Nicotine exposure and the progression of chronic kidney disease: role of the α7-nicotinic acetylcholine receptor

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Rezonzew G, Chumley P, Feng W, Hua P, Siegal GP, Jaimes EA. Nicotine exposure and the progression of chronic kidney disease: role of the α7-nicotinic acetylcholine receptor. Am J Physiol Renal Physiol 303: F304–F312, 2012. First published May 2, 2012; doi:10.1152/ajprenal.00661.2011.—Clinical studies have established the role of cigarette smoking as a risk factor in the progression of chronic kidney disease (CKD). We have shown that nicotine promotes mesangial cell proliferation and hypertrophy via nonneuronal nico-

tinic acetylcholine receptors (nAChRs). The α7-nAChR is one of the most important subunits of the nAChRs. These studies were designed to test the hypothesis that nicotine worsens renal injury in rats with 5/6 nephrectomy (5/6Nx) and that the α7-nAChR subunit is required for these effects. We studied five different groups: Sham, 5/6Nx, 5/6Nx + nicotine (Nic; 100 μg/ml dry wt), 5/6Nx + Nic + α7-nAChR blocker methyllicaconitine (MLA; 3 mg·kg−1·day−1·sq), and Sham + Nic. Blood pressure was measured by the tail-cuff method, and urine was collected for proteinuria. After 12 wk, the rats were euthanized and kidneys were collected. We observed expression of the α7-nAChR in the proximal and distal tubules. The administration of nicotine induced a small increase in blood pressure and resulted in cotinine levels similar to those found in the plasma of smokers. In 5/6Nx rats, the administration of nicotine significantly increased urinary protein excretion (100%), worsened the glomerular injury score and increased fibronectin (~50%), NADPH oxidase 4 (NOX4; ~100%), and transforming growth factor-β expression (~200%). The administration of nicotine to sham rats increased total proteinuria but not albuminuria, suggesting direct effects on tubular protein reabsorption. These effects were prevented by MLA, demonstrating a critical role for the α7-nAChR as a mediator of the effects of nicotine in the progression of CKD.

extracellular matrix; reactive oxygen species; tobacco

CIGARETTE SMOKING IS AN IMPORTANT risk factor for emphysema, atherosclerosis, and cancer and is the most important cause of preventable morbidity and mortality in the United States (3). In addition, epidemiological studies (26, 35) have demonstrated that cigarette smoking is an independent risk factor in the progression of chronic kidney disease (CKD) of different etiologies including hypertension and diabetes.

The mechanisms by which cigarette smoking accelerates the progression of CKD are, however, not well understood. The gas phase of cigarette smoke contains high concentrations of short-lived reactive oxygen species, nitric oxide, and free radicals of organic compounds (31). In addition, it contains varying amounts of more stable substances including nicotine, which, besides its addictive properties (39), plays an important role in the pathogenesis of tobacco-induced disease including atherosclerosis (14) and pulmonary fibrosis (34). Moreover, and as we have previously shown, nicotine worsens glomerular injury in a rat model of acute nephritis (21) and promotes extracellular matrix deposition in a mouse model of diabetic nephropathy (20).

Nicotine mediates its effects via the activation of nicotinic acetylcholine receptors (nAChRs) that function as agonist-regulated Ca2+ channels. The nAChRs are transmembrane oligomers consisting of five subunits and are expressed by neuronal as well nonneuronal cells including human mesangial cells (20), endothelial cells, and vascular smooth muscle cells (15, 24). Of these subunits, the α7-nAChR subunit in particular is critical for several of the cholinergic actions mediated by nAChRs in macrophages, vascular smooth muscle cells, and cancer cell lines (7, 33).

In these studies, we tested the hypothesis that the α7-nAChR plays a major role as mediator of the effects of nicotine in the progression of renal disease. For these studies, we utilized a rat model of 5/6 nephrectomy, a well-characterized, and validated model of CKD that closely mimics CKD in humans (37). In these studies, we demonstrate expression of the α7-nAChR and that nicotine worsens renal injury in this model of CKD that can be prevented by α7-nAChR blockade, suggesting that this receptor subunit is essential as mediator of the actions of nicotine in the progression of CKD. In addition, we demonstrate that the effects of nicotine are accompanied by increases in oxidative stress, NADPH oxidase 4 (NOX4), and transforming growth factor (TGF)-β.

METHODS

Eight-week-old male Sprague-Dawley rats were purchased from Charles River (Boston, MA) and maintained under controlled conditions of light, temperature, and humidity. All rats had baseline blood pressure measurements and urine collections and were subjected to either sham surgery or 5/6 nephrectomy (5/6Nx). The rats were divided in the following groups: group 1 (Sham): sham-operated rats drinking tap water (n = 8); group 2 (5/6Nx): Rats with 5/6Nx on tap water (n = 7); group 3 (5/6Nx + Nic): rats with 5/6Nx and receiving nicotine in the drinking water (21) (100 μg/ml; n = 8); group 4 (5/6Nx + Nic + MLA): rats with 5/6Nx, receiving nicotine in the drinking water (100 μg/ml) and receiving the α7-nAChR blocker methyllicaconitine (23) (MLA; 3 mg·kg−1·day−1 via osmotic mini-pump; n = 8); group 5 (Sham + Nic): sham-operated rats receiving nicotine in the drinking water (100 μg/ml; n = 8); group 6 (5/6Nx + MLA): rats with 5/6Nx and receiving MLA (3 mg·kg−1·day−1); and group 7 (Sham + Nic + MLA): sham-operated rats receiving nicotine in the drinking water (100 μg/ml) and MLA (3 mg·kg−1·day−1). The rats were maintained in their respective treatment groups for 12 wk after surgery and euthanized under deep anesthesia by exsanguination via cardiac puncture followed by cervical dislocation. Blood was
collected for cotinine serum and creatinine measurements and kidneys for Western blot analysis and histology.

Blood pressure was measured biweekly in all groups by the tail-cuff method (CODA-Kent Scientific). Urine collections were also performed biweekly in all groups in metabolic cages. The animals were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. The studies were approved by the Institutional Animal Care and Use Sub-Committee at the University of Alabama at Birmingham.

Nephrectomies. The 5/6 nephrectomies (5/6Nx) were performed in two stages while the animals were under general anesthesia (isoflurane 2.5%) using the full sterile technique (40). In the first stage, and via a retroperitoneal approach, a subtotal nephrectomy was performed in which both poles of the left kidney were surgically excised and the stumps ligated. Gel-Foam was used for hemostasis. A week later, a total right nephrectomy was performed after ligation of the renal vessels and the ureter.

Western blot analysis. Western blots were performed as previously described (22). Briefly, 100 mg of kidney cortex were homogenized in 300 µl of homogenization buffer (20 mmol/l Tris-HCl pH 7.4, 140 mmol/l NaCl, 10 mmol/l Na pyrophosphate, 10 mmol/l Na fluoride, 2 mmol/l Na orthovanadate, 3 mmol/l EDTA, and 10% glycerol). Protease inhibitor cocktail (Sigma P8340) was added to the homogenization buffer before use. The resulting lysates were centrifugated for 20 min at 13,200 rpm at 4°C. The supernatants were collected, and protein concentration was quantified by Bio-Rad assay.

For immunoblotting, 15–20 µl of homogenate were separated by SDS-PAGE (10–15% acrylamide gel) and transferred to a nitrocellulose membrane (Bio-Rad; 0.2 µm). The blots were incubated with antibodies against fibronectin (Sigma, St Louis, MO), nitrotyrosine (Millipore Billerica, MA), α-7 (Abcam, Boston, MA), and NOX 4 (Novus, Littleton, CO). β-Actin (Sigma) was used to control for loading. The blots were washed and incubated with goat anti-rabbit antibody (Jackson), and the signal was detected by luminol chemoluminescence (Millipore, Billerica, MA).

Immunofluorescence. Formalin-fixed, paraffin-embedded rat kidney cortex sections (5 µm) were deparaffinized and antigen retrieval performed on all sections using Vector antigen retrieval solution. Sections used to identify specific tubules were incubated for 15 min. Each with Avidin blocking reagent and Biotin blocking reagent. Sections used to identify proximal tubules were incubated with biotinylated Lotus tetragonolobus lectin (1:200) in PBS for 30 min. Sections used to identify distal tubules and collecting duct were incubated with biotinylated peanut agglutinin (1:1,000) in PBS for 30 min (4). All sections were then washed and incubated with streptavidin Texas red (1:200) in PBS for 30 min. Biotinylated reagents and streptavidin TXR were purchased from Vector Laboratories (Burlingame, CA). Sections were blocked with PBS + 1% bovine serum and 5% goat serum for 1 h at room temperature then incubated with primary antibody to fibronectin (Sigma), α-7 (Abcam), or NOX4 (Novus) at a 1:200 dilution in blocking buffer, overnight at 4°C in a humidified chamber. Sections were then washed three times for 5 min with PBS and incubated with secondary antibody, Alexa Fluor 488-labeled goat anti-rabbit (Molecular Probes, Portland, OR) at 1:400 dilution in blocking buffer, 1 h at room temperature. Sections were then washed three times for 5 min with PBS and mounted with coverslips. Image acquisition was performed on a Leica DM6000 epifluorescence microscope (Leica Microsystems, Bannockburn, IL) with a Hamamatsu ORCA ER cooled CCD camera and SimplePCI software (Compix, Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence. Identical sections used as controls were processed, either without primary or secondary antibodies present during identical incubation conditions.

Proteinuria. Proteinuria was measured by the Lowry method and adjusted by urinary creatinine (Cayman, Ann Arbor, MI). Urinary albumin concentrations were measured using a rat albumin ELISA quantitation kit from Bethyl (Montgomery, TX) and adjusted for urinary creatinine.

Serum creatinine. Serum creatinine was measured by mass spectrometry at the O'Brien Kidney Center Core Facilities at the University of Alabama at Birmingham.

Glotterular injury score. Glomerular injury score was measured in trichrome-stained kidney slides by one of us, an experienced pathologist purposely blinded to the different experimental conditions and utilizing a 0+ to 4+ scale as previously described (32). All glomeruli available in each slide (n = 32–263) were analyzed, and the data are expressed as the percentage of glomeruli injured at every level.

Urinary isoprostanes. Urinary F2-isoprostanes were measured by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions and adjusted for protein content (Lowry).

Statistical analysis. All data are expressed as the means ± SE. Statistical analysis was performed by ANOVA (Statistix 8 Analytical Software, Tallahassee, FL). Significance was considered when P < 0.05.

RESULTS

Renal expression of nicotinic α7 receptors. As shown in Fig. 1, we observed strong expression of the α7-nAChR in sham rats as assessed by Western blot. The expression of the α7-nAChR was reduced in 5/6Nx rats drinking tap water or on nicotine compared with sham rats. The administration of the α7-nAChR inhibitor MLA resulted in significant increases in the expression of α7-nAChR while the administration of nicotine to sham animals led to a twofold reduction (Fig. 1). By immunofluorescence, we observed strong expression of this receptor in the proximal tubules (Fig. 2B) and at a lower degree in the distal tubules (Fig. 2C). We did not observe significant

Fig. 1. Cortical expression of the α7-nicotinic acetylcholine receptors (nAChRs). A: representative Western blot for the α7-nAChR and β-actin, which was used to control for loading. B: densitometry data analysis for cortical α7-nAChR expression (n = 6–7 per group; *P < 0.05 vs. Sham). 5/6Nx, 5/6 nephrectomy; Nic, nicotine; MLA, methyllycaconitine; OD, optical density.
expression of this receptor subunit in the glomerulus or intrarenal vasculature in any of the groups (Fig. 2D).

Hemodynamic and metabolic effects of nicotine. The administration of nicotine in the drinking water at the concentrations used for these studies resulted in serum concentrations of cotinine, a stable metabolite of nicotine, similar to those found in the plasma of active smokers (16; Table 1). The serum levels of cotinine in rats with 5/6Nx were, however, slightly higher compared with those obtained in sham animals. The urinary excretion of cotinine was lower in rats with 5/6Nx compared with sham rats, indicating impaired cotinine excretion in rats with reduced renal function. The administration of nicotine to either sham or 5/6Nx rats did not result in significant changes in final body weight (Table 1). However, the administration of MLA to 5/6Nx rats resulted in lower final weights especially in rats concomitantly receiving nicotine (Table 1).

Rats with 5/6Nx had a small and nonsignificant increase in systolic blood pressure compared with sham rats. The administration of nicotine to either sham or 5/6Nx rats resulted in further increases in blood pressure (Table 1). The administration of the α7-nAChR receptor blocker MLA induced a small reduction in blood pressure in sham or 5/6Nx rats on nicotine but not in rats on tap water (Table 1).

**Effects of nicotine on proteinuria and renal injury.** As previously described by others, 5/6Nx resulted in significant increases in urinary protein excretion compared with sham rats (40). The administration of nicotine to 5/6Nx rats induced further and significant increases in proteinuria as expressed as protein/creatinine ratio (Fig. 3), which were evident as early as 2 wk after 5/6Nx. Blockade of the α7-nAChR with MLA in rats with 5/6Nx and receiving nicotine resulted in significantly lower urinary protein excretions. Interestingly, the administration of nicotine to sham rats also resulted in significant increases in urinary protein excretion that were more evident in the last 2 wk of the experimental protocol (Fig. 3). As shown in Table 2, these effects of nicotine on protein/creatinine ratios were not due to changes in the urinary excretion of creatinine. In addition, we measured albumin excretion in the last urine collection. As shown in Table 2, the urinary excretion of albumin was increased in 5/6Nx rats, further increased by the administration of nicotine and reduced by MLA. However, and in contrast with the measurements of total protein excretion, the administration of nicotine to sham rats did not modify the urinary excretion of albumin, indicating that the proteinuria observed in these rats was tubular in origin. In light of these results, we performed additional experiments in two extra

### Table 1. Weight, systolic blood pressure, and serum and urinary cotinine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5/6Nx</th>
<th>5/6Nx + Nic</th>
<th>5/6Nx + Nic + MLA</th>
<th>Sham + MLA</th>
<th>5/6Nx + MLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight at death, g</td>
<td>431.5 ± 30.0</td>
<td>424.3 ± 23.1</td>
<td>397.1 ± 12.8+†</td>
<td>398.4 ± 17.0</td>
<td>468.4 ± 15.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>135 ± 8.6</td>
<td>151 ± 7.1</td>
<td>148 ± 5.4</td>
<td>150 ± 6.8</td>
<td>132 ± 11.0‡</td>
</tr>
<tr>
<td>Serum cotinine, mg/ml</td>
<td>Nondetectable</td>
<td>Nondetectable</td>
<td>67.2 ± 2.7</td>
<td>73.3 ± 2.0</td>
<td>55.4 ± 1.0§</td>
</tr>
<tr>
<td>Urinary cotinine, µg/day</td>
<td>Nondetectable</td>
<td>Nondetectable</td>
<td>285.5 ± 31</td>
<td>228.6 ± 23</td>
<td>719.4 ± 267§</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Sham; †P < 0.05 vs. 5/6 nephrectomy (5/6Nx); ‡P < 0.05 vs. 5/6Nx + nicotine (Nic); §P < 0.05 vs. 5/6Nx + Nic and 5/6Nx + Nic + methyllycaconitine (MLA).
groups: Sham + Nicotine + MLA and 5/6Nx + MLA. As shown in Table 2, the administration of MLA did not modify the urinary excretion of either total protein or albumin in Sham rats on nicotine or 5/6Nx rats on tap water.

The glomerular injury score was increased in rats with 5/6Nx compared with sham rats and significantly reduced by MLA. As shown in Fig. 4, there were more injured glomeruli with scores of 2+ and 3+ in rats with 5/6Nx+Nic compared with 5/6Nx. The administration of MLA resulted in a significant improvement in glomerular injury as shown by a reduction in the number of glomeruli with 2+ and 3+ scores and an increase in the number of glomeruli with 0+ and 1+ scores. Although the administration of nicotine to sham animals resulted in significant increases in urinary protein excretion, the injury score of these rats was similar to the sham rats. Even though the administration of MLA did not significantly modify the urinary protein excretion in 5/6Nx rats on tap water, it did improve glomerular injury score in these rats (Fig. 4).

As expected, rats with 5/6Nx had significant increases in serum creatinine that were not further increased by nicotine, in spite of a worse injury score, but were normalized by MLA (Table 2). The administration of nicotine to sham rats resulted in lower serum creatinines. Treatment with MLA in this group normalized serum creatinines but had no effect on 5/6Nx rats on tap water (Table 2). To better assess renal function, we measured creatinine clearance (CrCl) in all groups. As shown in Table 2, the administration of MLA did not modify creatinine clearance (CrCl) in 5/6Nx rats on tap water. Sham rats on nicotine had significantly higher CrCl but had no effect on CrCl in 5/6Nx rats on tap water. Treatment with MLA resulted in a significant increase in the number of glomeruli with 0+ and 1+ scores.

**Effects of nicotine on fibronectin expression.** To determine the effects of nicotine on the production of extracellular matrix, we measured fibronectin protein expression in renal cortex homogenates. As shown in Fig. 5A, 5/6Nx resulted in a significant increase in fibronectin expression as assessed by Western blot that was further increased by the administration of nicotine and significantly ameliorated by MLA. In sham rats, the administration of nicotine did not cause significant changes in fibronectin expression (Fig. 5A). In addition, we determined that the administration of MLA had no significant effects on fibronectin expression in sham rats on nicotine (Fig. 5B) or 5/6Nx rats on tap water (Fig. 5C). By immunofluorescence (Fig. 6), we determined that most of the fibronectin expression was interstitial and perivascular although the glomeruli of 5/6Nx + nicotine rats also had increased glomerular expression of fibronectin (Fig. 6D). In addition we measured the cortical expression of TGF-β a well-recognized mediator of fibrosis and extracellular matrix production (42). As shown in Fig. 7, 5/6Nx alone did not result in significant increases in TGF-β expression but the administration of nicotine resulted in a twofold increase in TGF-β that was prevented by MLA. The administration of nicotine to sham rats did not have any effect on cortical TGF-β expression.

**Effects of nicotine on oxidative stress.** Renal injury in models of CKD such as 5/6Nx is associated with increases in oxidative stress (40). To determine whether nicotine administration results in further oxidative stress, we measured the levels of nitrotyrosine, a well-validated marker of oxidative stress, in the cortex of the different experimental groups. In our studies, 5/6Nx resulted in a small, albeit significant, increase in nitrotyrosine expression as assessed by Western blot (Fig. 8). The administration of nicotine to 5/6Nx rats resulted in further increases in nitrotyrosine expression that, however, was only partially and not significantly reduced by MLA. The adminis-

### Table 2. Serum creatinine, creatinine clearance, and urinary creatinine, protein excretion, and albumin

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6Nx</th>
<th>5/6Nx + Nic</th>
<th>5/6Nx + Nic + MLA</th>
<th>Sham + Nic</th>
<th>Sham + Nic + MLA</th>
<th>5/6Nx + MLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.26±0.01</td>
<td>0.62±0.19*</td>
<td>0.51±0.06*</td>
<td>0.26±0.03*†</td>
<td>0.14±0.02*‡</td>
<td>0.32±0.01#</td>
<td>0.56±0.06*</td>
</tr>
<tr>
<td>Creatinine clearance, mg·kg⁻¹·min⁻¹</td>
<td>7.1±0.9</td>
<td>2.7±0.40*</td>
<td>3.8±0.4*</td>
<td>10.3±1.0*‡</td>
<td>10.6±2.4*‡</td>
<td>6.2±0.48</td>
<td>2.7±0.4</td>
</tr>
<tr>
<td>Urinary creatinine week 12, mg/day</td>
<td>13.6±1.2</td>
<td>7.7±1.8*</td>
<td>9.6±0.96</td>
<td>12.9±1.0#</td>
<td>9.8±1.8</td>
<td>11.3±0.8</td>
<td>11.2±1.2</td>
</tr>
<tr>
<td>Urinary protein excretion week 12, mg/day</td>
<td>39.1±11.9</td>
<td>122.7±13.6*</td>
<td>162.3±30.3*</td>
<td>91±10*‡</td>
<td>101±31*‡</td>
<td>131±18*</td>
<td>123.7±22*</td>
</tr>
<tr>
<td>Urinary albumin, mg/day</td>
<td>0.54±0.3</td>
<td>3.29±2.0*‡</td>
<td>36.67±13*</td>
<td>13.31±5.6*‡</td>
<td>0.1±0.02#‡</td>
<td>0.65±0.33</td>
<td>13.5±4.8*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Sham; †P < 0.05 vs. 5/6Nx; ‡P < 0.05 vs. 5/6Nx + Nic; §P < 0.05 vs. Sham + Nic.
tration of nicotine to sham rats did not induce any significant changes in nitrotyrosine.

NOX4 is one of the most important sources of reactive oxygen species in the kidney cortex, and its expression and activity are increased in diverse models of kidney injury (12, 18). We observed high basal levels of NOX4 in sham rats, which were not significantly different in rats with 5/6Nx but markedly upregulated by nicotine and normalized by α7nAChR blockade (Fig. 9). The administration of nicotine to sham rats did not modify the cortical expression of NOX4. In addition, we measured the urinary excretion of F2-isoprostanes as markers of oxidative stress. The 5/6Nx did not result in a significant increase in the urinary excretion of isoprostanes; however, the administration of nicotine resulted in large increases in urinary isoprostanes that were normalized by MLA (Fig. 10). The administration of nicotine to sham-operated animals had no effect on the urinary excretion of isoprostanes.

**DISCUSSION**

Epidemiologic studies suggest that cigarette smoking accelerates the rate of progression of CKD of diverse etiologies including diabetes mellitus (26, 35), hypertension (2, 17), lupus nephritis (41), polycystic kidney disease, IgA nephropathy, and postkidney transplantation (27). Moreover, in experimental studies we and others have demonstrated that the exposure to environmental tobacco smoke worsens renal injury in mouse models of renal injury such as diabetic nephropathy (5) and aging (30). However, and in spite of the clinical and experimental evidence the mechanisms responsible for these effects have not been identified.

Nicotine is one of the biologically active and stable compounds present in large concentrations in tobacco that can be acquired through active and passive smoking (6). In addition to its addictive properties, nicotine promotes atherosclerosis and angiogenesis (14) and as we have previously demonstrated nicotine worsens glomerular injury in the anti-Thy1 model of acute proliferative glomerulonephritis (21) and worsens proteinuria and extracellular matrix expansion in db/db mice, a well-known model of diabetic nephropathy (36). In the current studies, we have demonstrated that activation of the α7-nAChR is responsible in large part for the harmful effects of nicotine in renal injury. In these studies, we utilized a model of renal ablation (5/6Nx) that closely mimics CKD in humans and is characterized by progressive decline in renal function, glomerulosclerosis, interstitial fibrosis, and proteinuria (40).

We determined that the administration of nicotine in the drinking water and at concentrations that result in serum levels similar to those found in the plasma of smokers (16), resulted in increased proteinuria, increased glomerular injury and interstitial fibronectin and TGF-β production, and increased oxidative stress in rats with 5/6Nx. The administration of nicotine to

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**Fig. 4. Glomerular injury score.** Glomerular injury score was assessed in all groups and the results presented as percent and severity of damaged glomeruli on a 0+ to 4+ scale where 0 is no damage [*P < 0.05 vs. 5/6Nx, 5/6Nx + Nic, and 5/6Nx + Nic + MLA; †P < 0.05 vs. 5/6Nx; ‡P < 0.05 vs. 5/6Nx + Nic].

**Fig. 5. Cortical fibronectin expression.** A, B, and C: representative Western blot for fibronectin and β-actin, which was used to control for loading. D: densitometry data analysis for cortical fibronectin expression (n = 6–7 per group; *P < 0.05 vs. 5/6 Nx and 5/6Nx + Nic; #P < 0.05 vs. 5/6Nx; †P < 0.05 vs. 5/6Nx + Nic).
sham and 5/6Nx rats resulted in small increases in blood pressure as assessed by the tail-cuff method. Although these increases in blood pressure were small, we cannot rule out that some of the effects of nicotine on renal injury are at least in part mediated by changes in blood pressure.

Moreover, and although the administration of nicotine worsened renal injury, it did not result in a significant change in renal function as assessed by either serum creatinine or by CrCl. Of interest, the administration of nicotine to sham rats resulted in a significant increase in CrCl, which was prevented by MLA. These results suggest that nicotine may have hemodynamic effects on the glomerular microcirculation that result in glomerular hyperfiltration. Indeed, large epidemiological studies (13) have shown that smokers have a higher glomerular filtration rate and an elevated risk for proteinuria compared with nonsmokers. We hypothesize that the chronic administration of nicotine is associated with abnormal autoregulation of the glomerular microcirculation and that the resultant hyperfiltration may be playing a role in the accelerated progression of renal injury. In 5/6Nx rats receiving nicotine and treated with MLA, we also observed significant increases in glomerular protein expression as assessed by immunofluorescence.

![Fig. 6. Fibronectin expression by immunofluorescence.](image)

![Fig. 7. Transforming growth factor (TGF)-β expression in kidney lysates.](chart)

![Fig. 8. Nitrotyrosine expression.](chart)
the expression of this receptor subunit. Alternatively that the in vitro cell culture conditions favor the production in these cells. In the current studies, however, we observed strong expression of the nAChR in proximal tubules and at some degree in distal tubules. Although we observed predominantly vascular expression of the α7-nAChR, we did not detect glomerular expression of this receptor subunit. We (20) have previously shown that cultured human mesangial cells express several nAChRs subunits including the α7-nAChR, and that nicotine promotes mesangial cell proliferation and fibronectin production in these cells. In the current studies, however, we did not detect glomerular expression of α7-nAChR expression, suggesting differences in expression according to the species or alternatively that the in vitro cell culture conditions favor the expression of this receptor subunit.

In our current studies, we utilized the compound MLA to block the α7-nAChR. MLA binds potently (Kᵦ around 2 nM) to α-bungarotoxin-binding sites (9) and has been classified as a competitive antagonist of α7-containing nAChRs (23). For these studies, we utilized similar concentrations as reported by others that result in specific blockade of the α7-nAChR and without any evident toxicity (38). In our studies, the administration of this inhibitor was well tolerated, did not result in significant changes in blood pressure, and resulted in significant reductions in proteinuria, glomerular injury score, fibronectin expression, and TGF-β production. These results therefore suggest that activation of the α7-nAChR plays a major role as mediator of the deleterious effects of nicotine in this model of CKD. Although we observed predominantly tubular expression of the α7-nAChR, blockade of the α7-nAChR with MLA reduced both glomerular injury as well as interstitial fibronectin expression. Based on these results and the experimental evidence supporting the role for the proximal tubules as a source of profibrotic and proinflammatory cytokines (11), we hypothesize that tubular activation of the α7-nAChR might results in increased production of these mediators resulting in increased glomerular and interstitial injury. The administration of MLA, however, especially to 5/6Nx rats on nicotine, resulted in lower body weights. We hypothesize that in these rats MLA may be having an effect on metabolic rate and/or caloric intake and will be investigated in future studies in our laboratory.

Renal ablation alone was associated with reduced α7-nAChR expression as assessed by Western blot. Although the mechanisms for this reduction are unclear, we speculate that they may be related, at least in part, to the reduction in total tubular mass a result of the surgical ablation of kidney mass. The administration of nicotine to sham rats was also associated with reductions in the expression of the α7-nAChR, while the administration of MLA increased its expression, suggesting a negative feedback by nicotine on the expression of this receptor subunit.

In previous studies, we (18) determined that the administration of nicotine to wild type C57BL/6 mice had no significant effects on albuminuria or renal injury. In the current studies, the administration of nicotine for 12 wk to sham-operated rats, resulted in significant increases in urinary protein excretion without concomitant changes in glomerular injury score, fibronectin, or TGF-β expression. In light of these results, we measured urinary albumin excretion in the last urine collection from all groups. As shown in Table 2, 5/6Nx resulted in a 10-fold increase in urinary albumin excretion that was further increased 10-fold by the administration of nicotine. However, the administration of nicotine to sham rats did not modify the urinary excretion of albumin. Given the strong expression of the α7-nAChR in the proximal tubules and the role of the proximal tubule in megalin-mediated protein reabsorption (25), we hypothesize that the administration of nicotine may be affecting the tubular reabsorption of filtered proteins (i.e., via megalin) resulting in increases in low-molecular weight pro-

![Fig. 9. NADPH oxidase 4 (NOX4) expression. A: representative Western blot for NOX4 and β-actin, which was used to control for loading. B: densitometry data analysis for cortical NOX4 expression (n = 6–7 per group; *P < 0.05 vs. Sham, 5/6Nx, and 5/6Nx + Nic; †P < 0.05 vs. 5/6Nx + Nic).](http://ajprenal.physiology.org/)

![Fig. 10. Urinary isoprostanes. Administration of nicotine to 5/6Nx rats resulted in a twofold increase in TGF-β as assessed by ELISA in kidney cortex lysates. MLA prevented the increase in TGF-β, suggesting that these effects are mediated by the α7-nAChR (*P < 0.05 vs. all other groups).](http://ajprenal.physiology.org/)
teinuria but not albuminuria in sham animals and at the same contributing in part to the increases in protein excretion and albuminuria in 5/6Nx rats. Interestingly, the administration of MLA did not prevent the increases in proteinuria induced by nicotine in sham animals, suggesting that the α7-nAChR does not mediate these effects. The study of the potential mechanisms involved is the subject of ongoing studies in our laboratory.

NOX is considered to be the most important source of reactive oxygen species in the kidney. Three different NOX isoforms are expressed in the kidney cortex (NOX4, NOX2, and NOX1). Of these, NOX4 is the most abundant NOX in the kidney (1). Induction of NOX4 mRNA expression is observed in response to inflammation (28), endoplasmic reticulum stress (29), diabetes mellitus (12) and activation of the renin angiotensin system (19). In our studies the administration of nicotine increased oxidative stress in 5/6Nx as demonstrated by increases in NOX4 expression and nitrotyrosine expression and urinary isoprostanes. The increases in NOX4 and urinary isoprostanes induced by nicotine in 5/6Nx rats were significantly reduced by α7-nAChR blockade; however, nitrotyrosine was only partially and nonsignificantly reduced suggesting the presence of other NOX4-independent mechanisms that lead to increased oxidative stress in these rats.

In conclusion, in these studies, we have identified the role of the α7-nAChR as an essential mediator of the effects of nicotine in renal injury. The administration of nicotine to rats with 5/6Nx resulted in increased proteinuria and a worse glomerular injury score. In addition, we determined that these effects are accompanied by increased oxidative stress, increased NOX4 and TGF-β expression. Most of the effects of nicotine were reversed by blockade of the α7-nAChR with MLA. These studies unveil novel mechanisms that mediate the deleterious effects of smoking in the progression of CKD and may result in the development of novel therapeutic strategies in the treatment and prevention of CKD in patients unsuccessful in their efforts to quit tobacco smoking.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


