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Research Paper

# Cholinergic anti-inflammatory pathway inhibits neointimal hyperplasia by suppressing inflammation and oxidative stress

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ABSTRACT

Neointimal hyperplasia as a consequence of vascular injury is aggravated by inflammatory reaction and oxidative stress. The α7 nicotinic acetylcholine receptor (α7nAChR) is a orchestrator of cholinergic anti-inflammatory pathway (CAP), which refers to a physiological neuro-immune mechanism that restricts inflammation. Here, we investigated the potential role of CAP in neointimal hyperplasia using α7nAChR knockout (KO) mice. Male α7nAChR-KO mice and their wild-type control mice (WT) were subjected to wire injury in left common carotid artery. At 4 weeks post injury, the injured aortae were isolated for examination. The neointimal hyperplasia after wire injury was significantly aggravated in α7nAChR-KO mice compared with WT mice. The α7nAChR-KO mice had increased collagen contents and vascular smooth muscle cells (VSMCs) amount. Moreover, the inflammation was significantly enhanced in the neointima of α7nAChR-KO mice relative to WT mice, evidenced by the increased expression of tumor necrosis factor-α/interleukin-1β, and macrophage infiltration. Meanwhile, the chemokines chemokine (C-C motif) ligand 2 and chemokine (CXC motif) ligand 2 expression was also augmented in the neointima of α7nAChR-KO mice compared with WT mice. Additionally, the depletion of superoxide dismutase (SOD) and reduced glutathione (GSH), and the upregulation of 3-nitrotyrosine, malondialdehyde and myeloperoxidase were more pronounced in neointima of α7nAChR-KO mice compared with WT mice. Accordingly, the protein expression of NADPH oxidase 1 (Nox1), Nox2 and Nox4, was also higher in neointima of α7nAChR-KO mice compared with WT mice. Finally, pharmacologically activation of CAP with a selective α7nAChR agonist PNU-282987, significantly reduced neointima formation, arterial inflammation and oxidative stress after vascular injury in C57BL/6 mice. In conclusion, our results demonstrate that α7nAChR-mediated CAP is a neuro-physiological mechanism that inhibits neointima formation after vascular injury via suppressing arterial inflammation and oxidative stress. Further, these results imply that targeting α7nAChR may be a promising interventional strategy for in-stent stenosis.

## 1. Introduction

Neointimal hyperplasia is a complicated cellular and molecular response characterized by aggressive proliferation following mechanical vascular injury, such as angioplasty and stenting, endarterectomy, and vein bypass graft failure [1]. This disorder leads to a narrowing of the arterial lumen known as restenosis, which limit the safety and efficacy of percutaneous transluminal coronary angioplasty and necessitates the need for retreatment [2,3]. Currently, it has been widely accepted that the abnormal proliferative phenotype of vascular smooth muscle cells (VSMCs) in the intimal region plays a key role in the development of neointimal hyperplasia after vascular injury. VSMCs always exhibit a contractile phenotype with little proliferation/migration and extracellular matrix (ECM) production under normal condition, but turn to a

*Abbreviations:* 3-NT, 3-nitrotyrosine; α7nAChR, α7 nicotinic acetylcholine receptor; α-SMA, α-smooth muscle actin; CAP, cholinergic anti-inflammatory pathway; CCL2, chemokines chemokine (C-C motif) ligand 2; CNS, central nervous system; CXCL2, chemokine (CXC motif) ligand 2; GSH, reduced glutathione; IL-1β, interleukin-1β; KO, knockout; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; WT, wild type; VSMCs, vascular smooth muscle cells

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synthetic phenotype after vascular injury [2,3]. Importantly, the vascular injury-induced inflammatory response, chemokines induction and oxidative stress, critically contribute to the VSMC phenotype switch and neointimal hyperplasia [4,5]. Many redox signaling factors, including nuclear factor 2 (Nrf2) [6], peroxiredoxin [7], and heme oxygenase-1 (HO-1) [8], play key roles in neointimal hyperplasia after vascular injury [4,9,10].

Nicotinic acetylcholine receptors (nAChRs) are a group of cholinergic ligand-gated ion channels that respond to the neurotransmitter acetylcholine. They also respond to many types of chemical compounds such as nicotine. The nAChRs are mainly expressed in the central nervous system (CNS) and regulate diverse biological function of CNS [11]. The α7 nicotinic ACh receptor (α7nAChR), which is also known as cholinergic receptor nicotinic α7 subunit (*CHRNA7*), is one of the most common receptors expressed in the CNS [12,13]. The α7nAChR is characterized by its rapid desensitization and high calcium permeability in cholinergic neurotransmission [12,13]. A large number of studies have pointed out that α7nAChR is not only involved in cognitive functions such as memory and learning, but also implicated in neurological disorders such as Alzheimer's diseases, Parkinson's disease, depression, and schizophrenia [12].

Interestingly, recent investigations discovered that α7nAChR is widely expressed in peripheral non-nerve cells such as lymphocytes, monocytes and macrophages, and plays an indispensable role in the "cholinergic anti-inflammatory pathway (CAP)", which refers to a physiological neuro-immune mechanism that limits innate immune function in a ACh-dependent manner [14–16]. CAP has a major contribution in alleviating both acute and chronic inflammatory pathologies such as endotoxemia and inflammatory bowel disease [14–16]. We previously reported that α7nAChR-medaited CAP is a potent protective mechanism in many disease states of cardiovascular system, including endothelial dysfunction [17], hypertension [18,19], shock [20] and vascular aging [21]. However, whether CAP participates in the development of neointimal hyperplasia after vascular injury has not been studied yet.

In the present study, we examined the function of CAP in vascular injury using α7nAChR knockout mice model, and explored the potential effects of α7nAChR deletion on inflammation and oxidative stress in the injured vascular wall.

## 2. Methods

### 2.1. Animal

The α7nAChR KO mice (*Chrna7*$^{tm1Bay}$, number 003232) and wild type control mice (C57BL/6) were purchased from Jackson laboratory and described in our previous studies [18,21]. The α7nAChR KO mouse strain used in this study was backcrossed to C57Bl/6 for at least six generations. The mice were bred and housed in temperature-controlled cages under a 12/12-h light/dark cycle with free access to water and chow in Tongji University Animal Core. Animals were used in accordance with the Tongji University institutional guidelines for animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

### 2.2. Arterial injury model

Transluminal arterial injury model was induced as described previously [22]. For all surgical procedures, the mice were anesthetized by intraperitoneal injection with pentobarbital sodium (50 mg/kg). Surgery was carried out using a dissecting microscope (SMZ-800, Nikon, Tokyo, Japan). A guide wire (0.38 mm in diameter) was inserted into the left common carotid artery of 8-week-old male WT or α7nAChR-KO mice. The wire was left in place for 1 min to denude and dilate the artery. Carprofen (5 mg/kg) was used for analgesia, administered subcutaneously daily for 3 days following surgery.

### 2.3. Drug administration

The C57BL/6 mice were divided into three groups: uninjured group, injured group and injured + PNU-282987 group. The mice in injured group underwent wire-injury as described above, while the mice in uninjured group underwent sham-operation without wire insertion. The mice in injured + PNU-282987 group underwent wire-injury and were administrated with PNU-282987 for 4 weeks. For PNU-282987 treatment, the PNU-282987 (Sigma-Aldrich, #P6499) was dissolved in 0.4% DMSO in saline was injected intraperitoneally once a day at 9 a.m. ~ 11 a.m. (1 mg/kg/d). This does was chosen according to our previous study [18,21]. The mice in injured group and uninjured groups also received injection with vehicle (0.4% DMSO in saline) at the same time.

### 2.4. Blood pressure measurement, tissue sampling and serum basal parameters

At 4 weeks post injury, the mice were subjected to measurement of blood pressure according to our previous report [18]. Then, mice were fasted overnight and weighted. The mice were then euthanized by intraperitoneal administration of an overdose of pentobarbital sodium (150 mg/kg, i.p.). The blood was obtained for isolating serum to determine fasting glucose and cholesterol using an automatic biochemistry analyzer (Hitachi 7020). For histological and immunohistochemistry analysis, the mice at death were perfused with 0.9% NaCl solution for 5 min followed by perfusion fixation with 4% paraformaldehyde in PBS (pH 7.4) for 15 min. The left (injured) and right (uninjured) common carotid arteries were carefully excised and further fixed in 4% paraformaldehyde overnight at 4 °C, and embedded in paraffin. For biomedical analysis, another set of mice with transluminal arterial injury were injected with pentobarbital sodium (150 mg/kg, i.p.) and perfused with 0.9% NaCl solution for 1 min to flush the blood. Then, their carotid arteries were excised swiftly and stored at −80 °C.

### 2.5. Histological examination

Hematoxylin and eosin (H&E) staining was used to assess morphological changes. Paraffin-embedded tissues were cut to sections (8 μm) and then paraffin was removed with xylen and tissues were washed with ethanol [23]. Then, the sections were stained in hematoxylin and eosin according to standard procedures. Collagen distribution in the aortic wall was evaluated by Masson staining [24]. Briefly, sections (8 μm) were dewaxed and rehydrated, followed by counterstaining with Weigert's iron hematoxylin (5–10 min), followed by Masson's trichrome staining solution. Sections were washed in 1% acetic acid (1 min) and dehydrated with alcohol and xylene using standard procedures.

### 2.6. Immunohistochemistry

Immunohistochemistry was performed as described previously [25–27]. For immunohistochemistry experiments, frozen 8-μm-thick sections were fixed in 4% paraformaldehyde. The sections were blocked by 8% normal goat serum for 4 h and then incubated in specific primary antibodies. After being washed three times by PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies. Staining is visualized using substrate diaminobenzidine. The following antibodies were used: α-smooth muscle actin (α−SMA, #ab7817, Abcam, Cambridge, UK, 1: 600 dilution), PCNA (clone PC10, Millipore, Milford, MA, USA, 1;1000 dilution), tumor necrosis factor-α (TNF-α, #ab9635, Abcam, Cambridge, UK, 1: 1000 dilution), interleukin-1β (IL-1β, #MAB4012, R&D Systems, Minneapolis, MN, USA, 1: 1000 dilution), CD68 (#MA5-13324, Invitrogen, Carlsbad, CA, USA, 1: 2000 dilution), 3-nitrotyrosine (3-NT, #sc-32757, 1: 4000 dilution) malondialdehyde (MDA, #ab6463, 1: 2000 dilution), myeloperoxidase (MPO, #PA5-16672, Invitrogen, 1: 2500 dilution), chemokine (C-C