Evaluation of the Effects of Nicotine on Mammalian Target of Rapamycin Complex 2 and Signal Transducer and Activator of Transcription 3 Genes Expression in a Mouse Model of Allergic Asthma: An experimental study

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Abstract

Background & Aims: Allergic diseases have increased in the last decade worldwide and researchers have been trying to introduce new strategies and drugs to treat these types of diseases. Nicotine shows anti-inflammatory properties and the studies have revealed that it can reduce the inflammation and the allergic responses. The mammalian target of rapamycin (mTOR) is a multifunctional protein kinase that forms two complexes in the signaling pathway. It has been shown that mTOR Complex 2 (mTORC2) tends to promote the immune response toward Th2. Also, the studies have indicated that the signal transducer and activator of transcription 3 (STAT3) is an essential transcription factor in anti-inflammatory responses and nicotine exert its anti-inflammatory effects using the STAT3 signaling pathway.

Materials & Methods: In this experimental Study, we investigated the effects of nicotine on the expression RICTOR-mTORC2 and STAT3 genes in a mouse model of allergic asthma. The mice were sensitized using ovalbumin and alum and 2 weeks later treated tree times with nicotine in the concentration of 10 mg/kg every other day. The mice were challenged with ovalbumin aerosols on days 35, 38 and 41 and sacrificed the next day.

Results: Our results showed that nicotine treatment resulted in down-regulation of RICTOR-mTORC2 expression. Also, the results indicated that nicotine could up-regulate the expression of STAT3.

Conclusion: Such data proposed that nicotine administration may decrease allergic responses and the inflammation in the airways of the allergic mice by down-regulating the expression of RICTOR-mTORC2 and up-regulating the expression of STAT3 genes.

Keywords: Allergy, Nicotine, mTORC2, STAT3

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Introduction

Researches have been indicated that in the recent years there has been an increasing trend in the occurrence of allergic diseases such as allergic asthma (1). Treatment of these types of diseases sometimes can be complicated and difficult due to the side effects and

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also the drug resistance (2, 3). Therefore, introducing new drugs is beneficial for the treatment of such diseases. Asthma is not just a T helper 2 (Th2) -type disease but Th2 cells are the main cells of the allergic asthma because they are the core producers of the cytokines involved in asthma pathogenesis (4). Studies have shown that the activation of the mammalian target of rapamycin (mTOR) signaling pathway is involved in the allergic responses of the immune system (5). The mTOR is a protein kinase that plays variety of important roles in immune and non-immune cells including cell metabolism, proliferation, differentiation and activation (6, 7). Two different complexes of the mTOR have been recognized to date, which have distinct functions including mTORC1 and mTORC2. Each mTOR complex has essential components that are important in signaling processes. The essential component of mTORC1 is called RAPTOR and the essential component of mTORC2 is called RICTOR. The mTOR signaling pathway is important in immune cells which can regulate and/or alter the immune responses (8). Studies indicated that mTORC1 has a tendency to promote Th1 immune response while mTORC2 promotes immune responses toward Th2 (9, 10). Another important transcription factor is called signal transducer and activator of transcription 3 (STAT3) which is critical for the regulation of the apoptosis and the immune responses (11, 12). STAT3 is very important in inflammation and researches have shown that the deletion of STAT3 gene resulted in enhanced inflammatory response and in other words STAT3 is a negative regulator of the inflammation (13, 14). Nicotine is a natural alkaloid that stimulates the parasympathetic nervous system which has attracted researchers’ attention due to its unique effects on the immune responses (15). Researches have been shown that nicotine can act as an anti-inflammatory agent and ameliorate the inflammation. Nicotine applies its effects through nicotinic acetylcholine receptors (nAChRs) and mainly via α7 subunit (16-18). In the current study, we investigated the effects of nicotine on RICTOR-TORC2 and STAT3 genes expression to find more information about the mechanisms involved in anti-inflammatory effects of nicotine in a mouse model of allergic asthma.

**Material and Methods**

**Animals:**

In this experimental study, six-to-eight-week-old healthy male BALB/c mice were used for the experiments. The mice purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran) and were housed one week before the experiments with free access to food and water. All the experiments were conducted in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences, Urmia, Iran (Ethics committee approval No. IR.umsu.rec.1393.208).

**Allergy induction, treatment, and challenge protocol:**

The mice were sensitized twice on days 0 and 7 using 10 µg of ovalbumin (OVA) (Grade V, Sigma-Aldrich, United States) and 100 µl of alum intraperitoneally. A group received nicotine with a dose of 10 mg/kg in 100 µl sterile saline (Santa Cruz Biotechnology, United States) on days 21, 23, and 25 subcutaneously. The control group was injected only with 100 µl sterile saline. The mice have challenged with OVA (5 percent in pyrogen-free saline) aerosols on days 35, 38 and 41 for 20 minutes using an ultrasonic nebulizer (Omron NE-U17, Japan). Twenty-four hours after the last challenge, the mice were killed to perform different experiments (16, 19-21). For all the experiments 5 mice per group were used.

**Real time polymerase chain reaction:**

To study the expression of mTORC2-RICTOR and STAT3 genes, we conducted real-time polymerase chain reaction (qRT-PCR). The mice were challenged one time on day 35 and sacrificed 24 hours later. The
spleens of the mice were removed aseptically and the splenocytes were extracted using an insulin syringe and cold pyrogen-free saline. The erythrocytes were lysed using cold ammonium chloride 0.9 percent for 5 minutes and the cells were washed twice with cold sterile saline. After the final washing, the cell suspension was centrifuged and the supernatant was discarded. The cell pellet was utilized for the extraction of total RNA using an extraction kit according to the manufacture’s instruction (Gene All, South Korea). Briefly, 1 × 10⁷ cells were added to 1 ml of the RiboX solution and then, 200 microliter of chloroform was added to the micro-tubes and the micro-tubes were kept on ice for 15 minutes. Then, the tubes centrifuged for 15 minutes (10000 rpm, 4 °C). The upper phase was extracted and transferred into a RNase free micro-tube. Afterward, the RB1 solution with the same volume of the upper phase was added to the micro-tube. 700 microliter of the mixture transferred into the RNA filter columns and centrifuged for 30 seconds (10000 rpm). Then, 500 microliter of the SW1 solution was added to the column and centrifuged for 30 seconds (10000 rpm). In final stage, 500 microliter of the RNW solution and 40 microliter of nuclease-free water were added to the tube and centrifuged for 3 minutes. The purity of the extracted RNA was determined by measuring the ratio of optical density at 260 nm to 280 nm. The isolated RNA was reverse transcribed using the random hexamer primers and a reverse transcriptase kit according to the manufacture’s instruction (Gene All, South Korea). Briefly, 4 microliter of extracted RNA, 1 microliter of the dNTPs mixture (10mM), 1 microliter of the random hexamer and 8 microliter of nuclease-free water were added to a sterile RNase free micro-tube and incubated for 5 minutes in 65 °C. Then, the mixture incubated on ice for 5 minutes and the Buffer MDTT and RTASE buffer, the transcriptase and RNase inhibitor were added to the tube. A polymerase chain reaction (PCR) performed with following criteria: 10 minutes in 25 °C, 60 minutes in 55 °C and 5 minutes in 85 °C. The amplification of the cDNA performed using a SYBR-green PCR master mix kit (Ampliqon, Denmark) according to the protocol provided by the manufacturer. RT-PCR was performed by the specific primers for RICTOR-mTORC2, STAT3 and β-actin 2 (Table 1). The conditions by which RT-PCR was performed for the above mentioned genes include: initial denaturation; 95 °C for 51 s, annealing; 60.5 °C for 60 s and also β-actin 2 was used as an internal control. We used the 2-ΔΔCT formula for calculation of the relative quantitation (22-24).

Table 1. Primers sequences to evaluate the expression of mTORC2 and STAT3 in the splenocytes of allergic BALB/c mice

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin2</td>
<td>Forward 5 CGTTGACATCCGTAAGACC 3’</td>
<td>285</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CAGTAAACAGTCCGCCTAGAA 3’</td>
<td></td>
</tr>
<tr>
<td>mTORC2(RICTOR)</td>
<td>Forward 5’ GGAGCACACGGATGACAAT 3’</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ TCTAAGGGGTGTGGATCTGG 3’</td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>Forward 5’ CTGGCGGTGGGTGTTAGA 3’</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ ATGGAAAGGCTATGCTGT 3’</td>
<td></td>
</tr>
</tbody>
</table>

mTORC2, mammalian target of rapamycin complex 2, STAT3, Signal transducer and activator of transcription 3, RICTOR, essential component of mTORC2
Statistical analysis:
Analyzing the data was performed using one-way analysis of variance (ANOVA) and followed by Tukey post hoc test. The analyzing was performed using SPSS software (Version 20). A p-value of less than 0.05 was considered significant. Data presented as mean ± standard error of mean (SEM).

Results
Allergic asthma induction:
The results showed that our protocol could induce allergic asthma efficiently. As it’s illustrated in Fig. 1 there was a significant difference between the control and allergic mice. The inflammation in the airways was significantly higher in the sensitized mice compared to those in control group. We recently showed that anti-inflammatory effects of nicotine are dose dependent and nicotine in concentration of 10 mg/kg had significantly higher suppressive effects on allergic responses in comparison with 1 mg/kg concentration. Since we observed better results using 10 mg/kg of nicotine, so in the current study we used the 10 mg/kg concentration to investigate its effects on mTORC2 and STAT3 genes expression.

Expression of mTORC2 and STAT3 genes in the splenocytes:
To evaluate the effects of nicotine on RICTOR-mTORC2 and STAT3 genes, we analyzed the expression patterns of the genes in splenocytes of allergic BALB/c mice. As it’s shown in Fig.2 there is a significant reduction in the expression of RICTOR-mTORC2 mRNA in the mice treated with nicotine in comparison with control group (P = 0.026). On the other hand, as it’s illustrated in Fig. 3, mRNA expression of STAT3 significantly increased in nicotine-treated mice compared to the control group (P = 0.031 and P=0.017). Therefore, nicotine could down-regulate RICTOR-mTORC2 expression while it’s up-regulated STAT3 expression.

Fig. 1. Effects of nicotine administration on the lung tissue inflammation of the allergic BALB/c mice. (A) The control group that received pyrogen-free saline only. A sever inflammation in the lung tissue can be observed. (B) Administration of Nicotine in concentration of 10 mg/kg reduced the inflammation dramatically. (C) A digital image of the lung tissue of the naive mice which did not treat with any substance.
Fig. 2. Effects of nicotine administration on mTORC2 gene expression in splenocytes of allergic BALB/c mice. Nicotine treatment in concentration of 10 mg/kg significantly down-regulated mTORC2 mRNA expression (P = 0.026). Data presented as mean ± SEM. * P value ≤ 0.05 is significant.

Fig. 3. Effects of nicotine administration on STAT3 gene expression in splenocytes of allergic BALB/c mice. Nicotine treatment in concentration of 10 mg/kg significantly up-regulated STAT3 mRNA expression (P = 0.031 and P = 0.017). Data presented as mean ± SEM. * P value ≤ 0.05 is significant.

Discussion
Allergic inflammation is a characteristic indication of some allergic diseases including asthma in which there is an accumulation of the inflammatory cells around the airways (22). Therefore, as expected, extensive studies have been performed to decrease the inflammation in allergic asthma. As it’s mentioned before, asthma is more than a Th2-type disease and the
researches have been indicated that T helper 1 (Th1), T helper 17 (Th17), and T helper 9 (Th9) cells also involved in the pathogenesis of the disease (23-25). Therefore, new strategies for the treatment of allergic asthma and decreasing the inflammation in the airways are required. Our previous study showed that nicotine may be considered as a new medicine for the treatment of allergic asthma and the results showed that nicotine reduced the inflammation in the airways of allergic mice, interleukin-4 (IL-4) production, allergen-specific immunoglobulin E (IgE) concentration while increased transforming growth factor beta/interleukin 4 (TGF-β/IL-4) ratio and proliferation of regulatory T cells (Tregs). In the current study, the main purpose was to investigate the effects of nicotine on the expression of RICTOR-mTORC2 and STAT3 genes.

mTOR is a multifunctional protein kinase. Two mTOR signaling complexes (mTORC1 and mTORC2) have been identified and distinct roles described for each complex. The role of the complexes in the immune responses has been studied extensively. Researches have revealed that mTORC1 is important in developing Th1 immune responses and mTORC2, unlike the first complex, tends to progress Th2 immune responses (9, 10). Since Th2 is the main response in allergic diseases, so it’s possible that mTORC2 will be up-regulated in these types of diseases. Huang and colleagues showed that interleukin-4 signals via mTORC2 (26). Since in the allergic diseases, expression and production of IL-4 are up-regulated and the cytokine signaling is conducted via mTORC2 so it’s possible that the gene expression of mTORC2 is up-regulated too. Our results showed that the mice in control group had higher RICTOR-mTORC2 gene expression than the naive mice and the group that treated with nicotine had lower RICTOR-mTORC2 gene expression. So, it’s possible that down-regulation of RICTOR-mTORC2 by nicotine resulted in less signaling of IL-4 and subsequently less allergic response. This possible mechanism confirms our previous finding that nicotine reduced IL-4 production by the splenocytes of allergic mice (16). These results also are in line with the results reported by Huang et al. Down-regulation of RICTOR-mTORC2 by nicotine subsequently may decrease promoting of the immune responses toward Th2 and as a result, the inflammation and allergic response reduced in the treated mice.

STAT3 is a member of the STAT gene family that its role in cellular responses has been studied widely (27). Although relatively specific activities in response to some limited cytokines have been reported for other STAT family members, many biological functions have been reported for STAT3 in response to various ranges of the cytokines. As it’s mentioned before STAT3 is a negative regulator of the inflammation and its presence is vital for anti-inflammatory responses of the immune system (8, 28). Besides, it’s essential for lymphocytes differentiation and proliferation and also the regulation of many immune functions. Two Studies by Sui and Hosur have indicated that nicotine may apply its anti-inflammatory effects via STAT3 signaling pathway. Therefore, it’s possible that the expression of the STAT3 gene be down-regulated in the allergic diseases since the anti-inflammatory responses are impaired in allergic patients (29). Our results showed that STAT3 gene expression has decreased in the mice of the control group compared to the naive mice however the decrement was not significant statistically. Furthermore, our result showed that nicotine has up-regulated STAT3 expression in comparison with the mice of the control group and therefore, these results are in line with the studies of Sui and Hosur (30, 31). Up-regulation of STAT3 gene by nicotine may potentiate anti-inflammatory responses and subsequently reduce allergic responses and allergic inflammation in the treated mice.

We believe that this study has some limitations such as the lake of measuring the protein levels of the genes
and limited number of studied genes. We hope these limitations will be addressed in the following studies.

**Conclusion**

Altogether, our results indicated that nicotine may apply its anti-inflammatory effects by down-regulating of RICTOR-mTORC2 expression and therefore, decreasing the progression of the immune response toward Th2 and also by up-regulating of STAT3 expression and thus, enhancing the anti-inflammatory responses of the immune system. However, to confirm these results further studies about other proteins involved in the signaling pathways are required.

**Acknowledgments**

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**Abbreviations**

- Mammalian target of rapamycin = mTOR
- Mammalian target of rapamycin complex 1 = mTORC1
- Mammalian target of rapamycin complex 2 = mTORC2
- Signal Transducer and Activator of Transcription 3 = STAT3
- T helper 2 = Th2
- T helper 17 = Th17
- T helper 9 = Th9
- Nicotinic acetylcholine receptors = nAChRs
- Hematoxylin–eosin staining = (H & E)
- Bronchoalveolar lavage fluid = BALF
- Interleukin-4 = IL-4
- Interleukin-18 = IL-18
- Transforming growth factor beta = TGF-β
- Tumor necrosis factor alpha = TNF-α
- Intercellular adhesion molecule 1 = ICAM-1

**References**

11. Jie Z, Dinwiddie DL, Senft AP, Harrood KS. Regulation of STAT signaling in mouse bone marrow derived dendritic