Nuclear factor E2 related factor (NRF2) inhibits mast cell-mediated allergic inflammation via SIRT4-mediated mitochondrial metabolism

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Background: Mast cell (MC)-mediated inflammation is essential for allergic rhinitis, and nuclear factor E2 related factor (NRF2) is found to inhibit inflammation. This study investigated whether NRF2 could inhibit MC inflammation and its molecular mechanisms concerning SIRT4.

Methods: Real-time quantitative PCR (RT-qPCR) and western blot were used to detect gene expression, and Elisa kit was used to detect the content of histamine and inflammatory cytokines in the medium of MCs, and Seahorse XF instrument was used to measure the mitochondrial metabolism of MCs. Knockdown SIRT4 and establish SIRT4 overexpression of HMC-1 cells to study the function of SIRT4.

Results: As an activator of NRF2, 4-Octyl Itaconate increases not only NRF2 expression but also increases SIRT4 expression. Although 4-Octyl Itaconate could reduce the histamine release and degranulation of MCs, which was induced by compound 48/80, SIRT4 knockdown decreased the inhibition of 4-Octyl Itaconate. Similarly, 4-Octyl Itaconate inhibited the secretion of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) by MCs, which was induced by LPS, but SIRT4 knockdown decreases the inhibition of 4-Octyl Itaconate. Also, the up-regulation of SIRT4 significantly inhibited mitochondrial metabolism in MCs and inhibited SIRT1 and P-p65 protein expression after inducing by 100 ng/mL LPS for 1 hour.

Conclusions: NRF2 inhibits MC degranulation and MC-mediated inflammation by promoting SIRT4, and SIRT4 overexpression inhibits the mitochondrial metabolism of MCs.

Keywords: Nuclear factor E2 related factor (NRF2); SIRT4; mast cells (MC); allergic rhinitis; inflammation


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Introduction

Allergic rhinitis is a type I allergy that mediates sensitization of mast cells (MC) by IgE antibodies after exposure to an allergen in atopic individuals (1). Statistics show that the prevalence of allergic rhinitis in the United States is 5–22%, and it costs about $7 billion annually to treat allergic rhinitis (2). In China, the prevalence of allergic rhinitis also shows a significantly increasing trend; about 30 million people yearly (3). Although allergic rhinitis does not threaten the patient’s life safety, a series of rhinitis clinical symptoms including nasal itching, stuffy nose, sneezing, and runny nose can seriously affect the patient’s quality of life and cause personal and social-economic burden (4,5).

MCs are cells derived from CD4+ spinal cord hematopoiesis, which enter the peripheral blood from the bone marrow and eventually migrate to the corresponding
sites to differentiate and mature (6). MCs play an essential role in the pathogenesis of allergic rhinitis, and allergens specifically bind to sensitized MC surface FeRI, triggering degranulation of MCs, releasing inflammatory mediators and cytokines associated with type I allergies, causing vasodilatation, increased vascular permeability, and other inflammatory responses (7,8). From the relationship between MCs and allergic rhinitis, inhibition of histamine and other related inflammatory cytokines by MCs can ease the symptoms of allergic rhinitis to some extent, which is one foundation for the development of drugs to treat allergic rhinitis (9).

Nuclear factor E2 related factor (NRF2) is an important transcription factor in the CNC family, which plays an anti-inflammatory role in regulating inflammatory response(10). Previous studies have found that activation of NRF2 inhibits substance P-induced MC activation and degranulation (11) and that the NRF2/HO-1 pathway is involved in regulating human MC-mediated allergic inflammation (12). However, previous studies only proved that NRF2 was involved in regulating the occurrence of allergic rhinitis and human MC-mediated allergic inflammation. The molecular mechanism of NRF2 in regulating inflammation in allergic rhinitis is still unclear. Whether NRF2 regulates MC-mediated allergic inflammation by regulating mitochondrial metabolism, whether this is related to SIRT4 is also unknown. In this study, we found NRF2 upregulated SIRT4 expression, and upregulated SIRT4 inhibited mitochondrial metabolism and NF-κB signaling pathways, thereby inhibiting human MC-mediated allergic inflammation. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/apm-20-1848).

Methods

Reagents and instruments

4-Octyl Itaconate (HY-112675) was bought from MedChemExpress (USA). Lipopolysaccharide (LPS, 00-4976-03), RPMI-1640 medium (61870044), and Fetal bovine serum (FBS, 10437028) were bought from ThermoFisher SCIENTIFIC (USA). Antibody for NRF2 (ab137750), SIRT4 (ab10140), SIRT1 (ab110304), p65 (ab16502), P-p65 (ab86299), GAPDH (ab181602) and Elisa kit for histamine (ab213975), TNF-α (ab100654), IL-1β (ab46052), IL-6 (ab100712) and IL-8 (ab46032) were all purchased from ABCAM (UK). PrimeScript RT Reagent Kit with gDNA Eraser (RR047B, Takara, Japan) and GoTaq® qPCR Master Mix (A6002, Promega, China). XFe24 extracellular flux assay kits (B35616) were bought from Agilent (USA). Nuclear and Cytoplasmic Protein Extraction Kit were bought from Sangon Biotech (China).

PCR instrument (S-1000, BIO-RAD, China), Fluorescence quantitative polymerase chain (FQD-96A, Hangzhou Bori Technology Co., Ltd., China), Extracellular Flux Analyzer (XFe-24, Agilent, USA), Microscope (DMI3000, Leica, German).

Cell culture and transfection

HMC-1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and were cultured with RPMI-1640 plus 10% FBS in 37 °C and 5% CO₂. Si-SIRT4 was synthesized by Sangon Biotech (China) and was directly transfected into cells with Lipofectamine™ 2000, and the cell could be used for research after si-SIRT4 transfer for 24 hours and verified by PCR and western blot. The SIRT4 overexpressing HMC-1 cell line was set up by Genmeditech and verified by PCR and western blot.

Real-time quantitative PCR (RT-qPCR)

Levels of miRNA/mRNA were detected by RT-qPCR as previously described (13). Reverse transcription program: 37 °C-15 min, 85 °C-5 second, 4 °C-until the end; RT-qPCR parameter settings: 95 °C for 30 seconds, 40 cycles of 90 °C for 5 seconds, and 65 °C for 30 seconds. PCR primer: NRF2-F: 5’-TTCCCCTGTCACATCGGAG-3’, NRF2-R: 5’-TTCTGTTGCATACGGTCTAAATC-3’; SIRT4-F: 5’-GAATCGGGCAATACGACTACA-3’, SIRT4-R: 5’-GCACCAGCTACGAAGTTTCTCG-3’; GAPDH-F: 5’-TAGCCGCTAGCGGTAT-3’, GAPDH-R: 5’-CGGGGCTATGGCTAGCGAG-3’.

Western blot

Protein levels were analyzed by the western blot, as described previously (13). The blocking solution was 5% TBST diluted skim milk powder and blocked at room temperature for 1 hour. The primary antibody [NRF2 (ab137750, 1:1,000), SIRT4 (ab10140, 1:500), SIRT1 (ab110304, 1:1,000), p65 (ab16502, 1:500), P-p65 (ab86299, 1:500), GAPDH (ab181602, 1:3,000)] was diluted with blocking solution and incubated for 2 hours at room temperature, and the secondary antibody was diluted with blocking solution.
and incubated for 1 hour at room temperature. ImageJ (NIH, USA) was used to analyze the gray value of the protein band, and GAPDH normalized the protein.

**Toluidine blue staining**
HMC-1 cells (2×10⁶) were inoculated into a 6-well plate. After drug treatment, the medium was removed, washed three times with PBS, and 1 ml of toluidine blue solution was added to each well. After staining for 30 seconds, the toluidine blue solution was discarded. The color separation was conducted for 10 seconds with a 95% ethanol solution, and then the ethanol solution was discarded and washed once with PBS. Remove the coverslip onto the slide, then observe and take a picture with a microscope.

**Mitochondrial metabolism**
Mitochondrial respiratory metabolism of MCs is assessed by Seahorse XF instruments, as previously described (14). Briefly, oxygen consumption rate (OCR) and spare respiratory capacity (SRC) were measured using XFe-24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA, USA) and XFe24 extracellular flux assay kits (B35616, Agilent, Santa Clara, CA, USA) to test mitochondrial oxidative phosphorylation reaction according to the instructions. The final concentration of Oligomycin, FCCP, and Rotenone/ Antimycin A was 1.0, 0.5, and 0.5 μM, respectively. After the experiment was completed, collected the total protein extracted from each well and determined the concentration. We normalize the OCR/SCR values with protein concentration. In this study, wild-type and SRT4 overexpressing HMC-1 were collected after 100 ng/mL LPS stimulation for 1 hour to assess mitochondrial metabolism.

**Statistical analysis**
SPSS20.0 analyzed the data in its present study. Multiple groups were compared with the one-way ANOVA and the Duncan test as post hoc tests. And P<0.05 means a significant difference.

**Results**

**NRF2 activator, 4-Octyl Iaconate upregulated SIRT4 expression**
4-Octyl Iaconate was the activator of NRF2 (15) and was added to the medium of MCs to upregulate NRF2 expression. As shown in Figure 1A, 4-Octyl Itaconate was found to be not significantly cytotoxic to MCs when the dose less than 200 μM, but 250 μM 4-Octyl Itaconate was significantly toxic to MCs, so we chose 200 μM as the working concentration in our study. Four hours after adding 4-Octyl Iaconate to the medium of MCs, we collected the cells and detected gene expression. We found (Figure 1B,C) as an NRF2 activator, 4-Octyl Iaconate could promote NRF2 mRNA and protein expression. 4-Octyl Itaconate also promotes SIRT4 gene expression.

**SIRT4 inhibits histamine release and degranulation of MCs**
Compound 48/80 promotes histamine release and degranulation in MCs, and inhibition of histamine release and degranulation from MCs is essential to treat allergic rhinitis. As shown in Figure 2A, 4-Octyl Iaconate could significantly upregulate SIRT4 expression in MCs with treatment by 48/80, and si-SIRT4 reduced SIRT4 protein expression with treatment by 48/80. Also, 4-Octyl Iaconate could significantly decrease the release of histamine (Figure 2B) and degranulation from MCs after inducing by 48/80 (Figure 2C), but si-SIRT4 increased them.

**SIRT4 inhibits LPS-induced inflammation in MCs**
TNF-α, IL-1β, IL-6, and IL-8 are common inflammatory cytokines and are essential for allergic rhinitis. We detect the content of TNF-α, IL-1β, IL-6 and IL-8 in the medium of MCs after inducing by LPS, and found that (Figure 3) 4-Octyl Iaconate could significantly decrease the content of TNF-α, IL-1β, IL-6 and IL-8 in the medium of MCs after inducing by LPS, but si-SIRT4 increased them.

**SIRT4 inhibits mitochondrial respiration and NF-κB pathway in MCs**
Seahorse XF instrument was used to measure the mitochondrial metabolism of MCs, and we found (Figure 4A) upregulated SIRT4 could significantly decrease basal OCR and SRC of MCs, it showed that upregulated SIRT4 inhibited the mitochondrial metabolism of MCs. Upregulated SIRT4 decreased SIRT1 protein expression and P-p65 protein expression after inducing by 100 ng/mL LPS for one hour (Figure 4B,C).
Discussion

NRF2 is a crucial transcription factor in the CNC family, is located at the 2q31 locus, and it has six different functional regions, named Neh1-Neh6, which are widely present in various tissues and organs of the body and interact with antioxidant elements ARE to activate downstream gene transcription further to regulate antioxidant and anti-inflammatory proteins (16). Previous studies have found that exerts anti-inflammatory function in vitro and in vivo models (17,18), such as NRF2 activated AMP-activated protein kinase (AMPK) can inhibit the expression of LPS-induced inflammatory genes by promoting nuclear transfer and phosphorylation of NRF2, thereby improving the survival rate of LPS-induced mice (18). In addition, Lee et al. found that 3,4,5-Trihydroxycinnamic acid inhibits lipopolysaccharide (LPS)-induced inflammation by Nrf2 activation in vitro and improves survival of mice in LPS-induced endotoxemia model in vivo (19). And Qi et al. found that isorhamnetin could decrease the expression of PGE2, NO, IL-6 and IL-8 in human gingival fibroblasts, but NRF2 knockdown reversed the anti-inflammatory effects of isorhamnetin, which indicated that NRF2 was necessary for isorhamnetin to inhibit inflammation in human gingival fibroblasts (20).

In this study, we found 4-Octyl Itaconate (15), a specific activator of NRF2, upregulated not only NRF2 in MCs but also upregulated SIRT4. SIRT4 is an essential member of the Sirtuin family of proteins localized to mammalian cell
Figure 2 Effect of SIRT4 on histamine release and degranulation in mast cells. (A) SIRT4 protein expression in mast cells with different treatments. (B) The content of histamine in the medium of mast cells with different treatments. (C) Toluidine blue staining was used to observe mast cell degranulation, and (a) (WT) was an HMC-1 cell without any treatment (100×); (b) (48/80) was 10 μg/mL compound 48/80 was added to HMC-1 cell culture medium for 2 hours; (c) (4-OC) was 10 μg/mL compound 48/80 was added to HMC-1 cell culture medium for 2 hours after adding 200 μM 4-Octyl Itaconate for 4 hours; (d) Si-SIRT4 was 10 μg/mL, and the compound 48/80 was added to the HMC-1 cell culture medium for 2 hours after transferring to si-SIRT4 for 24 hours. Three independent repetitions per experiment. *, P<0.05 and ***, P<0.001 vs. WT group; #, P<0.05; ##, P<0.01; ###, P<0.001 vs. 48/80 groups; $$, P<0.01; $$$, P<0.001 vs. 4-OC group.

Previous studies have shown that SIRT1, SIRT3, and SIRT6, members of the Sirtuin family, are being found to be involved in regulating inflammatory responses by sensing the NAD/NADH energy axis (14,21). Although there are few studies on regulating SIRT4 and inflammation, SIRT4 can inhibit amino acids, fatty acid catabolism, and promote fatty acid anabolism by catalyzing the deacetylation, de-glutamylation, ADP ribosylation, lipidation or regulation of substrate expression of substrates, thereby regulating the biological effects of glucose tolerance and insulin antagonism (22). Therefore, we hypothesized that NRF2 might regulate the cellular energy supply by upregulating SIRT4 and then taking part in regulating the inflammatory response.

MC degranulation refers to a functional state of MCs. Their high-affinity FcεR1 binding sensitizes MCs to allergen-specific IgE. When the same allergen reappears and crosslinks cell surface IgE, FcεR1 activates MCs via signal transduction and releases intragranular active medium in the granules to induce type I hypersensitivity (23,24). Previous studies have shown that (25,26) allergens act on MCs to activate and release the active mediator is the direct cause of allergic/allergic reactions, while compound 48/80 is a tool for degranulation of MCs. We found NRF2 inhibited compound 48/80-induced MC degranulation and histamine release, but this inhibitory effect was significantly
Figure 3 Effect of SIRT4 on the expression of inflammatory cytokines induced by LPS in mast cells. (A,B,C,D) The content of TNF-α (A), IL-1β (B), IL-6 (C), and IL-8 (D). Three independent repetitions per experiment. *, P<0.05; **, P<0.01 and ***, P<0.001 vs. WT group; ###, P<0.01; ####, P<0.001 vs. LPS group; $$$, P<0.01, $$$$, P<0.001 vs. 4-OC group.

Figure 4 Effects of SIRT4 expression on mitochondrial metabolism and NF-κB pathway in mast cells. (A) Seahorse XF instrument was used to measure the mitochondrial metabolism in different HMC-1 cells after inducing by 100 ng/mL LPS for 1 hour. (B) SIRT4, SIRT1, p65, and P-p65 protein were detected in HMC-1 cells by western blot after inducing by 100 ng/mL LPS for 1 hour. Three independent repetitions per experiment. ****, P<0.001 vs. WT group.
reduced in SIRT4 knockdown MCs. These findings showed that SIRT4 functioned as a downstream protein of NRF2 and was involved in MC degranulation by regulating NRF2.

LPS is a significant component in the cell wall of Gram-negative bacteria and can induce inflammation in the body tissues and is one of the primary pathogens of inflammatory diseases in the body. And previous studies have shown that lipopolysaccharide-induced inflammatory responses in cells can establish in vitro cellular inflammatory models (27,28). In this study, we used LPS to stimulate MCs to produce an inflammatory response to set up an in vitro cellular inflammatory model. We found NRF2 inhibited LPS-induced MC-mediated allergic inflammation, but this inhibition was significantly reduced in SIRT4 knockdown MCs. These findings suggested that SIRT4 functioned as a downstream protein of NRF2 involved in regulating MC-mediated allergic inflammation by NRF2. Previous studies have shown that many intermediate proteins and cellular inflammatory factors need to be synthesized during the inflammatory process, which required an abundant supply of energy (29,30), and SIRT1, SIRT3, and SIRT6 be involved in regulating inflammation by regulating cellular mitochondrial metabolism (14,21). And SIRT4 was found mainly regulated glutamine metabolism in mitochondria, and glutamine was the primary source of the supplemental tricarboxylic acid cycle which as a raw material for α-ketoglutaric (31), so SIRT4 was an “engine” that provided energy support for cells to perform various biological functions (32,33). Therefore, SIRT4, a protein localized to mitochondria, is also suspected to be involved in regulating MC-mediated allergic inflammation by regulating mitochondrial metabolism.

We showed a SIRT4 overexpressing MC line. We found that the up-regulation of SIRT4 significantly reduced the base OCR and SRC of a MC, suggesting that the up-regulation of SIRT4 has an inhibitory effect on mitochondrial metabolism. As we have already mentioned above, the main function of SIRT4 discovered so far times is to regulate glutamine metabolism in mitochondria, and glutamine as raw material of α-oxoglutarate is the main source for supplementing the TCA cycle (34), and is an “engine” that provides energy support for inflammation (35). Study showed that the loss of SIRT4 resulted in the dysregulation of mammalian target of rapamycin complex 1 (mTORC1) expression and loss of stability for cAMP responsive element binding2 (CREB2), thereby increasing GDH activity and glutamine metabolism (36), Glutamine is a metabolite necessary for synthesis and secretion of inflammatory factors, and synthesis and secretion of inflammatory factors is one of characteristics of tumor cell metabolism (35,36). Also, we found that SIRT4 overexpression could significantly increase SIRT1 expression and inhibit p65 protein phosphorylation. SIRT1 (silent information regulator 1) is a histone deacetylase widely expressed in human cells. It carried out important biological functions by deacetylating multiple transcription factors, including p33 (37), UCP2 (38), P300 (39) and NF-κB (40). p65 is an important component of NF-κB, which only functions after it is acetylated. In inflammatory responses, SIRT1 deacetylated p65, thus inhibiting the transcription of TNF-α, IL-6, and other inflammatory genes downstreem of NF-κB (40) p65, is an important protein in the TLR/NF-κ signaling pathway. Its phosphorylation-mediated translocation (from the cytoplasm to the nucleus) is an important marker of the activation of NF-κB signaling. NF-κB, also known as a κ-binding nuclear factor, belonged to the Rel protein family and was an important signal transduction molecule involved in the inflammatory reaction (41).

Conclusions

Overexpression of NRF2 inhibits MC degranulation and MC-mediated inflammation by upregulating the expression of SIRT4 in MCs in vitro, and SIRT4 overexpression inhibits mitochondrial metabolism of MCs. NRF2 may be a potential target to treat allergic rhinitis by inhibiting MC mitochondrial metabolism.

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Footnote

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