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Original investigation

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Nicotine Enhances High-Fat Diet-Induced Oxidative Stress in the Kidney

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Abstract

Introduction: Life expectancy of an obese smoker is 13 years less than a normal weight smoker, which could be linked to the increased renal risk imposed by smoking. Both smoking—through nicotine (NIC)—and obesity—by free fatty acid overload—provoke oxidative stress in the kidney, which ultimately results in development of chronic kidney injury. Their combined renal risk, however, is virtually unknown. We tested the hypothesis that chronic NIC exposure worsens renal oxidative stress in mice on high-fat diet (HFD) by altering the balance between expression of pro-oxidant and antioxidant genes.

Methods: Nine-week-old male C57Bl/6J mice consumed normal diet (ND) or HFD and received either NIC (200 μg/ml) or vehicle (2% saccharine) in their drinking water. Body weight, plasma clinical parameters, renal lipid deposition, markers of renal oxidative stress and injury, as well as renal expression of the pro-oxidant p66shc and the antioxidant MnSOD were determined after 12 weeks.

Results: NIC significantly augmented levels of circulating free fatty acid, as well as lipid deposition, oxidative stress and sublethal injury in the kidneys of mice on HFD. In addition, NIC exposure suppressed HFD-mediated induction of MnSOD while increased expression of p66shc in the kidney.

Conclusions: Tobacco smoking or the increasingly popular E-cigarettes—via NIC exposure—could worsen obesity-associated lipotoxicity in the kidney. Hence, our findings could help to develop strategies that mitigate adverse effects of NIC on the obese kidney.

Implications: Life expectancy of an obese smoker is 13 years less than a normal weight smoker, which could be linked to the increased renal risk imposed by smoking. NIC—the main component of tobacco smoke, E-cigarettes and replacement therapies—links smoking to renal injury via oxidative stress, which could superimpose renal oxidative stress caused by obesity. Our results substantiate this scenario using a mouse model of diet induced obesity and NIC exposure and imply the augmented long-term renal risk in obese smokers. Also, our study may help to develop strategies that mitigate adverse effects of NIC on the obese kidney.

Introduction

Several studies have revealed adverse effects of smoking on kidney health.[1–3] Nicotine (NIC)—a major component of tobacco smoke,[4] E-cigarettes,[5] and NIC replacement therapies[6]—links smoking to kidney injury[7] by inducing oxidative stress.[8–10] Studies have demonstrated that chronic exposure to NIC exacerbates outcomes of chronic[11–13] and acute kidney injury.[8] We have also shown that chronic NIC exposure augments mitochondrial production of reactive oxygen species (ROS) and consequent injury[8] by transcriptional

activation of the pro-oxidant p66shc in cultured renal proximal tubule cells.[14]

Obesity increases the risk of lipotoxic kidney injury,[15] which initiates a cascade of events leading to chronic kidney injury and ultimately end-stage renal disease. Mediators of lipotoxicity are non-esterified free fatty acids (FFA), levels of which are chronically elevated in obese persons.[16] In the kidney, FFA accumulation elicits lipotoxic effects on proximal tubule cells[17] by increasing production of ROS[18] and consequent mitochondrial dysfunction.[15] Previously, we have shown that oleic acid increases mitochondrial ROS production and consequent mitochondrial-depolarization-dependent injury via transcriptional activation of the pro-oxidant p66shc in cultured renal proximal tubule cells.[19]

Many clinicians consider obesity a life-style choice and many people choose smoking to lose weight[20,21] unaware that the life expectancy of an obese smoker is 13 years less than a non-obese smoker.[21] One adverse effect of smoking in obesity which is not fully recognized is increased dyslipidemia,[22,23] which may further enhance obesity-related renal risk. Clinical studies have shown obesity and smoking as independent risk factors for renal damage, but the precise mechanism of their combined effects is not well understood. It is plausible that chronic NIC exposure augments high-fat diet (HFD)-associated renal oxidative stress similar to that observed in the liver.[24]

Oxidative stress is the result of excessive production of ROS and/or impaired activation of the antioxidant defense system.[25] HFD induces antioxidant responses in the kidneys of mice[26]; however, smoking or NIC exposure attenuates it.[27,28] Whether smoking/NIC exposure affects renal oxidative stress in obesity is virtually unknown.

Accordingly, we hypothesize that chronic NIC exposure exacerbates HFD-associated renal oxidative stress by changing the balance between pro-oxidant and antioxidant responses. This hypothesis was tested in mice that were fed on HFD and received NIC or vehicle in their drinking water and compared to those on a normal diet (ND).

Materials

Animals, Diet, Chronic NIC Exposure

Nine-week-old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and divided into the following groups (6–6 animals): normal diet+vehicle (ND+vehicle), high-fat diet+vehicle (HFD+vehicle), normal diet+nicotine (ND+NIC), and high-fat diet+nicotine (HFD+NIC). ND (13% kcal from fat; TD.07055) and HFD (39.7% kcal from fat; TD.07054) were purchased from Harlan Laboratories (Madison, WI). The animals had free access to food and drinking water for 12 weeks. The drinking water contained either 2% saccharine (vehicle) or 200 μg/ml NIC-bitartrate in 2% saccharine.[8] Our previous studies have shown that 4-week-exposure to this NIC regimen increases plasma and renal cotinine content (a stable metabolite of NIC) to a level similar to that found in long-time smokers.[8] Animals were weighed weekly and their water consumption was determined and averaged as ml/mouse/d. At the end of 12 weeks mice were subjected to an overnight fast and sacrificed in accordance with the IACUC regulations. Plasma and kidneys were collected and stored at −80°C.

Clinical Chemistry

The following parameters were measured from the plasma according to the manufacturers' recommendations: creatinine (QuantiChrome Creatinine Assay kit from Bioassay Systems, Hayward, CA), FFA (Serum/Plasma fatty acid kit for detection of nonesterified fatty acid from ZenBio, Research Triangle Park, NC), glucose (Cayman Chemical, Ann Arbor, MI), cholesterol (Wako Diagnostics, Mountain View, CA), and cotinine (Cotinine Direct ELISA kit, Calbiotech, Spring Valley, CA).

Renal Histology

At the time of sacrifice, a midsagittal section of an excised kidney was performed. One of the halves was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin (H&E). Images were viewed and captured at ×20 and/or ×10 magnification using an Olympus BX41 microscope with digital camera (Olympus America, Inc, Melville, NY). The extent of lipid deposition and evidence of microvesicular and macrovesicular fatty changes were assessed qualitatively by visual inspection of the prepared slides.

Renal Oxidative Stress and Injury

Extent of renal oxidative stress was determined from kidney lysates using the "OxiSelect HNE-His adduct ELISA" kit and the "8-iso-Prostaglandin F2a Assay" from Cell Biolabs, Inc, San Diego, CA. Renal KIM-1 content—a marker of renal tubular injury[29]—was determined from kidney lysates using the "Quantikine mouse TIM-1/KIM-1/HAVCR immunoassay" kit (R&D Systems, Minneapolis, MN).

Preparation of Lysates and Western Blotting

Kidney lysates were prepared in RIPA buffer as described elsewhere.[8] 100 μg of total kidney lysates were separated on a 4%–12% NuPAGE Novex Bis-Tris gradient mini gel (Life Technologies, Grand Island, NY) and transferred to a PVDF membrane by using iBlot (Life Technologies). Blots were hybridized with the primary anti-MnSOD (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p66shc antibody (Nanotools USA/Axxora, San Diego, CA) followed by the relevant HRP-labeled secondary antibody (Cell Signaling Technology, Danvers, MA). Equal loading was determined by rehybridization with actin (Millipore, Billerica, MA). Bands were visualized by Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL), and exposed to an X-ray film (Midwest Scientific, St Louis, MO). Films were digitized and analyzed by Un-Scan-It Version 6.1 software (Silk Scientific, Orem, UT).

Statistical Analysis

Continuous variables are expressed as means and *SD*s. One-way analysis of variance (ANOVA) with Holm-Sidak post hoc test was used to evaluate the differences between groups. Differences between means were considered significant if $P < .05$. All analyses were performed using the SigmaStat 3.5 (Systat, San Jose, CA) software package.

Results

NIC Exposure Significantly Augments Plasma FFA and Renal Lipid Deposition not Only in Mice Fed on HFD but Also in Those on ND

One group of mice was kept on ND that received either NIC (ND+NIC) or vehicle (ND+vehicle), while the other group was fed a HFD and received NIC (HFD+NIC) or vehicle (HFD+vehicle) for 12

weeks. Body weight and fluid consumption were monitored weekly. As Supplementary Figure 1A shows, HFD significantly increased body weight as early as 1 week after starting HFD compared to mice that received ND. In the NIC+HFD group, the increase in body weight was somewhat delayed (Supplementary Figure 1B) reaching significance around week 4. Overall, during the 12-week-period the HFD+vehicle group gained 15 ± 2.7 g in body weight while the ND+vehicle group only 4 ± 0.44 g. In contrast, the HFD+NIC group gained only 12 ± 2.14 g in body weight as opposed to 3.5 ± 1.13 g in the ND+NIC group. We also calculated weekly weight gain in the HFD group less weekly weight gain in the ND group with or without NIC. Supplementary Figure 1C shows that HFD-induced weight gain in the HFD+NIC group is somewhat less than in the HFD+vehicle group, although the difference is not statistically significant. Similar results are evident for the differences in ND+NIC and ND+vehicle groups. Even though we found fluctuation in water consumption (Supplementary Figure 2, A and B) the differences were not significant (Supplementary Figure 1D).

Clinical parameters such as plasma FFA, glucose and cholesterol along with cotinine (a stable NIC metabolite) were also determined after 12 weeks. Table 1 depicts the results: levels of cotinine were high in the plasma of NIC-exposed mice. As expected, HFD increased FFA content that was further increased by NIC. In fact, NIC mildly—although not significantly—increased FFA levels in ND-fed mice. In addition, other parameters such as glucose and cholesterol were elevated in HFD mice, the extent of the latter was also augmented by NIC (Table 1). These results are consistent with the development of obesity.

Renal histology of mice subjected to HFD/ND+vehicle or HFD/ND+NIC showed no evidence of pathological changes to the glomeruli or renal tubules (Figure 1, A and B and Supplementary Figure 3, A–B). However, in those mice exposed to a HFD, microvesicular and macrovesicular fatty changes were appreciated extending from the subcapsular area to an approximate depth of 2/3 of the renal cortex (Figure 1A). Furthermore, in the group placed on HFD+NIC, these changes occurred on a more substantial scale (Figure 1B). Importantly, FFA content of kidney lysates was significantly higher in the HFD+NIC than in the HFD+VEH group (Figure 1C). In fact, NIC itself mildly increased renal FFA content (ND+NIC), though the fat deposition was not evident by histology (Supplementary Figure 2B). Since both FFA and NIC provoke renal oxidative stress and consequent injury,[8,15] our data imply that NIC augments HFD-associated renal oxidative stress and lipotoxicity.

NIC Exposure Exacerbates HFD-Induced Renal Oxidative Stress and Sublethal Injury

The extent of oxidative stress was assessed by determining HNE-His adduct and 8-isoprostane content (markers of lipid peroxidation and hence, markers of tissue oxidative stress) in kidney lysates after 12 weeks. Figure 2A shows that HFD increases HNE-His adduct levels in the kidney, which is further augmented by NIC. It is important to note, that NIC itself also increases renal oxidative stress (Figure 2A). Renal 8-isoprostane levels showed changes similar to HNE (Figure 2B).

In parallel with oxidative stress, the content of KIM-1—a known marker of renal tubular injury[30]—in kidney lysates showed a similar pattern: HFD significantly enhances renal KIM-1 expression. Though slightly, NIC alone also enhances it. Importantly, NIC exposure further augments the HFD-dependent increase in renal KIM-1 (Figure 2C). On the other hand, renal function (measured by serum creatinine content) was unaffected (Table 1). These data show a close correlation between plasma/renal FFA content and renal tubular lipid deposition, as well as renal oxidative stress and (sublethal) renal injury.

NIC Exposure Augments Expression of the Pro-Oxidant p66shc and Attenuates Expression of the Antioxidant MnSOD in the Kidneys of Mice on HFD

We analyzed expression of p66shc and MnSOD in kidney lysates by Western blotting. Figure 3, A–C shows that renal expression of p66shc moderately but significantly increased in ND+NIC, as well as in HFD mice, which was further augmented in the presence of NIC. In contrast, while MnSOD expression was elevated by HFD, it was suppressed by chronic NIC exposure (Figure 3, B–D). These results suggest that chronic NIC exposure may exacerbate renal oxidative stress by augmenting p66shc-dependent pro-oxidant signaling while suppressing antioxidant (MnSOD) responses.

Discussion

Obesity—currently recognized as an epidemic in both the United States and throughout the world—is linked to an increase in the incidence of chronic kidney injury both in adults and children.[31–33] The potential mechanism for renal lipotoxicity includes FFA-dependent production of ROS[31] and consequent increase in mitochondrial membrane permeability, that subsequently depolarizes the mitochondria and results in cell injury.[34] Previously, we have demonstrated that oleic acid—by increasing mitochondrial ROS production—depolarizes the mitochondria and leads to injury of cultured renal proximal tubule cells.[19] Similar scenario could exist in the kidneys of mice on HFD: both plasma FFA levels (Table 1) and renal lipid deposition (Figure 1) are elevated, which is possibly responsible for the observed renal oxidative stress and (sublethal) injury (Figure 2). Whether this injury is due to mitochondrial depolarization needs further investigation.

Table 1. Parameters in the Plasma of ND- or HFD-Fed Mice That Received Either Vehicle (VEH) or Nicotine (NIC)

	ND+VEH	HFD+VEH	ND+NIC	HFD+NIC
Cotinine (ng/ml)	ND	ND	128 ± 12	134 ± 20
FFA (μM)	145 ± 8.7	985 ± 145[a]	185 ± 21	1375 ± 107[a,b]
Glucose (mg/dL)	138 ± 15.9	268 ± 30[a]	153 ± 14	297 ± 40.7[a]
Cholesterol (mg/dL)	3.9 ± 0.13	16.7 ± 1.4[a]	4.3 ± 0.18	21 ± 1.9[a,b]
Creatinine (mg/dL)	0.39 ± 0.09	0.4 ± 0.1	0.37 ± 0.06	0.4 ± 0.08

HFD = high-fat diet; FFA = free fatty acids; ND = normal diet.

[a]$P < .05$ compared to ND+VEH.

[b]$P < .05$ compared to HFD+VEH, $n = 6$.

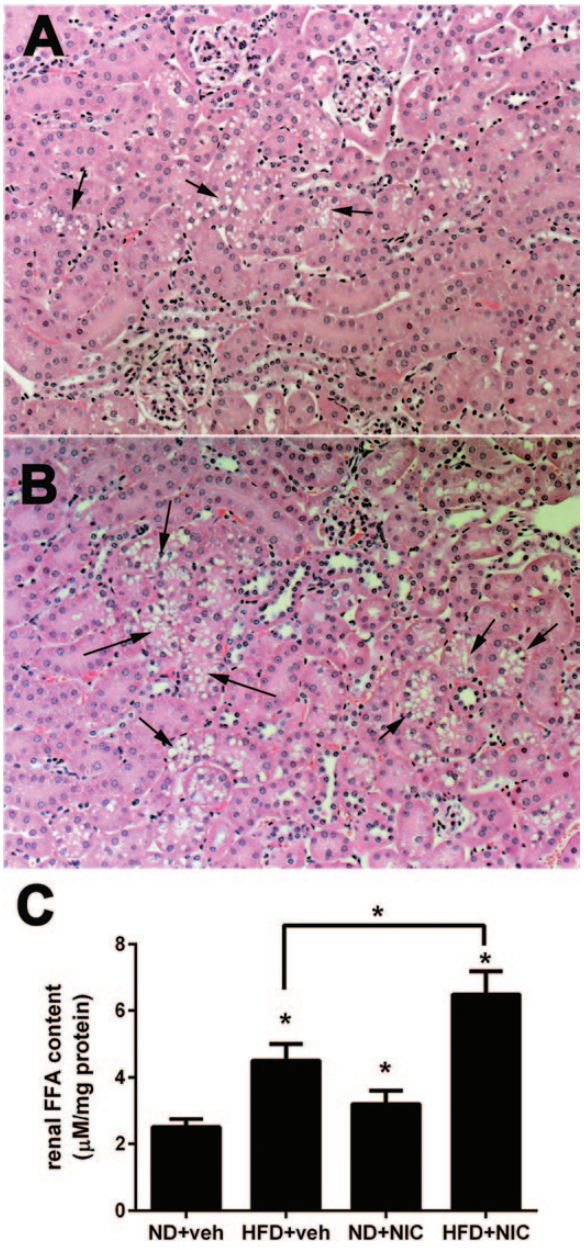

Figure 1. Lipid deposition in the kidney of mice on high-fat diet (HFD) in the presence or absence of nicotine (NIC). Formalin-fixed and paraffin-embedded kidneys from mice on HFD+vehicle (A) and HFD+NIC (B) were processed and stained with hematoxylin and eosin (H&E) and subjected to microscopic evaluation for lipid deposition and evidence of microvesicular and macrovesicular fatty changes. Slides shown here are typical representatives. Magnification: ×20. Arrows show lipid deposition. (C) Free fatty acid (FFA) content of kidney lysates were determined as described in the Materials section. $n = 6$, $^*P < .05$ compared to normal diet (ND)+vehicle or as indicated.

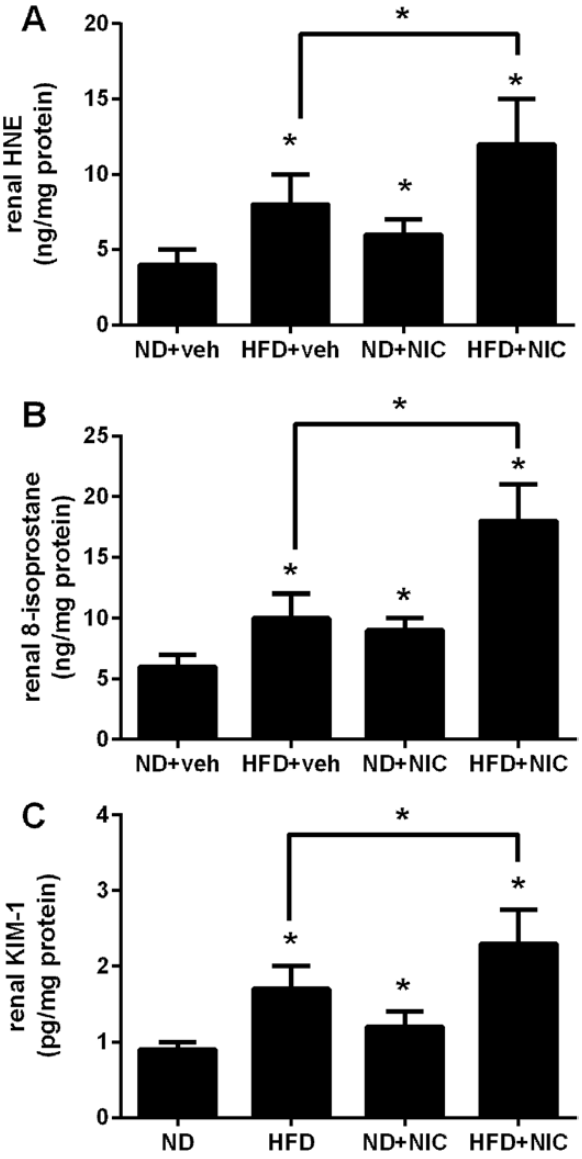

Figure 2. Oxidative stress and injury in the kidney. (A) Mice were fed on normal diet (ND) or high-fat diet (HFD) in the presence or absence of vehicle or nicotine (NIC) for 12 weeks. Oxidative stress was demonstrated by determining levels of HNE-his adduct (A) or 8-isoprostane (B) content of the kidney lysates. Values are given as mean ± *SD*, $n = 6$, $^*P < .05$ compared to ND+vehicle, or as indicated. (C) Kidney injury was demonstrated in lysates by determining KIM-1 content. Values are given as mean ± *SD*, $n = 4$, $^*P < .05$ compared to ND+vehicle or as indicated.

Previously, we demonstrated that oleic acid activates the promoter of the pro-oxidant p66shc gene leading to excess mitochondrial ROS generation and consequent mitochondrial depolarization-dependent injury of cultured renal proximal tubule cells.[19] It has been shown that p66shc—in response to oxidative stress—is translocated to the mitochondrial intermembrane space where it binds and oxidizes cytochrome *c*.[35,36] The consequence is excessive generation of ROS, which in turn depolarizes the mitochondria.[35,36] Interestingly, HFD elevates renal expression of p66shc (Figure 3A), which may be due to FFA-mediated induction of its promoter as we observed in cultured renal proximal tubule cells.[19] Hence, the observed renal oxidative stress and consequent injury (Figure 2) are likely linked to p66shc-dependent mitochondrial ROS generation and consequent mitochondrial depolarization, as we published earlier.[19]

Even though obesity is preventable by life-style changes, many people choose smoking to lose weight[20,21] oblivious to the fact that the life expectancy of an obese smoker is 13 years less than a normal weight smoker.[21] In our experiments chronic NIC exposure slightly (although not significantly) decreased HFD-associated weight gain (Supplementary Figure 1), which is somewhat at odds with a

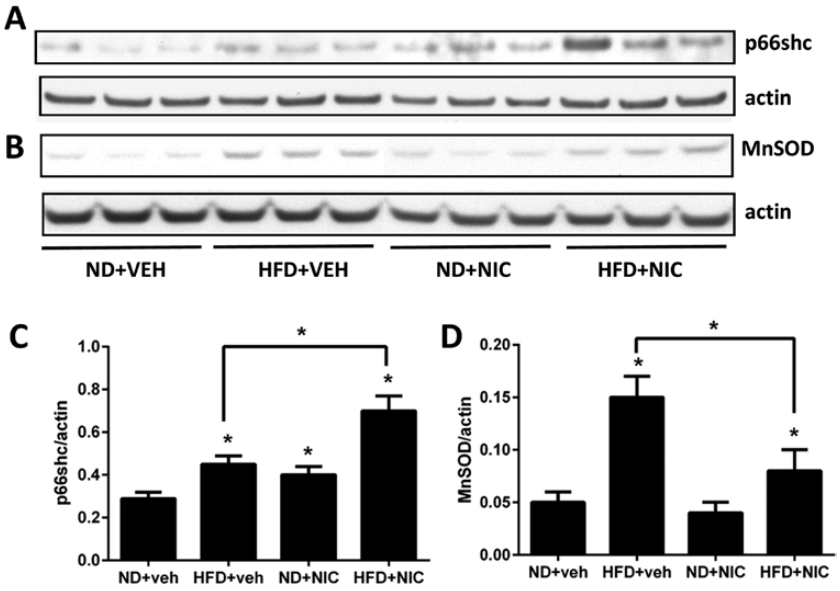

Figure 3. Expression of the pro-oxidant p66shc and the antioxidant MnSOD in the kidney. (A) 100–100 μg kidney lysates were subjected immunoblotting with an anti-p66shc antibody and after stripping with an anti-actin antibody. Samples shown are representatives from 6–6 animals. ND+veh: normal diet+vehicle; HFD+veh: high-fat diet+vehicle; ND+NIC: normal diet+nicotine; HFD+NIC: high-fat diet+nicotine. (C) Densitometric results of p66shc immunoblotting: levels of p66shc were normalized to actin expression. *$P < .05$ compared to ND+vehicle or as indicated. (B) In a separate experiments, levels of MnSOD were also determined by Western blotting; equal loading was detected by rehybdridizing the blot with an anti-actin antibody. (D) Densitometric results of MnSOD immunoblotting: levels of MnSOD were normalized to actin expression. *$P < .05$ compared to ND+vehicle or as indicated.

significant decrease in body weight of mice on HFD after treatment with NIC as shown by others.[37] The differences are probably due to using a different HFD (60% fat calories vs. 39% in our study). Also, the cited study did not determine serum cotinine levels, and as such, we were unable to compare whether the extent of NIC exposure was of similar magnitude. We have seen a trend of weight loss in the presence of NIC, and we believe that increasing the fat calories in the diet (up to 60%) may result in significant weight loss. Regardless, the main point (ie, adverse effects of chronic NIC exposure on the kidney in HFD mice) is clear from our studies.

Smoking itself is an independent risk factor that increases the risk of progression of chronic kidney disease.[3] Smoking in obesity increases dyslipidemia,[22] diabetes, insulin resistance, cardiovascular disease[21] and potentially augments renal risk, which is not always fully recognized. Our results indicate that NIC augments circulating FFA content (Table 1) and renal tubular lipid deposition in mice on HFD (Figure 1B). Our previous in vitro studies demonstrated that deleterious effects of lipids in cultured renal proximal tubule cells are dose-dependent,[19] hence, the higher lipid deposition in the kidney predicts more injury. Indeed, the extent of sublethal injury is significantly higher in HFD+NIC than in ND+NIC mice (Figure 2C). In our model, the observed renal injury is sublethal, it does not cause either histologic (Figure 1, A and B) or functional (Table 1; plasma creatinine levels) changes. Since it was previously shown that chronic NIC exposure–despite increasing renal oxidative stress and renal expression of KIM-1—results in only sublethal injury—this is not fully surprising.[8] A recent study by Wicks et al.[38] demonstrates that the C57Bl/6J mouse strain is relatively resistant to diet-induced obesity-related kidney injury, which is in line with our findings. Further studies are needed to determine whether longer exposure to HFD+NIC or using other mouse strain could lead to morphological renal damage, in addition to the elevation in markers of oxidative stress and sublethal injury.

We have shown that NIC increases renal oxidative stress and injury in mice and in cultured renal proximal tubule cells,[8] which is at least partly attributed to NIC-dependent induction of the p66shc promoter.[14] Hence, it is not surprising that NIC further increases HFD-induced expression of p66shc (Figure 3A), as well as oxidative stress (Figure 2). Whether deleterious effects of NIC on HFD is attributed to increased p66shc expression, needs further in vivo and in vitro evaluation.

Oxidative stress prevails when the balance of pro-oxidant and antioxidant responses are impaired. Our studies showed that chronic NIC exposure augments HFD-induced oxidative stress (Figure 2, A and B), which could be at least partly due to p66shc-mediated ROS generation.[14,19,39] We also showed that while HFD increases expression of MnSOD—and hence, antioxidant responses—in the kidney, chronic exposure to NIC suppresses it (Figure 3, B–D). MnSOD is the major antioxidant in the mitochondria, which protects against oxidative stress in the kidney.[40] Conversely, deficiency in MnSOD induction results in increased oxidative stress and injury. Indeed, renal oxidative stress and injury are augmented in the kidneys from mice on NIC+HFD (Figure 2) while MnSOD expression is diminished (Figure 3, B–D). Expression of MnSOD is regulated via its transcription, which may involve p66shc-dependent suppression of MnSOD.[41,42] Further in vivo and in vitro studies are needed to determine whether (1) p66shc is involved in transcriptional suppression of the MnSOD promoter and (2) this suppression is attributed to the increase in oxidative stress. In addition, studies are needed to determine whether NIC interferes with specific signaling along this antioxidant pathway or the superimposed oxidative stresses (NIC+HFD) represent a threshold beyond which the antioxidant capacity is impaired.

Obesity is associated with other complications such as hyperglycemia.[43] We found that HFD significantly increases fasting glucose levels in mice, although chronic NIC exposure did not worsen

that (Table 1). Whether longer HFD+NIC exposure actually worsens hyperglycemia and hyperglycemia contributes to the observed renal oxidative stress, needs further verification. Studies have also shown that obesity[44] and smoking/NIC exposure[45] contribute to development of hypertension and subsequent renal injury. Our unrelated studies did not show changes in blood pressure in animals on chronic NIC regimen (data not shown). Further studies are needed to determine whether chronic NIC exposure causes or worsens hypertension in HFD+NIC mice, which could contribute to renal oxidative stress and consequent long-term renal injury.

Some studies suggest that the bitter taste of NIC may influence water intake in female rats,[46] potentially causing dehydration-induced renal oxidative stress.[47] Those experiments, however, used 50 to 100 times higher concentration of NIC (10–20 mg/ml as opposed to 200 µg/ml in our study). Additionally, we used male mice. Even though the weekly water consumption fluctuated, there were no statistically significant differences between vehicle and NIC groups in weekly water uptake (Supplementary Figure 2, A and B). Hence, dehydration-induced renal oxidative stress is unlikely in our study.

Our studies offer means to ameliorate smoking/NIC-exposure-associated worsening of renal oxidative stress and consequent development and progression of chronic kidney injury in obese individuals. We believe that our results have high translational relevance because: (1) many obese people choose smoking to lose weight and therefore, refuse to quit,[21] and (2) the popularity of E-cigarettes for recreational use is increasing at an alarming rate,[5] which may increase the renal risk not only in the obese/overweight but in the general population, as well.

Supplementary Material

Supplementary Figures 1–3 can be found online at http://www.ntr.oxfordjournals.org

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Declaration of Interests

None declared.

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