


The Changing Regulation of Autophagy in Atherosclerosis in ApoE Gene Knockout Mice

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Abstract: Aim To investigate the changes of autophagy in different stages of atherosclerosis (AS) in Apolipoprotein E gene knockout (ApoE^{-/-}) mice. **Methods** 6-weeks-old male ApoE^{-/-} mice (No.=40) were randomly divided into two groups and fed with common adaptability diet for 2 weeks. The mice of *the control group* (No.=10) received a sham operation and the common diet for another 8 weeks. While *the model* mice (No.=30) received a right common carotid artery cannulation and randomly subdivided into three groups (*the 2 weeks, the 4 weeks and the 8 weeks*) and fed with the high fat diet separately for 2 weeks, 4 weeks and 8 weeks. The blood samples obtained from femoral arteries were studied via the biochemical analysis. The right common carotid arteries were split out for histopathological study. Real-time quantitative polymerase chain reaction (qRT-PCR) and the western blot were used to detect the relative expression levels of mRNA and protein about mTOR. **Results** As the operation time prolonged the lipid levels especially TG and LDL_c were time relative increased. The histopathological analysis results showed that there was a small amount of cells infiltrated in the common carotid artery in *the 2 weeks*, although the wall was still unspoiled. The vascular wall in *the 4 weeks* was messy and there was thrombus in the vascular lumen. The thickness of the right common carotid artery in *the 8 weeks* was higher than others and its elastic membranes significantly decreased. The qRT-PCR and western blot detection suggested the mRNA and protein expression of mTOR in *the 4 weeks* was higher than *the 2 weeks* and *the 8 weeks*, and the expression in *the 8 weeks* was also higher than those in the *2 weeks*. **Conclusions** Autophagy was continuously stimulated during AS formation, however, the levels of autophagy will decrease after reaching the peak at a time.

Keywords: Autophagy; mTOR; Atherosclerosis; ApoE^{-/-} mice

Introduction

AS has been one of the most dangerous diseases among the world and it also is the reason for some fatal diseases, such as stroke and coronary heart disease^[1, 2]. The cause of AS is unclearly now, however, there are some theories about it, like the oxidative stress, the

immune and the inflammation^[3-6]. Autophagy is a cellular housekeeping process which can swallow and degrade the damaged proteins and other cellular macromolecular particles^[7, 8]. Autophagy can be regulated by a series of signaling molecules and one of them is the mammalian target of rapamycin (mTOR). If

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Published at: <http://www.ijsciences.com/pub/issue/2016-11/>

DOI: 10.18483/ijSci.1146; Online ISSN: 2305-3925; Print ISSN: 2410-4477



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there are inadequate nutrients or in the presence of mTOR inhibitors (eg.rapamycin), mTOR is not activated and autophagy is induction^[9-11].

According to the published research, autophagy is stimulated after the AS happened. Perrotta I^[12] found autophagosome can be found in all kinds of cells which involved in AS. Ouimet M et al^[13] found that autophagy can regulate cholesterol efflux from macrophage foam cells in vitro experiments. However, We don't know the detailed mechanisms of autophagy in the whole atherosclerosis. On the basis of our previous studies about the animal model of atherosclerosis, we use ApoE^{-/-} mice to establish the AS model to explore the changes of autophagy in different times in common carotid artery AS and speculate the possible roles of autophagy in different stages of AS.

Materials and methods

1.1 Animals

The SPF levels ApoE^{-/-} mice (male) and its high-fat diet (0.25% cholesterol +15% fat) was purchased from Beijing HFK Bioscience. All mice were feed separately (22~25°C)with free drinking water and feeding.

1.2 Reagents

Reverse transcription Kit No.RR047a, Real time fluorescence quantitative reagent kit No.420a, TRIZOL, RIPA lysis solution, 0.45 μm PVDF film was purchased from TAKARA BIOTECHNOLOGY(DALIAN). The qRT-PCR primer (mTOR and GAPDH) was purchased from Sheng Gong in Shanghai. The antibody was purchased from CST Corporation (America). The ECL chemical luminous liquid was purchased in Wuhan Dr. de Biological Engineering Co., Ltd.

1.3 The AS animal model

40 ApoE^{-/-} mice were randomly divided into 2 groups (*the control* with 10 mice and *the model* with 30 mice) after 2 weeks adaptive diet. Mice in *the control* received a sham operation and continue the normal diet for 8 weeks. All mice in *the model* received right

common carotid artery outer silicone tube placement to accelerate the formation of AS animal model and then all mice were divided into 3 groups on average (*the 2 weeks, the 4 weeks and the 8 weeks*) and respectively feed 2 weeks, 4 weeks and 8 weeks with the high-fat diet.

1.4 Tissue pathological section

The common carotid artery isolated from the ApoE^{-/-} mice was washed with physiological saline and then immediately immersed in 30% of formaldehyde Solution. The artery was dehydrated in ascending series of ethanol, cleared in xylene and embedded in paraffin before making paraffin section and the next thing was xylene dewaxing, gradient ethanol hydration, hematoxylin-eosin staining, then gradient alcohol and xylene dehydration and mounting.

1.5 The qRT-PCR

The total RNA was isolated from the common carotid artery using Trizol reagent and the RNA concentration was tested by the spectrophotometer. Next, the quantitative RNA (about 1ug) was used to detect the relative mRNA expression levels of mTOR. Finally, the relative mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ ^[14] method and the mRNA levels of GAPDH were used as an internal control. The producer was pre-denaturation at 95°C for 30s, denaturation at 95°C for 5s and annealing at 60°C for 20s, totally 40 cycles. The mTOR primers was that: forward 5'-GCCCTCACCTCACAAAGACAT-3' and reverse 5'-GCTCTCTCACCCAGCAGAAC-3'. The GAPDH primers was that: forward 5'- TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and reverse 5'- AAA TGA GCC CCA GCC TTC ATG-3'.

1.6 The western blot

Tissue was washed with pre-cooled PBS for 2 times and then lysed in RIPA buffer with protease inhibitors. After centrifugation at 12000g for 10 minutes at 4°C, the protein in supernatant was collected and the protein concentration was quantified by BCA method. Total 20 ug protein was separated by SDS-PAGE (80v for

30min in concentration gel and 120v for 1h in separation gel) and transferred onto PVDF membranes (incubated overnight with primary antibodies). Then, membranes were washed three times with TBST and incubated with secondary antibodies for 1 h at room temperature. Finally, the membranes were washed with TBST for three times again and visualized using an ECL western blotting substrate Kit. The gray value was analyzed by Image J software.

1.7 Data analysis

SPSS 19.0 was used for the statistical analysis, and the single factor variance analysis was used to analyze

statistical differences. $P < 0.05$ was accepted as significant. The data were expressed as the mean \pm sd.

Result

2.1 The detection of blood about ApoE^{-/-} mice

The detection indicated the level of TG, TC, LDL_c in the model was higher than the control ($P < 0.05$) and the level was gradually increased with the extension of the high fat feeding time after the common carotid artery cannula operation. It means the level of TG, TC, LDL_c in the 4 weeks was higher than the 2 weeks ($P < 0.05$) but lower than the 8 weeks ($P < 0.05$).

Table 1. The detection of blood fat levels in ApoE^{-/-} mice (mmol/L, mean \pm sd)

Groups	n	TG	TC	LDL _c
the control	10	1.51 \pm 0.45	10.06 \pm 1.12	1.23 \pm 0.90
the 2 weeks	10	3.77 \pm 1.33*	23.63 \pm 0.80*#	7.84 \pm 1.27*#
the 4 weeks	10	3.47 \pm 1.41*	25.75 \pm 0.72*#	9.42 \pm 1.12*#
the 8 weeks	10	3.79 \pm 1.25*	27.18 \pm 0.54*#	16.96 \pm 4.13*#

* $P < 0.05$, compared with the control, # $P < 0.05$, compared among the model.

2.2 The histopathological changes about the common carotid artery in ApoE^{-/-} mice

The histological specimens with hematoxylin and eosin (HE) staining showed the artery vessel wall in *the control* was thin and the elastic membranes were continuously and arranged closely. The structure of the common carotid artery in *the 2 weeks* was similar to the control but the vessel wall was thickened in somewhere and contained a small number of cells between the elastic membranes. The structure of the common carotid artery in *the 4 weeks* was very messy and was destroyed heavily. The thickness of the artery in *the 8 weeks* was the biggest among the control and infiltrated lots of foam cells and inflammatory cells in the wall.

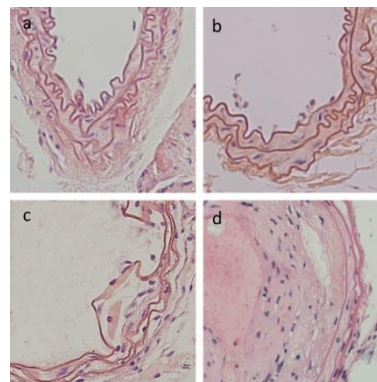


Figure 1. Pathological observation of the common carotid artery with hematoxylin-eosin staining in ApoE^{-/-} mice (HE \times 400)

2.3 The mRNA expression of mTOR

The study of qRT-PCR showed the mRNA expression of mTOR in *the control* was higher than the expression in *the 4 weeks and the 8 weeks* ($P < 0.05$). The mRNA expression of mTOR in *the 8 weeks* was higher than *the 4 weeks* but lower than *the 2 weeks* ($P < 0.05$).

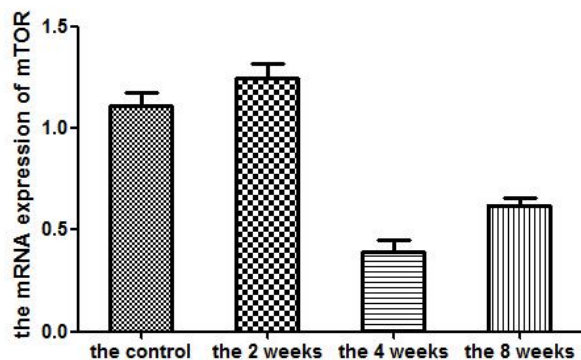


Figure 2. The mRNA expression of mTOR (mean \pm sd, n=10)

2.4 The expression mTOR

The analysis of western blot bands suggested the p-mTOR/mTOR in *the control* was higher than *the 4 weeks* and *the 8 weeks* ($P < 0.05$) and the ratio in *the 8 weeks* was higher than *the 4 weeks* but lower than *the 2 weeks* ($P < 0.05$).

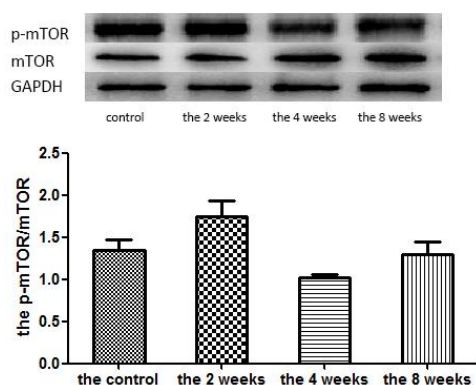


Figure 3. The expression of mTOR (mean \pm sd, n=10)

Discuss

Autophagy is an housekeeping process involved in the degradation of long-lived proteins and excess or dysfunctional organelles. Autophagy usually keeping in the low levels under normal conditions in most cells and it usually regarded as a survival mechanism^[15, 16]. The occurrence of autophagy is guided by a series of signaling pathways and the most classical signaling pathway is a signal system that takes the mammalian target of rapamycin (mTOR) as the center. The mTOR can suppress autophagy by phosphorylating autophagy related gene 13 (ATG13) and decreasing the activity of

ATG1 kinase in normal circumstances. The mTOR protein kinase is the target of the antifungal drug rapamycin so it can induce autophagy by inhibiting the activity of mTOR, dephosphorylating ATG13 and activating ATG1^[5, 7, 17].

Recent studies suggest that autophagy plays an important role in the formation and development of AS. Such as, Verheye et al^[18] found autophagy presents in the macrophages and smooth muscle cells in AS plaques by using scanning electron microscopy and immunohistochemistry methods. However, the specific mechanism of autophagy in AS was unknown and The specific changes of the level of autophagy in AS was unclearly. So in order to explore the specific changes of autophagy in the whole process of AS we established the AS model with 3 time points through ApoE^{-/-} mice.

We found the autophagy levels was rising in the early hours of AS formation through this research and our previous studies. Compared with *the 2 weeks*, the vascular elastic membrane structure of common carotid artery in *the 4 weeks* was destroyed and the number of cells in the blood vessel wall was also increased and the relative expression level of mRNA and protein of autophagy related protein mTOR was both decreased between these two time points. So we thought autophagy is constantly activated after the AS formation to protect the blood vessel from hurting and other studies have also proved this point. Such as, Martinet et al^[19] thought that oxidized low density lipoprotein (ox-LDL), inflammation and metabolic stress can promote the autophagy in the plaque cells and plaque cell autophagy is a cellular device that prevents the external oxidative stress by degrading the damaged cells. However, the levels of autophagy will not always rise but reduce at a time. In this research we found the relative expression level of mRNA and protein of autophagy related protein mTOR in *the 8 weeks* was higher than *the 4 weeks*. De Meyer et al^[20] found that oxidative damage of the lysosomal membrane will lead to the release of lysosomal hydrolases, which causes damage of cytosolic proteins

and organelles and finally induces apoptosis. Li W et al^[21] found the expression of ATG5 in advanced AS was lower than in the early stage and the expression of p62 in the advanced AS was higher than in the early by immunohistochemistry. In conclusion, we suggested autophagy will not always rise in the whole stage of AS and it will reduce at a peak.

The level of autophagy was not the same in the formation and development of AS. Autophagy was continuously activated in the AS formation period and autophagy can eliminate the lipid drops, inflammatory factor and nonfunctional proteins and organelles to protect vessels from damage. With the deepening of the AS, autophagy was affected by many factors, such as apoptosis, the levels of autophagy and its protective effect was continuously reduced. This research indicated the changing regulation of autophagy in the whole stages of AS and it not only gives a reference for the next research about autophagy and AS but provide a new idea and method for the treatment of AS.

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