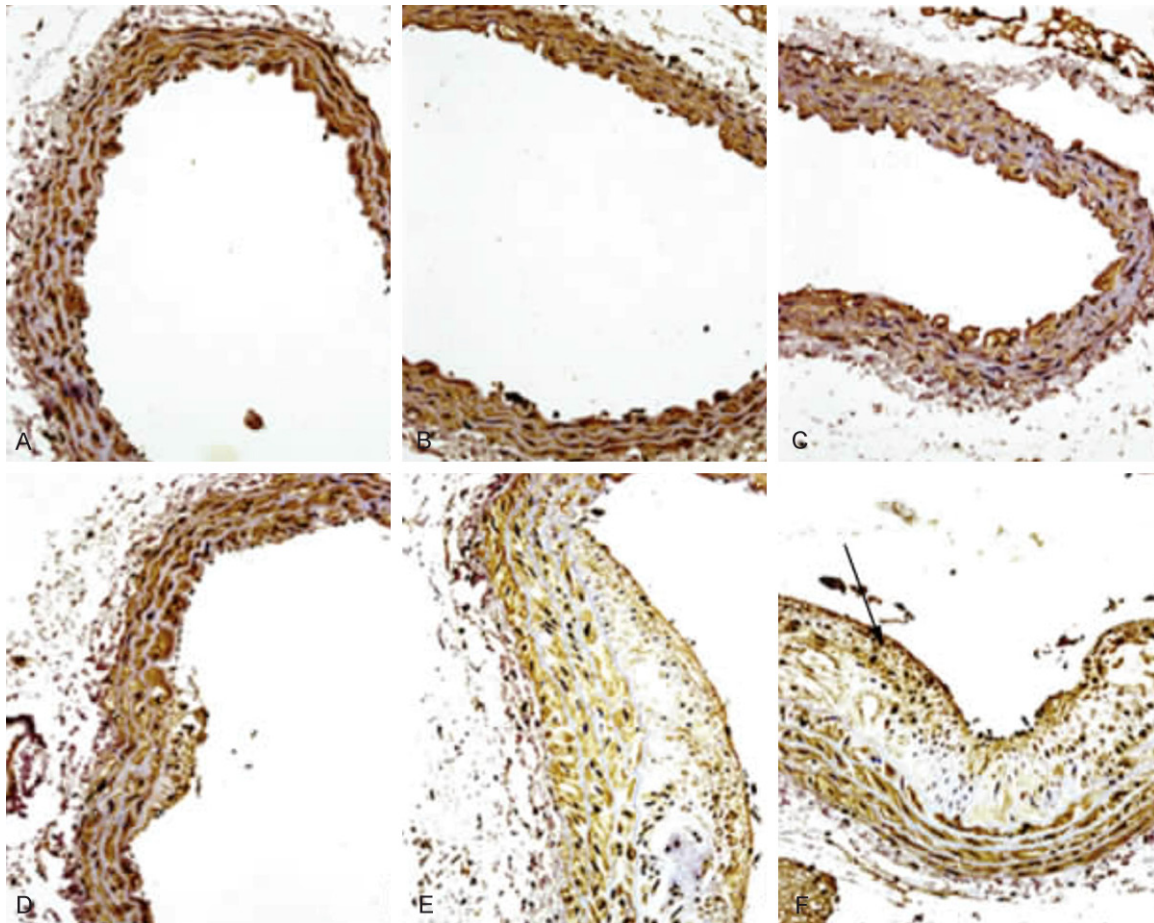
### Pathological and structural changes of aortic atherosclerotic plaques in mice at different ages

ApoE<sup>-/-</sup> mice aged 20 weeks old have already formed fatty plaques, with integral fibrous cap containing small lipid cores and a small number of foam cells. Fibrous cap mainly comprises VSMCs, elastic fibers and considerable collagenous fibers. In the 27-week-old ApoE<sup>-/-</sup> mice, there were atherosclerotic plaques, accumulated foam cells and attenuated, partly ruptured fibrous cap. The 33-week-old ApoE<sup>-/-</sup> mice suffered from obvious progression of athero-

**Table 2.** Blood lipid level changes ( $\bar{x} \pm s$ , n=5)

Blood lipid level	Control			Model		
	20-week-old	27-week-old	33-week-old	20-week-old	27-week-old	33-week-old
TG (mmol/L)	1.42±0.24	1.37±0.37	1.41±0.11	2.22±0.14 <sup>a</sup>	2.42±0.15 <sup>a</sup>	2.65±0.16 <sup>a,b</sup>
TC (mmol/L)	4.41±0.34	4.01±0.22	4.21±0.24	9.71±0.24 <sup>a</sup>	15.91±0.72 <sup>a,b</sup>	20.31±1.12 <sup>a,b</sup>
LDLC (mmol/L)	1.85±0.14	2.12±0.12	2.15±0.22	3.67±0.72 <sup>a</sup>	7.09±0.94 <sup>a,b</sup>	11.12±1.54 <sup>a,b</sup>
HDLC (mmol/L)	1.17±0.31	0.97±0.30	1.15±0.32	1.07±0.20	1.32±0.33	1.27±0.30

<sup>a</sup>: P<0.01, compared with the control group at the same age; <sup>b</sup>: P<0.01, compared with the 20-week-old model group.

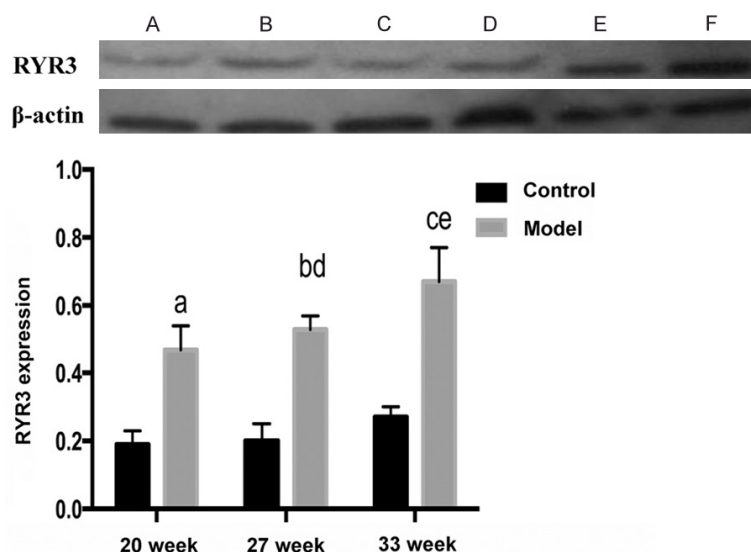


**Figure 4.** RYR3 expressions detected by immunohistochemical staining ( $\times 200$ ). A. 20-week-old control group; B. 27-week-old control group; C. 33-week-old control group; D. 20-week-old model group; E. 27-week-old model group; F. 33-week-old model group. Arrow: Area in which RYR3 was positively expressed.

sclerotic plaques, apoptosis of a large number of VSMCs and disintegration of foam cells. Meanwhile, there were large amounts of amorphous necrotic materials deep inside the ruptured plaques, accompanied by evident luminal stenosis also. In contrast, the control mice at all tested ages were free from plaques, and VSMCs in the middle layer hardly changed (Figures 1-3). Masson staining showed that with increasing age, a large amount of willow-shaped cholesterol crystals formed and fibrous

tissues (blue) gradually decreased in the plaques of ApoE<sup>-/-</sup> mice. In the meantime, the fibrous cap attenuated (Figure 3).

With elapsed time, the ratio of plaque area/lumen area and that of lipid area/plaque area increased, while less extracellular matrix, as the main component of the fibrous cap, was synthesized, and the ratio of collagen area/plaque area also dropped (P<0.05). Particularly, these were vulnerable plaques (Table 1).



**Figure 5.** Aortic RZR3 expressions detected by Western blot. A. 20-week-old control group; B. 27-week-old control group; C. 33-week-old control group; D. 20-week-old model group; E. 27-week-old model group; F. 33-week-old model group. a:  $P < 0.01$ , compared with the 20-week-old control group; b:  $P < 0.01$ , compared with the 27-week-old control group; c:  $P < 0.01$ , compared with the 33-week-old control group; d:  $P < 0.01$ , compared with the 20-week-old model group; e:  $P < 0.05$ , compared with the 27-week-old model group.

#### Blood lipid levels

After feeding with high-fat diet, the model group had significant higher TC, TG and LDLC levels than those of the control group ( $P < 0.01$ ), but the HDLC levels were similar (Table 2).

#### RZR3 protein expression levels

Compared with control mice at the same age, RZR3 expression was significantly different in the atherosclerotic plaques of ApoE<sup>-/-</sup> mice, and such level further rose with increasing age ( $P < 0.05$ , Figures 4 and 5).

#### Discussion

In 1992, ApoE<sup>-/-</sup> mice were independently produced in the laboratories at Rockefeller University (USA) and Leiden University (Netherlands) using gene targeting [5, 6]. The mice have the same predilection sites of atherosclerotic plaques as those of human, and also undergo plaque maturation from fatty streaks to fibrous cap covering, so they are an ideal animal model for studying atherosclerosis.

Acute coronary syndrome mainly results from atherosclerotic plaque instability that is determined by composition and structure but not size [7]. As one of the most important cellular

components participating in atherosclerotic plaque, VSMCs promote the onset and progression of atherosclerosis upon changes. Involved in the entire process of atherosclerotic plaque formation, vascular remodeling is closely associated with the proliferation and apoptosis of VSMCs. Their proliferation mainly drives intimal thickening, while their apoptosis plays a crucial role in plaque vulnerability [8]. Plaque rupture is mainly attributed to decreased synthesis of extracellular matrix in VSMCs and (or) increased degradation by proteolytic enzymes. When the number of apoptotic cells exceeds that of proliferative ones, less collagen is secreted, and plaques are prone to rupture due to attenuation of fibrous cap.

Intracellular calcium ions are involved in cell proliferation and apoptosis, and closely related with vascular remodeling [9]. Increase in the concentration of free  $Ca^{2+}$  is conducive to cell proliferation, but excessive increase and even exhaustion of calcium reservoir in the endoplasmic reticulum can lead to cell apoptosis. Upon mechanical and inflammatory stimuli,  $IP_3R$  in the calcium reservoir of VSMCs is activated, and  $Ca^{2+}$  is released from the endoplasmic reticulum into the cytoplasm. With exhaustion of the intracellular calcium reservoir,  $Ca^{2+}$  channels on the cell membrane were activated, allowing  $Ca^{2+}$  to enter the cytoplasm. This process is referred to as calcium influx [10].

Located in  $Ca^{2+}$ -releasing channels on the sarcoplasmic reticulum, RZR is extremely sensitive to intracellular  $Ca^{2+}$  concentration, i.e. the channels are activated by 1-10  $\mu\text{mol/L}$   $Ca^{2+}$ , whereas such activation is inhibited by  $>10$   $\mu\text{mol/L}$   $Ca^{2+}$  [11]. RZR1, RZR2 and RZR3 are the three subtypes of RZR. RZR exists in almost all types of muscle cells. RZR1 mainly induces skeletal muscle excitation, RZR2 realizes myocardial contraction, and RZR3 is mainly expressed in mouse VSMCs [12]. When  $Ca^{2+}$  enters the cell membrane through L-shaped channels,  $IP_3R$ , RZR3 and RZR2 on the sarcoplasmic reticulum are activated, and  $Ca^{2+}$  flows



to the cytoplasm, also known as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. At certain  $\text{Ca}^{2+}$  concentration, RYSR near the sarcoplasmic reticulum is activated, which further activates  $\text{K}^+$  channels on the cell membrane, triggers the production of spontaneous transient outward currents, regulates membrane potential, induces membrane repolarization, and finally decreases intracellular  $\text{Ca}^{2+}$  [13, 14].

In this study, ApoE<sup>-/-</sup> mice suffered from fatty streaks, early plaque formation, atherosclerotic plaques until fibrous atherosclerotic plaques with increasing age. VSMCs, which proliferated at the beginning of atherosclerotic plaque formation, underwent considerable apoptosis during the formation of vulnerable plaques, accompanied by decreased synthesis and secretion of extracellular collagenous fibers, attenuation of fibrous cap, disintegration of foam cells, accumulation of extracellular lipids and severe lumen stenosis. In contrast, the control mice were free from plaques. RYSR expression gradually increased in ApoE<sup>-/-</sup> mice from 20 to 33 weeks old, which was positively and negatively correlated with plaque area/lumen area and collagen area/plaque area respectively. However, RYSR expression of control mice did not change dynamically, also with significantly lower levels than those of model mice at the same age. Therefore, RYSR functions in the onset and progression of atherosclerotic plaques by participating in vascular remodeling.

Massaelli et al. found in young mice that insufficient oxidized low-LDL induced functional changes of RYSR, and that chronic low level weakened the functions [15, 16]. In this study, the serum cholesterol level of ApoE<sup>-/-</sup> mice was significantly higher than that of control mice, and the levels of TC and LDLC as well as lipid area also rose with increasing age. Hence, abnormal expression of RYSR protein and elevated blood lipid levels simultaneously participated in atherosclerosis and plaque formation. In summary, RYSR is a key molecule involved in the formation of atherosclerotic plaques, and also one of the targets for treating the plaques and acute coronary syndrome. However, the correlation between blood lipid metabolism and RYSR-mediated  $\text{Ca}^{2+}$  signals, and their effects on the formation of atherosclerotic plaques still need in-depth studies. Atherosclerosis can feasibly be delayed by interfering with the above processes.

## Disclosure of conflict of interest

None.

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## References

- [1] Rainbow RD, Macmillan D, McCarron JG. The sarcoplasmic reticulum  $\text{Ca}^{2+}$  store arrangement in vascular smooth muscle. *Cell Calcium* 2009; 46: 313-322.
- [2] Berra-Romani R, Mazzocco-Spezia A, Pulina MV, Golovina VA.  $\text{Ca}^{2+}$  handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. *Am J Physiol Cell Physiol* 2008; 295: C779-790.
- [3] Van Assche T, Franssen P, Gans PJ, Herman AG, Bult H. Altered  $\text{Ca}^{2+}$  handling of smooth muscle cells in aorta of apolipoprotein E-deficient mice before development of atherosclerotic lesions. *Cell Calcium* 2007; 41: 295-302.
- [4] Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y, Kodama T, et al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 1997; 386: 292-6.
- [5] Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Versteuyt JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992; 71: 343-353.
- [6] Nakajima K, Mita T, Osonoi Y, Azuma K, Takasu T, Fujitani Y, Watada H. Effect of Repetitive Glucose Spike and Hypoglycaemia on Atherosclerosis and Death Rate in Apo E-Deficient Mice. *Int J Endocrinol* 2015; 2015: 406394.
- [7] Zhang J, Zhou LS, Shi H, Liang Y. [Progress of the Vulnerable Plaques's Components in Imaging Research]. *Chinese Journal of Arteriosclerosis* 2013; 03: 285-288.
- [8] Tucka J, Yu H, Gray K, Figg N, Maguire J, Lam B, Bennett M, Littlewood T. Akt1 regulates vascular smooth muscle cell apoptosis through FoxO3a and Apaf1 and protects against arterial remodeling and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2014; 34: 2421-2428.
- [9] Feng LX, Ge CJ. [Role of intracellular calcium ion transport protein in the formation of vulnerable atherosclerotic plaques]. *Journal of Cardiovascular and Pulmonary Diseases* 2012; 05: 636-638.

- [10] Cheng KT, Leung YK, Shen B, Kwok YC, Wong CO, Kwan HY, Man YB, Ma X, Huang Y, Yao X. CNGA2 channels mediate adenosine-induced  $\text{Ca}^{2+}$  influx in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 2008; 28: 913-918.
- [11] Rizzuto R, Marchi S, Bonora M, Aguiari P, Bononi A, De Stefani D, Giorgi C, Leo S, Rimessi A, Siviero R, Zecchini E, Pinton P.  $\text{Ca}^{2+}$  transfer from the ER to mitochondria: when, how and why. *Biochim Biophys Acta* 2009; 1787: 1342-1351.
- [12] Jaggar JH, Porter VA, Lederer WJ, Nelson MT. Calcium sparks in smooth muscle. *Am J Physiol Cell Physiol* 2000; 278: C235-256.
- [13] Chiang DY, Kongchan N, Beavers DL, Alsina KM, Voigt N, Neilson JR, Jakob H, Martin JF, Dobrev D, Wehrens XH, Li N. Loss of microRNA-106b-25 cluster promotes atrial fibrillation by enhancing ryanodine receptor type-2 expression and calcium release. *Circ Arrhythm Electrophysiol* 2014; 7: 1214-1222.
- [14] Lohn M, Jessner W, Furstenau M, Wellner M, Sorrentino V, Haller H, Luft FC, Gollasch M. Regulation of calcium sparks and spontaneous transient outward currents by RyR3 in arterial vascular smooth muscle cells. *Circ Res* 2001; 89: 1051-1057.
- [15] Massaeli H, Austria JA, Pierce GN. Lesions in ryanodine channels in smooth muscle cells exposed to oxidized low density lipoprotein. *Arterioscler Thromb Vasc Biol* 2000; 20: 328-334.
- [16] Massaeli H, Austria JA, Pierce GN. Overexpression of SERCA2 Atpase in vascular smooth muscle cells treated with oxidized low density lipoprotein. *Mol Cell Biochem* 2000; 207: 137-141.