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The pro-oxidant gene p66shc increases nicotine exposure-induced lipotoxic oxidative stress in renal proximal tubule cells

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Abstract. Nicotine (NIC) exposure augments free fatty acid (FFA) deposition and oxidative stress, with a concomitant increase in the expression of the pro-oxidant p66shc. In addition, a decrease in the antioxidant manganese superoxide dismutase (MnSOD) has been observed in the kidneys of mice fed a high-fat diet. The present study aimed to determine whether the pro-oxidant p66shc mediates NIC-dependent increases in renal oxidative stress by augmenting the production of reactive oxygen species (ROS) and suppressing the FFA-induced antioxidant response in cultured NRK52E renal proximal tubule cells. Briefly, NRK52E renal proximal tubule cells were treated with 200 μM NIC, 100 μM oleic acid (OA), or a combination of NIC and OA. The expression levels of p66shc and MnSOD were modulated according to genetic methods. ROS production and cell injury, in the form of lactate dehydrogenase release, were subsequently detected. Promoter activity of p66shc and MnSOD, as well as forkhead box (FOXO)-dependent transcription, was investigated using reporter luciferase assays. The results demonstrated that NIC exacerbated OA-mediated intracellular ROS production and cell injury through the transcriptional activation of p66shc. NIC also suppressed OA-mediated induction of the antioxidant MnSOD promoter activity through p66shc-dependent inactivation of FOXO activity. Overexpression of p66shc and knockdown of MnSOD had the same effect as treatment with NIC on OA-mediated lipotoxicity. These data may be used to generate a therapeutic means to ameliorate renal lipotoxicity in obese smokers.

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Introduction

Non-esterified free fatty acids (FFAs) impose lipotoxic effects on the kidney (1) by increasing production of reactive oxygen species (ROS) (2), thus leading to mitochondrial dysfunction (3). Although obesity is preventable, many people use smoking as a means to lose weight (4,5). Nicotine (NIC) is a major component of tobacco smoke (6) and E-cigarettes (7), and is responsible for the association between smoking and kidney injury (8) through increases in oxidative stress (9). Therefore, it is possible that FFA- and NIC-associated oxidative stresses are superimposed, resulting in enhanced oxidative stress in the kidneys of obese smokers. A previous *in vivo* study revealed that NIC exposure exacerbates high-fat diet (HFD)-associated FFA deposition and oxidative stress in mouse kidneys (10).

It has previously been reported that NIC and oleic acid (OA) increase the transcription of the pro-oxidant p66shc gene, resulting in elevated mitochondrial ROS production and consequent mitochondrial depolarization-dependent injury in cultured renal proximal tubule cells (11,12). Our recent study demonstrated that NIC exposure increased oxidative stress with a concomitant increase in p66shc expression in the kidneys of mice fed a HFD compared with those fed a normal diet (10). Therefore, it is possible that NIC exacerbates FFA-mediated oxidative stress, in part, due to additive effects on p66shc. Furthermore, oxidative stress is not solely the result of excessive production of ROS; it may also be due to impaired activation of the antioxidant defense system (13). Our previous study revealed that while HFD increases expression of the antioxidant manganese superoxide dismutase (MnSOD), its expression was reduced in the kidneys of mice exposed to NIC (10). Notably, p66shc is important for the direct production of mitochondrial ROS (11,12) and the suppression of MnSOD in non-renal cells (14-16).

Therefore, the present study hypothesized that NIC exposure would exacerbate renal oxidative stress and consequent renal lipotoxicity via a p66shc-dependent increase in ROS production and suppression of MnSOD expression in renal proximal tubule cells.

Materials and methods

Cell line and treatment. The NRK52E renal proximal tubule cell line was purchased from American Type Culture Collection

(Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in an atmosphere containing 5% CO_2. Cells were treated with 200 μM NIC (Sigma-Aldrich, St. Louis, MO, USA), 100 μM OA (Sigma-Aldrich), or with a combination of NIC and OA.

Modulation of p66shc and MnSOD expression. NRK52E cells were transfected with a p66shc expression plasmid to overexpress (OE) or a short hairpin (sh) p66shc plasmid to knockdown (k.d.) p66shc expression (17,18) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. These plasmids were prepared in our laboratory. In order to suppress MnSOD expression, NRK52E cells were transfected with a shMnSOD plasmid (Addgene, Inc., Cambridge, MA, USA) using Lipofectamine® 3000 transfection reagent, according to the manufacturer's protocol. Cells were transfected with the aforementioned plasmids prior to treatment.

Determination of intracellular ROS production. NRK52E cells were cultured in T25 flasks and transfected with the aforementioned plasmids. Following trypsinization, cells were counted and loaded with the oxidant-sensitive dye 2',7'-dichlorofluorescein-diacetate (100 μM; Thermo Fisher Scientific, Inc.) as previously described (17). The cells were incubated for 30 mins at 37˚C and the dye was washed away with fresh Hanks' balanced salt solution (Sigma-Aldrich). The cells were then seeded at a density of $0.2x10^6$ cells/well and were treated with 200 μM NIC, 100 μM OA, or with a combination of NIC and OA for 24 h. ROS production was determined by recording the increase in fluorescence at 485 nm_{exc}/530 nm_{em} in 30-min-intervals for up to 120 min using a microplate reader (FluoroCount, Packard; Molecular Devices, LLC, Sunnyvale, CA, USA). ROS production was calculated as: Change in fluorescence / 30 min / $0.2x10^6$ cells, and was expressed as a percentage of the corresponding untreated values.

Determination of cell injury. The extent of cell injury was determined using the fluorescent CytoTox-ONE™ Homogenous Membrane Integrity assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, cells cultured in 96-well plates were transfected/treated as required and lactate dehydrogenase (LDH) content in the supernatant was compared with total cellular LDH content. LDH release was calculated as percentage of total LDH content (19). In some experiments, cells were pretreated with 10 μM N-acetyl-cysteine (NAC: Sigma-Aldrich) for 30 min prior to treatment with NIC and OA.

Reporter luciferase assay. NRK52E cells were cultured in 24-well plates and were transfected with the following reporter luciferase plasmids: p66shc-promoter luciferase (20) provided by Dr Irani (Cardiovascular Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, USA), MnSOD promoter-reporter-luciferase (21) or a luciferase plasmid that harbors 6 canonical forkhead box (FOXO) binding sites (6xDBE) to determine FOXO-dependent transcription (22) provided by Dr Burgering (Department of Molecular Cancer Research, University Medical Center Utrecht, Utrecht, Netherlands) together with *Renilla* luciferase (Promega Corporation) using Lipofectamine® 3000 reagent. Firefly and *Renilla* luciferase activity levels were determined after 24 h using the Dual Luciferase assay kit (Promega Corporation). Luciferase activity levels were calculated as a ratio of the firefly and *Renilla* activities and expressed as a percentage of the control (untreated cells) values.

Preparation of cell lysate and Western blotting. Cell lysates were prepared in radioimmunoprecipitation assay buffer, as described previously (18). Protein content of the lysates was determined using Pierce Bicinchoninic Acid Assay (cat. no. 23225; Pierce; Thermo Fisher Scientific, Inc., Rockford, IL, USA) according to the manufacturer's protocol. Protein samples (100 μg) were separated on a 4-12% NuPAGE Novex®Bis-Tris gradient mini gel (Thermo Fisher Scientific, Inc.) and were transferred to a polyvinylidene fluoride membrane using iBlot (Thermo Fisher Scientific, Inc.). Blots were blocked for 1 h at room temperature in Tris-buffered saline-0.5% Tween (TBST) containing 5% dried milk. After blocking, the blots were washed three times for 5 min in TBST at room temperature. Blots were subsequently hybridized with the following primary antibodies diluted in TBST containing 5% nonfat dry milk overnight at 4˚C: Mouse anti-MnSOD (1:100; cat. no. sc-137254; Santa Cruz Biotechnology, Inc., Dallas, TX) or rabbit anti-shc (1:1,000; cat. no. 610082; BD Biosciences, San Jose, CA, USA). Subsequently, blots were washed three times for 5 min in TBST at room temperature and were then incubated with horseradish peroxidase-labeled anti-mouse (1:5,000; cat. no. 7076S) or anti-rabbit (1:5,000; cat. no. 7074S) secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) for 45 min at room temperature. Blots were washed a further three times for 5 min with TBST at room temperature and were incubated with Pierce Enhanced Chemiluminescence Western Blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) for 1 min at room temperature, before being exposed to an X-ray film (Midwest Scientific, St. Louis, MO, USA). Films were digitized and analyzed using Un-Scan-It™ Version 6.1 software (Silk Scientific, Orem, UT, USA). Each blot was stripped with Restore PLUS Western Blot stripping buffer (cat. no. 46430; Thermo Scientific, Inc.) for 20 min at 37˚C and was washed three times for 5 min with TBST at room temperature. The stripped blots were rehybridized with an anti-actin antibody (1:20,000; cat. no. MAB1501; EMD Millipore, Billerica, MA, USA) for 40 min at room temperature, followed by washing and hybridization with a secondary mouse antibody as aforementioned.

Statistical analysis. All experiments were performed in triplicate. Continuous variables are expressed as the mean ± standard deviation. One-way analysis of variance with Holm-Sidak post-hoc test was used to evaluate the differences between groups. All analyses were performed using GraphPad InStat version 3 (GraphPad, La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exposure to NIC augments FFA-dependent induction of the p66shc promoter. Our previous studies reported that NIC and OA induced p66shc promoter activity in renal proximal tubule

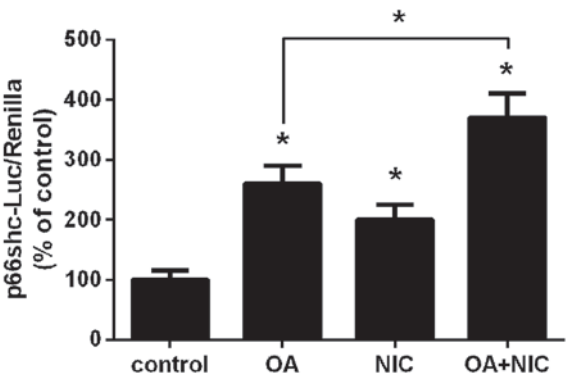

Figure 1. NIC increases FFA-dependent induction of p66shc promoter actvity. NRK52E cells were transfected with a p66shc-promoter luciferase and *Renilla* luciferase plasmid, and were treated with 100 μM OA, 200 μM NIC or with their combination. n=3, *P<0.05 vs. control or as indicated. NIC, nicotine; FFA, free fatty acids; OA, oleic acid.

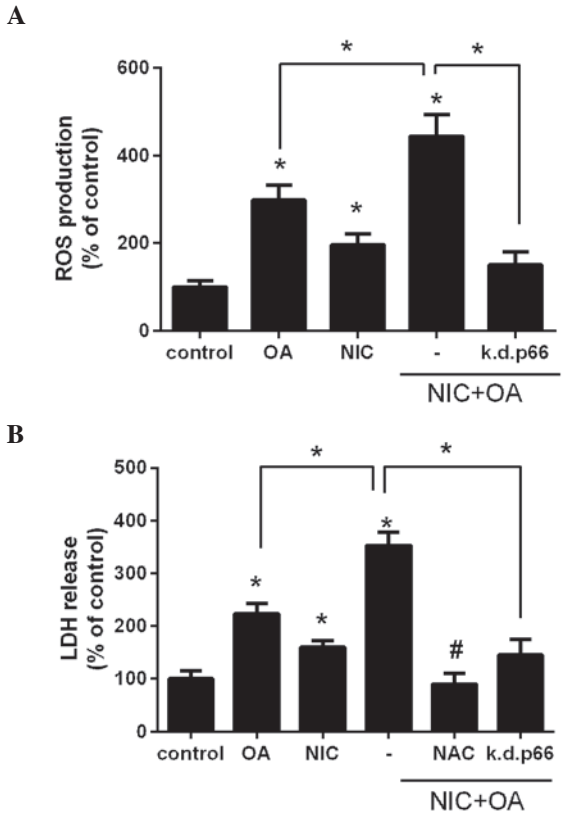

Figure 2. NIC exposure exacerbates the lipotoxic effects of OA via p66shc in NRK52E cells. (A) ROS production was increased in the wild-type cells treated with NIC + OA compared with the k.d.p66shc-transfected cells, n=3. *P<0.05 vs. control or as indicated. (B) LDH release was greater in cells treated with OA, NIC and NIC + OA compared with the control. Cell injury was determined by means of LDH release in cells that were treated with 100 μM OA, 200 μM NIC or with their combination. A group of cells was also pre-treated with 10 μM NAC prior to treatment with NIC + OA. *P<0.05 vs. control or as indicated; #P<0.01 vs. NIC + OA-treated cells. ROS, reactive oxygen species; OA, oleic acid; NIC, nicotine; k.d.p66shc, p66shc knockdown; LDH, lactate dehydrogenase; NAC, N-acetylcysteine.

cells (11,12). Therefore, it is possible that their combined application is additive. In the present study, NRK52E cells transfected with a p66shc-promoter luciferase plasmid (20) together with *Renilla* luciferase, were treated with either 100 μM OA, 200 μM NIC, or with a combination of NIC and OA. As shown in Fig. 1, treatment with OA (P<0.05) and NIC (P<0.05) significantly increased activity of the p66shc promoter compared with the control group. In addition, when both treatments were applied simultaneously, the activity levels were significantly greater compared with the OA only group (P<0.05).

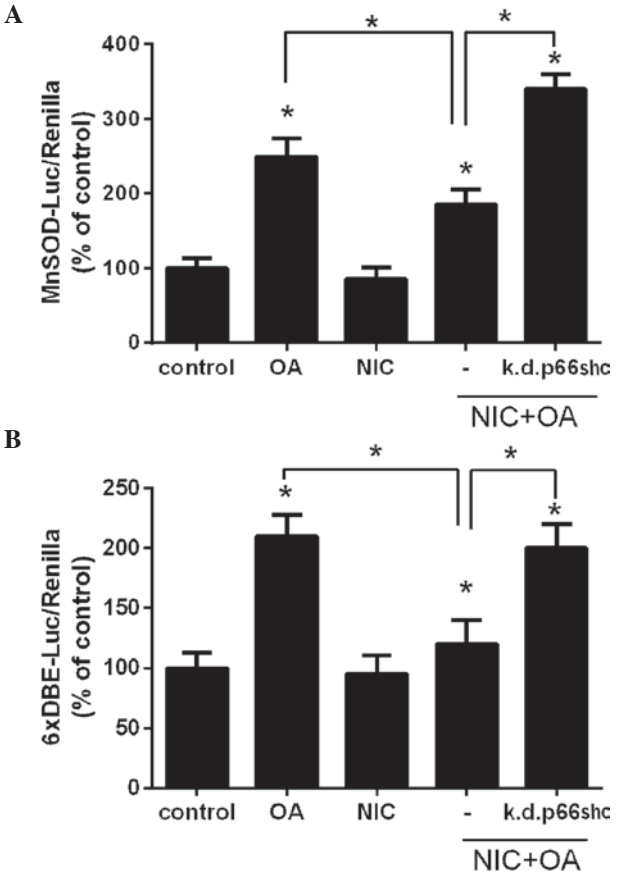

Figure 3. NIC attenuated FFA-dependent induction of MnSOD via p66shc-mediated reduction of FOXO activity. (A) Treatment with OA increased MnSOD promoter activity compared with in the control group *P<0.05 vs. control or as indicated. (B) NIC + OA treatment increased FOXO promoter activity. n=3, *P<0.05 vs. control or as indicated. MnSOD, manganese superoxide dismutase; OA, oleic acid; NIC, nicotine; k.d.p66shc, p66shc knockdown; 6xDBE, luciferase plasmid that harbors 6 canonical forkhead box binding sites; FOXO, forkhead box; FFA, free fatty acids;.

NIC exacerbates FFA-induced ROS production and consequent injury in a p66shc-dependent manner. Previously, we determined that NIC and OA induced ROS production and subsequent cell injury via the activation of p66shc in cultured renal proximal tubule cells (11,12). In order to determine whether their co-application is additive, NRK52E cells were treated with either 100 μM OA, 200 μM NIC or with their combination, and intracellular ROS production was determined. As presented in Fig. 2A treatment with OA and NIC significantly increased ROS production compared with the control and OA only groups (P<0.05). In addition, transfection with the k.d.p66shc plasmid significantly reduced NIC + OA-dependent ROS production (P<0.05; Fig. 2A). These findings suggest that the adverse effects of NIC on OA-mediated ROS release are p66shc-dependent.

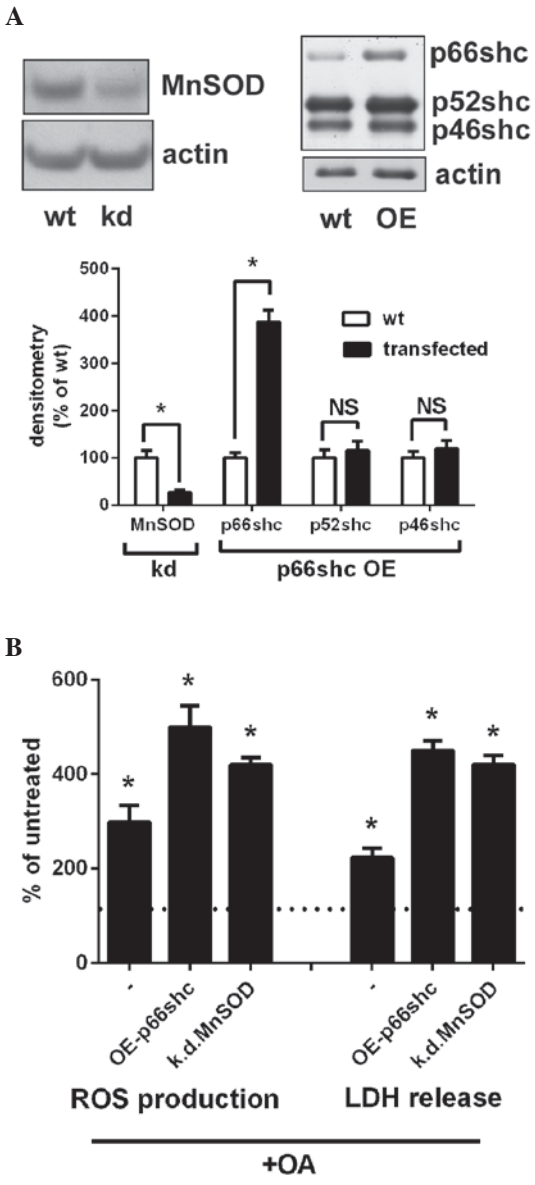

Figure 4. Overexpression of p66shc and knockdown of MnSOD emulates the adverse effects of NIC on OA-mediated lipotoxicity. (A) Expression levels of MnSOD and p66shc were determined by western blotting. β-actin was used as an internal control. Densitometric analysis confirmed the knockdown of MnSOD and overexpression of p66shc. *P<0.05 as indicated; NS, not significant. Levels of MnSOD or shc isoforms were calculated as ratios compared to actin levels and expressed as a percentage of the corresponding value (ratio) in wt cells. Data are from three independent experiments. (B) Overexpression of p66shc and transfection with a k.d.MnSOD plasmid resulted in increased ROS production and LDH release. *P<0.05 vs. control. The dotted line represents untreated (100%) values. wt, wild-type cells; kd, knockdown; OE, overexpression; k.d.MnSOD, manganese superoxide dismutase knockdown; ROS, reactive oxygen species; LDH, lactate dehydrogenase; OA, oleic acid.

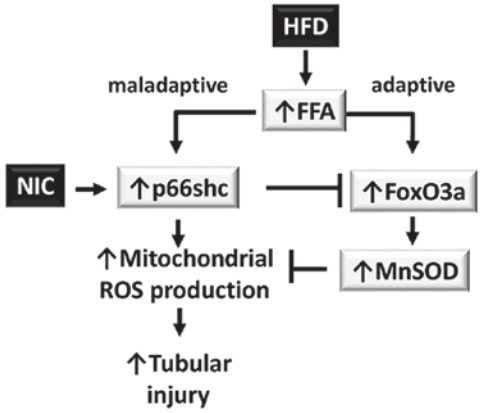

Figure 5. Contribution of p66shc to the adverse effects of NIC exposure on renal lipotoxicity. Both FFA and NIC transcriptionally activate p66shc, which exerts dual function. It increases ROS production directly via a mitochondrial mechanism and indirectly via suppressing transcription of the antioxidant MnSOD through inhibiting FoxO3a activation. The net result is increased oxidative injury of renal proximal tubule cells. HFD, high-fat diet; FFA, free fatty acid; NIC, nicotine; FOXO3a, forkhead box O3; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species.

The present study also aimed to determine whether an increase in ROS production by NIC + OA treatment led to increased cell injury. Cells were treated with OA, NIC or their combination, and LDH release was determined. As presented in Fig. 2B, treatment with OA and NIC significantly increased LDH release, and thus cell injury, compared with the control group (P<0.05). Furthermore, LDH release was significantly increased in the NIC + OA group compared with the OA only group (P<0.05). Pretreatment of the cells with the antioxidant NAC (10 μM) significantly attenuated OA + NIC-induced cell injury, thus suggesting that NIC + OA-dependent injury may be mediated through ROS. In addition, transfection with k.d.p66shc significantly reduced the release of LDH compared with the NIC + OA treatment group(P<0.05; Fig. 2B), which suggests that the adverse effects of NIC on OA-mediated cell injury are p66shc-dependent.

NIC attenuates FFA-dependent induction of MnSOD via p66shc-mediated inactivation of FOXO activity. The present study demonstrated that p66shc acts as a mediator of the adverse effects of chronic NIC exposure on OA-dependent lipotoxicity (Fig. 2). In our previous study we reported that NIC mitigates HFD-dependent induction of MnSOD in the mouse kidney, which also coincides with increased p66shc expression (10). Therefore, the present study aimed to investigate whether p66shc is responsible for suppression of MnSOD. Accordingly, NRK52E cells were transfected with a reporter luciferase plasmid that harbors the promoter of the MnSOD gene (21) together with a *Renilla* luciferase plasmid, and were treated with either 100 μM OA, 200 μM NIC or with their combination. After 24 h cell luciferase activity was determined. As presented in Fig. 3A, treatment with OA significantly elevated the MnSOD promoter activity compared with the control group (P<0.05). However, MnSOD promoter activity was suppressed in the NIC treatment group. Subsequently, NRK52E cells were co-transfected with a shp66shc plasmid, to knockdown p66shc expression, in conjunction with MnSOD-luc reporter/*Renilla* luciferase plasmids, and the cells were treated with NIC + OA. As shown in Fig. 3A, knockdown of p66shc rescued the OA-dependent induction of MnSOD in the presence of NIC.

The MnSOD promoter is primarily regulated through the forkhead FOXO3a transcription factor (23). Previous studies have revealed that p66shc suppressed MnSOD promoter activity through the inactivation of FOXO3a in non-renal cells (14,16). In the present study, NRK52E cells were transfected with

a 6xDBE-luc plasmid that contains 6 copies of the canonical FOXO binding site (22) together with a *Renilla* luciferase plasmid, and were subsequently treated with NIC, OA or with their combination. As presented in Fig. 3B, OA treatment significantly induced 6xDBE-luciferase activity compared with in the control and the NIC + OA treatment groups ($P<0.05$). However, this activity was suppressed by NIC, which was similar to its effects on the MnSOD promoter (Fig. 3A). Notably, k.d.p66shc reduced this suppression (Fig. 3B).

Overexpression of p66shc or suppression of MnSOD emulates the adverse effects of NIC on OA-mediated lipotoxicity. The present study indicated that the upregulation of p66shc and p66shc-mediated suppression of MnSOD may be responsible for the adverse effects of NIC on OA-mediated lipotoxicity. To demonstrate this, endogenous MnSOD expression was suppressed by ~70% through transfection of cells with an shMnSOD plasmid (Fig. 4A). In addition, transfection with a p66shc expression vector increased the levels of p66shc by 400%, whereas the levels of the other shc isoforms (p52shc and p46shc) remained unchanged. Subsequently, these cells were treated with 100 μM OA, and ROS production and LDH release were determined. As shown in Fig. 4B, overexpression of p66shc (OE-p66shc group) significantly increased ROS production compared with the OA-treated group ($P<0.05$; Fig. 4B). In addition, knockdown of MnSOD (k.d.MnSOD group) significantly augmented OA-dependent ROS production compared with the control group ($P<0.05$; Fig. 4B). The effects of k.d.MnSOD and OE-p66shc were similar to the effects of NIC on cell injury and ROS levels (Fig. 2).

Discussion

Obesity is an epidemic in the United States and other western countries, and is associated with the increased incidence of chronic kidney disease in adults and children (24-26). The potential mechanism underlying renal lipotoxicity includes FFA-dependent production of ROS (24), which increases mitochondrial permeability transition (27) leading to depolarization of the mitochondria and cell death (28). OA is a major circulating FFA in obese individuals (29), and our previous study demonstrated that it increases transcription of the pro-oxidant p66shc gene, which contributed to increased ROS production and subsequent injury in cultured renal proximal tubule cells (12).

Although obesity is preventable, many people often adopt smoking as a practice to lose weight (4,5); however, they are often oblivious to the fact that the life expectancy of an obese smoker is 13 years less compared with a smoker at a normal weight (5). Adverse effects of smoking on obesity include increased dyslipidemia (30), diabetes, insulin resistance, cardiovascular disease (5) and an increased renal risk, the mechanisms of which remain to be fully elucidated.

Smoking is an independent risk factor that augments the risk of the increased development and progression of chronic kidney disease via oxidative stress (31). NIC is a major tobacco alkaloid (6) that is responsible for the association between smoking and kidney injury (8). Our previous study demonstrated that NIC augments oxidative stress via the transcriptional activation of p66shc, which may be responsible for NIC-mediated ROS production and consequent cell injury in renal proximal tubule cells (11).

A previous study reported that smoking may exacerbate the obesity-dependent renal risk (32). Furthermore, NIC in combination with HFD augments mitochondrial abnormalities (33) and oxidative stress in the liver (34), which exacerbates the severity of HFD-induced hepatic lipotoxicity (34). Conversely, to the best of our knowledge, the impact of NIC on HFD-associated renal lipotoxicity has not been thoroughly investigated. Our previous study demonstrated the adverse effects of NIC exposure on HFD-associated fat deposition and oxidative stress in the kidneys of mice (10). Our present *in vitro* experiments recapitulated this scenario: NIC treatment augmented OA-dependent ROS production and resulted in increased injury (Fig. 2). In addition, it was demonstrated that NIC-induced injury may be mitigated by knockdown of the p66shc gene (Fig. 2). This is not surprising, since NIC and OA exert renotoxicity via the induction of p66shc expression in cultured renal proximal tubule cells (11,12). Induction of the p66shc promoter was increased in cells treated with the combination of NIC and OA (Fig. 1), which is in agreement with our previous study in NIC + HFD mice (10). Our previous study demonstrated that NIC increased p66shc promoter activity via hypomethylation or p53 (11). In addition, it has previously been reported that the expression levels of p53 in renal tubular cells are enhanced in obese mice (35) and a genome-wide increase in DNA methylation has been observed in obese children (36). Whether these pathways act in conjunction to enhance the effects of p66shc and thus increase NIC+HFD/FFA-associated renal oxidative stress remains to be determined.

Increased oxidative stress may be only partially due to an increase in ROS production; it may also be the result of impaired antioxidant responses. The present study demonstrated that NIC increased OA-associated ROS production and suppressed OA-dependent induction of MnSOD expression in a p66shc-dependent manner (Fig. 3A); these findings are in agreement with the results of our previous study in mice fed a HFD (10). To the best of our knowledge, this is a novel observation in renal cells and the molecular mechanism underlying this suppression remains to be elucidated. Previous studies have demonstrated that p66shc is able to suppress transcription of MnSOD via inactivation of FOXO3a in various non-renal cells (14-16). The results of the present study supported this scenario, since OA treatment increased the activity of a FOXO reporter (Fig. 3B), which was abolished by NIC. Notably, knockdown of p66shc expression mitigated the negative effects of NIC (Fig. 3B), thus suggesting that p66shc may be responsible for the observed suppression.

The present study suggested that NIC exerted its adverse effects by promoting an increase in p66shc-mediated ROS release and p66shc-induced MnSOD antioxidant suppression. The overexpression of p66shc and knockdown of MnSOD had similar effects to NIC treatment on OA-associated renal lipotoxicity (Fig. 4).

In conclusion, HFD induces maladaptive and adaptive responses via increased renal FFA deposition. The maladaptive responses are triggered by transcriptional activation of the p66shc gene, which increases mitochondrial ROS production and leads to tubular injury in the kidney. This maladaptive response is compensated by the adaptive response: FOXO-dependent

induction of the antioxidant gene MnSOD. In the presence of NIC, transcription of p66shc is increased; therefore, the balance is shifted to a primarily maladaptive response (Fig. 5).

The overall rate of cigarette smoking in the United States has been reported to be in decline (37); however, there has been an increase in the use of alternative NIC delivery products, including E-cigarettes (38). Perceived as being a safe alternative to cigarettes (39), the popularity of E-cigarettes is increasing at an alarming rate (7), which may represent an additional renal risk to obese smokers. The results of the present study may offer a therapeutic means to ameliorate the adverse effects of NIC exposure in obese individuals. Future animal studies are required to explore therapeutic interventions that aim to modify p66shc expression.

Acknowledgements

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