Acetylcholinesterase Inhibition Attenuates the Development of Hypertension and Inflammation in Spontaneously Hypertensive Rats

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BACKGROUND
It is hypothesized that chronic increase of availability of acetylcholine, resulting from the effect of antiacetylcholinesterases, may prevent autonomic imbalance and reduce inflammation yielding beneficial effects for cardiovascular disorders in hypertension. The effect of long-term administration of antiacetylcholinesterase agents with central and/or peripheral action, i.e., donepezil and pyridostigmine, were investigated on arterial pressure (AP), sympathovagal balance, plasma cytokine levels, and cardiac remodeling in spontaneously hypertensive rats (SHR).

METHODS
Chronic treatment with donepezil or pyridostigmine started before the onset of hypertension. AP was measured by plethysmography every 4 weeks. At the end of 16 weeks of treatment, methylatropine was used to evaluate the cardiac vagal tone; AP and pulse interval (PI) variability were also evaluated followed by plasma and heart collection for analysis.

RESULTS
Pyridostigmine, which does not cross the blood–brain barrier, increased cardiac vagal tone, and reduced cardiomyocyte diameter and collagen density, but did not affect the AP and plasma cytokine levels. Donepezil, which crosses the blood–brain barrier, attenuated the development of hypertension, increased cardiac vagal tone, and improved AP and PI variability. Likewise, donepezil reduced the plasma levels of tumor necrosis factor-α, interleukin 6, and interferon γ, besides reducing cardiomyocyte diameter and collagen density.

CONCLUSIONS
Donepezil attenuated the development of hypertension in SHR probably involving antiinflammatory effects, indicating that acetylcholinesterase inhibition yields beneficial effects for antihypertensive therapy.

Keywords: autonomic dysfunction; blood pressure; donepezil; hypertension; pharmacological therapy; pyridostigmine; vagal stimulation.

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Autonomic dysfunction in arterial hypertension is characterized by enhanced sympathetic activity and withdrawal of parasympathetic control.1 Current treatments focus on strategies for attenuating the sympathetic modulation of the cardiovascular system. Although the protective effect of the parasympathetic system on the cardiovascular system is not often taken into account, it may lead to an interesting alternative approach for antihypertensive therapy.2-5 The inflammatory reflex is a proposed reflex circuit that includes sensory and motor arms centered in the vagus nerve.6,7 The efferent signaling culminates in the T cell release of acetylcholine, which interacts with alpha 7 nicotinic acetylcholine receptors on immunocompetent cells to inhibit the release of cytokines from macrophages.5,7 Thereby, drugs that augment acetylcholine availability at the neuroeffector junction may be expected to have antiinflammatory effects. Antiacetylcholinesterase agents prevent the hydrolysis of acetylcholine, prolonging the availability of acetylcholine in cholinergic nerve endings and increasing the efficiency of cholinergic transmission.8 Acetylcholinesterase inhibitors, such as donepezil (DON) and pyridostigmine (PYR), have been used in experiments to increase parasympathetic activity, and the results showed improved cardiac function and attenuated cardiac remodeling in a rat model of heart failure.2,5,9 PYR is a reversible antiacetylcholinesterase agent that does not cross the blood–brain barrier and acts specifically in peripheral synaptic clefts.8 DON crosses the blood–brain barrier and has been used in the treatment of Alzheimer’s disease.10 Reale et al.11 demonstrated that a 1-month administration of DON to patients with Alzheimer’s disease led to reduced levels of oncostatin M, interleukin (IL)-6, and IL-1 in peripheral blood mononuclear cells, suggesting a possible antiinflammatory effect of this centrally acting antiacetylcholinesterase agent. DON also produced antiinflammatory effects in apolipoprotein E-knockout mice that showed suppressed expression of monocyte chemoattractant protein-1 and tumor necrosis factor-α (TNF-α) in the aorta.3

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The immune system plays a role in the development and maintenance of hypertension; thus, we hypothesized that the increased availability of acetylcholine in the synaptic cleft that results from chronic treatment with an antiacetylcholinesterase inhibitor (DON or PYR) may prevent autonomic imbalance, reduce inflammation, and produce beneficial effects on the cardiovascular disorders observed in spontaneously hypertensive rats (SHR). Therefore, we investigated the effects of a long-term (16 weeks) administration of antiacetylcholinesterase agents with central and/or peripheral action (DON and PYR, respectively) on sympathovagal balance, plasma cytokine levels, and cardiac remodeling in SHR.

METHODS

Animals

All experimental procedures adhered to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Copyright © 1996 by the National Academy of Sciences), and were approved by the Committee of Ethics in Animal Research of the School of Medicine of Ribeirão Preto, University of São Paulo, SP, Brazil (Protocol no. 126/2011). Experiments were performed in male SHR and Wistar–Kyoto rats (WKY) supplied by the Multidisciplinary Center for Biological Research (CEMIB), UNICAMP, Campinas, SP, Brazil. The animals were housed individually with free access to food and water and were maintained on a 12-h light-dark cycle.

Experimental protocol

DON or PYR treatment was started before the onset of hypertension, when the SHR were 5 weeks old. The rats were anesthetized with isoflurane (induction: 5%; maintenance 1.5–3%) and implanted with subcutaneous osmotic minipumps (Alzet, Model 2004) between the scapulae to administer pyridostigmine bromide (Valeant Pharmaceutical Co., Waltham, MA, USA) at 1.4 mg/kg/day, the concentration required to inhibit plasma acetylcholinesterase activity by ~50%, or donepezil (Biotang Inc., Waltham, MA, USA) at 1.4 mg/kg/day, the concentration required to inhibit ~60% of brain acetylcholinesterase activity. The drugs were administered for 16 weeks, and the osmotic minipumps were changed 4 times during this period. No adverse outcomes induced by PYR or DON were observed during the administration to the rats. Age-matched control SHR and WKY rats underwent the same surgical procedure. The development of hypertension was accompanied by indirect measurements of systolic arterial pressure (SAP) every 4 weeks by the tail-cuff method. At the end of the treatment, the animals were anesthetized with isoflurane (induction: 5%; maintenance 1.5–3%) and instrumented with polyethylene catheters in the femoral artery and vein. After 24h of recovery, the arterial pressure (AP) was recorded directly from the femoral artery of conscious rats. The basal AP was recorded for 1h followed by a bolus injection of methylatropine (1 mg/kg, iv; Sigma-Aldrich, ST Louis, MO, USA). The increase in mean heart rate (HR) produced by methylatropine was considered the cardiac vagal tone, as described previously. Fifteen minutes after the administration of methylatropine, a bolus of a solution with propranolol (2 mg/kg iv; Sigma-Aldrich) was given to the rats, and the AP was continuously recorded during another 15 minutes. The HR measured when both autonomic blockers were administered was considered the intrinsic HR. After the hemodynamic recordings, the rats were killed by a sodium pentobarbital overdose (Rhobifarma Indústria Farmacêutica Ltda, Hortolândia, SP, Brazil). The heart was collected to analyze myocyte size and interstitial collagen density. Basal AP recordings were used to examine SAP and pulse interval (PI) variability.

Arterial pressure and heart rate measurement

The arterial catheter was connected to a pressure transducer (MLT844; ADInstruments, Sydney, Australia). The pulsatile blood pressure was amplified (ML224; ADInstruments), fed to an IBM personal computer connected to a PowerLab system (ML866/P; ADInstruments) and continuously sampled (2 kHz). Beat-by-beat time series of the SAP were generated. The PI and HR were measured from successive diastolic PIs.

Systolic blood pressure and pulse interval variability analysis

The SAP and PI variability analysis was performed in the time and frequency domains by the computer program CardioSeries (v1.0; http://sites.google.com/site/cardioseries). The variance was calculated for use as the time-domain parameter. Frequency-domain analyses were performed on 15-minute stationary series. The 15-minute series were divided into 34 segments with 512 points each, with an overlap of 50% (Welch Protocol). The stationary values of each segment were visually examined, and the artifacts were excluded. Each stationary segment was subjected to spectral analysis using a fast Fourier transform algorithm after the application of a Hanning window. The SAP and PI spectra were integrated for bands with low (LF: 0.2–0.75 Hz) and high (HF: 0.75–3 Hz) frequencies. The mean power of the spectra in both frequency bands, LF and HF, was used to express the LF and HF power from the SAP and PI spectrum for each rat. The relative power (%) of the spectra in each frequency band and the ratio between the powers of LF and HF (LF/HF) were then determined.

Morphological and morphometric analysis

The hearts were cut transversely, fixed in phosphate-buffered 10% formalin, and embedded in paraffin. Each block was serially cut 6 μm from the midventricular surface. The sections were stained with hematoxylin and eosin or picrosirius red. The minor diameter of the myocytes was measured using Leica Qwin software (Leica Imaging Systems, Cambridge, UK) in conjunction with a Leica microscope.
video camera, and an online computer. Approximately 30 values were obtained per rat. The mean value was then calculated. To estimate the volume fraction (%) of collagen in picrosirius red-stained sections, a medium-power light-microscopy field was quantitatively examined. For each heart, 15 fields per rat were randomly selected and analyzed using Leica Qwin software (Leica Imaging Systems, Cambridge, UK), and the mean value was calculated.

Plasma norepinephrine and cytokines Assay

Rats were killed by decapitation after the end of 16 weeks of treatment with the antiacetylcholinesterase agents. Blood samples were collected, centrifuged, and the plasma was stored at −80 °C until the assays were conducted. Norepinephrine (NE) was analyzed by high performance liquid chromatograph as previously described.16 TNF-α, IL-6, and interferon (IFN)-γ were measured by ELISA according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Summary data are presented as the mean ± SEM and the level of significance was set at $P < 0.05$. Comparisons between groups of animals or treatments were made by 2-way analysis of variance. The Student–Newman–Keuls test was used for post hoc comparisons.

RESULT

Arterial pressure and heart rate

Figure 1 shows representative AP recordings from WKY rat and SHR from control, SHR + PYR or SHR + DON group. PYR did not change SAP. However, DON delayed the onset and reduced the hypertensive levels later in life (Figure 1, panels A and B). Basal HR (Table 1) was higher in control SHR than that in the WKY rats; nevertheless, DON reduced the basal HR in the SHR + DON group to a level that was similar to the level in WKY rats, while PYR had no effect.

Sympathovagal balance

The HR response to the pharmacological blockade of muscarinic cholinergic receptors revealed impairment of the vagal tone in the control SHR (Table 1). SHR + DON and SHR + PYR groups showed higher vagal tone (tachycardic response induced by methylocaripine) as compared to the SHR group (Table 1), confirming that both acetylcholinesterase inhibitors were able to produce cardiac parasympathetic activation. Besides, SHR + PYR showed a higher intrinsic HR than that of the control SHR or SHR + DON (Table 1). Analysis of the SAP and PI variability in the time (variance) and frequency (spectral analysis) domains is shown in Figure 2. The control SHR displayed elevated SAP variance and LF power compared with those in the WKY rats (Panels A and B). The control SHR also exhibited a reduced PI HF

![Figure 1](https://academic.oup.com/ajh/article-abstract/28/10/1201/2743214)

**Figure 1.** Panel A shows typical recordings of basal pulsatile and mean arterial pressure (white line) from conscious Wistar–Kyoto (WKY) rat, control spontaneously hypertensive rat (SHR), and SHR treated with pyridostigmine (PYR) or donepezil (DON). Panel B displays the systolic arterial pressure (SAP) measured by the tail-cuff method during the development of hypertension (0, 4, 8, and 12 weeks of treatment) and the SAP measured directly from the femoral artery at the end of the study (16 weeks of treatment). WKY: $n = 10$; control SHR: $n = 7$; SHR + PYR: $n = 6$; SHR + DON: $n = 7$. *$P < 0.05$ compared with WKY group; †$P < 0.05$ compared with control SHR group. ‡$P < 0.05$ compared with SHR + PYR group.
power as well as high PI LF power and LF/HF ratio (Panels D, E, and F). DON reduced both the SAP variance and the LF power (Panels A and B) and also normalized the PI LF, HF, and LF/HF power (Panels D, E, and F). The PI variance was similar among the 4 groups studied (Panel C). The SAP and PI variability of SHR + PYR did not differ from those of the control SHR. Plasma NE (Table 1) was significantly higher in the control SHR than that in the WKY rats; however, it was not changed by PYR or DON.

**Plasma cytokines**

The plasma levels of TNF-α, IL-6, and IFN-γ were elevated in the control SHR (Figure 3, panels A, B, and C). The chronic administration of DON reduced the TNF-α, IL-6, and IFN-γ levels, while PYR did not produce the same effect (Figure 3).

**Cardiac remodeling**

The minor diameter of the myocytes (Figure 4, panels A and C) and the collagen density (Figure 4, panels B and D) were greater in the control SHR than in the WKY rats. Both the minor diameter of the myocytes and the collagen density were attenuated by the chronic administration of PYR or DON (Figure 4).

**DISCUSSION**

This study showed that the long-term administration of DON, an anticholinesterase agent that crosses the blood–brain barrier, attenuated the development of hypertension, improved the cardiovascular autonomic modulation, reduced the plasma proinflammatory cytokine levels and prevented the cardiac remodeling in SHR, providing evidence that the blockade of acetylcholinesterase is capable of producing beneficial effects for the treatment of hypertension. PYR, an anticholinesterase agent that does not cross the blood–brain barrier, also improved cardiac vagal tone and prevented cardiac remodeling, although it was unable to reduce high blood pressure and plasma cytokine levels.

The cardiac parasympathetic activation produced by DON and PYR was confirmed by the higher tachycardic response induced by the atropine test. Although both of the tested acetylcholinesterase inhibitors elicited the same increase in cardiac vagal tone, only DON was able to attenuate the development of hypertension in SHR, suggesting that
this antihypertensive effect cannot be attributed to a direct improvement in cardiac parasympathetic tone. Furthermore, only DON showed an antiinflammatory effect, which may have played a role in the attenuation of the hypertension. Inflammation in some particular regions of the brain, which are essential for AP control, is associated with alterations in cardiovascular autonomic modulation and the development of arterial hypertension.4,17 Cytokines can be produced locally in the brain by glia and neurons.4 Evidence suggests that cytokines that are produced peripherally and in the brain contribute to the pathophysiology of hypertension.17 Intracerebroventricular infusion of minocycline, a broad spectrum antibiotic from the tetracycline family, decreased the expression of proinflammatory cytokines (IL-6, IL-1, and TNF-α) in the paraventricular nucleus, attenuated the AP increase and reduced the plasma NE in rats that were chronically infused with angiotensin II.4 Acetylcholine attenuates tissue inflammation by decreasing the release of proinflammatory cytokines;5,7 therefore, it is possible that the increased availability of acetylcholine in the synaptic cleft that resulted from the central inhibition of acetylcholinesterase may produce antiinflammatory effects in the areas of the central nervous system controlling AP, attenuating the development of hypertension. However, this antihypertensive effect does not seem to be mediated by a reduction in sympathetic hyperactivity because DON treatment did not change plasma NE level. On the other hand, the central nervous system plays a role in AP regulation via several neurohumoral factors, such as angiotensin II and vasopressin,18 which might also be involved. Awasthi et al.19 demonstrated that DON reduced the brain angiotensin II converting enzyme activity and oxidative stress elevation induced by

Figure 3. Levels of plasma tumor necrosis factor-alpha (TNF-α, panel A), interleukin-6 (IL-6, panel B) and interferon gamma (IFN-γ, panel C) in Wistar-Kyoto rats (WKY, n = 8), control spontaneously hypertensive rats (SHR, n = 7) and SHR treated with pyridostigmine (PYR, n = 8) or donepezil (DON, n = 7). *P < 0.05 compared with the WKY group; †P < 0.05 compared with the control SHR; ‡P < 0.05 compared with SHR + PYR group.

Figure 4. Photomicrographs and bar graphs of the minor diameter of myocytes (Panels A and C) and collagen density (Panels B and D) from the left ventricle of Wistar-Kyoto rats (WKY; n = 8), control spontaneously hypertensive rats (SHR; n = 6) and SHR treated with pyridostigmine (PYR; n = 6) or donepezil (DON; n = 8). *P < 0.05 compared with the WKY group; †P < 0.05 compared with the control SHR. Bar = 50 µm.
colchicine. Furthermore, peripheral mechanisms, in addition to the central mechanisms, should be taken into consideration because the release of cytokines by macrophages and T cells promotes vasoconstriction, vascular remodeling and sodium retention, leading to increased AP.\textsuperscript{18}

The administration of acetylcholinesterase inhibitors with central action has been shown to decrease proinflammatory cytokines levels in various experimental models of inflammation;\textsuperscript{37} however, the anti-inflammatory effects of peripherally acting acetylcholinesterase inhibitors are not well understood.\textsuperscript{17} SHR chronically treated with PYR did not show reduced levels of plasma cytokines. During murine endotoxemia, peripheral administration of galantamine, a centrally acting acetylcholinesterase inhibitor, reduced serum TNF levels and protected against lethality.\textsuperscript{38} These effects were mediated through brain muscarinic receptors and required an intact vagus nerve.\textsuperscript{20} Also during endotoxemia, physostigmine, an acetylcholinesterase inhibitor that penetrates the blood–brain barrier, reduced capillary leakage and the interaction between leukocytes and the endothelium.\textsuperscript{21} However, neostigmine, a quaternary acetylcholinesterase inhibitor that cannot cross the blood–brain barrier, failed to exert protective effects against the histopathologic organ injury produced by endotoxemia\textsuperscript{22} and failed to suppress the lung inflammatory response induced by mechanical ventilation in mice.\textsuperscript{23} It was also demonstrated that the antiinflammatory effects produced by galantamine during endotoxemia were blocked by atropine sulfate, a muscarinic receptor antagonist that crosses the blood–brain barrier, but not by atropine methyl nitrate, which does not cross the blood–brain barrier.\textsuperscript{20} These data suggest that the antiinflammatory effects produced by acetylcholinesterase inhibitors are related to a central cholinergic pathway, which explains why PYR did not produce anti-inflammatory effects. Moreover, other mechanisms should be taken into account, since it has been demonstrated that DON may act inhibiting the renin–angiotensin system and the oxidative stress in the central nervous system and also in the aorta.\textsuperscript{3,19}

The chronic administration of DON prevented the increase in HR observed in control SHR compared to normotensive WKY, and this effect may be attributed to the improvement in vagal tone. In contrast, the same vagal tone increment caused by PYR did not prevent the increase in HR in this group, which may be attributed to the higher intrinsic HR. A PYR-induced increase in intrinsic HR has been reported previously in rats with heart failure; however, the underlying mechanism was not clear.\textsuperscript{24}

It is well established that the adverse impact of hypertension on cardiovascular risk depends not only on the increase in absolute AP values but also on the augmented AP variability.\textsuperscript{25} Accumulating evidence indicates that an increase in AP variability is associated with the development, progression, and severity of cardiac, vascular, and renal damage and with an increased risk of cardiovascular events and mortality independently of the elevated AP.\textsuperscript{26} The SHR + DON group showed reduced SAP variance and LF power. The degree of AP oscillation mainly reflects the influence of central and reflex autonomic modulation, the elastic properties of arteries and the effects of humoral factors, such as angiotensin II, bradykinin, endothelin-1, insulin, and nitric oxide.\textsuperscript{26} DON also increased the PI HF power, indicating an increase in cardiac vagal modulation. It has been documented that parasympathetic modulation predominates over the sympathetic modulation of the sinoatrial node,\textsuperscript{27} consistent with the reduced PI LF and LF/HF ratio observed in SHR + DON.

The increase in left ventricular mass in hypertensive patients, particularly in its concentric form, has been shown to be associated with a significant increase in the all-cause mortality for all levels of AP.\textsuperscript{28} DON and PYR reduced the left ventricular cardiomyocyte diameter and collagen density in SHR. These protective effects do not appear to be dependent on the reduction in AP or proinflammatory cytokines levels since PYR and DON produced similar results. Chronic administration of DON\textsuperscript{29} or PYR\textsuperscript{30} attenuated the cardiac remodeling in a heart failure model. The cardiac hypertrophy in hypertension is associated with increased stiffness of large arteries.\textsuperscript{29} Inanaga et al.\textsuperscript{31} demonstrated that DON attenuated the atherogenesis in apolipoprotein E-knockout mice that were fed a high-fat diet with or without angiotensin II stimulation, possibly through antioxidative and antiinflammatory effects. Furthermore, acetylcholine dilates arterial vessels by activating endothelial nitric oxide synthase.\textsuperscript{30} It is possible that the increase in nitric oxide production is mediated by acetylcholinesterase inhibition. Therefore, the inhibition of acetylcholinesterase and the resulting increase in acetylcholine availability may have pronounced effects on the vascular bed, decreasing the cardiac afterload and preventing cardiac remodeling. However, clinical evidence has also demonstrated that sympathetic hyperactivity is related to the damage in target organs observed in hypertension.\textsuperscript{31} In the heart, parasympathetic modulation exceeds sympathetic effects, most likely through 2 independent mechanisms: (i) acetylcholine can act on the muscarinic receptors situated in sympathetic presynaptic nerve endings, thus inducing a reduction in NE release,\textsuperscript{32} and (ii) cholinergic stimulation may inhibit the increase in intracellular cAMP,\textsuperscript{33} thus inhibiting the adrenergic signaling cascade.\textsuperscript{37} It has been shown that PYR was effective at preventing the hypertrophic effect of isoproterenol in neonatal cardiomyocytes maintained in culture.\textsuperscript{34} Hence, the sympathetic drive inhibition in the heart produced by parasympathetic stimulation may also explain the DON- and PYR-induced attenuation of cardiac remodeling.

DON is the most used member of the acetylcholinesterase inhibitors class for treating Alzheimer's disease.\textsuperscript{35} Clinical trials that examined the safety and tolerability of donepezil for patients with Alzheimer's disease did not report changes in AP.\textsuperscript{36,37} Nevertheless, studies that examined the association between acetylcholinesterase inhibitor usage and cardiovascular mortality in patients with Alzheimer's disease reported that the acetylcholinesterase inhibitor users had a reduced risk of myocardial infarction and cardiovascular mortality than nonusers.\textsuperscript{38,39} This association strengthened with increasing the acetylcholinesterase inhibitor dose.\textsuperscript{38} The findings of this study are in line with these previous data, supporting the notion that acetylcholinesterase inhibitors that cross the blood–brain barrier have beneficial effects for improving cardiovascular outcomes.

In conclusion, the long-term (16 weeks) administration of DON that began during the prehypertensive phase in
SHR attenuated the development of hypertension probably involving antiinflammatory effects, providing evidence that a pharmacological approach involving acetylcholinesterase inhibition yields beneficial effects for antihypertensive therapy.

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DISCLOSURE

The authors declared no conflict of interest.

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