Chronic nicotine exposure exacerbates acute renal ischemic injury

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1Division of Pediatric Nephrology, Department of Pediatrics, 2Department of Physiology and Biophysics, and 3Division of Nephrology, Department of Medicine, University of Mississippi Medical Center, Jackson, Mississippi; and 41st Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

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Arany I, Grifoni S, Clark JS, Csongradi E, Maric C, Juncos LA. Chronic nicotine exposure exacerbates acute renal ischemic injury. Am J Physiol Renal Physiol 301: F125–F133, 2011. First published April 20, 2011; doi:10.1152/ajprenal.00041.2011.—Recent epidemiological reports showed that smoking has a negative impact on renal function and elevates the renal risk not only in the renal patient but perhaps also in the healthy population. Studies suggested that nicotine, a major tobacco alkaloid, links smoking to renal dysfunction. While several studies showed that smoking/chronic nicotine exposure exacerbates the progression of chronic renal diseases, its impact on acute kidney injury is virtually unknown. Here, we studied the effects of chronic nicotine exposure on acute renal ischemic injury. We found that chronic nicotine exposure increased the extent of renal injury induced by warm ischemia-reperfusion as evidenced by morphological changes, increase in plasma creatinine level, and kidney injury molecule-1 expression. We also found that chronic nicotine exposure elevated markers of oxidative stress such as nitrotyrosine as well as malondialdehyde. Interestingly, chronic nicotine exposure alone increased oxidative stress and injury in the kidney without morphological alterations. Chronic nicotine treatment not only increased reactive oxygen species (ROS) production and injury but also exacerbated oxidative stress-induced ROS generation through NADPH oxidase and mitochondria in cultured renal proximal tubule cells. The resultant oxidative stress provoked injury through JNK-mediated activation of the activator protein (AP)-1 transcription factor in vitro. This mechanism might exist in vivo as phosphorylation of JNK and its downstream target c-Jun, a component of the AP-1 transcription factor, is elevated in the ischemic kidneys exposed to chronic nicotine. Our results imply that smoking may sensitize the kidney to ischemic insults and perhaps facilitates progression of acute kidney injury to chronic kidney injury.

smoking; oxidative stress; JNK/AP-1 activation

SMOKING IS A MAJOR AND PREVENTABLE contributor to excess morbidity and mortality in the United States. Although the pathological role of smoking in the development of cardiovascular diseases, cancer, or chronic obstructive pulmonary diseases is widely studied, its impact on kidney function has only recently been recognized (38). Epidemiological studies have concluded that smoking accelerates the rate of progression of renal failure to end-stage renal disease in the renal patient (36). It also elicits a negative impact on renal function and may elevate the risk of chronic renal injury even in the healthy population (5, 37). However, while it has been suggested that smoking may have a negative impact on experimental and human radiocontrast-induced nephropathy (17, 28), whether smoking has a detrimental effects on acute kidney injury (AKI) is unknown. This is important not only because of the increased mortality that is associated with AKI but also because AKI itself is an important risk factor for the development and progression of chronic kidney disease (34). Thus smoking may exacerbate AKI-induced tubulointerstitial injury and progression to chronic renal disease.

The mechanisms of smoking-related renal damage are poorly understood but are likely due to both vascular and tubular effects. For instance, smoking-induced oxidative stress leads to endothelial dysfunction (35) and vascular injury (36). Indeed, studies have found increased renal vascular resistance (11, 45), decreased glomerular filtration rate (GFR), and biochemical evidence of smoking or chronic nicotine-induced renal toxicity, even in the absence of histological changes (26). Increased oxidative stress and morphological abnormalities have also been observed in the proximal tubular epithelium after exposure to chronic cigarette smoke or nicotine (NIC) (8–10, 12), and low-grade damage of proximal tubules has also been observed among chronic smokers in the general population (16, 20). These alterations may sensitize the kidney to acute ischemic AKI.

While the harmful effects of smoking may be due to many different components of tobacco smoke, one of the more likely culprits is the alkaloid NIC (19). NIC is excreted by glomerular filtration and tubular secretion (19) and has been found in high concentration in the serum and kidneys of smokers (reviewed in Ref. 19). Chronic exposure to NIC increases oxidative stress in the kidney (22, 43), cultured proximal tubule (25), or mesangial cells (23), thus linking smoking and NIC to renal injury (23). Consequently, smoking or chronic NIC exposure might exacerbate acute renal injury through increasing oxidative stress as observed in experimental radiocontrast-induced AKI (17).

Therefore, our first aim was to determine whether chronic NIC exposure exacerbates ischemia-reperfusion-induced oxidative stress and kidney injury in mice and in cultured mouse proximal tubule cells. We then performed in vitro studies to determine the mechanism by which chronic NIC exposure enhanced oxidative stress and consequent tubular injury.

MATERIALS AND METHODS

Animals, chronic NIC exposure, and renal eschima-reperfusion. Nine- to 10-wk-old male C57Bl/6j mice (Jackson Laboratories) were divided into two groups (n = 12/group). The first group of mice received nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) in a 2% saccharine solution at 200 μg/ml concentration as their drinking source for 4 wk as suggested by others (6). The second group received a 2% saccharine solution for 4 wk. Our pilot studies showed that this length of NIC exposure was needed to reproducibly mimic plasma cotinine levels in C57BL/6j mice that are comparable to those found in chronic smokers (19). Eight animals from each group were sub-
ected to 18 min of warm ischemia followed by 24-h reperfusion. Briefly, renal ischemia was imposed under pentobarbital sodium anesthesia as described elsewhere (40). Kidneys were exposed through an abdominal incision and subjected to bilateral ischemia by clamping both renal pedicles with nontraumatic vascular microclamps (Micro Aneurysm clip, straight, 10 mm, 125-g pressure, RS-5426, Roboz Surgical Instruments, Rockville, MD). Mice were kept on a heating pad, and their rectal temperature was monitored to maintain the body temperature at 37°C. After 18 min the clamps were removed, reperfusion of the kidneys was visually confirmed, and the incisions were closed. After the surgery, the animals were monitored for recovery then returned to their cages and allowed free access to food and water with NIC or saccharine, respectively. Four animals from each group (saccharine or NIC) underwent a sham operation; i.e., kidneys were exposed similar to the ischemia-reperfusion group, but renal pedicle clamping was not employed. Twenty-four hours after the ischemia, all animals were euthanized, blood was drawn, and the kidneys were removed for further purposes such as histological and molecular studies. All these procedures were done in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Cotinine content of serum and the kidney. Serum and kidney cotinine content was determined by a Cotinine ELISA kit (Calbiotech, Spring Valley, CA) according to the manufacturer’s recommendations.

Renal histology. Formalin-fixed and paraffin-embedded kidneys were assessed for tubulointerstitial injury in 5-μm paraffin sections stained with periodic acid-Schiff (PAS) according to a standard protocol as described elsewhere (29). Accordingly, 60 randomly selected fields of view of the renal cortex/medulla (at magnification of ×200) in 4–4 sections were subjected to a semiquantitative scoring with a light microscope with a scale of 0–4 (grade 0, normal; grade 1, affected area <10%; grade 2, affected area 10–25%; grade 3, affected area 25–75%; and grade 4, affected area >75%).

Plasma creatinine assay. A QuantiChrom Creatinine Assay kit was used (BioAssay Systems, Hayward, CA) as recommended by the manufacturer.

Malondialdehyde assay. A malondialdehyde (MDA) assay kit was provided by Northwest Life Science Specialties (Vancouver, WA). Equal amounts of kidney lysates (200 μg) were processed according to the manufacturer’s recommendation, and the MDA content was determined spectrophotometrically and expressed as nanomoles per milligram protein.

Cell line and establishment of oxidative injury in vitro. The immortalized mouse proximal tubule line (TKPTS) (15) was used. Oxidative injury was induced by treatment of cells with 200 μM H2O2 (3). Some cells were pretreated with 200 μM NIC (Sigma-Aldrich) for 24 h before treatment with H2O2. The dose of NIC was chosen based on our preliminary studies that showed that this dose exacerbated oxidative stress-related injury in vitro.

Adenoviral infection. TKPTS cells grown in 24-well plates were infected with 50 multiplicities of infection/ml dominant-negative JNK (ΔNJK) (24) or control adenovirus (Ad-Null, Vector BioLabs, Philadelphia, PA) for 24 h as described elsewhere (3). The ΔNJK adenovirus was a kind gift of Dr. H. Kaneto (Osaka University).

Assessment of cell viability/injury in vitro. Cell injury was assessed by a fluorescent CytoTox-One Homogenous Membrane Integrity Assay Kit (Promega, Madison, WI). Briefly, after the appropriate treatment an aliquot of the growth medium was removed and saved. The monolayer was lysed according to the manufacturer’s recommendations and lactate dehydrogenase (LDH) content was determined by a fluorescent substrate in both the medium and cell lysate. LDH release was calculated as a percentage of LDH content in the medium compared with the total LDH content (medium+lysate). We also determined the extent of cell viability by a LIVE/DEAD Viability/ Cytotoxicity Assay Kit (Invitrogen). The kit contains two fluorescent dyes: calcein AM, which is retained by live cells and emits green fluorescence, and ethidium-1 (EthD-1), which is taken up by damaged cells but excluded by live cells (it emits red fluorescence). Briefly, cells grown in six-well plates were treated as described, and monolayers were washed with PBS and stained with calcein AM and EthD-1 for 20 min. After repeated washing with PBS, red and green fluorescence was observed under a fluorescence microscope. Calcein exhibits green fluorescence, and it is a marker of intact cells. EthD-1 exhibits red fluorescence that indicates compromised membrane integrity.

Fluorescence microscopy. For imaging of live cells, a Nikon Eclipse TS100Fi inverted fluorescence microscope equipped with CY3, FITC, and DAPI filters was used at ×10–40 magnification. Images were captured by a DS Cool Camera head (DS-Qi1) using NIS Elements for Basic Research 3.0 software (Nikon).

Protein isolation, Western blotting. Kidneys were removed and homogenized in a RIPA buffer as described earlier (2). Similarly, monolayers of cells were lysed in a RIPA buffer. SDS-PAGE and Western blotting were performed using conventional techniques as described elsewhere (2).

Measurement of reactive oxygen species production in vitro. Intracellular generation of reactive oxygen species (ROS) was determined by a microplate assay using oxidant-sensitive 2′,7′-dichlorofluorescin-diacetate (DCFDA; Invitrogen, Grand Island, NY). Cells grown in T25 flasks were pretreated as needed and isolated with trypsinization and then loaded with 100 μM DCFDA in HBSS for 30 min at 37°C. After incubation, the dye was washed away with fresh HBSS and placed in wells of a 96-well plate (0.5 × 10⁶ cells/well). H2O2 was added to the wells, and the increase in fluorescence was monitored in a fluorescence plate reader (Fluorocount, Packard) at 485 nm/530 nm excitation/emission. ROS production was calculated as changes in fluorescence/30 min/0.5 × 10⁶ cells and expressed as the percentage of untreated values. To assess the source of generated ROS, chronic NIC-exposed TKPTS cells were pretreated with the xanthine oxidase inhibitor allopurinol (100 μM), the NADPH inhibitor diphenilenediodium (5 μM), or the mitochondrial inhibitor antimycin A (10 μM) for 30 min before treatment with 200 μM H2O2 to similar to that described elsewhere (1).

Reporter luciferase assay. TKPTS cells were transfected with a pAP-1-Luc plasmid (Agilent Technology Wilmington, DE) together with a Renilla luciferase plasmid (Promega) using Xfect reagent (Clontech, Mountain View, CA) according to the manufacturer’s recommendations. Twenty-four hours after transfection, cells were treated as needed and 24 h later the firefly and Renilla luciferase activities were determined by a Dual Luciferase Assay Kit (Promega).

Statistical analysis. Continuous variables were expressed as means and SD. Statistical differences between the treated and control groups were determined by Student’s t- or Mann-Whitney rank sum tests. Differences between means were considered significant if P < 0.05. All analyses were performed using the SigmaStat 3.5 software package (Sysstat, San Jose, CA).

RESULTS

Chronic NIC exposure exacerbates renal ischemia-reperfusion injury in mice and oxidative stress-induced injury in cultured renal proximal tubule cells. We adopted a model of chronic NIC exposure using C57Bl/6J mice as described by others (6). Plasma cotinine levels in the NIC-exposed mice (Table 1) were comparable to that found in the serum of chronic smokers (150–300 ng/ml) (19). In addition, the kidneys also contained significant amounts of cotinine (Table 1). NIC did not alter basal plasma creatinine levels but caused a moderate increase in expression of kidney injury molecule-1 (KIM-1), a known marker of renal proximal tubular injury (27), in kidney lysates (Fig. 1), suggesting that mild kidney injury may be present.

Subjecting the animals to 18 min of warm ischemia caused significant AKI, as demonstrated by an increase in plasma creatinine (Table 1) and in renal KIM-1 expression (Fig. 1).
Chronic NIC exacerbated ischemia-reperfusion-induced AKI; it caused plasma creatinine and renal KIM-1 expression to increase to a greater extent than in the saccharin-treated mice.

Chronic NIC also exacerbated ischemia-reperfusion-induced renal morphological changes. The extent of tubulointerstitial injury was assessed and scored in 5-μm paraffin sections stained with PAS according to a standard protocol. While no injury was observed in the saccharine+sham and NIC+sham animals (Fig. 2, A and B), prominent injury to tubular epithelial cells was observed in the saccharine+ischemia-reperfusion (Fig. 2C and Table 1: injury score 1.85 ± 0.14) and NIC+ischemia-reperfusion (Fig. 2D and Table 1: injury score 2.59 ± 0.3) groups, with the degree of injury being greater in the nicotine+ischemia-reperfusion group (Fig. 2D and Table 1). The injury was most apparent in the outer medullary and juxtapamedullary region of the renal cortex.

Next, we studied the effects of chronic NIC exposure (24 h) on an established in vitro model of oxidative injury in mouse renal proximal tubule cells (TKPTS) (2, 3). Accordingly, TKPTS cells were pretreated with 200 μM NIC for 24 h before treatment with 200 μM H₂O₂. Twenty-four hours after treatment with H₂O₂, the extent of LDH release and calcein/EthD-1 uptake was determined. As seen in Fig. 1C, oxidative stress (H₂O₂ treatment) significantly increased LDH release, which was further increased by chronic pretreatment with NIC. We also assessed the extent of injury by calcein/EthD-1 staining. While calcein uptake (green fluorescence) is a sign of live/intact cells, EthD-1 uptake (red fluorescence) is a sign of impaired cell membrane integrity. Values were expressed as percentage of cells with EthD-1 uptake compared with the total number of cells. Figure 2, E and F, shows that EthD-1 uptake was significantly increased upon exposure to H₂O₂ (from <1% to 7.5 ± 1.2%), which was further aggravated by pretreatment with NIC (25.3 ± 3.5%: Fig. 2H). Interestingly, chronic NIC exposure alone moderately but significantly increased both LDH release (Fig. 1C) and EthD-1 uptake (Fig. 2G: 9.2 ± 1.5%). These data suggest that chronic NIC exposure alone is mildly toxic to proximal tubule cells in vitro. These in vitro findings corroborate with KIM-1 results in vivo (Fig. 1A and B), suggesting a mild injury in renal cells upon chronic NIC exposure both in vivo and in vitro.

Chronic NIC exposure increases ischaemic injury by exacerbating oxidative stress in the kidney as well as in cultured renal proximal tubule cells. Renal nitrotyrosine expression, a marker of oxidative stress, was determined by Western blotting. Nitrotyrosine expression was elevated in the ischemic kidneys from saccharine-treated mice, which further increased in the ischemic kidneys from NIC-exposed mice (Fig. 3, A and B). Chronic NIC exposure alone also elevated expression of nitrotyrosine. In addition, MDA content was also determined. Figure 3C shows that renal MDA content was significantly higher in ischemic kidneys from NIC-exposed mice compared with their saccharine-exposed counterparts. Interestingly, MDA content of kidneys from NIC-exposed mice was also higher than in their saccharine-treated counterparts. These results suggest the presence of a mild oxidative stress in the kidneys of NIC-treated mice and significant exacerbation of oxidative stress in the ischemic kidneys.

Next, TKPTS cells were pretreated or not with 200 μM NIC for 24 h and 200 μM H₂O₂-induced ROS production was determined. As Fig. 4A shows, chronic NIC pretreatment significantly increased H₂O₂-induced ROS production in an additive manner. In addition, chronic NIC exposure itself also increased ROS production (Fig. 4A), which underscores the in vivo findings (Fig. 3).

As the main source of ROS in renal cells is xanthine oxidase, NADPH oxidase, or the mitochondria, we employed various inhibitors, similar to our study published earlier (1), to assess the contribution of those pathways to NIC+H₂O₂-induced ROS generation in vitro. Figure 4B shows that while the xanthine oxidase inhibitor allopurinol does not affect NIC+H₂O₂-induced ROS production, then both the NADPH oxidase inhibitor diphenyldiamidine and mitochondrial inhibitor antymycin A significantly inhibited it. Thus we concluded that both NADPH oxidase and the mitochondria are important players in ROS generation. To determine whether NIC-induced oxidative stress mediates injury, cells were pretreated with 100 μM N-acetyl-cysteine (NAC) before treatment with NIC+H₂O₂ and LDH release was determined. In these experiments, the antioxidant NAC abrogated NIC+H₂O₂-induced LDH release (Fig. 4C).

Chronic NIC exposure aggravates acute renal ischemia- or oxidative stress-induced stress kinase signaling in the kidney or in cultured renal proximal tubule cells. Oxidative stress activates (phosphorylates) various MAPKs, including JNK both in the kidney (13, 21) and in cultured renal proximal tubule cells (3, 21), which, through the transcription factor AP-1 (14, 30), is involved in injury (3, 13, 21). Accordingly, we analyzed kidney lysates from ischemic or sham-operated mice for phosphorylation of JNK by Western blotting. Representative blots in Fig. 5A show increased phosphorylation of JNK in the ischemic kidneys, which was exacerbated upon chronic exposure to NIC. Interestingly, chronic NIC exposure alone also elevated JNK phosphorylation in the kidney (Fig. 5, A and B), suggesting a higher level of stress in the kidneys of....
We also found that the ROS scavenger NAC attenuated NIC+/H2O2-induced phosphorylation of JNK (Fig. 6A) but dnJNK inhibition of JNK function did not significantly inhibit NIC+/H2O2-induced ROS production (Fig. 6B). These results suggest that phosphorylation of JNK is the consequence but not the cause of NIC+/H2O2-induced ROS production.

Activated JNK, possibly through AP-1, mediates injury of renal proximal tubule cells. Further experiments were designed to demonstrate the role of JNK and its downstream target, AP-1, in NIC+-oxidative stress-induced injury in vitro. Accordingly, TKPTS cells were infected with a dnJNK adenovirus as described earlier (3) and treated with 200 μM NIC for 24 h

NIC-treated animals. These results suggest that chronic NIC exposure provokes stronger stress, and consequent prodeath, responses to ischemia in the kidneys. In addition, phosphorylation of c-jun, a component of the AP-1 transcription factor (44), is also aggravated in the ischemic kidneys from NIC-treated animals (Fig. 5, C and D).

Fig. 1. Chronic nicotine (NIC) exposure exacerbates injury of the kidney after acute ischemia-reperfusion (IR) and cultured proximal tubule cells after treatment with H2O2. A: expression of kidney injury molecule (KIM)-1 in kidney lysates from C57BL/6J mice that underwent renal IR injury was determined by Western blotting. One group received saccharine, while the other NIC as described in MATERIALS AND METHODS. B: densitometry of results from A. Values are means ± SD of ratios of KIM-1/actin; n = 4 (sham) and n = 8 (IR). *P < 0.05 compared with sham. #P < 0.05 compared with saccharine group. @P < 0.05 compared with the saccharine group. C: TKPTS cells were pretreated or not with 200 μM NIC for 24 h before treatment with 200 μM H2O2. Lactate dehydrogenase (LDH) release was determined 24 h after treatment with H2O2. Values represent percentage of released LDH and expressed as means ± SD; n = 3. *P < 0.05 compared with untreated (none).

Fig. 2. Effects of chronic NIC exposure on kidney morphology and integrity of cultured proximal tubule. Sections of kidneys form saccharine+ sham (A), saccharine+IR (B), NIC+ sham (C), and NIC+IR (D) were stained with PAS and evaluated for injury as described in MATERIALS AND METHODS. TKPTS cells were treated or not with 200 μM NIC for 24 h followed by 200 μM H2O2 for 3 h and stained with calcein/ethidium as described in MATERIALS AND METHODS. Pictures are representatives of 3 independent experiments. E: control, untreated. F: H2O2-treated; G: NIC alone; H: NIC+H2O2.
followed by treatment with 200 μM H2O2. LDH release was determined 24 h later. As is shown in Fig. 4C, dnJNK significantly inhibited NIC+H2O2-induced injury. dnJNK also inhibited H2O2- as well as NIC-induced LDH release (data not shown).

In addition, NIC or H2O2 alone significantly increased activity of the AP-1-luciferase reporter, which was further increased by their combination (NIC+H2O2). Importantly, all those AP-1 activities were significantly attenuated by dnJNK.

Fig. 3. Chronic NIC exposure increases oxidative stress in the kidney. A: expression of nitrotyrosine (NTyr) in kidney lysates from C57BL/6J mice that underwent renal IR injury was determined by Western blotting. One group received saccharine while the other NIC as described in MATERIALS AND METHODS. B: densitometry of results from A. Values are means ± SD of NTyr/actin ratios; n = 4 (sham) and n = 8 (IR). *P < 0.05 compared with sham, #P < 0.05 compared with saccharine group. @P < 0.05 compared with the saccharine group. C: renal malondialdehyde (MDA) content of kidney lysates from C57BL/6J mice that underwent renal IR injury was determined by spectrophotometry as described in MATERIALS AND METHODS. One group received saccharine while the other NIC as described in MATERIALS AND METHODS. Values represent MDA content in nmol/mg protein and expressed as means ± SD; n = 4 (sham) and n = 8 (IR). *P < 0.05 compared with sham. #P < 0.05 compared with saccharine group. @P < 0.05 compared with saccharine group.

Fig. 4. Chronic NIC exposure exacerbates H2O2-induced reactive oxygen species (ROS) production and resultant injury of cultured proximal tubule cells. A: TKPTS cells were pretreated or not with 200 μM NIC for 24 h, and then 200 μM H2O2-induced ROS production was determined. Values represent ROS production as percentage of the corresponding untreated values and are expressed as means ± SD; n = 3. *P < 0.05 compared with untreated. B: NIC-exposed TKPTS cells were pretreated with the xanthine oxidase inhibitor allopurinol (Allo; 100 μM), the NADPH inhibitor diphenilenediodium (DPI; 5 μM), or the mitochondrial inhibitor antimycin A (AntA; 10 μM) for 30 min before treatment with 200 μM H2O2. ROS production was expressed as percentage of NIC+H2O2-treated value; n = 3; means ± SD. *P < 0.05 compared with NIC+H2O2-treated value. C: TKPTS cells were pretreated with 100 μM N-acetylcysteine (NAC) 1 h before treatment with 200 μM NIC for 24 h followed by 200 μM H2O2 for 24 h; then, LDH release was determined. Similarly, some cells were infected with a dominant-negative JNK (dnJNK) adenovirus for 24 h before treatment with 200 μM NIC for 24 h followed by 200 μM H2O2. In these cells, LDH release was also determined. Values represent the percentage of the control values and are expressed as means ± SD; n = 3. *P < 0.05 compared with untreated.
Fig. 5. Chronic NIC exposure exacerbates acute renal ischemia-induced phosphorylation of JNK and c-jun in the mouse kidney. A: levels of phospho-JNK and JNK in kidney lysates from C57BL/6J mice that underwent renal IR injury were determined by Western blotting. One group received saccharine while the other NIC as described in MATERIALS AND METHODS. B: densitometry of results from A. Values are expressed as means ± SD of ratios of pJNK/JNK; n = 4 (sham) and n = 8 (IR). *P < 0.05 compared with sham. #P < 0.05 compared with saccharine group. @ P < 0.05 compared with the saccharine group. C: levels of phospho-c-jun and actin were also determined as described in A. D: densitometry of results from C. Values are expressed as means ± SD of p-c-jun/actin ratios; n = 4 (sham) and n = 8 (IR). *P < 0.05 compared with sham. #P < 0.05 compared with saccharine group. @ P < 0.05 compared with the saccharine group.

(Discussion)

NIC is a major component of cigarette smoke (19); it has been found in high concentration in the blood (19) and kidneys of chronic smokers (19, 48). It is excreted by glomerular filtration and tubular secretion (19). Thus the renal tubules are exposed to high levels of NIC and/or its major metabolite, cotinine, which may cause direct tubular toxicity. In fact, the presence of NIC or cotinine highly correlates with smoking-induced toxicity (26), suggesting that NIC is likely an important component of smoking-induced renal injury (23). Consequently, the main goal of the present study was to assess the effect of nicotine exposure, as that seen in smokers, on AKI.

In the present study, we used a model of chronic NIC exposure. We selected this model because it has the advantage that 1) the animals get a “bolus” of NIC every time they drink water (like smokers do when they smoke), and 2) their cotinine concentrations stabilize at levels that are similar to those found in chronic smokers. We and others (18) have found that an exposure period of at least 2–3 wk is necessary to consistently reach these levels. Our model went on for 4 wk, a time frame in which blood and tissue levels of cotinine levels are elevated. As before, we found that the cotinine levels in blood and kidney were significantly elevated (Table 1); the plasma levels were comparable to that found in chronic smokers (19). These increases in cotinine levels were associated with a moderate but significant increase in renal KIM-1 expression in the kidneys exposed to NIC (Fig. 1, A and B), whereas plasma creatinine levels and kidney morphology were unchanged (Table 1). Thus chronic NIC administration appeared to cause early tubular injury without altering GFR or the morphological appearance of the kidneys. This is consistent with studies in humans that have reported that smokers exhibit mild injury of the proximal tubules (16, 20). Further studies are needed to demonstrate whether the integrity of the actin cytoskeleton or activity of endoplasmic reticulum chaperones are involved in this stage of injury (4) as those events may predispose the kidney to further ischemic injury.

The more salient finding of our study was that chronic exposure to NIC exacerbated the severity of acute renal ischemia-reperfusion injury; plasma creatinine levels and renal expression of KIM-1 increased more than in untreated controls (Table 1). Moreover, renal ischemia-reperfusion-induced morphological alterations were worse in the mice that received NIC (Figs. 1A and 2, Table 1). It is interesting to note that previous studies have reported the opposite; NIC ameliorated ischemia-reperfusion-induced AKI (41, 50, 51). However, those studies are not directly comparable to ours because they only exposed their mice to NIC acutely (immediately beforesubjecting them to AKI). The shorter exposure to NIC (to NIC naive mice/rats) results in significantly lower cotinine levels in the plasma, which may result in different effects and signals. In our study, we not only found that chronic NIC exacerbated AKI in vivo, but we found the same in vitro. Accordingly, chronic NIC treatment of mouse renal proximal tubule cells increased H2O2-induced LDH release (Fig. 1C) as well as EthD staining (Fig. 2, E–H).

Increased production of ROS and the consequent increase in the incidence and severity of several diseases is evident (46). Chronic NIC exposure increases ROS production in mesangial (23) and proximal tubule (25) cells in vitro. In animal models, chronic exposure to cigarette smoke or NIC significantly increased oxidative stress in the kidney and pretreatment with...
antioxidants prevented renal injury (9, 22, 32, 43). These results clearly connect smoking/chronic NIC to oxidative injury. In our animal model, chronic exposure to NIC increased both basal and acute ischemia-induced oxidative stress in the kidney, as evidenced by increased renal expression of nitrotyrosine (Fig. 3, A and B) and renal MDA content (Fig. 3C). Our in vitro studies also confirmed these observations: H$_2$O$_2$-induced ROS production is further increased upon chronic pretreatment with NIC and NIC itself increased ROS production (Fig. 4A). The source of this NIC-mediated ROS could be the NAPDH oxidase system (18) or the mitochondria, which we confirmed by utilizing pathway-specific inhibitors (Fig. 4B). Our in vitro studies established a clear connection between NIC-induced enhancement of ROS production and oxidative injury: pretreatment with the ROS scavenger NAC attenuated NIC+$H_2$O$_2$-induced LDH release (Fig. 4B).

Ischemia-reperfusion injury activates the stress kinase JNK through ROS production (14, 30), which is involved in injury of renal proximal tubules in the ischemic kidney (13, 14, 39). The activated JNK increases activity of the transcription factor AP-1 during oxidative stress or renal ischemia-reperfusion injury both in vitro and in vivo (31, 42) and as such supports injury (14, 30). Several studies demonstrated that chronic NIC treatment activates JNK signaling (47, 49) in various cell types, but its effect on the kidney and on renal ischemia is virtually unknown.

Our study shows that chronic NIC exposure exacerbates acute renal ischemia-induced phosphorylation of JNK but also elevates its baseline phosphorylation (Fig. 5, A and B). The elevated stress kinase activation in the chronic NIC-exposed kidneys is probably due to the increased oxidative stress (Fig. 3), and it may also explain the persistent mild injury (Fig. 1A). Our in vitro studies also evidenced that the activated JNK plays a crucial role in injury (Fig. 1C) through activation of AP-1 (Fig. 6C). We also determined that chronic NIC exposure-associated ROS is essential for phosphorylation of JNK and not vice versa (Fig. 6, A and B). A similar scenario might exist in vivo: chronic NIC exposure, in addition to JNK (Fig. 5, A and B), also aggravates phosphorylation of c-jun (Fig. 5, C and D), a component of the AP-1 transcription factor (44).

Our in vitro data on tubular cells demonstrated that NIC has a toxic effect on renal epithelia. However, smoking and NIC also have vascular effects. Thus it is possible that chronic NIC exposure was affecting AKI via affecting blood pressure and/or renal hemodynamics. While we did not study this possibility in the current study, we have separate experiments in which we
look at the chronic effects of NIC on blood pressure and intrarenal hemodynamics in Sprague-Dawley rats. We have found that chronic NIC exposure does not alter either blood pressure or renal hemodynamics (unpublished observations). Similar observations were reported by Hua et al. (18), who found no changes in systolic blood pressure after chronic NIC administration in control or diabetic mice. Thus we think that it is unlikely that deleterious effects that are mediated by chronic NIC are secondary to changes in basal blood pressure or intrarenal hemodynamics. Thus these results, taken together with our in vitro data, suggest that NIC may exacerbate renal injury, at least in part, via direct tubular effects.

Our results are summarized in Fig. 7: chronic NIC exposure exacerbates acute renal ischemia-reperfusion-induced production of ROS, which in turn aggravates phosphorylation of JNK. The resultant increase in JNK activation leads to increased p65SHC-mediated mitochondrial dysfunction in renal proximal tubule cells during oxidative injury. Am J Physiol Renal Physiol 298: F1214–F1221, 2010.


