Renoprotective effects of long-term oral nicotine in a rat model of spontaneous proteinuria

Pramod K. Agarwal,1 Jacob van den Born,3 Harry van Goor,2 Gerjan Navis,3 Rijk O. B. Gans,1 and Stephan J. L. Bakker1

1Internal Medicine, 2Department of Pathology, and 3Nephrology, University Medical Center Groningen, Groningen, The Netherlands

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Nicotine exerts its anti-inflammatory effects via α-7 nicotinic acetylcholine receptors (α-7nAchR) (6, 9, 14), which are present on macrophages and peritubular capillaries in the kidney (40, 58). It is well recognized that proteinuria leads to renal inflammation and fibrosis, most likely via tubular activation by filtered proteins, leading to the production of a cascade of mediators by tubular cells, including chemotactants for leukocytes such as monocyte chemoattractant protein (MCP-1) (11). The consequence is an influx of monocytes and macrophages, which mediate renal inflammation and fibrosis (26).

Renal inflammation is seen in many proteinuric renal diseases. Chronic renal inflammation is related to a progressive decline in kidney function, glomerulosclerosis, interstitial damage (37), and resistance to renoprotective therapy (20). Recruited monocytes and macrophages orchestrate inflammation-related damage. A reduction of inflammation by immuno-suppressive drugs has been shown to slow down renal dysfunction with a reduction in glomerulosclerosis and interstitial inflammation (3, 7, 18, 19).

Smoking is a risk factor for development and progression of diabetic nephropathy, for progression of chronic kidney disease to end-stage kidney disease (1, 29) and also for graft failure in renal transplant patients (1). Cigarette smoke contains many compounds of a hydrophilic, lipophilic, and amphiphilic nature, which together are supposed to mediate cigarette smoking-related renal damage (27). Whether nicotine contributes to the harmful effects of cigarette smoke on renal function is not clear. Nicotine is a major constituent of cigarette smoke and used widely in oral form to help subjects quit smoking (38, 39, 41–43, 54). Previous studies have shown nicotine to be clinically beneficial in many diseases in which an inflammatory component plays a role (4). Notably, nicotine has been shown as beneficial in ulcerative colitis (46), sepsis (50), hypersensitivity pneumonitis (5), renal ischemic-reperfusion injury (40), and experimental type 1 diabetes (21).

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We found that long-term oral nicotine slows down the rise in proteinuria, slows down the loss of kidney function, and reduces glomerular injury and inflammatory cellular infiltration in a rat model of spontaneous proteinuria.

MATERIALS AND METHODS

Animals and housing. Inbred 20-wk-old male MWF rats (n = 46) were obtained from Harlan. All animals were housed in temperature-controlled 12:12-h light-dark cycle rooms and had free access to food and drinking water. All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1996), and the protocol was approved by University Medical Center Groningen Animal Ethical Committee.

Experimental setup. After acclimatization for 2 wk, animals were trained for tail-cuff blood pressure measurement (CODA 6, Kent Scientific) daily for another 2 wk. At 24 wk of age, baseline blood pressure was measured, venous blood was withdrawn, and 24-h urine was collected in metabolic cages. For baseline histology, six rats were killed at the age of 24 wk. The other animals were randomly assigned to four different groups. Rats were given either of three different concentrations [20 mg/l (n = 10; N20), 60 mg/l (n = 10; N60), and 100 mg/l (n = 10; N100)] of nicotine mixed with 0.5% sodium saccharin as a sweetening agent ad libitum in drinking water. The control group (n = 10; CON) only received water sweetened with 0.5% sodium saccharin. Treatment continued for 28 wk (until 52 wk of age). One animal in N60 was euthanized prematurely because of tooth malformation due to which the animal was unable to eat and lost weight. One animal in N20 was euthanized because of a bone tumor. The tumor formation is unlikely to be related to nicotine since it was a sporadic case which might be related to aging. Both animals were excluded from all analyses.

Nicotine intake and cotinine measurement. The amount of nicotine intake per day was equal to 24-h water intake of individual rats in metabolic cages × the concentration of nicotine in water. The cumulative dose of nicotine intake was calculated by calculating the area under the curve of nicotine intake over 28 wk of treatment. Cotinine is a major and stable metabolite of nicotine and used to quantify smoking (17). Cotinine was measured in plasma to quantify the amount of nicotine by ImmunoMetric Assay IMMULITE 2000 (Siemens). The range of measurement was >10 to <500 ng/ml.

Clinicopathological parameters. Blood pressure was measured in trained awake animals by the tail-cuff method. Twenty-four-hour urine and venous blood were collected every 4 wk until the end of the study. Twenty-four-hour urine was collected and stored at −20°C.
until analyses were performed. Venous blood was collected with heparin and stored at −80°C. Urinary and plasma creatinine and urinary protein were measured by a multitest analyzer system (Roche Modular; Hoffmann-La Roche, Basel, Switzerland). Creatinine clearance was calculated from 24-h urinary volume, plasma, and urinary creatinine (49).

Reduction in UPE after 28 wk of nicotine treatment at the end of the experiment was calculated as percent difference in urinary protein excretion (UPE) in nicotine-treated animals relative to the non-nicotine-treated control group.

Euthanasia and organs. Animals were euthanized under isoflurane-induced general anesthesia by cutting the diaphragm. Animals were flushed with 0.9% NaCl, the kidneys were harvested, weights were measured, and the organs were partly stored in −80°C and partly fixed in 4% formalin followed by embedding in paraffin.

Morphological analysis. Four-micrometer-thick formalin-fixed paraffin sections were deparaffinized and stained by periodic acid-Schiff (PAS) for quantification of focal segmental glomerulosclerosis (GS). GS was semiquantitatively scored in a blinded fashion by determining the level of mesangial expansion and focal adhesion in each quadrant in a glomerulus and expressed on a scale from 0 to 4. If 25% of the glomerulus was affected, it was scored as 1, 50% as 2, 75% as 3 and 100% as 4. In total, 50 glomeruli/kidney were analyzed, and the total GS score was calculated by multiplying the score by the percentage of glomeruli with the same GS score. The sum of these scores gives the total GS score from 0 to 200 (49).

Immunohistochemistry. Deparaffinized and rehydrated sections (4 µm) were subjected to heat-induced antigen retrieval by overnight incubation in a 0.1 M Tris·HCl buffer (pH 9.0) at 80°C. Endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 30 min, and sections were incubated with ED1 antibody to visualize monocytes/macrophages (Serotec, Oxford, UK) or anti-smooth muscle actin (SMA; A5228, Sigma-Aldrich, Zwijndrecht, The Netherlands) to show myofibroblasts, anti-desmin antibody to show podocyte injury (Novus Biologicals), and anti-podocin antibody to show changes in glomerular podocin content (Sigma) for 60 min at room temperature. Binding of the antibody was detected using sequential incubations with horseradish peroxidase (HRP)-labeled rabbit anti-mouse and HRP-labeled Swine anti-rabbit antibodies (Dako), both for 30 min. Peroxidase activity was developed using 3-amino-9-ethylcarbazole (AEC) for 15 min. Sections were counterstained with hematoxylin. Monocyte/macrophage influx and myofibroblasts were quantified in 50 cortical and outer medullary areas using an image processing and analysis program (ImageJ), magnified 200 times in a standardized and blinded fashion. Desmin and podocin positivity was quantified in a similar fashion. The results are presented as the percentage of positively stained area relative to the total area measured. Arteries were excluded from analysis.

Quantitative real-time RT-PCR. Total RNA was extracted from 25–30 mg of frozen rat kidney tissue using an RNeasy minikit from Qiagen. cDNA was synthesized using 1 µg of RNA and a QuantiTect Reverse Transcriptase kit (catalog no. 205313, Qiagen). PCRs were

Fig. 2. Effect of oral nicotine on the development of glomerulosclerosis. Development of focal segmental glomerulosclerosis (GS) was reduced by oral nicotine. At baseline, no GS was present (A). After 28 wk of treatment, the control group developed GS (B), which is dose dependently downmodulated by 20 (C), 60 (D), and 100 mg/l (E) oral nicotine. Photos are representative photomicrographs of periodic acid-Schiff (PAS) staining. Magnification ×400. Quantification of GS in the various groups is depicted (F). #No GS. ***P < 0.001. **P = 0.02.
performed in a 10-μl reaction containing 6.6 ng of RNA and using a 2X Sensimix CYBR Green mastermix kit (catalog no. QT 650, Qiagen). Quantitative real-time (qPCR) was performed in a C1000 Thermal cycle (Bio-Rad). On-demand primers for GAPDH, MCP-1, and VCAM-1 were obtained from Qiagen. Results are expressed relative to CON after euthanasia at 52 wk of age after normalizing for GAPDH, a housekeeping gene.

Statistical analyses. Statistical analysis was performed using SPSS 14. Areas under the curve and figures were made by GraphPad Prism (version 4.00; GraphPad Software, San Diego, CA). Parametric values are expressed as means ± SE. Nonparametric values are expressed as medians (Inter Quartile Range) and were log transformed for further analysis. ANOVA with a post hoc Tukey’s test for comparison of treatment groups and controls at 52 wk and a general linear model for repeated measurements for comparison of the time course of proteinuria between treatment groups were used. P < 0.05 was considered statistically significant.

RESULTS

Nicotine intake and clinicopathological parameters. Parameters concerning nicotine intake and clinicopathological markers are shown in Table 1. Cumulative nicotine intake was calculated from concentrations added to drinking water and the volume of water intake. Nicotine intake was significantly different among the three treatment groups, and there was no nicotine intake in the CON group. Similarly, there was a dose-dependent difference in plasma cotinine concentrations among the treatment groups and no detectable plasma cotinine in the CON group. The CON group had significantly higher plasma triglyceride compared with N60 and N100. There were no differences in body weight, water intake, and systolic blood pressure among the groups during the experiment.

Renal parameters. Plasma creatinine increased from 26 ± 2 μmol/l at 24 wk of age in the baseline group to 50 ± 7 μmol/l at 52 wk of age after 28 wk of placebo with sodium saccharin (CON) (table 1, P = 0.003). After 28 wk of nicotine treatment, groups N60 and N100 had significantly lower serum creatinine (31 ± 1 and 32 ± 2 μmol/l, respectively, both P < 0.05) than the CON group. To certify that observed differences are the consequence of changes in kidney function and not changes in muscle mass, creatinine clearance was calculated. Nicotine treatment improved kidney function dose dependently compared with CON (P = 0.003) (Fig. 1A). UPE increased spontaneously in MWF rats during the experiment, with values of 38 (22–57) mg/24 h and 140 (105–159) in the CON group at

Fig. 3. Effect of oral nicotine on podocyte damage. Podocyte damage was evaluated after staining for podocin. At baseline, there was less podocytopathy (A). After 28 wk of treatment, the control group developed higher podocytopathy (B), which was dose dependently downmodulated by 20 (C), 60 (D), and 100 mg/l (E) oral nicotine. Photos are representative photomicrographs of podocin staining. Magnification ×200. Quantification of % of glomerular podocin staining in the various groups is depicted (F). *P < 0.05. **P < 0.01.
the end of the experiment ($P < 0.001$). Although no impressive differences were achieved, nicotine treatment significantly mitigated the rise in UPE by 10, 20, and 16% in the N20, N60, and N100 groups, respectively ($P = 0.03$ vs. CON) (Fig. 1B).

**Glomerular histomorphological analysis**. PAS staining was done to quantify GS. MWF rats developed de novo GS over time. Similarly, there were decreases in podocin and increases in desmin over time. At the end of the study, CON had higher GS than the nicotine-treated groups ($P < 0.001$). Long-term nicotine treatment led to a significant (~50%) reduction in GS score in the N60 and N100 groups vs. CON (both $P = 0.02$) (Fig. 2). Similarly, at the end of the study, the CON group had higher desmin deposition and lower podocin staining than the nicotine-treated groups (both $P < 0.01$, Figs. 3 and 4, respectively).

**Renal infiltration of monocyte/macrophages and myofibroblasts**. Nicotine treatment of the N60 and N100 groups significantly reduced the amount of ED1-positive macrophages in the kidney compared with CON ($P = 0.001$) (Fig. 5). Myofibroblasts were stained with $\alpha$-SMA and represent a major cell type involved in interstitial matrix deposition. Figure 6 shows that N60 ($P = 0.01$) and N100 animals ($P < 0.05$) had significantly reduced $\alpha$-SMA positivity in the kidneys compared with CON.

**qPCR analysis**. Since the influx of ED-1-positive monocytes and macrophages was reduced upon oral nicotine, we evaluated by qPCR renal expression of MCP-1 and VCAM-1, the former being a major chemoattractant and the latter being an important adhesion molecule for monocytes and macrophages. The expression of both MCP-1 and VCAM-1 were higher in CON at the end of the study compared with baseline (both $P < 0.01$, Fig. 7, A and B, respectively). Nicotine treatment dose dependently lowered the expression of total MCP-1 in the N20, N60, and N100 groups. However, levels in the N60 ($P < 0.01$ vs. CON) and N100 groups ($P < 0.01$ vs. CON) reached statistically significance. Moreover, nicotine treatment lowered the expression of total VCAM-1 as well in N60 ($P < 0.05$ vs. CON) and N100 animals ($P < 0.05$ vs. CON).

**DISCUSSION**

Our results demonstrate that long-term oral nicotine improves renal function and morphology and reduces renal inflammation in the MWF rat. As anticipated in this model, the

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**Fig. 4.** Effect of oral nicotine on glomerular desmin deposition. At baseline, there was less desmin deposition in glomeruli (A). After 28 wk of treatment, the control group developed higher desmin deposition (B), which was dose dependently downmodulated by 20 (C), 60 (D), and 100 mg/l (E) oral nicotine. Photos are representative photomicrographs of desmin staining. Magnification $\times200$. Quantification of % of glomerular desmin deposition in the various groups is depicted (F). *$P < 0.05$. **$P < 0.01$. 

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CON group developed loss of kidney function, GS, and tubulointerstitial inflammation. The glomerular as well as interstitial damage was reduced by oral nicotine in a dose-dependent manner. Improvement was also seen at the mRNA level by downregulation of the inflammatory markers MCP-1 and VACM-1.

Nicotine and cigarette smoking have been shown to elicit an acute systemic adrenergic response, resulting in increases in blood pressure and heart rate in both healthy subjects and renal patients (36). However, it is questionable whether these effects are sustained in the long term. In an animal study, the same group found no effects on blood pressure of long-term administration of nicotine in combination with cigarette smoke extract (27). Consistent with these findings, we did not find any statistically significant change in blood pressure in our long-term animal study. Whereas our assessment of blood pressure by tail-cuff is not the gold standard, it seems implausible that an effect on blood pressure would go unnoticed altogether.

On one hand, nicotine is known to induce endothelial nitric oxide synthase (eNOS), which is vasodilatory in isolated, perfused kidney (13), while on the other hand it has been shown that nicotine attenuates the renal vasodilatory responsiveness to some vasodilator pathways, particularly when in combination with cyclosporine (12). Therefore, a direct effect on glomerular pressure by either efferent vasodilatation, afferent vasoconstriction, or both cannot be excluded, in particular because UPE was slightly higher in CON groups than in nicotine-treated groups. The lowering of UPE was persistent throughout the experiment. This lowering in UPE might have contributed to the observed renoprotective effects of oral nicotine. Of note, the attenuation of renal vasodilatory responsiveness by nicotine may the decrease renoprotective properties of nicotine, in particular when combined with drugs that further decrease this responsiveness, like cyclosporine.

It is known that nicotinic receptors are present on macrophages, peritubular capillaries, dendritic cells, and vascular smooth muscle cells (4, 6, 52). Previous studies have shown that nicotine reduces monocyte/macrophage influx in tissues via the α7NACHR pathway and reduces endothelial activation (51, 56). Nicotinic agonists attenuate the activation of macrophages (10). We also showed that macrophage infiltration was reduced in nicotine-treated animals. Various cytokines, including IFN-γ, IL-6, and lymphocyte activation, are implicated in the activation of macrophages (32, 53). Nicotine is known to downregulate these inflammatory cytokines, and this effect could play a role in the reduction of glomerulosclerosis and interstitial fibrosis (8, 9, 47). We observed reduced glomerulosclerosis and lower density of myofibroblasts. These obser-
vations indicate an antifibrotic effect of nicotine. However, we cannot discriminate between a direct nicotinic anti-inflammatory effect and a secondary nicotinic effect via a reduction in UPE. We investigated the therapeutic effects of oral nicotine using three different doses. There was a clear reduction of GS, UPE, podocytopathy, desmin deposition, ED-1 influx, and α-SMA accumulation at N20, which is in between CON and N60. With the exception of the effect on α-SMA, for doses of 60 and 100 mg/l the effects were more or less similar, suggesting that there is a saturation of the effect of nicotine beyond 60 mg/l.

Few studies have investigated the role of nicotine in rodent models of kidney disease. Although findings of our study are consistent with many studies, some have even reported opposite results. On one hand, our findings are in line with findings of Yeboah et al. (55) and Sadis et al. (40), where they found anti-inflammatory effects of oral nicotine in rat models of ischemia-reperfusion-induced kidney damage. On the other hand, a deteriorating effect of nicotine was found in mice models for diabetes (16) and ischemia-reperfusion injury (2). How might these differences be explained? First of all, it is always cumbersome to compare rodent models with respect to strain differences (mouse vs. rat) and source of delivery, sex (male vs. female), age, and dosage scheme of the drug. More important is the realization that the effects of nicotine are evaluated in different models of renal disease. Our model, the MWF rat, is a proteinuric model (48). That means that proteinuria is the driving force behind renal damage. Proteinuria can be reduced by reducing glomerular pressure. Most likely, nicotine reduced glomerular pressure and/or reduced proteinuria, and consequently renal function and morphology and renal inflammation are reduced. The studies that reported negative effects of oral nicotine used diabetic mice and mice that underwent ischemia-reperfusion (45, 46). Driving forces behind renal damage in these models are hyperglycemia and lack of oxygen, respectively, which apparently use other activation and signaling routes that are less sensitive to interference with nicotine. Moreover, the MWF rat model used by us demonstrates more chronic inflammation compared with the db/db mice and the chronic phase of ischemia-reperfusion. This might explain why anti-inflammatory actions of nicotine were clearer in our model.

Smoking is a risk factor for cardiovascular diseases, kidney disease, and lung cancer (1, 44). Nicotine is one of the most addictive components of cigarette smoke. Our study indicates that oral nicotine, besides reducing direct harmful effects from...
cigarette smoking and overcoming the addiction of smoking, reduces inflammation in kidneys. However, nicotine binds to all nicotinic receptors and could lead to systemic side effects. Binding of nicotine to neural nicotinic receptors makes it addictive in nature. Caution should be taken in prescribing nicotine due to its potential cardiovascular side effects. Further research is necessary to assess whether nicotine without concomitant exposure to other constituents normally present in cigarette smoke is harmful from a cardiovascular perspective (31, 57). Thus other specific α-7nAchR agonists could be of therapeutic use, particularly if these compounds would be devoid of addiction effects.

To the best of our knowledge, long-term studies with nicotine are lacking despite nicotine’s being used chronically in clinical settings. Therefore, we investigated nicotine usage in a long-term experiment and found that nicotine is renoprotective in the MWF rat model of spontaneous proteinuria. It should, however, be realized that these renoprotective effects may be limited to the model we used. MWF rats have spontaneous progressive proteinuria and glomerular podocytopathy (23, 48). Therefore, it cannot be excluded that similar beneficial effects are not present in other models of progressive proteinuria and kidney disease. This deserves additional experimentation. Kidney function was measured with creatinine clearance to avoid potential effects of a change in muscle mass (30). We found that nicotine treatment improved creatinine clearance. However, kidney function as measured by creatinine clearance is not the gold standard, especially in rodents since there is a considerable amount of tubular secretion of creatinine. Measuring renal function with exogenous renal markers, such as inulin, iothalamate, or iohexol, is considered to be the gold standard, but this is time consuming, invasive, and cumbersome (45). It can therefore not be excluded that preserved creatinine clearance is due to nicotine-induced tubular secretion of creatinine rather than preserved glomerular filtration. Our conclusion of the renoprotective effects of nicotine is, however, not based solely on the improvement in creatinine clearance but also on the concomitant moderate attenuation of UPE and, more importantly, on the concomitant prevention of deterioration of renal histological parameters. Furthermore, although we show that oral nicotine results in lowering of proteinuria and renal inflammation, the exact mechanism responsible for renoprotection is not identified by our study and deserves further research, e.g., further studies with renal denervation.

In conclusion, our data show that long-term oral nicotine improves kidney function, reduces proteinuria, renal inflammation, and glomerulosclerosis in MWF rats. This novel action of nicotine could be evaluated as a potential additional therapeutic option for treating proteinuric and/or inflammatory kidney diseases.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Fig. 7. Effects of oral nicotine on mRNA expression values of inflammatory markers monocyte chemoattractant protein (MCP)-1 and VCAM-1. A: qPCR results for MCP-1. Control at week 52 had higher expression of MCP-1 than baseline rats. Nicotine treatment at the higher doses of 60 and 100 mg/l reduced the mRNA expression of MCP-1 compared with age-matched control. The quantifications are relative to control after normalization for GAPDH. **P < 0.01. B: qPCR results for VCAM-1. Control has higher expression of VCAM-1 than baseline rats. Nicotine treatment at the higher doses of 60 and 100 mg/l reduced the mRNA expression of MCP-1 compared with age-matched control. The quantifications are relative to control after normalization with GAPDH. ***P < 0.001. *P < 0.05.