Activation of Nicotinic Receptors Can Contribute to Endothelium-Dependent Relaxations to Acetylcholine in the Rat Aorta

Qian Zou, Susan W. S. Leung, and Paul M. Vanhoutte

Department of Pharmacology and Pharmacy, University of Hong Kong, Hong Kong SAR, China (Q.Z., S.W.S.L., P.M.V.); and Department of BIN Fusion Technology, Chonbuk National University, Jeonju, Korea (P.M.V.)

Received January 18, 2012; accepted March 12, 2012

ABSTRACT

Acetylcholine causes endothelium-dependent relaxations in the rat aorta. Both muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs) are expressed in endothelial cells. It is generally accepted that mAChRs are responsible for the endothelium-dependent relaxations evoked by acetylcholine. The present study was designed to investigate whether nAChRs can also be involved in such responses evoked by the cholinergic transmitter. Rings with or without endothelium of aortae of spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) normotensive rats were suspended in organ chambers for the measurement of isometric tension. In WKY aortae the muscarinic antagonist atropine abolished the relaxations to increasing concentrations of acetylcholine, confirming that mAChRs are responsible mainly for the response under control conditions. In SHR aortae, atropine caused only partial inhibition of the endothelium-dependent relaxations to acetylcholine; the remaining decreases in tension were inhibited by the nicotinic antagonist mecamylamine, which did not significantly affect the response in the absence of atropine in either SHR or WKY preparations. Thus, when mAChRs are inhibited, nAChRs mediate relaxation to the cholinergic transmitter in the SHR but not the WKY aorta. Nicotine, a direct agonist of the nicotinic receptor, induced endothelium-dependent relaxations in both SHR and WKY rats via the activation of α7-nAChRs, but not by mecamylamine-sensitive nicotinic receptors (α3 subtype). The acetylcholine-induced, atropine-insensitive relaxations and those to nicotine both involve the phosphatidylinositol 3-kinase/akt pathway. The present study demonstrates that the activation of nAChRs can contribute to acetylcholine-induced, endothelium-dependent relaxations in the aortae of hypertensive animals and suggests that these receptors may contribute to the endothelium-dependent regulation of vascular tone.

Introduction

The endothelium, the thin monolayer of cells that lines the interior surface of all blood vessels, modulates vascular tone by releasing endothelium-derived relaxing factor (EDRF) and contracting factors that help to control the tone of the underlying vascular smooth muscle. Nitric oxide (NO) is the best characterized EDRF and plays a key role in protecting the vascular wall (Boulander, 1999; Furchgott, 1999; Moncada and Higgs, 2006; Taddei et al., 2006; Vanhoutte et al., 2009). Acetylcholine and physiological stimuli (physical forces, circulating hormones, platelet products, and prostaglandins) stimulate the release of EDRFs to evoke endothelium-dependent relaxations in both SHR and WKY rat aortae of hypertensive animals and suggests that these receptors may contribute to the endothelium-dependent regulation of vascular tone.

ABBRiEVATIONS: EDRF, endothelium-derived relaxing factor; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; bp, base pairs; DHβE, dihydro-β-erythroidine hydrobromide; EC, endothelial cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IkCa, intermediate-conductance calcium-activated potassium channel; l-NAME, N-nitro-l-arginine methyl ester; mAChR, muscarinic acetylcholine receptor; MAP, mitogen-activated protein; MEK, MAP kinase kinase; nAChR, nicotinic acetylcholine receptor; NO, nitric oxide; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; SHR, spontaneously hypertensive rat; SKCa, small-conductance calcium-activated potassium channel; WKY, Wistar Kyoto; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzo- 3,4-coumarin-4-one; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole; TRAM-34, 1-[(2-chlorophenyl)di phenylimethyl]-1H-pyrazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-amino phenylthio]butadiene; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-triethenio-1,7-metheno-7H-dibenzo[b,j] [1,5,12,16]tetraazaacyclotricosine-5,13-dilium dibromide.
nerve fibers, the myogenic, and the neurogenic component (Boulanger et al., 1994; Wessler and Kirkpatrick, 2008). However, not all muscarinic receptor subtypes expressed in given blood vessels contribute to the vasodilator response to acetylcholine. Pharmacological experiments with subtype-prefering agents and functional studies using gene-targeted mice deficient in one of three receptor subtypes (M1, M2, and M3) demonstrate that the M2 subtype is the main mediator of endothelium-dependent vasodilatations in response to acetylcholine (Boulanger et al., 1994; Gericke et al., 2011). In addition to muscarinic receptors, a number of neuronal-type nAChR subunits (α2, α3, α4, α5, α6, α7, α9, α10, β2, β3, and β4) have been identified in the endothelium (Brüggmann et al., 2003). Patch-clamp studies demonstrate that human endothelial cells express functional nAChRs (Wu et al., 2009), and nAChRs can mediate proliferation, survival, migration, and tube formation in vitro as well as angiogenesis in vivo (Wang et al., 2001, 2003). Nicotine, a potent nAChR agonist, promotes angiogenesis through the activation of non-reuronal nAChRs (Wang et al., 2003; Wu et al., 2009). Mecamylamine, a nonselective inhibitor of nAChRs, can completely and reversibly inhibit the resulting endothelial network formation. Similar results have been obtained with α-bungarotoxin, a selective inhibitor of α7 nAChRs (Wu et al., 2009). In addition to its angiogenic effect, activation of α7 nAChRs may regulate the release of EDRFs. Thus, the aim of the present study was to verify the hypothesis that nAChRs can contribute to the endothelium-dependent relaxations evoked by the cholinergic transmitter.

Materials and Methods

All animal experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong and were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Animal and Tissue Preparation. Male spontaneously hypertensive rats (SHR; 8, 18, and 36 weeks old) and Wistar-Kyoto (WKY) rats (36 and 60 weeks old) were used. They were housed in a room with standardized temperature (21 ± 1°C) and exposed to a 12-h dark-light cycle. The rats had free access to a standardized diet (LabDiet 5053; PMI Nutrition, St. Louis, MO) and tap water. They were anesthetized with pentobarbital sodium (30 mg/kg) intraperitoneally before sacrifice. Their thoracic aortae were isolated and placed immediately into ice cold Krebs-Ringer buffer with the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, and 11.1 mM glucose (control solution). The adhering fat and connective tissue were removed. The aortae were then cut into rings (approximately 3 mm in length). In some rings, the endothelium was removed mechanically by gently rubbing the intimal surface of the rings with a syringe needle.

Isometric Force. The rings were suspended in organ chambers that contained 5 ml of control solution aerated with 95% O2 and 5% CO2 gas mixture, pH 7.4, at 37°C. Each ring was connected to a force transducer (model MLT0201/D, ADInstruments, Colorado Springs, CO) for isometric tension recording. The rings were stretched to an optimal tension of 2.5 g (determined in preliminary experiments; data not shown) and allowed to equilibrate for 90 min. They then were contracted twice with 60 mM KCl to obtain a submaximal reference contraction. The aortic rings were incubated with vehicle, mecamylamine (nAChR inhibitor; 10−6 M) (Bacher et al., 2009), atropine (nAChR inhibitor; 10−6 M) (Clark, 1926), mecamylamine plus atropine, dihydro-β-erythroidine hydrobromide (DHβE; selective antagonist at α4-nAChRs; 10−6 M) (Roegge and Levin, 2006), α-bungarotoxin (selective antagonist at α7-nAChRs; 10−6 M) (Wu et al., 2009), α-conotoxin (an antagonist at α7 and α9 nAChRs; 10−5 M) (Johnson et al., 1995), N-nitro-l-arginine methyl ester (l-NAME; NO synthase inhibitor; 10−5 M) (Rees et al., 1990), or 1-[2-chlorophenyl]imidophenylmethyl]-1H-pyrazole (TRAM-34; intermediate-conductance calcium-activated potassium channel (IKCa) inhibitor; 5 × 10−7 M) (Ghiaus et al., 2005), and 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-8H-indolo[2,3,b]benzofuro[4,5,6-h10,11,12,16]tetrazaasarcocrypticine-5,13-dium dibromide (UCL 1684; small-conductance calcium-activated potassium channel (SKCa) inhibitor; 5 × 10−7 M) (Ghiaus et al., 2005). To investigate the signaling mechanisms underlying the nicotine- and acetylcholine-induced endothelium-dependent relaxations, rings were incubated with 2-(4-morpholinyl)-8-phenyl-1H-benzopyran-4-one hydrochloride (LY294002; phosphatidylinositol 3-kinase (PI3K) inhibitor; 10−6 M), 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580; p38 mitogen-activated protein (MAP) kinase inhibitor; 10−6 M), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059; selective inhibitor of the MAP kinase kinase MEK1; 10−6 M) and 1,4-diamino-2,3-dicyano-1,4-bis-[2-aminoethyl]hexadiene (U0126; selective inhibitor of MEK1 and MEK2; 10−6 M) (Wu et al., 2009; Banquet et al., 2011).

After 40 min of incubation, the rings were contracted with phenylephrine (10−5 M) and then exposed to cumulatively increasing concentrations of acetylcholine (10−7 to 10−5 M) or nicotine (10−7 to 10−5 M). All experiments were performed in the presence of indomethacin (nonselective cyclooxygenase inhibitor; 10−6 M) (Lüscher and Vanhouute, 1986) to prevent the formation of vasoactive prostaglandins. The concentrations of the inhibitors used in this study were selected either from preliminary experiments (data not shown), previous experience in the laboratory, or the literature.

Detection of nAChR Subunit mRNA using PCR. TRIZol reagent (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions to extract RNA from aortic samples. First-strand cDNA synthesis was performed by using a PrimeScript II First Strand cDNA Synthesis Kit (Takara, Dalian, China). Gene information for the nAChR subunits was obtained from the National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore), and all primers sequences (Table 1) were designed by using the GenScript Real Time PCR Primer Design Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore), and all primers sequences (Table 1) were designed by using the GenScript Real Time PCR Primer Design Center (http://www.genscript.com/ssl-bin/app/primer). The PCR assay was carried out by using 46 μl of PCR SuperMix, 1 μl of each primer (10−6 M), and 2 μl of reverse-transcribed product by using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: 95°C for 30 s for denaturing, 95°C for 1 min for annealing, and 72°C for 1.5 min for Taq activity. The PCR was performed for 40 cycles with a final 10-min extension step. The resulting PCR mix was then analyzed by electrophoresis on 1.2% agarose gels containing GelRed Nucleic Acid Stain (Biotium, Hayward, CA).

Chemicals. Acetylcholine, atropine, α-conotoxin, choline, indomethacin, l-NAME, LY294002, mecamylamine, TRAM-34, UCL 1684, and U0126 were purchased from Sigma (St. Louis, MO). PD98059 was purchased from Merck (Whitehouse Station, NJ). SB203580 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 4-DAMP, DHβE, and α-bungarotoxin were purchased from Tocris Bioscience (Ellisville, MO). A stock solution of indomethacin was prepared in a 5 × 10−3 M sodium bicarbonate solution. All other compounds were prepared in deionized water. The concentrations are given in molar (M) in the bath solution.

Data Analysis. The relaxations induced by acetylcholine or nicotine are expressed as a percentage of the sustained phenylephrine-induced contractions. The effective concentrations causing 50% of the response and the maximal relaxations to either acetylcholine or nicotine obtained in the absence and presence of inhibitors were calculated by Prism version 5 (GraphPad Software Inc., San Diego, CA) from
the individual concentration response curves (Tables 2 and 3). Results are presented as means ± S.E.M. with n referring to the number of rats used. Statistical analysis was performed by Student’s t test for comparison of two groups or two-way analysis of variance. The statistical analysis and the area under the curve calculations were performed with Prism version 5. P values < 0.05 were considered to indicate statistically significant differences.

Results

**nAChR Subunits.** The α2, α3, α4, α5, α6, α7, α9, α10, and β2 subunits of nicotinic receptors were abundantly expressed in both SHR and WKY aortae (Fig. 1).

**Acetylcholine-Induced Relaxations.** Acetylcholine caused relaxations in rings with endothelium of all experimental groups tested; such relaxations were not observed in preparations in which the endothelium had been removed mechanically (Figs. 2 and 3; Table 2). The protein expression of endothelial NO synthase was measured by Western blotting in rings with and without endothelium to further assess the functional integrity of the endothelium; it was significantly lower in rings without endothelium compared with those with endothelium (data not shown).

In aortae of 36-week-old SHR (Fig. 2; Table 2), the relaxations to acetylcholine in mecamylamine-treated rings were similar to those obtained in control preparations; however, the relaxations were reduced but not abolished by atropine. The remaining responses and maximal relaxations in the presence of atropine approximated 50% of the relaxations observed in untreated control preparations, and the responses were abolished by the addition of mecamylamine. Likewise, in aortae of both 8- and 18-week-old SHR (Fig. 3; Table 2), the remaining responses in the atropine-treated rings were similar to those obtained in preparations of 36-week-old SHR and abolished by the addition of mecamylamine.

In aortae of 36-week-old WKY rats (Fig. 3; Table 2), acetylcholine-induced relaxations in mecamylamine-treated (to inhibit nAChRs) (Bacher et al., 2009; Kirshenbaum et al., 2011) rings were not significantly different from those observed in control preparations, but were abolished by atropine (10^-5 M; to inhibit mAChRs) (Clark, 1926). The maximal relaxations were not significantly different in rings treated with atropine alone and with mecamylamine. In aortae of 60-week-old WKY rats (Fig. 3; Table 2), the acetylcholine-induced relaxations in the different treatment groups were not significantly different from those obtained in preparations of 36-week-old animals.

**Species.** In SHR aortae treated with 4-DAMP (preferential M3− mAChR inhibitor; 10^-7 M) (Boulanger et al., 1994), the remaining responses and maximal relaxations to acetylcholine were comparable with those observed in atropine-treated rings (Fig. 4; Table 2). The atropine-insensitive relaxations to acetylcholine were not affected significantly by treatment with α-bungarotoxin (selective antagonist at α7-nAChRs; 10^-6 M) (Wu et al., 2009), α-conotoxin (antagonist at α7 and α9 nAChRs; 10^-6 M) (Johnson et al., 1995), DHβE (selective antagonist at α4-nAChRs; 10^-5M) (Roegg and Levin, 2006), or UCL1684 plus TRAM-34 (SKCa and IKCa inhibitors, respectively; 5 × 10^-7 M) (Gluais et al., 2005), whereas they

### Table 1

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sense</th>
<th>Antisense</th>
<th>Product Length</th>
<th>Accession Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2</td>
<td>AAAAGGAGGAGAGAAGAGGAGG</td>
<td>CAGACGCTGCAAGATGATG</td>
<td>222</td>
<td>NM_133420.1</td>
</tr>
<tr>
<td>α3</td>
<td>TTAGCCTTGCCAGGCTCA</td>
<td>GCGCCACATCTCGGGGGAC</td>
<td>171</td>
<td>NM_02805.2</td>
</tr>
<tr>
<td>α4</td>
<td>TAAATACGACTCACTATAGGAAGCCGAGGCCGCTCTTGAG</td>
<td>GCTGGGACACACTGCTGAC</td>
<td>274</td>
<td>NM_024354.1</td>
</tr>
<tr>
<td>α5</td>
<td>ACCCTACCAATTGCAACC</td>
<td>GACCCAAAAGCCCATCTGGA</td>
<td>146</td>
<td>NM_024354.1</td>
</tr>
<tr>
<td>α6</td>
<td>TGGTTTGAAGCCACCAAA</td>
<td>GCTGTCGCTTAAACCTTCGTG</td>
<td>142</td>
<td>NM_017078.2</td>
</tr>
<tr>
<td>α7</td>
<td>TAAATACGACTCACTATAGGAAGCCGAGGCCGCTCTTGAG</td>
<td>GCTGGGACACACTGCTGAC</td>
<td>274</td>
<td>NM_024354.1</td>
</tr>
<tr>
<td>α9</td>
<td>CACGAAACGGCGCAATGGAT</td>
<td>GCCCGCATCAGCAGCAGG</td>
<td>207</td>
<td>NM_012832.3</td>
</tr>
<tr>
<td>α10</td>
<td>TGGTTTGAAGCCACCAAA</td>
<td>GCTGTCGCTTAAACCTTCGTG</td>
<td>195</td>
<td>NM_022930.1</td>
</tr>
<tr>
<td>β2</td>
<td>GCTAGCGGTCGATGAACTGTC</td>
<td>GAGGAGTCGAGGACACATAG</td>
<td>180</td>
<td>NM_019297.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTCATTCAACCACCCTGAGA</td>
<td>CGGCATACGCAGAAGCTG</td>
<td>299</td>
<td>AF106860</td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>pEC_{50}</th>
<th>R_{Max}</th>
<th>pEC_{50}</th>
<th>R_{Max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR, 36 weeks</td>
<td></td>
<td></td>
<td>WKY Rats, 36 weeks</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>-7.41 ± 0.45</td>
<td>73.56 ± 13.74</td>
<td>-6.64 ± 0.52</td>
<td>61.66 ± 10.39</td>
</tr>
<tr>
<td>Atropine</td>
<td>-7.97 ± 0.52</td>
<td>34.94 ± 13.85</td>
<td>-7.17 ± 1.27</td>
<td>4.77 ± 9.92</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>-6.65 ± 0.53</td>
<td>70.05 ± 10.95</td>
<td>-6.39 ± 0.32</td>
<td>57.86 ± 13.03</td>
</tr>
<tr>
<td>Atropine + mecamylamine</td>
<td>-4.98 ± 0.69</td>
<td>-19.60 ± 0.28</td>
<td>-7.05 ± 1.17</td>
<td>9.76 ± 4.68</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>-7.87 ± 0.44</td>
<td>24.10 ± 6.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + t-NAME</td>
<td>-7.61 ± 0.87</td>
<td>10.32 ± 3.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + UCL 1684</td>
<td>-7.86 ± 1.09</td>
<td>33.79 ± 6.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + α-conotoxin</td>
<td>-5.98 ± 1.04</td>
<td>25.70 ± 12.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + α-bungarotoxin</td>
<td>-5.90 ± 1.99</td>
<td>23.73 ± 0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + DHβE</td>
<td>-9.62 ± 0.05</td>
<td>27.47 ± 9.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + LY294002</td>
<td>-6.65 ± 0.76</td>
<td>12.80 ± 5.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + PD98059</td>
<td>-7.35 ± 1.52</td>
<td>32.78 ± 10.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + SB203580</td>
<td>-10.35 ± 3.21</td>
<td>21.84 ± 11.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine + U0126</td>
<td>-8.09 ± 1.28</td>
<td>23.98 ± 5.61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
weakened by \( \text{L-NAME} \) (Fig. 4; Table 2). In the presence of atropine, the maximal relaxations to acetylcholine were significantly reduced by \( \text{L-NAME} \) but not altered by other inhibitors.

**Nicotine-Induced Relaxations.** In both SHR and WKY rat aortae, nicotine induced relaxations in preparation with, but not without, endothelium. The nicotine-induced relaxations were not affected significantly by atropine, mecamylamine, or the combination of these two inhibitors. The relaxations were not affected by DH\( \beta \)E but were partially inhibited by \( \alpha \)-conotoxin and significantly inhibited by \( \alpha \)-bungarotoxin. The maximal relaxations to nicotine were reduced to 60% of those observed in the vehicle group by \( \alpha \)-bungarotoxin in both SHR and WKY rats. The responses were almost abolished by \( \text{L-NAME} \) and were partially inhibited by TRAM-34 plus UCL 1684 in WKY aortae, but they were not significantly affected by this combination in SHR preparations (Fig. 5; Table 3). The \( EC_{50} \) values of nicotine in the absence and presence of the different inhibitors tested were similar between SHR and WKY rats (Table 3).

**Signaling Mechanisms.** In SHR aortae, the remaining relaxations to acetylcholine in the presence of atropine were

---

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>SHR, 36 weeks</th>
<th>WKY Rats, 36 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( pEC_{50} )</td>
<td>( R_{\text{Max}} )</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-6.15 ± 0.68</td>
<td>52.24 ± 9.13</td>
</tr>
<tr>
<td>Atropine</td>
<td>-6.37 ± 0.54</td>
<td>54.25 ± 0.78</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>-6.31 ± 0.58</td>
<td>46.45 ± 22.85</td>
</tr>
<tr>
<td>Atropine + mecamylamine</td>
<td>-6.41 ± 0.40</td>
<td>47.41 ± 5.63</td>
</tr>
<tr>
<td>L-NAME</td>
<td>-5.73 ± 0.76</td>
<td>16.58 ± 3.88</td>
</tr>
<tr>
<td>UCL 1684 + TRAM-34</td>
<td>-6.51 ± 0.61</td>
<td>49.92 ± 1.9</td>
</tr>
<tr>
<td>( \alpha )-Conotoxin</td>
<td>-6.54 ± 0.45</td>
<td>33.22 ± 7.32</td>
</tr>
<tr>
<td>( \alpha )-Bungarotoxin</td>
<td>-6.41 ± 0.34</td>
<td>27.25 ± 3.95</td>
</tr>
<tr>
<td>DH( \beta )E</td>
<td>-6.91 ± 0.57</td>
<td>43.10 ± 11.5</td>
</tr>
<tr>
<td>LY294002</td>
<td>-6.53 ± 0.32</td>
<td>14.03 ± 4.52</td>
</tr>
<tr>
<td>PD98059</td>
<td>-5.60 ± 1.78</td>
<td>38.98 ± 6</td>
</tr>
<tr>
<td>SB203580</td>
<td>-5.46 ± 1</td>
<td>25.09 ± 6.28</td>
</tr>
<tr>
<td>U0126</td>
<td>-7.58 ± 0.24</td>
<td>23.38 ± 8.67</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** PCR measurements of messenger RNAs encoding for \( \alpha_2, \alpha_3, \alpha_4, \alpha_5, \alpha_6, \alpha_7, \alpha_9, \alpha_{10}, \) and \( \beta_2 \) nicotinic acetylcholine receptor subunits in SHR (top) and WKY (bottom) aortae with endothelium. Results are representative of four to six experiments.
reduced significantly by LY294002 (PI3K inhibitor; $10^{-6}$ M), but not affected significantly by PD98059 (MEK1 inhibitor; $10^{-6}$ M), SB203580 (p38 MAP kinase inhibitor; $10^{-6}$ M), or U0126 (MEK1/2 inhibitor; $10^{-6}$ M) (Wu et al., 2009; Banquet et al., 2011). The nicotine-induced relaxations were significantly prevented by incubation with LY294002 in WKY aortae and partially inhibited in SHR rats. The relaxations were not affected by the other inhibitors (Fig. 6; Table 3). The maximum of the remaining relaxation to acetylcholine and the response to nicotine was reduced to the same extent by LY294002 (Tables 2 and 3).

**Discussion**

In both male SHR (8, 18, and 36 weeks old) and WKY (36 and 60 weeks old) aortae, the acetylcholine-induced relaxations were similar to the control responses in the presence of mecamylamine, indicating that muscarinic receptors are mainly responsible for endothelium-dependent relaxations to acetylcholine under control conditions. The main finding of the present study was that when muscarinic receptors were inhibited with atropine a substantial remaining relaxation to acetylcholine persisted in the SHR but not the WKY aorta. The remaining relaxations were not affected by $\alpha$-bungarotoxin and $\alpha$-conotoxin, indicating that the $\alpha 7$ and $\alpha 9$ subunits of nicotinic receptors are not responsible for the atropine-insensitive relaxations to acetylcholine (Johnson et al., 1995; Wu et al., 2009). The latter were abolished by the NO synthase inhibitor L-NAME but not by the SK$_{Ca}$ and IK$_{Ca}$ inhibitors UCL 1684 and TRAM-34, indicating that they are mediated by NO but do not involve endothelium-dependent hyperpolarization (Féletou and Vanhoutte, 2007).

The atropine-insensitive responses observed in the SHR aorta are likely to be mediated through the activation of nicotinic receptors. This conclusion is based on the observation that mecamylamine, a nonselective inhibitor of nicotinic receptors (Kirshenbaum et al., 2011), prevented the remaining relaxations. This unexpected response cannot be attributed to premature aging of blood vessel walls as a consequence of hypertensive process in the SHR (Küng and Lüscher, 1995; Taddei et al., 1997), because similar remaining responses were observed in the aortae of both 8- and 18-week-old SHR rats and no such remaining response were observed in the aortae of 60-week-old WKY rats exposed to atropine. These results indicate the presence of functional nicotinic receptors at different stages of hypertension in the SHR. Hence, the response seems to be specific for the hypertensive strain and is not age-dependent. The effect observed in hypertensive animals could be explained by either parallel redundant mechanisms or cross-talk between muscarinic and nicotinic receptors. The observation that mecamylamine caused a modest, albeit not significant, reduction in the potency of acetylcholine indicates that nicotinic receptors may also function to mediate endothelium-dependent relaxations. However, when the two parallel pathways are available muscarinic receptors are sufficient to mediate endothelium-dependent relaxations, and only when muscarinic receptors are inhibited/occupied by atropine do nicotinic receptors become apparent and contribute to endothelium-dependent relax-
A similar type of redundancy occurs between M1 and M3 muscarinic receptors in salivary glands, where both subtypes are functional, but the activation/presence of either one is sufficient to mediate robust salivary output (Gautam et al., 2004). An alternative explanation would be that muscarinic receptors can tonically inhibit nicotinic receptor. A similar interaction between muscarinic and nicotinic receptors has been demonstrated in rat hippocampal interneurons where activation of M1 receptors, through a phospholipase C-, calcium-, and protein kinase C-dependent signal transduction cascade, reduced the amplitude of the α7 nicotinic receptor-mediated responses (Shen et al., 2009). That M3 muscarinic receptors are responsible for atropine-sensitive inhibitory response to acetylcholine is demonstrated by the observation that the M3 preferential muscarinic receptor antagonist 4-DAMP (Boulanger et al., 1994) can unmask identical remaining responses as atropine. The fact that when M3 muscarinic receptors are occupied by atropine nicotinic receptors can mediate endothelium-dependent relaxations to acetylcholine only in preparations of hypertensive rats but not in those of normotensive controls suggests that hypertension may unmask a compensatory mechanism making the latter receptors “functional” when they are relieved from the inhibitory effect of the former. Such compensatory mechanism may result either from loss of or reduction in responsiveness to muscarinic receptors during the development of hypertension. The present observations do not permit further speculations concerning the mechanism underlying the interaction between M3 muscarinic and nicotinic receptors.

To verify whether functional endothelial nicotinic receptors may be unmasked by atropine in the SHR aorta, the response to nicotine was examined. The present study demonstrates that this prototypical agonist at nicotinic receptors can induce endothelium-dependent relaxations in both SHR and WKY rat aortae, which, as could be expected, were not affected by atropine. The nicotine-induced relaxations were not sensitive to either mecamylamine or DHβE. Nicotine has a high affinity to the α4β2 subunit (Paradiso and Steinbach, 2003), and DHβE is a selective antagonist of α4β2 nicotinic

Fig. 4. The effect of acetylcholine on phenylephrine-induced contraction in SHR aortic rings incubated with vehicle, atropine (10⁻⁵ M), or 4-DAMP (10⁻⁵ M) (a) or vehicle, atropine (10⁻⁵ M), atropine plus L-NAME (10⁻⁵ M), atropine plus UCL1684 and TRAM-34 (5 × 10⁻⁷ M), atropine plus α-conotoxin (10⁻⁶ M), atropine plus α-bungarotoxin (10⁻⁶ M), or atropine plus DHβE (10⁻³ M) (b). All rings were incubated with indomethacin (10⁻⁵ M) to prevent the formation of vasoactive prostanoids. Data are expressed as area under the curve (n = 6–10). *, P < 0.05 versus atropine.

Fig. 5. The effect of nicotine on phenylephrine-induced contraction in aortic rings with or without endothelium of 36-week-old male SHR (a) and WKY rats (b). The preparations were incubated with: vehicle (control), atropine (10⁻⁵ M), mecamylamine (10⁻⁴ M), atropine plus mecamylamine, α-conotoxin (10⁻⁶ M), α-bungarotoxin (10⁻⁶ M), DHβE (10⁻³ M), L-NAME (10⁻⁵ M), and TRAM-34 plus UCL 1684 (5 × 10⁻⁷ M). All rings were incubated with indomethacin (10⁻⁵ M) to prevent the formation of vasoactive prostanoids. Data are expressed as area under the curve (n = 6–10). *, P < 0.05 versus vehicle.
Acetylcholine induced relaxation

![Graph](image1)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Area under the curve (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>200</td>
</tr>
<tr>
<td>Atropine + LY294002</td>
<td>150</td>
</tr>
<tr>
<td>Atropine + PD98059</td>
<td>100</td>
</tr>
<tr>
<td>Atropine + SB203580</td>
<td>50</td>
</tr>
<tr>
<td>Atropine + U0126</td>
<td>0</td>
</tr>
</tbody>
</table>

Nicotine induced relaxation

![Graph](image2)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Area under the curve (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200</td>
</tr>
<tr>
<td>LY294002</td>
<td>150</td>
</tr>
<tr>
<td>PD98059</td>
<td>100</td>
</tr>
<tr>
<td>SB203580</td>
<td>50</td>
</tr>
<tr>
<td>U0126</td>
<td>0</td>
</tr>
</tbody>
</table>

WKY (36 weeks, male)

![Graph](image3)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Area under the curve (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200</td>
</tr>
<tr>
<td>LY294002</td>
<td>150</td>
</tr>
<tr>
<td>PD98059</td>
<td>100</td>
</tr>
<tr>
<td>SB203580</td>
<td>50</td>
</tr>
<tr>
<td>U0126</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 6.** Relaxations to cumulative concentrations of acetylcholine (in the presence of atropine, $10^{-5}$ M) of 36-week-old SHR (a) or cumulative concentrations of nicotine of 36-week-old SHR (b) and 36-week-old WKY (c) aortic rings contracted with phenylephrine and incubated with LY294002 ($10^{-6}$ M), SB203580 ($10^{-6}$ M), PD98059 ($10^{-6}$ M), or U0126 ($10^{-6}$ M). All rings were incubated with indomethacin ($10^{-5}$ M) to prevent the formation of vasoactive prostanoids. Data are expressed as areas under the curve ($n = 5–6$). * $P < 0.05$ versus atropine (a) or control (c).

receptors (Roeggé and Levin, 2006). Mecamylamine is a commonly used nonselective antagonist, which, however, has a relatively higher affinity for the $\alpha_3\beta_4$ subunit compared with other nicotinic receptor subunits (Papke et al., 2001; Eguchi et al., 2007). Thus, the nicotine-induced endothelium-dependent relaxations are not likely caused by the activation of either $\alpha_4\beta_2$ or $\alpha_3\beta_4$ subunits, although these subunits are present in the rat aorta. However, the nicotine-induced relaxations were inhibited by $\alpha$-bungarotoxin and $\alpha$-conotoxin, demonstrating the involvement of $\alpha_7$-nAChRs, a conclusion in line with the abundant expression of this subunit in the studied preparation (Johnson et al., 1995; Wu et al., 2009). Taken together, the present findings indicate that if the nicotine-induced endothelium-dependent relaxations are mediated via $\alpha_7$ nicotinic receptors they are not affected by the state of muscarinic receptors, whereas the acetylcholine-induced atropine-insensitive relaxations are mediated through mecamylamine-sensitive nicotinic receptors, most likely the $\alpha_3$ subunits.

The nicotine-induced relaxations were also abolished by the NO synthase inhibitor L-NAME, indicating that they are mediated mainly by endothelium-derived NO. However, in the WKY aorta, the response to nicotine was inhibited partially by TRAM-34 plus UCL 1684, suggesting that endothelium-dependent hyperpolarization may also contribute to the response in the normotensive strain (Félétou and Vanhoutte, 2007).

Because both the remaining responses to acetylcholine and the nicotine-induced relaxations were caused mainly by the release of NO, the signaling mechanisms that lead to the activation of endothelial NO synthase were probed pharmacologically. In human coronary artery endothelial cells, nicotinic receptors can activate three typical survival signaling pathways: PI3K/AKT, Janus tyrosine kinase 2/signal transducer and activator of transcription 3, and extracellular signal-regulated kinase 1/2 MAP kinases (Smedlund et al., 2011). The present results suggest that both relaxations to nicotine and those to acetylcholine in the presence of atropine are mediated mainly by the PI3K/AKT signaling cascade, but not the other pathways tested, because both responses were reduced only by LY294002, an established inhibitor of PI3K (Johnson et al., 1995; Wu et al., 2009). Thus, endothelial nicotinic receptors can activate the PI3K/AKT pathway, which consequently activates endothelial NO synthase in the endothelium, leading to the production of NO and inducing vasodilatation.

In summary, in the SHR aorta endothelium-dependent relaxations evoked by acetylcholine result mainly from the activation of muscarinic receptors under control conditions. However, when the $M_3$ muscarinic receptors are inhibited/occupied by atropine, nicotinic receptors can also contribute to endothelium-dependent relaxations in that preparation. Nicotine can induce endothelium-dependent relaxations via the activation of $\alpha_7$ nicotinic receptors in both SHR and WKY aortae. The acetylcholine-induced, atropine-insensitive relaxations and those to nicotine both involve the PI3K/AKT pathway and are caused by the release of endothelium-derived NO. These findings provide further evidence showing that nicotinic receptors can modulate physiological and pathophysiological processes in non-neuronal tissues and organ systems (Wang et al., 2004; Egleton et al., 2008; Mimura et al., 2010). In addition to their involvements in angiogenesis, oxidative and phagocytic activity of macrophages, and the proinflammatory and proapoptotic actions of cytokines on endothelial cells (Lee and Cooke, 2011), nicotinic receptors...
may contribute to the endothelium-dependent regulation of vascular tone.

**Authorship Contributions**

**Participated in research design:** Zou and Vanhoutte.

**Conducted experiments:** Zou.

**Performed data analysis:** Zou.

**Wrote or contributed to the writing of the manuscript:** Zou, Leung, and Vanhoutte.

**References**


H1602–H1608.

- Conotoxin ImI exhibits subtype-specific nicotinic acetylcholine receptor blockade: preferential inhibition of homomeric \( \alpha_7 \) and \( \alpha_9 \) receptors. *Mol Pharmacol* 48:194–199.


Shen JX, Tu B, and Yakel JL (2009) Inhibition of \( \alpha \)7-containing nicotinic ACh receptors by muscarinic M1 ACh receptors in rat hippocampal CA1 interneurons in slices. *J Physiol* 587:1033–1042.


Address correspondence to: Dr. Susan W. S. Leung, Department of Pharmacology and Pharmacy, University of Hong Kong, 2/F, Laboratory Block, Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China. E-mail: swsleung@hkucc.hku.hk

nAChRs Contribute to Endothelium-Dependent Relaxations 763