Cholinergic Anti-Inflammatory Pathway

Dysfunction of the Cholinergic Anti-Inflammatory Pathway Mediates Organ Damage in Hypertension

Dong-Jie Li, Roger G. Evans, Zhong-Wei Yang, Shu-Wei Song, Pei Wang, Xiu-Juan Ma, Chong Liu, Tao Xi, Ding-Feng Su, Fu-Ming Shen

Abstract—Inflammatory responses are associated with the genesis and progression of end-organ damage (EOD) in hypertension. A role for the α7 nicotinic acetylcholine receptor (α7nACHR) in inflammation has recently been identified. We tested the hypothesis that α7nACHR dysfunction contributes to hypertensive EOD. In both spontaneously hypertensive rats (SHRs) and rats with abdominal aorta coarctation–induced hypertension, atropine–induced tachycardia was blunted compared with normotensive controls. Both models of hypertension were associated with deficits in expression of the vesicular acetylcholine transporter and the α7nACHR in cardiovascular tissues. In hypertension induced by abdominal aorta coarctation, deficits in aortic vesicular acetylcholine transporter and α7nACHR were present both above and below the coarctation site, indicating that they were independent of the level of arterial pressure itself. Hypertension in 40-week-old SHRs was associated with cardiac and aortic hypertrophy. Morphological abnormalities consistent with EOD, along with elevated tissue levels of proinflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and interleukin-6) were observed in the heart, kidney, and aorta. Chronic treatment of SHRs with the α7nACHR agonist PNU-282987 relieved EOD and inhibited tissue levels of proinflammatory cytokines and activation of nuclear factor κB. Greater serum levels of proinflammatory cytokines and more severe damage in the heart, aorta, and kidney were seen in α7nACHR−/− mice subjected to 2-kidney-1-clip surgery than in wild-type mice. A deficit in the cholinergic anti-inflammatory pathway appears to contribute to the pathogenesis of EOD in models of hypertension of varying etiology. This pathway may provide a new target for preventing cardiovascular disease resulting from hypertension. (Hypertension. 2011;57:298-307.) • Online Data Supplement

Key Words: acetylcholine • α7 nicotinic acetylcholine receptor • inflammation • hypertension • end-organ damage

Hypertension is a major risk factor for myocardial infarction, heart failure, stroke, and kidney dysfunction. End-organ damage (EOD), including cardiac hypertrophy and myocyte dysfunction, vascular remodeling, and renal lesions, is a crucial mediatory link between hypertension and the development of these cardiovascular events. Therefore, a better understanding of the mechanisms leading to hypertensive EOD could provide new avenues for prevention of cardiovascular events. Inflammation is very important in the genesis and development of EOD. Furthermore, the renin–angiotensin system can contribute to EOD, at least partly by promoting inflammation. However, cardiovascular inflammation and EOD can also occur without renin–angiotensin system activation, so other mechanisms must also operate. Recent evidence indicates that neuronal α7 nicotinic cholinergic systems influence inflammatory responses by controlling the release of tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 and that nonneuronal acetylcholine synthesis and release machinery are downregulated in inflammation. Vagal activation can suppress inflammation. For example, vagal stimulation blunted the release of TNF-α from macrophages and decreased mortality induced by endotoxin in rats. The anti-inflammatory actions of vagal efferent activity appear to be mediated chiefly by acetylcholine (ACh) acting at the α7 nicotinic acetylcholine receptor (α7nACHR). The vesicular acetylcholine transporter (VACHT) mediates the loading of ACh into secretory organelles in neurons, thereby making ACh available for release. Thus, if impairment of cholinergic anti-inflammatory pathways contributes to development of EOD during hypertension, then dysfunction of the α7nACHR and/or the VACHT seems likely.

Hypertension is associated with impaired arterial baroreflex function. The high frequency component of heart rate variability is reduced in human hypertension, indicating impairment of cardiac vagal drive. Importantly, impaired baroreflex function and reduced vagal tone are associated with increased risk for cardiovascular disease, but the mechanistic link between reduced vagal tone and increased inflammation can suppress inflammation. For example, vagal stimulation blunted the release of TNF-α from macrophages and decreased mortality induced by endotoxin in rats. The anti-inflammatory actions of vagal efferent activity appear to be mediated chiefly by acetylcholine (ACh) acting at the α7 nicotinic acetylcholine receptor (α7nACHR). The vesicular acetylcholine transporter (VACHT) mediates the loading of ACh into secretory organelles in neurons, thereby making ACh available for release. Thus, if impairment of cholinergic anti-inflammatory pathways contributes to development of EOD during hypertension, then dysfunction of the α7nACHR and/or the VACHT seems likely.

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cardiovascular risk is largely unknown. In the present study, we tested the hypothesis that dysfunction in \(\alpha7\nAChR\)-mediated signaling contributes to development of EOD in hypertension. If this mechanism is important, it should operate across a range of models of hypertension of differing etiology. Thus, we used 3 hypertensive animal models, including spontaneously hypertensive rats (low renin), pressure-overload hypertensive rats (coarctation hypertension, high renin), and 2-kidney-1-clip (2K1C) surgery-induced hypertension (high renin).

**Methods**

**Animals**
Male Sprague–Dawley rats, Wistar–Kyoto (WKY) rats, and spontaneously hypertensive rats (SHRs) and \(\alpha7\nAChR^{-/-}\) mice were housed in a 12/12-hour light/dark cycle with free access to food and water. All the animals used in this work received humane care in compliance with the institutional animal care guidelines and the *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health.

**Abdominal Aorta Coarctation–Induced Hypertension in Rats**
Partial abdominal aorta coarctation was produced as described previously\(^{20}\) to induce hypertension in the upper part of the systemic circulation (see the online Supplement available at http://hyper.ahajournals.org).

**Renovascular Hypertension in Mice**
2K1C hypertension was induced in male \(\alpha7\nAChR^{-/-}\) mice and wild-type (WT) mice (20 to 25 g), as described previously.\(^{21,22}\) Silver clips (0.12-mm opening width, Exidel SA, Lausanne, Switzerland) were used to narrow the left renal artery (see the online Supplement).

**Blood Pressure and Heart Rate Measurement**
Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were continuously recorded in conscious rats as described previously.\(^{23,24}\) Blood pressure-overload hypertensive rats (coarctation hypertension–induced hypertension–induced hypertension (high renin).

**Morphological Examination**
Injury to heart, kidney, and aorta was examined morphologically, as described previously.\(^{25}\) Glomerular sclerosis score provided a semi-quantitative evaluation of glomerular damage in the right kidney, as described previously (see the online Supplement).

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction Analysis**
These analyses were performed as described in previous publications (see the online Supplement).\(^{27,28}\)

**Protein Extraction and Western Blot Analysis**
Whole or nuclear protein extractions and Western blot analysis of \(\alpha7\nAChR\), VACHT, and nuclear factor (NF)-\(\kappa\)B were performed as described previously (see the online Supplement).\(^{27,28}\)

**Quantification of Tissue Inflammatory Factors**
Levels of TNF-\(\alpha\), IL-1\(\beta\), and IL-6 were determined by ELISA, as described previously (see the online Supplement).\(^{28}\)

**Histological Analysis by Light and Electron Microscopy**
These analyses were performed as described previously (see the online Supplement).\(^{25}\)

**Chronic Treatment With PNU-282987**
Four groups of rats (8 in each) were included in this experiment: WKY rats, WKY rats treated with PNU-282987, SHRs, and SHRs treated with PNU-282987. PNU-282987 is a selective agonist of the \(\alpha7\nAChR\). From 36 weeks of age, PNU-282987 (0.38 mg/kg, IP; Sigma)\(^{29}\) or its vehicle (0.4% DMSO in saline) was administered daily for 28 days. Rats were then killed under deep anesthesia, and the right kidney, aorta, and heart were excised for Western blot, ELISA, and histological analyses.

**Statistical Analysis**
All values are expressed as means±SEM. Results were analyzed by paired (within-group) or unpaired (between 2 groups) Student t test or ANOVA, followed by Tukey test (among 3 or more groups). Two-sided \(P<0.05\) was considered statistically significant.

**Results**

**Basal Arterial Pressure and Vagal Tone in SHRs**
To assess vagal tone, the cardiovascular effects of atropine were determined in SHRs and age-matched WKY rats (Table S1 in the online Supplement). Atropine significantly increased HR but not SBP or DBP. Atropine-induced tachycardia was significantly less in hypertensive SHRs than WKY rats (ie, at 20 and 40 but not 4 weeks of age), indicating a deficit in cardiac vagal tone in hypertensive SHRs. Baroreceptor reflex sensitivity, which also reflects vagal tone, was also reduced in SHRs at 20 and 40 but not 4 weeks of age (Table S2).

**Gene and Protein Expression of VACHT and \(\alpha7\nAChR\) in Heart, Kidney, and Aorta of SHRs**
To evaluate cholinergic function in SHRs at the molecular level, we assessed protein expression of the VACHT and \(\alpha7\nAChR\). VACHT protein expression and both mRNA and protein expression of the \(\alpha7\nAChR\) were all less in the heart, kidney, and aorta of SHRs than in age-matched WKY rats at 20 and 40 weeks of age but not at 4 weeks of age (Figure 1).

**Vagal Tone, \(\alpha7\nAChR\), and VACHT in Coarctation-Induced Hypertension Rats**
To examine the role of increased arterial pressure itself in the altered expression of \(\alpha7\nAChR\) and VACHT in hypertension, expression of these factors was examined in aorta coarctation–induced hypertension (CH) rats. As expected, 16 weeks after surgery to induce CH, SBP measured from the carotid artery was significantly greater in CH rats compared with sham-operated controls (Table S3), whereas SBP measured from the femoral artery was similar in the 2 groups of rats (Table S3). That is, in CH, SBP was increased within the thoracic aorta but not the abdominal aorta. Nevertheless, \(\alpha7\nAChR\) and VACHT protein expression were less in both the thoracic and abdominal aorta of CH rats compared with sham-operated controls (Figure 2). Atropine caused less tachycardia in CH rats than in sham-operated rats (Table S3).
Cardiovascular Inflammation and EOD in SHRs

In WKY, levels of IL-1β and TNF-α, but not IL-6, varied among the heart, kidney, and aorta. Compared with age-matched WKY rats, levels of IL-1β, IL-6, and TNF-α were all greater in the heart, kidney, and aorta of 40-week-old SHRs (Figure 3A through 3C). Evidence of EOD, including cardiac hypertrophy (Figure 3D), renal atrophy (Figure 3E), and aortic hypertrophy (Figure 3F), was observed in SHRs.

Inflammation and EOD Induced by Hypertension in α7nAChR−/− Mice

To further investigate the role of the α7nAChR in hypertensive EOD, α7nAChR−/− mice and WT controls were rendered hypertensive by clipping 1 renal artery. After sham surgery, arterial pressure and levels of proinflammatory cytokines were similar in the 2 genotypes. Eight weeks after the 2K1C operation, SBP was similar in WT and α7nAChR−/− mice, albeit within the hypertensive range (145 to 150 mm Hg). At this time point, levels of serum IL-1β, IL-6, and TNF-α were greater in α7nAChR−/− mice compared with WT mice (Figure 4).

There was evidence of more severe EOD in α7nAChR−/− mice compared with WT controls 8 weeks after 2K1C surgery, including left ventricular hypertrophy and glomerular sclerosis (Figure 4). No glomerular sclerosis was observed in sham-operated α7nAChR−/− mice and WT mice.

Histological examination did not demonstrate morphological differences between WT and α7nAChR−/− mice after sham surgery (Figure 5). However, there were clear differences in the level of EOD induced by 2K1C hypertension in SHRs.
α7nAChR−/− mice compared with WT controls. In 2K1C-WT mice, renovascular hypertension was associated with irregular arrangement and mild degeneration of cardiac myocytes, vagueness of the cardiac myocyte mitochondrial membrane, irregular arrangement of vascular smooth muscle cells, mild degeneration of vascular endothelial cells, focal degeneration of the renal tubular epithelium, and mild hyperplasia of the glomerular mesangium. These lesions were considerably more severe in 2K1C-α7nAChR−/− mice than WT mice (Figure 5).

Effects of PNU-282987 on Cardiovascular Inflammation and EOD in SHRs
In SHRs, no significant changes in SBP, DBP, or HR were observed after single acute intravenous doses of PNU-282987 of 0.38 or 3.8 mg/kg (Table S4). Chronic PNU-282987 administration (0.38 mg/kg per day for 28 days, IP) in SHRs also did not significantly alter arterial pressure or heart rate (Table S5) or expression of α7nAChR protein in heart, kidney, aorta, or macrophages (Figure S1 in the online Supplement). However, levels of some inflammatory factors in tissues of SHRs were reduced by PNU-282987 treatment. Levels of IL-1β, IL-6, and TNF-α in SHR tissues were greater than those in WKY. Lesser levels of these factors were observed in the aorta of the PNU-282987–treated SHRs compared with vehicle-treated SHRs. In the heart, lesser levels of TNF-α were found in PNU-282987–treated SHRs compared with vehicle-treated SHRs, whereas levels of IL-1β and IL-6 were not significantly altered by PNU-282987 treatment. In the kidney, lesser levels of IL-1β and IL-6, but not TNF-α, were observed in PNU-282987–treated SHRs compared with vehicle-treated SHRs. There were no significant differences in the levels of these proinflammatory cytokines between WKY and WKY treated with PNU-282987 (Figure 6).

At the level of light microscopy, left ventricular damage in vehicle treated SHRs was characterized by irregular arrange-
To further explore the intracellular mechanisms underpinning the anti-inflammatory effects of PNU-282987, we investigated the effects of this agent on NF-κB signaling in SHRs and WKY rats (Figure 8). Compared with WKY rats, the nuclear NF-κB p65 subunit was conspicuously enhanced in tissues of SHRs, whereas the cytosolic IkB was decreased, indicating the NF-κB system was activated in SHRs. Treatment of PNU-282987 attenuated nuclear NF-κB p65 subunit levels and upregulated cytosolic IkB levels in SHRs (Figure 8). These observations indicate that activation of the α7nAChR by PNU-282987 suppresses inflammation, at least partly, through inhibiting activation of the NF-κB signaling pathway.

Discussion

Three major new findings arose from the present work. Firstly, vagal tone and expression of both the VACHT and the α7nAChR were significantly less in both CH rats and SHRs compared with normotensive control rats. Importantly, in CH, impaired VACHT and α7nAChR expression was observed in the aorta both above and below the coarctation. Thus, α7nAChR-mediated signaling is impaired systemically in multiple forms of hypertension. Secondly, serum levels of proinflammatory cytokines and EOD in 2K1C hypertension were greater in α7nAChR−/− mice than WT controls. Thus, inflammation and EOD are enhanced in the absence of α7nAChR-mediated signaling. Thirdly, we found that the increased tissue levels of proinflammatory cytokines and the EOD observed in 40-week-old SHRs could be blunted by chronic treatment with the selective α7nAChR-agonist PNU-282987. Thus, EOD can be blunted by enhancing α7nAChR-mediated signaling. These α7nAChR-mediated beneficial effects were associated with its ability to attenuate activation of the NF-κB signaling pathway in hypertension. Collectively, these findings indicate that diminished α7nAChR signaling is a major mechanism in the pathogenesis of cardiovascular inflammation and EOD in hypertension. The
The significance of these findings is 2-fold. Firstly, they provide a plausible mechanistic explanation for the association of reduced vagal tone with increased cardiovascular risk. Secondly, they indicate that the \( \alpha_7 \) nAChR may be a therapeutic target for prevention of hypertensive EOD.

We evaluated the function of cholinergic anti-inflammatory pathways using multiple methods. Firstly, we determined the tachycardic response to atropine, a classic index of cardiac vagal tone. Although the response to atropine is mediated by blockade of muscarinic receptors rather than nicotinic receptors, it provides an integrated measure of basal ACh release and muscarinic receptor signaling in the heart. In accord with previous studies indicating impaired arterial baroreflex function and depressed baroreflex-mediated cardiac vagal responsiveness in essential hypertension, the tachycardia induced by atropine was significantly decreased in hypertensive SHRs compared with WKY rats and in rats with CH compared with sham-operated controls. Our present findings provide a mechanistic explanation for this finding, because cardiac VACHT expression was greatly blunted in both of these models of hypertension, consistent with the proposition that they are associated with reduced storage and release of ACh in the heart. Interestingly, the deficit in VACHT expression was not confined to the heart but was also observed in the kidney and aorta, tissues devoid of direct parasympathetic innervation. Thus, hypertension of multiple etiology appears to be associated with a systemic diminution in both neuronal and nonneuronal cholinergic signaling.

There is strong evidence that the \( \alpha_7 \) nAChR, expressed in primary immune cells and perhaps also in peripheral tissues, is a pivotal mediator of the cholinergic anti-inflammatory pathway. To understand the impact of arterial pressure per se on cholinergic signaling, \( \alpha_7 \) nAChR and VACHT expression were examined in multiple models of hypertension. Firstly, expression of the \( \alpha_7 \) nAChR and VACHT in the heart, kidney, and aorta was compared between age-matched WKY rats and SHRs. \( \alpha_7 \) nAChR and VACHT expression in

**Figure 5.** Histological analysis of EOD in WT and \( \alpha_7 \) nAChR \(^{-/-}\) mice (n=6 per group) 8 weeks after surgery to induce 2K1C hypertension. Representative light and electron micrographs of heart (A), kidney (B), and aorta (C) indicate that 2K1C-induced hypertension caused more severe organ damage in \( \alpha_7 \) nAChR \(^{-/-}\) mice than in WT mice. EC indicates endothelial cells.
these tissues was consistently less in SHRs with established hypertension (20 and 40 weeks of age) than in age-matched WKY rats, but not in prehypertensive SHRs (4 weeks of age). These observations support the concept that it is hypertension, rather than other factors associated with the genotypic differences between SHRs and WKY rats, that leads to downregulation of the α7nAChR and VAChT in SHRs. To further assess the influence of arterial pressure on α7nAChR and VAChT expression, a pressure-overload rat model was prepared by abdominal arterial coarctation. In this model, α7nAChR and VAChT were equally downregulated in aortic segments upstream and downstream of the coarctation. Thus, rather than exerting a local effect dependent on the level of arterial pressure within each specific vascular territory, hypertension appears to exert a systemic effect to suppress cholinergic signaling.

It is well accepted that chronic hypertension results in decreased vagal function. The finding that both vagal tone and expression of the VAChT were reduced, in both SHRs and CH rats with established hypertension, raises the possibility that the vagal dysfunction induced by hypertension might subsequently result in downregulation of the α7nAChR. However, it is difficult to envisage how vagal dysfunction could affect cholinergic signaling in tissues devoid of parasympathetic innervation such as the kidney and aorta. One possible explanation is the existence of nonneuronal cholinergic anti-inflammatory pathways in these tissues. It is well established that nonneuronal cholinergic systems contribute to multiple cellular functions including proliferation, differentiation, migration, and ion and water movement. Dysfunction of nonneuronal cholinergic systems appears to be involved in the pathogenesis of many diseases, including some inflammatory conditions. Our findings in the kidney and aorta suggest the existence of nonneuronal cholinergic anti-inflammatory pathways in these tissues, which limit signaling through proinflammatory cytokines and consequently organ damage.

The α7nAChR has been proposed as a therapeutic target for control of inflammatory disorders. Therefore, we investigated the role of the α7nAChR in inflammation and EOD in renovascular hypertension using α7nAChR−/− mice. After induction of 2K1C hypertension, α7nAChR−/− mice displayed greater serum levels of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and more severe EOD than WT mice. Thus, the α7nAChR exerts an anti-inflammatory influence in 2K1C hypertension.

**Figure 6.** Tissue levels of IL-1β, IL-6, and TNF-α in WKY rats and SHRs treated with vehicle or PNU-282987. Data are means±SEM (n=8 per group). *P<0.05, **P<0.01 compared with WKY rats; #P<0.05, ##P<0.01 compared with vehicle (unpaired t test).
We also examined the effects of chronic treatment of SHRs with PNU-282987, a selective α7nAChR agonist. Consistent with previous studies, levels of IL-6, IL-1β, and TNF-α in aorta and plasma were greater in adult SHRs than in age-matched WKY rats. Similar observations have been made in hypertensive patients. Our important new finding was that treatment with PNU-282987 did not reduce blood pressure but decreased the levels of these inflammatory factors in SHR tissues in most cases and greatly attenuated EOD as assessed by both light and electron microscopy. Thus, pharmacological rescue of the deficit in α7nAChR signaling in SHRs has anti-inflammatory effects. Collectively, these data from α7nAChR−/− mice and SHRs treated with PNU-282987 provide compelling evidence for a role of the cholinergic anti-inflammatory pathway, mediated via the α7nAChR, in the pathogenesis of hypertensive EOD.

An association between the α7nAChR and the NF-κB signaling pathway has recently been reported in neuronal cells. Our finding that chronic treatment of SHRs with the α7nAChR agonist PNU-282987 inhibited activation of NF-κB in heart, kidney, and aorta provides the first evidence for a critical role of the NF-κB signaling pathway in the anti-inflammatory influence of the α7nAChR in hypertension.

The importance of the renin–angiotensin system in cardiovascular disease is well established. With regard to the role of autonomic nervous system in hypertensive EOD, most attention has focused on the sympathetic nervous system, with little attention paid to the parasympathetic nervous system. In this study, we provide strong evidence that dysfunction of α7nAChR-mediated signaling, likely attributable to the combined effects of impaired ACh release and downregulation of the α7nAChR, contributes to EOD in multiple forms of hypertension through disinhibition of proinflammatory cascades.

**Perspectives**

Our findings provide a plausible mechanistic explanation for the well established association between impaired cardiac

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**Figure 7.** Histological analysis of EOD in WKY and SHRs (n=6 per group) treated chronically with PNU-282987 or its vehicle for 4 weeks. Representative light and electron micrographs of heart (A), kidney (B), and aorta (C) indicate that PNU-282987 ameliorated organ damage in SHRs while having little influence on WKY rats. EC indicates endothelial cells.
vagal tone, as evidence by reduced heart rate variability, and increased cardiovascular risk. That is, reduced heart rate variability may be an epiphenomenon associated with impaired activation of anti-inflammatory pathways downstream from α7nAChR activation. Activation of the α7nAChR may therefore represent a novel therapeutic strategy for limiting EOD in hypertension.

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Disclosures
None.

References


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Supplementary Materials

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Supplementary methods

Coarctation hypertension (CH)
Male Sprague Dawley rats (200-250g) were anesthetized with pentobarbital (40 mg/kg, i.p.). Via a medial laparotomy, the abdominal aorta was isolated below the diaphragm and near the exit of the superior mesenteric artery. A blunt needle (0.8 mm OD) was then placed along the side of the isolated aorta segment. A cotton thread was used to constrict the aorta. The needle was then removed, and the abdomen was sutured. After surgery, all rats were treated daily with penicillin (80,000U, i.m.) for three days.

Two kidney one clip (2K1C) operation
Male α7nAChR−/− mice and wild type (WT) mice (20-25 g) were anesthetized with a combination of ketamine (40 mg/kg, i.p.) and diazepam (6 mg/kg, i.p.). Via a left flank incision, a silver clip (0.12 mm opening width, Exidel SA, Lausanne, Switzerland) was placed on the left renal artery. After surgery, all mice were treated daily with penicillin (25,000 U, i.m.) for three days.

Blood pressure measurement
Rats were anesthetized with a combination of ketamine (40 mg/kg, i.p.) and diazepam (6 mg/kg, i.p.). A floating polyethylene catheter was inserted into the lower abdominal aorta via the left femoral artery for blood pressure (BP) and HR measurement, and another catheter was inserted into the left femoral vein for drug administration. The catheters were exteriorized through the interscapular skin. After a 2-day recovery period, the animals were placed in individual cylindrical cages containing food and water for BP recording. The aortic catheter was connected to a BP transducer via a rotating swivel that allowed the animals to move freely in the cage. In CH rats, supra-diaphragmatic arterial pressure was measured via the left common carotid artery while sub-diaphragmatic arterial pressure was measured via the left femoral artery, from 16 weeks after abdominal aorta coarctation. In 2K1C mice, SBP was measured using the tail-cuff technique 8 weeks after placement of the clip. Only mice with a SBP higher than 140 mmHg were used in this experiment.

To assess cardiac vagal tone in rats, SBP, DBP and HR were averaged over the 5 min periods before and after atropine injection (0.03 mg/kg, i.v.). To determine the acute effects of PNU-282987 (0.38 and 3.8 mg/kg, iv) on blood pressure, SBP, DBP and HR were recorded during the period one hour before and one hour after drug administration in conscious SHR.

Baroreflex sensitivity (BRS), which has been shown to mainly reflect vagal tone, was measured in rats using the method described previously. The principle of this method is to measure the prolongation of heart period in response to an elevation of SBP. A bolus injection of phenylephrine (1–5 μg/kg) was given to raise SBP between 20 and 40 mmHg. The relationship between heart period and SBP was determined using linear regression analysis to provide BRS (msec/mmHg).

Histological analysis by light and electron microscopy
The fresh heart, kidney and aorta were immersed in a 4% solution of paraformaldehyde in PBS and were fixed in this solution for 24 h. For light microscopy, tissues were washed,
dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 μm) were cut transversely, and then stained with hematoxylin and eosin (HE) for light microscopic investigation. For electron microscopy, tissues were fixed as described above and then postfixed with osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin. Samples were sectioned (50 nm), counterstained with uranyl acetate and lead citrate and observed with a Hitachi H-800 Transmission Electron Microscope (Hitachi, Japan).

**Morphological examination**

The animals were weighed and anesthetized with pentobarbital (40 mg/kg, i.p). The thoracic and peritoneal cavities were immediately opened. The right kidney, aorta and heart were excised and rinsed in cold physiological saline. The right kidney and the heart were gently blotted dry so that kidney weight and left ventricular weight were determined. At the same time, the aorta was cleaned of adhering fat and connective tissue. A 30-mm-long segment of thoracic aorta was harvested from just below the branch of the left subclavian artery, blotted dry and weighed. The ratio of left ventricular weight to body weight (LVW/BW), aortic weight to the length of aorta (AW/length) and right kidney weight to body weight (RKW/BW) were calculated. In the kidney, glomerulosclerosis scores (GSS) were calculated as follows: 0, no sclerosis; 1, < 25% sclerotic changes in glomerulus; 2, 25%–50%; 3, 50%–75%; and 4, > 75%.

**RNA extraction and real-time quantitative PCR analysis**

Total RNA was extracted from several rat tissues using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. The RNA was treated with RNase-free DNase I to reduce the risk for genomic DNA contamination and 2 μg RNA was reverse transcribed to cDNA using the M-MLV enzyme (Promega, Madison, WI). Real-time quantitative PCR was performed using the Chromo4™ real-time PCR detection system (Bio-Rad) and the SYBR Premix Ex Taq Mixture (Takara) with specific primers. The PCR reactions were initiated with denaturation at 95°C for 10 s, followed by amplification with 40 cycles at 95°C for 10 s, and annealing at 60°C for 20 s (two-step method). Finally, melting curve analysis was performed from 60°C to 85°C. Data were evaluated with Opticon Monitor™ version 3.0 software. The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. All samples were performed in triplicate. The relative expression of the target genes was normalized to the level of GAPDH in the same cDNA.

The following primers designed with Primer Express Software were used: rat α7nAChR (accession no. NM_012832.3), 5’ GGTCGTATATGGCCGTTTG 3’ (sense) and 5’ TGCGGTTGGCGATGTAGCG 3’ (antisense); rat GAPDH (accession no. NM_017008.3), 5’ AGACCTCTATGCCAACACAGTGC 3’ (sense) and 5’ GAGCCACCAA TCCACACAGGT 3’ (antisense).

**Protein extraction and western blot analysis**

Tissues were washed in PBS and homogenized in a Tris-HCl buffer (20 mmol/L, pH 7.5) containing EDTA (2 mmol/L), NP-40 (1% w/v), Triton-100 (1% w/v), PMSF (2 mmol/L), leupeptin (50 µg/ml), aprotinin (25 µg/ml), pesptatin A (10µg/ml) and dithiothreitol (DTT2 mmol/L). The supernatant was obtained by centrifugation at 11,000 g for 15 min
at 4°C. For studies of NF-κB signaling, nuclear protein was extracted using a Nuclear Protein Extraction Kit (Pierce Chemical Co.). The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China). Samples of approximately 30 µg were run on 10% SDS-PAGE. The proteins were electro-transferred to PVDF membranes. The PVDF membranes were incubated with primary antibody (anti-α7nAChR, Sigma-Aldrich, 1:5,000; anti-vesicular acetylcholine transporter (VACHT), Chemicon, 1:500; anti-p65 NF-κB, Santa-cruz, 1:250; anti-IκB, Santa-cruz, 1:200) for 2 hours at 25°C and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:1,000 dilution; Bethyl Laboratories Inc). The bound antibody was visualized on a Kodak Biomax film (Eastman Kodak, Rochester, NY) using a Supersignal substrate chemiluminescence detection kit (Pierce). The relative expression of the target proteins was normalized to the level of GAPDH, or lamin B, or actin in the same sample.

**Determination of tissue inflammatory factors levels by ELISA**

*Rats:* Forty-week-old SHR and WKY rats were anesthetized with pentobarbital (40 mg/kg, i.p). Then, the heart, kidney and aorta were harvested and washed three times in PBS, homogenized, centrifuged at 11,000 g at 4°C for 10 min, and the supernatant was obtained. Protein was quantified with a BCA Protein Assay Kit (Beyotime, China) and then the levels of interleukin 1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were measured with commercial ELISA kits (RapidBio Lab, Calabasas, CA, USA), following the instructions of the manufacturer. The detection limits of the assays were 5 pg/ml for IL-1β and TNF-α and 10 pg/ml for IL-6, with intraassay and interassay coefficients of variation of less than 10% in all cases.

*Mice:* Eight weeks after surgery to induce 2K1C hypertension, α7nAChR−/− mice and WT mice were anesthetized with pentobarbital (40 mg/kg, i.p). Blood samples were collected. Blood was centrifuged at 3,000 g for 15 min at 4°C to collect serum. The serum was kept at -80°C until analyzed. The levels of IL-1β, IL-6, and TNF-α were measured with commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). The detection limits of the TNF-α, IL-6 and IL-1β assays were, respectively, 5.1, 1.6 and 3.0 pg/ml. Intra and interassay coefficients of variation for all assays was ≤10%.

**Isolation of peritoneal macrophages**

To assess the effects of chronic treatment with PNU-282987 on α7nAChR expression in peritoneal macrophages, SHR aged 36 weeks old received a daily injection of PNU-282987 (ip, 0.38 mg/kg) or its vehicle (0.4% DMSO in saline) for 28 days. Rats were sacrificed after the last injection. Hanks' balanced salt solution was injected into the abdomen of each rat. Macrophages in Hanks' balanced salt solution were removed from the abdomen, collected by centrifugation, and cultured in DMEM containing 10% FBS for 4 h at 37°C in 5% CO2. Adherent cells were harvested, and approximately 95% of them were macrophages.
References


### Table S1. Arterial pressure and heart rate before and after administration of atropine (iv, 0.03 mg/kg) in WKY and SHR

<table>
<thead>
<tr>
<th>Group</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>HR (bpm)</th>
<th>ΔHR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>4 w (n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>128 ± 2</td>
<td>130 ± 5</td>
<td>81 ± 5</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>SHR</td>
<td>129 ± 6</td>
<td>132 ± 7</td>
<td>87 ± 4</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>20 w (n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>137 ± 4</td>
<td>139 ± 4</td>
<td>90 ± 2</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>SHR</td>
<td>178 ± 8</td>
<td>178 ± 9</td>
<td>120 ± 7</td>
<td>121 ± 7</td>
</tr>
<tr>
<td>40 w (n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>140 ± 2</td>
<td>142 ± 2</td>
<td>92 ± 3</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>SHR</td>
<td>175 ± 6</td>
<td>178 ± 4</td>
<td>119 ± 6</td>
<td>120 ± 4</td>
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</tbody>
</table>

Values are mean ± SEM. *P<0.01 vs Pre-atropine (paired t-test). †P<0.01 vs age-matched WKY (unpaired t-test). SBP, Systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Pre, Pre-atropine; Post, Post-atropine; bpm, beats per minute; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>HR (bpm)</th>
<th>BRS (ms/mmHg)</th>
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<tbody>
<tr>
<td>WKY (4 w)</td>
<td>7</td>
<td>127 ± 3</td>
<td>81 ± 3</td>
<td>455 ± 10</td>
<td>0.22 ± 0.04</td>
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<tr>
<td>SHR (4 w)</td>
<td>7</td>
<td>133 ± 4</td>
<td>88 ± 3</td>
<td>446 ± 20</td>
<td>0.26 ± 0.03</td>
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<tr>
<td>WKY (20 w)</td>
<td>8</td>
<td>137 ± 2</td>
<td>91 ± 3</td>
<td>310 ± 6</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td>SHR (20 w)</td>
<td>8</td>
<td>182 ± 6*</td>
<td>125 ± 5*</td>
<td>326 ± 11</td>
<td>0.32 ± 0.02*</td>
</tr>
<tr>
<td>WKY (40 w)</td>
<td>7</td>
<td>140 ± 2</td>
<td>93 ± 3</td>
<td>319 ± 7</td>
<td>0.65 ± 0.04</td>
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<tr>
<td>SHR (40 w)</td>
<td>7</td>
<td>178 ± 6*</td>
<td>123 ± 5*</td>
<td>340 ± 6</td>
<td>0.28 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *P<0.01 vs age-matched WKY (unpaired t-test). SBP, Systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; bpm, beats per minute; BRS, baroreflex sensitivity; WKY, Wistar–Kyoto rats; SHR, spontaneously hypertensive rats.
Table S3. Arterial pressure and heart rate before and after administration of atropine (iv, 0.03mg/kg) in sham-operated rats and rats with hypertension induced by abdominal aorta coarctation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham (16 w)</th>
<th>CH (16 w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-atropine</td>
<td>Post-atropine</td>
</tr>
<tr>
<td>SBP-above (mmHg)</td>
<td>127 ± 3</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>SBP-below (mmHg)</td>
<td>125 ± 6</td>
<td>121 ± 2</td>
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<tr>
<td>HR (bpm)</td>
<td>371 ± 6</td>
<td>404 ± 9 ‡</td>
</tr>
<tr>
<td>Δ HR (bpm)</td>
<td>34 ± 3</td>
<td>19 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of n=8 in each group. *P < 0.01 vs sham-operated group
Pre-atropine (unpaired t-test); †P < 0.01 vs SBP-above (paired t-test); ‡P < 0.01, § P < 0.05 vs Pre-atropine (paired t-test); ||P < 0.01 vs WKY (unpaired t-test). SBP-above, Systolic blood pressure measured at carotid artery; SBP-below, Systolic blood pressure measured at abdominal aorta; HR, heart rate; bpm, beats per minute; Sham, sham-operated rats; CH, coarctation hypertensive rats.
Table S4. Arterial pressure and heart rate after single acute doses of PNU-282987 (iv, 0, 0.38, 3.8mg/kg) or its vehicle in SHR

<table>
<thead>
<tr>
<th>Variable</th>
<th>PNU-282987 (mg/kg)</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>SBP (mmHg)</td>
<td>168 ± 4</td>
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<tr>
<td>DBP (mmHg)</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>334 ± 8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of n=10 per group. SBP, Systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; bpm, beats per minute; SHR, spontaneously hypertensive rats.
Table S5. Arterial pressure and heart rate in SHR and WKY after chronic PNU-282987 administration (ip, 0.38 mg/kg/day for 28 days)

<table>
<thead>
<tr>
<th>Variable</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>PNU-282987</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126 ± 6</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>89 ± 3</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>328 ± 10</td>
<td>335 ± 9</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of n=8 per group. SBP, Systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; bpm, beats per minute; WKY, Wistar–Kyoto rats; SHR, spontaneously hypertensive rats.
Figure S1. Effects of chronic treatment (4 weeks) with PNU-282987 on α7nAChR protein expression in WKY and SHR. PNU-282987 treatment did not significantly alter α7nAChR protein expression in tissues of heart, kidney and aorta from WKY (A) and SHR (B). Expression of α7nAChR in heart, kidney and aorta was significantly less in SHR compared to WKY rats (B). Protein levels of α7nAChR in peritoneal macrophages from SHR were not significantly altered by PNU-282987 treatment (C). Data are mean ± SEM (n=8 in each group). *P < 0.05 compared with WKY rats (Tukey’s test or unpaired t-test).