Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;49:1249-1257
DOI: 10.1159/000493332
Published online: 7 September, 2018
Accepted: 29 August, 2018

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Original Paper

Activation of M_3 Muscarinic Acetylcholine Receptors Delayed Cardiac Aging by Inhibiting the Caspase-1/IL-1β Signaling Pathway

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Key Words
M_3 muscarinic acetylcholine receptor • Cardiac aging • Caspase-1 • IL-1β

Abstract
Background/Aims: Because the prevalence of age-related cardiac impairment increases as the human lifespan increases, it is important to combat the effects of aging. Recently, the cardiac M_3 muscarinic acetylcholine receptor (M_3-mAChR) has been demonstrated to play important roles in cardiac development and in the pathogenesis of cardiac diseases. However, the role of M_3-mAChR in aging remains largely unknown. Therefore, the aim of this study was to investigate the involvement of M_3-mAChR in the progression of cardiac aging. ***Methods:*** We established a cardiac aging model in mice through subcutaneous injection with D-galactose at a dose of 100 mg/kg/day for 6 weeks. D-galactose was also used to induce aging in primary cultured neonatal mouse cardiomyocytes. The myocardium from mice was stained with hematoxylin and eosin for histological analysis. The protein expression levels of p53 and p21 were determined using western blotting. The mRNA and protein expression levels of M_3-mAChR, caspase-1, and interleukin (IL)-1β were determined using real-time PCR, immunohistochemical staining, and western blotting. ***Results:*** The expression of M_3-mAChR was down-regulated in the myocardium from aged mice and D-galactose-treated mice, while the expression levels of caspase-1 and its downstream molecule IL-1β were significantly increased. The M_3-mAChR agonist choline reduced the increase in caspase-1 in cardiomyocytes

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induced by D-galactose, which was reversed by the M_3-mAChR antagonist 4-DAMP. Moreover, 4-DAMP promoted D-galactose-induced cardiomyocyte aging, which was attenuated by a caspase-1 inhibitor. ***Conclusion:*** Activation of M_3-mAChR delayed cardiac aging by inhibiting the caspase-1/IL-1β signaling pathway.

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Introduction

According to the World Health Organization, the world's population aged 60 years and older is expected to total 2 billion by 2050, up from 841 million today [1]. Associated with a progressive loss of physiological integrity, aging is one of the greatest known risk factors for most human diseases [2]. Unfortunately, the exact mechanisms underlying the process of aging are still unclear. Recent studies have indicated that inflammation plays an increasingly important role in the rate of aging and age-related diseases [3, 4].

The aging process is accompanied by inflammatory activation and cytokine release, which are responsible for age-related cardiac dysfunction [5]. In particular, NOD-like receptor 3 (NLRP3), a key component of the inflammasome. is an important regulator of age-related inflammation [6]. Also, the NLRP3 inflammasome promotes the activation of caspase-1, which cleaves the inactive precursors of interleukin (IL)-1β and IL-18 and stimulates their secretion [7]. These cytokines provoke inflammatory responses and accelerate the process of aging [8].

The autonomic nervous system regulates heart function through transmitters and modulators binding to cell surface receptors. Among these cell surface receptors, the cardiac M_3 muscarinic acetylcholine receptor (M_3-mAChR) plays important roles in the maintenance of cardiac function and the generation and progression of cardiac diseases [9-11]. Through the activation of a delayed rectifying K^+ current I_{KM3} to modulate cardiac membrane repolarization, M_3-mAChR exerts anti-arrhythmia effects [12, 13]. M_3-mAChR enhances endogenous antioxidant capacity and decreases intracellular Ca^{2+} overload. The up-regulation of M_3-mAChR was also found to alleviate angiotensin II-induced cardiac hypertrophy [14, 15]. These findings indicate that M_3-mAChR contributes to the protection against cardiac injury.

The pathophysiological role of cardiac M_3-mAChR is currently under investigation. In a preliminary experiment, we found that M_3-mAChR expression was down-regulated in the myocardium of aged mice. Therefore, in the present study, we investigated the role of M_3-mAChR in the progression of cardiac aging and explored the underlying mechanisms.

Materials and Methods

Cardiac aging mouse model

Mice were injected subcutaneously with D-galactose at a dose of 100 mg/kg/day or the same volume of solvent for 6 weeks. After treatment, mice were sacrificed and the myocardium excised and used for experiments. The experimental protocols were approved by the Ethics Committee of Harbin Medical University, P. R. China. Use of animals conformed to the US National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996).

Hematoxylin and eosin staining

Mouse myocardial samples were fixed in 4% paraformaldehyde followed by dehydration. The processed samples were embedded in paraffin and cut into 5-μm thick sections using tissue-processing equipment. The sections were deparaffinized and stained with hematoxylin and eosin (HE) for histological analysis.

Immunohistochemical staining

Myocardial samples were fixed with 4% buffered paraformaldehyde, dehydrated, and embedded in paraffin. Sections (5 μm) were deparaffinized, rehydrated, and rinsed in distilled water. Antigen unmasking was carried out by microwave heating in citrate buffer for 20 min. Sections were immunostained with primary antibody against M_3-mAChR (Alomone, Jerusalem, Israel), caspase-1 (Cell Signaling Technology, Danvers, MA), or IL-1β (Cell Signaling Technology) at 4°C overnight. After incubation with the secondary antibody, the sections were stained with diaminobenzidine.

Isolation and culture of neonatal mouse cardiomyocytes

Neonatal mice (1–3 days old) were sterilized by 75% ethanol solution and then sacrificed. Hearts were extracted and transferred immediately into a Petri dish containing cold Dulbecco's modified Eagle medium (DMEM) (HyClone Technologies, Logan, UT). The ventricles were washed and minced into small pieces. The cells were dissociated at 37°C with trypsin-EDTA solution (Beyotime Institute of Biotechnology, Jiangsu, China). Following dissociation, cells were added to an equal volume of DMEM containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and kept at 4°C. After final collection, the resulting mixture was filtered and centrifuged for 3 min at 1500 rpm to obtain a pellet of cells. The cells were resuspended in culture medium and incubated for 1.5 h under a water-saturated atmosphere of 5% CO_2–95% air to allow the attachment of non-cardiomyocytes. The suspended cells were then collected, plated onto a new culture plate, and incubated under the same conditions as described above [16].

Real-time PCR

Total RNA was extracted from cells or tissues using Trizol reagent (Roche, Indianapolis, IN). Then, 500 ng RNA was reverse transcribed to cDNA using Reverse Transcription Master Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed using an ABI 7500 fast system (Applied Biosystems, Carlsbad, CA) with SYBR Green I (Toyobo). The sequences of the primer pairs are as follows. M_3-mAChR: forward 5'-GTCTGGCTTGGGTCATCTCCT-3', reverse 5'-GCTGCTGCTGTGGTCTTGGCT-3'; caspase-1: forward 5'-ACACGTCTTGCCCTCATTATCT-3', reverse 5'-ATAACCTTGGGCTTGTCTTTCA-3'; IL-1β: forward 5'- CCCTGCAGCTGGAGAGTGTGG -3', reverse 5'- TGTGCTCTGCTTGAGAGGTGCT -3'; and GAPDH: forward 5'-ATCACTGCCACCCAGAAGAC3', reverse 5'-TTTCTAGACGGCAGGTCAGG -3'. GAPDH served as an internal control. The relative quantification of gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Western blotting

Total protein was extracted from cells or tissues. The suspension was subjected to 10% acrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose filter. After blocking with 5% (w/v) non-fat milk dissolved in phosphate-buffered saline for 2 h, membranes were incubated at 4°C overnight with primary antibodies against p53 (Cell Signaling Technology), p21 (Cell Signaling Technology), M_3-mAChR (Alomone, Jerusalem, Israel), caspase-1 (Cell Signaling Technology), IL-1β (Cell Signaling Technology), and GAPDH (ZSGB-BIO, Beijing, China), followed by incubation with horseradish peroxidase-labeled goat anti-mouse IgG or anti-rabbit IgG (1:1000) (ZSGB-BIO) for 1 h. The intensities of immunoblot bands were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Data were analyzed with SPSS 13.0 software (SPSS Inc., Chicago, IL). Statistical comparisons between two groups were performed using Student's *t*-test. Statistical comparisons among multiple groups were performed using analysis of variance. A two-tailed *P*-value < 0.05 was considered statistically significant. Data are presented as mean and standard error of the mean.

Results

Expression of M_3-mAChR was down-regulated in the myocardium from aged mice

Several studies have demonstrated that the expression of M_3-mAChR is increased in patients with atrial fibrillation, atrial dilation, ventricular myocardial ischemia, and cardiac hypertrophy [13, 14, 17]. Whether the expression of M_3-mAChR is changed in the process

of aging has not been determined. Therefore, we compared aged mice (18–20 months old) with young mice (8–12 weeks old). HE staining showed the characteristics of the heart from young and aged mice (Fig. 1 A). The expression levels of p53 and p21, markers of aging, were significantly upregulated in the myocardium of aged mice (Fig. 1 B & C). Immunohistochemical staining, real-time PCR, and western blotting confirmed the down-regulation of M_3-mAChR in the myocardium from aged mice compared with young mice at both mRNA and protein levels (Fig. 1 D–F).

Expression levels of caspase-1 and IL-1β were up-regulated in the myocardium from aged mice

The caspase-1/IL-1β pathway is an essential signaling pathway for inflammation. It has been reported that the expression of caspase-1 was significantly increased in the process of

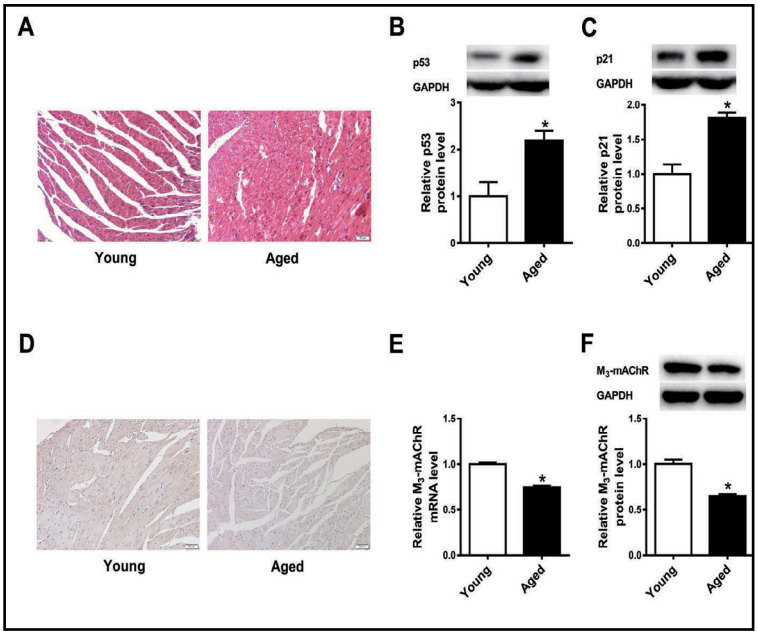

Fig. 1. Expression of M_3-mAChR was down-regulated in the myocardium from aged mice. A. Histopathological characteristics of the heart from young and aged mice. B. Protein expression of p53 in young and aged mice. C. Protein expression of p21 in young and aged mice. D. Immunohistochemical staining of M_3-mAChR in young and aged mice. E. mRNA expression of M_3-mAChR in young and aged mice. F. Protein expression of M_3-mAChR in young and aged mice. GAPDH served as an internal control. * P<0.05 vs. young group; n = 5.

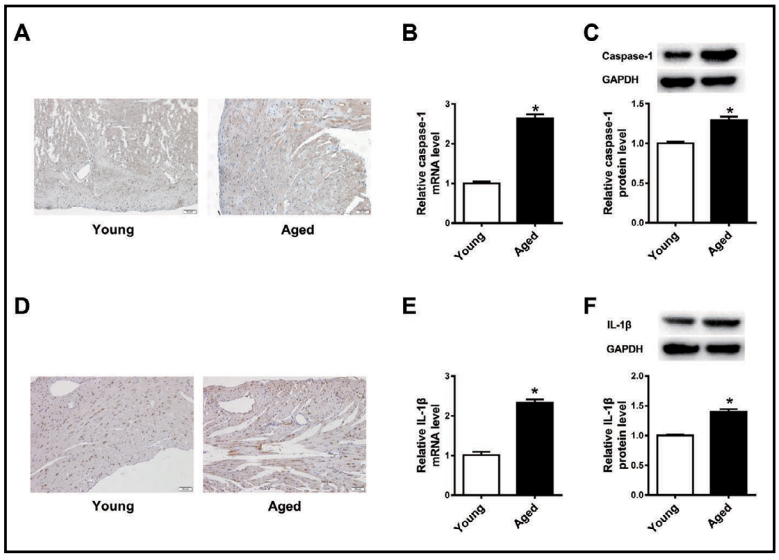

Fig. 2. Expression levels of caspase-1 and IL-1β were up-regulated in the myocardium of aged mice. A. Immunohistochemical staining of caspase-1 in young and aged mice. B. mRNA expression of caspase-1 in young and aged mice. C. Protein expression of caspase-1 in young and aged mice. D. Immunohistochemical staining of IL-1β in young and aged mice. E. mRNA expression of IL-1β in young and aged mice. F. Protein expression of IL-1β in young and aged mice. GAPDH served as an internal control. * P<0.05 vs. young group; n = 5.

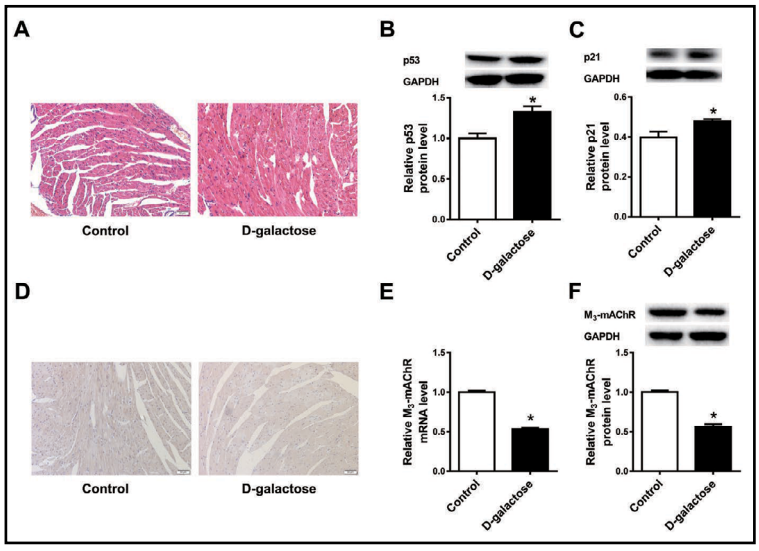

Fig. 3. Expression of M_3-mAChR was down-regulated in the myocardium from D-galactose-treated mice. A. Histopathological characteristics of the heart from control and D-galactose-treated mice. B. Protein expression of p53 in control and D-galactose-treated mice. C. Protein expression of p21 in control and D-galactose-treated mice. D. Immunohistochemical staining of M_3-mAChR in control and D-galactose-treated mice. E. mRNA expression of M_3-mAChR in control and D-galactose-treated mice. F. Protein expression of M_3-mAChR in control and D-galactose-treated mice. GAPDH served as an internal control. * $P<0.05$ vs. control group; n = 5.

aging [18]. A significant increase in caspase-1 immunostaining was found in myocardium from aged mice (Fig. 2 A). To further evaluate the expression of caspase-1, real-time PCR and western blotting experiments were performed. The results showed that the mRNA and protein levels of caspase-1 were up-regulated in aged mice relative to young mice (Fig. 2 B & C). Simultaneously, the expression of IL-1β was also up-regulated (Fig. 2 D–F).

Expression of M_3-mAChR was down-regulated in the myocardium from D-galactose-treated mice

D-galactose was used to establish a mouse model of cardiac aging. After treatment, mice were sacrificed and the cardiac tissues were harvested for subsequent experiments. HE staining showed the characteristics of the heart from control and D-galactose-treated mice (Fig. 3 A). Expression levels of the aging markers p53 and p21 were significantly up-regulated in the myocardium of D-galactose-treated mice (Fig. 3 B & C). The expression of M_3-mAChR was also down-regulated in D-galactose-treated mice (Fig. 3 D–F). These findings suggested the involvement of the decreased expression of M_3-mAChR in the process of cardiac aging.

Expression levels of caspase-1 and IL-1β were up-regulated in the myocardium from D-galactose-treated mice

Immunohistochemical staining, real-time PCR, and western blotting results showed that the expressions of caspase-1 and IL-1β were up-regulated at both mRNA and protein levels (Fig. 4 A–F). These findings suggested that increased expression of caspase-1 and IL-1β has a role in the process of cardiac aging.

Activation of M_3-mAChR reversed the effects of D-galactose on Caspsae-1 and IL-1β in cardiomyocytes

As the expression of M_3-mAChR was down-regulated in aged mice, we considered that M_3-mAChR may have a role in the process of cardiac aging. Choline chloride was used to activate M_3-mAChR, whereas 4-DAMP was used to inhibit M_3-mAChR. Cardiomyocytes were divided into the following four groups: control, D-galactose, D-galactose + choline (M_3-mAChR agonist), and D-galactose + choline + 4-DAMP (M_3-mAChR antagonist) groups. D-galactose up-regulated the expression levels of p53 and p21, which were attenuated by choline. However, 4-DAMP reversed the effect of choline (Fig. 5 A & B). The mRNA and protein levels of caspase-1 and IL-1β were evaluated. The increased expression of caspase-1

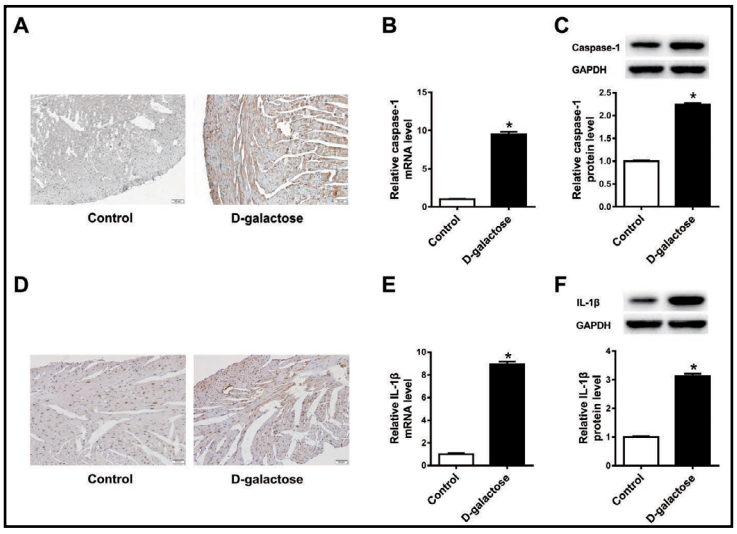

Fig. 4. Expression levels of caspase-1 and IL-1β were up-regulated in the myocardium from D-galactose-treated mice. A. Immunohistochemical staining of caspase-1 in control and D-galactose-treated mice. B. mRNA expression of caspase-1 in control and D-galactose-treated mice. C. Protein expression of caspase-1 in control and D-galactose-treated mice. D. Immunohistochemical staining of IL-1β in control and D-galactose-treated mice. E. mRNA expression of IL-1β in control and D-galactose-treated mice. F. Protein expression of IL-1β in control and D-galactose-treated mice. GAPDH served as an internal control. * P<0.05 vs. control group; n = 5.

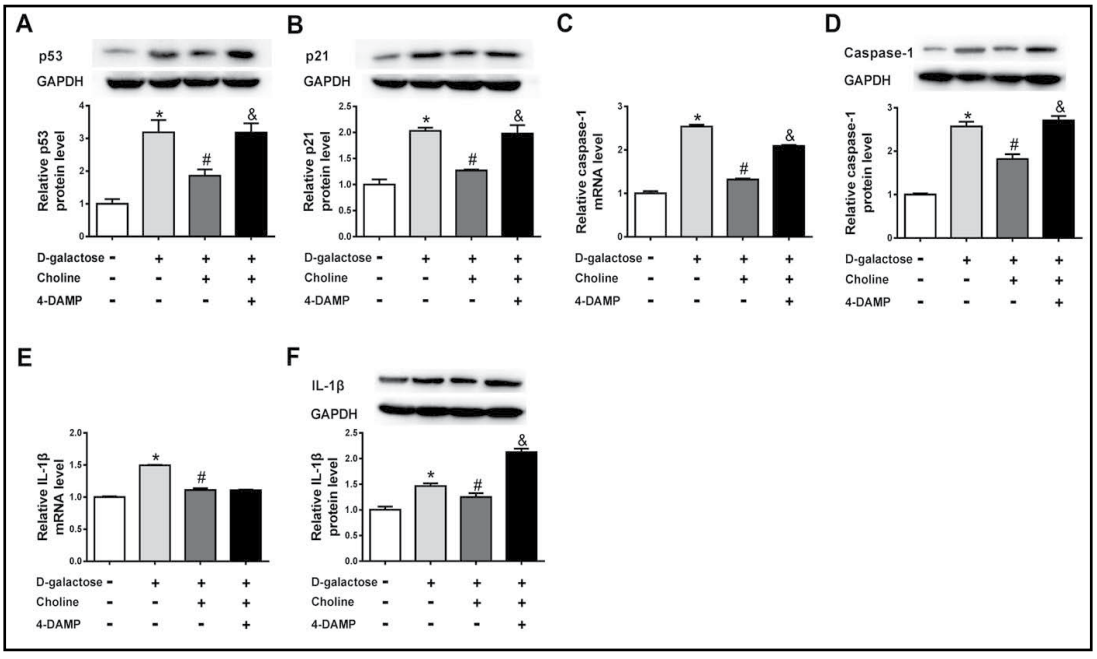

Fig. 5. Activation of M_3-mAChR reversed the effects of D-galactose on Caspsae-1 and IL-1β in cardiomyocytes. A. Protein expression of p53 in cardiomyocytes. B. Protein expression of p21 in cardiomyocytes. C. mRNA expression of caspase-1 in cardiomyocytes. D. Protein expression of caspase-1 in cardiomyocytes. E. mRNA expression of IL-1β in cardiomyocytes. F. Protein expression of IL-1β in cardiomyocytes. GAPDH served as an internal control. * P<0.05 vs. control group; # P<0.05 vs. D-galactose group; & P<0.05 vs. D-galactose + choline group; n = 3.

induced by D-galactose was inhibited by choline, and this was reversed by 4-DAMP (Fig. 5 C & D). The up-regulation of IL-1β, a downstream cytokine of caspase-1, was also effectively reversed by the M_3-mAChR agonist (Fig. 5 E & F). These results suggest that activation of M_3-mAChR can inhibit the caspase-1/IL-1β signaling pathway.

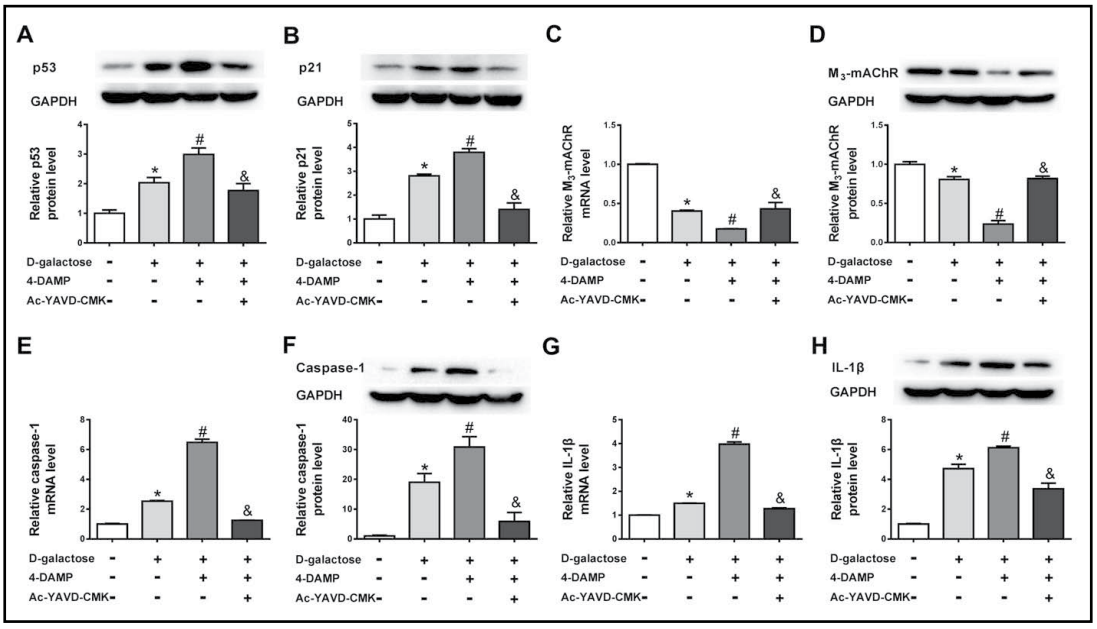

Fig. 6. Inhibition of M_3-mAChR promoted D-galactose-induced cardiomyocyte aging through the caspase-1/IL-1β signaling pathway. A. Protein expression of p53 in cardiomyocytes. B. Protein expression of p21 in cardiomyocytes. C. mRNA expression of M_3-mAChR in cardiomyocytes. D. Protein expression of M_3-mAChR in cardiomyocytes. E. mRNA expression of caspase-1 in cardiomyocytes. F. Protein expression of caspase-1 in cardiomyocytes. G. mRNA expression of IL-1β in cardiomyocytes. H. Protein expression of IL-1β in cardiomyocytes. GAPDH served as an internal control. * P<0.05 vs. control group; # P<0.05 vs. D-galactose group; & P<0.05 vs. D-galactose + 4-DAMP group; n = 3.

Inhibition of M_3-mAChR promoted D-galactose-induced cardiomyocyte aging through the caspase-1/IL-1β signaling pathway

To explore the mechanism underlying the role of M_3-mAChR in cardiomyocyte aging, neonatal mouse cardiomyocytes were divided into four groups: control, D-galactose, D- galactose + 4-DAMP, and D-galactose + 4-DAMP + Ac-YVAD-CMK (caspase-1 inhibitor) groups. The expression levels of p53, p21, caspase-1, and IL-1β were determined. The results showed that 4-DAMP promoted the effect of D-galactose. However, co-administration of D-galactose + 4-DAMP +Ac-YVAD-CMK attenuated the effect of D-galactose and 4-DAMP (Fig. 6). Therefore, inhibition of caspase-1 could reverse the effect of 4-DAMP in D-galactose-induced cardiomyocyte aging. These results suggest that M_3-mAChR acted at least partially through regulation of the caspase-1/IL-1β signaling pathway.

Discussion

As the global population continues to grow older, understanding of aging as a predominant risk factor for all cardiovascular diseases is essential for revealing the molecular pathogenesis of aging. Furthermore, discovering new therapeutic targets is vital in the prevention and treatment of age-related cardiac diseases. The activation of NLRP3 initiates an inflammatory cascade reaction, which occurs in the processes of aging and cardiac injury [19, 20]. During this process, caspase-1 cleaves the precursors, and stimulates the release, of IL-1β and IL-18, which causes an inflammatory reaction and accelerates aging [7]. It has also been reported that the expression of caspase-1 is increased during the process of aging [18, 21]. Moreover, inhibition of caspase-1 protects the heart from acute myocardial ischemic injury and hypertrophy [22].

The aging process is accompanied by a series of changes in the autonomic control of the cardiovascular system. M_3-mAChR plays an important role in cardiac function. Many recent studies have revealed the relationship between M_3-mAChR and cardiac diseases, including cardiac ischemia, pathological cardiac hypertrophy, cardiac arrhythmias, and heart failure [23]. M_3-mAChR was also involved in LPS-induced lung inflammation and fibroblast proliferation by mediating the NF-κB signaling pathway [24, 25]. These findings suggest that the blockade of M_3-mAChR exerts anti-inflammatory properties.

In this study, we found that the expression of M_3-mAChR was down-regulated in the myocardium from aged and D-galactose-treated mice, while the expression levels of caspase-1 and IL-1β were significantly up-regulated. Activation of M_3-mAChR reduced the increased expression of caspase-1 and IL-1β induced by D-galactose in cardiomyocytes. Furthermore, the inhibition of M_3-mAChR promoted the effect of D-galactose on cardiomyocytes. However, the caspase-1 inhibitor Ac-YVAD-CMK attenuated the effect of D-galactose and 4-DAMP.

To our knowledge, this is the first study that explored the expression, function, and molecular mechanism of M_3-mAChR in cardiac aging. Overall, our results suggest that down-regulation of M_3-mAChR occurs during the process of cardiac aging and the activation of M_3-mAChR delays cardiac aging by inhibiting the caspase-1/IL-1β signaling pathway. Therefore, activation of M_3-mAChR may be a new strategy to delay aging.

Acknowledgements

This work was supported by the China Postdoctoral Science Foundation (Grant No. 2013M541413) and National Natural Science Foundation of China (Grant No. 81401151).

Disclosure Statement

The authors declare no conflict of interests.

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