Chronic nicotine exposure augments renal oxidative stress and injury through transcriptional activation of p66shc

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Background. Chronic nicotine (Ch-NIC) exposure exacerbates ischemia/reperfusion (I/R)-induced oxidative stress and acute kidney injury (AKI), and mitochondrial production of reactive oxygen species (ROS) in cultured renal proximal tubule cells (RPTCs). Because Ser36-phosphorylated p66shc modulates mitochondrial ROS production and injury of RPTCs, we hypothesized that Ch-NIC exacerbates AKI by increasing stress-induced phosphorylation of p66shc.

Methods. We first tested whether Ch-NIC augments I/R-AKI-induced expression and phosphorylation of p66shc in vivo. We then examined whether knocking down p66shc, or impairing its Ser36 phosphorylation or binding to cytochrome c, alters the effects of Ch-NIC on oxidative stress (H2O2)-

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induced production of ROS, mitochondrial depolarization and injury in RPTCs in vitro.

**Results.** We found that Ch-NIC increased the expression of p66shc in the control and ischemic kidneys, but only increased its Ser36 phosphorylation after renal I/R. Knocking down p66shc or impairing phosphorylation of its Ser36 residue, via the S36A mutation (but not the phosphomimetic S36D mutation), blunted Ch-NIC + H2O2-dependent ROS production, mitochondrial depolarization and injury in RPTCs. Additionally, Ch-NIC + H2O2-dependent binding of p66shc to mitochondrial cytochrome c was attenuated by S36A mutation of p66shc, and impairing cytochrome c binding (via W134F mutation) abolished ROS production, mitochondrial depolarization and injury, while ectopic overexpression of p66shc (which mimics Ch-NIC treatment) augmented oxidant injury. We determined that Ch-NIC stimulates the p66shc promoter through p53- and epigenetic modifications (promoter hypomethylolation).

**Conclusions.** Ch-NIC worsens oxidative stress-dependent acute renal injury by increasing expression and consequent oxidative stress-dependent Ser36 phosphorylation of p66shc. Thus, targeting this pathway may have therapeutic relevance in preventing/ameliorating tobacco-related kidney injury.

**MATERIALS AND METHODS**

**In vivo experiments**

All experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals guidelines, and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Ten-week-old male C57Bl/6j mice (Jackson Laboratories) were randomized to receive either 200 µg/mL NIC in 2% saccharine or vehicle in their drinking water for 4 weeks as previously described [6], after which they underwent either 18 min warm renal ischemia followed by 6 h of reperfusion or a sham procedure. The kidneys were then removed and homogenized in a radioimmunoprecipitation assay (RIPA) buffer for immunoprecipitation and western blot analysis as described previously [23].

**Cell culture experiments: cells, cell transfection and Ch-NIC protocols**

Immortalized mouse proximal tubule cells (TKPTS) were used as described previously [21, 23]. Prolonged NIC (Ch-NIC) exposure was achieved by adding 200 µM NIC (Sigma-Aldrich, St Louis, MO) or vehicle (saline) to the culture media for 24 h, after which acute oxidative injury was induced by adding 400 µM H2O2 [24]. The role of p66shc and its Ser36 phosphorylation/cytochrome c binding in ROS production, mitochondrial depolarization or cell injury was determined by using p66shc knockdown TKPTS cells [23], and TKPTS cells that had been transfected with the serine phosphorylation mutant S36A-p66shc [23] or the cytochrome c-binding mutant W134F-p66shc [21]. Promoter activity of p66shc was determined after transfecting cells with a p66shc-promoter-luciferase [25] reporter. We used Lipofectamine 2000 (Invitrogen, Grand Island, NY) for transfection as suggested by the manufacturer. After completion of the Ch-NIC + H2O2 treatment, cell viability, intracellular ROS production, mitochondrial depolarization or luciferase activity were determined.

Monolayers of cells or kidney samples were lysed in a RIPA buffer (Promega, Madison, WI) for immunoprecipitation and western blot analysis.

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Viability, intracellular ROS production and mitochondrial depolarization

The viability of TKPTS cells was determined by lactate dehydrogenase (LDH) release (measured 24 h after adding H$_2$O$_2$ to the media) using the fluorescence ‘Cytotox-One Homogeneous Membrane Integrity’ kit (Promega, Madison, WI) [21]. Intracellular production of ROS was determined by the 2,7-dichloro-fluorescein-diacetate (DCFDA; Invitrogen, Grand Island, NY) [21]. Mitochondrial depolarization was determined after loading the cells with the fluorescent JC-1 (Invitrogen, Grand Island, NY) [21]. Intracellular production of ROS and mitochondrial depolarization were measured immediately after adding H$_2$O$_2$ to the media and the reaction was monitored for either 120 or 20 min for ROS and JC-1, respectively [21, 23].

Immunoprecipitation and western blotting

SDS–PAGE and western blotting were performed using conventional techniques [23]. For immunoprecipitation, 500 μg total cell or kidney lysates were incubated with the appropriate primary antibody overnight at 4°C by using the ‘Catch and Release v2.0 reversible immunoprecipitation system’ (Millipore, Charlottesville, VA). Immunoprecipitated proteins or 20–50 μg of kidney or cell lysates were separated on a 4–12% NuPAGE Novex Bis-Tris gradient mini gel (Invitrogen, Grand Island, NY) and transferred to a polyvinylidene fluoride membrane by using iBlot (Invitrogen, Grand Island, NY). Blots were hybridized with appropriate primary antibodies, 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). The following antibodies were used: anti-p66shc (Nanotools USA/Axxora, San Diego, CA), anti-pSer36p66shc (Abcam, Cambridge, MA), anti-cytochrome C and secondary antibodies (Cell Signaling Technology, Danvers, MA) as well as anti-actin (Millipore, Charlottesville, VA).

Reporter luciferase assay

To evaluate p66shc promoter activity, cells grown in 24-well plates were transfected with the p66shc promoter-luciferase plasmid [25], together with a Renilla luciferase plasmid (Promega, Madison, WI) by using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY). Twenty-four hours later, the cells were treated with 200 μM NIC for 24 h followed by 400 μM H$_2$O$_2$ in the presence or absence of 50 μM pifithrin-α (PIF) or 100 nM 5-aza-cytidine (5AZA). After 24 h, firefly and renilla luciferase activities were determined by using the Dual Luciferase assay kit (Promega, Madison, WI).

Statistical analysis

Continuous variables were expressed as mean plus standard deviation. Differences between treated and control groups were determined by one-way ANOVA analysis with the Holm–Sidak post hoc test. Differences between the means were considered significant if P < 0.05. All analyses were performed using SigmaStat 3.5 software (Systat, San Jose, CA).

RESULTS

Ch-NIC augments p66shc expression as well as its IR-AKI-induced Ser36 phosphorylation

We first determined whether Ch-NIC-induced exacerbation of I/R-AKI is associated with augmented Ser36 phosphorylation of p66shc in vivo. As shown in Figure 1A, Ch-NIC increased basal expression of p66shc similar to I/R-AKI. Together, Ch-NIC and I/R-AKI had an additive effect on p66shc expression. Despite increasing p66shc expression, Ch-NIC alone did not increase Ser36 phosphorylation of p66shc. However, it caused a synergistic increase of I/R-AKI-dependent Ser36 phosphorylation of p66shc (Figure 1B).

Ch-NIC affects the activity of the p66shc promoter

To assess whether Ch-NIC and H$_2$O$_2$ increase the transcription of p66shc, TKPTS cells were transfected with a luciferase reporter plasmid containing the promoter of human p66shc gene [25], together with a Renilla luciferase plasmid. These cells were treated with Ch-NIC, H$_2$O$_2$ or both and firefly (p66shc-Luc) as well as Renilla luciferase activities were determined. Both Ch-NIC and H$_2$O$_2$ treatment increased the activity of the p66shc promoter (Figure 2A). In addition, Ch-NIC exacerbated H$_2$O$_2$-dependent activity of the promoter. In separate experiments, we found that this upregulation of the p66shc promoter by NIC and H$_2$O$_2$ is dose-dependent (data not shown).

Because previous studies including our own have suggested that the p66shc promoter is upregulated by p53 [25, 26] and hypomethylation [27, 28], we next tested whether these mediators are implicated in Ch-NIC + H$_2$O$_2$-mediated p66shc induction. Cells transfected with the p66shc-Luc/Renilla plasmids were treated with either the p53 inhibitor PIF (50 μM) or the DNA methylase inhibitor 5AZA (100 nM), followed by Ch-NIC or H$_2$O$_2$ as before. We found that PIF inhibited, while 5AZA increased NIC- and H$_2$O$_2$-dependent activation of the p66shc promoter (Figure 2B). These results suggest that Ch-NIC- and H$_2$O$_2$-mediated induction of the p66shc promoter p53- and DNA hypomethylation-dependent.

Ch-NIC exacerbates ROS production, mitochondrial depolarization and injury via p66shc

We next investigated whether this increase in p66shc expression and Ser36 phosphorylation plays a role in Ch-NIC-induced exacerbation of renal cell injury. For this, we first tested whether knockdown of p66shc would block the deleterious effects of Ch-NIC. We used a p66shc knockdown cell line that was derived from TKPTS cells. This line exhibits less ROS production, mitochondrial depolarization and injury in response to H$_2$O$_2$ treatment than its corresponding vector-transfected counterpart [21]. As shown in Figure 3A, knockdown of p66shc significantly attenuated NIC + H$_2$O$_2$-dependent ROS production, mitochondrial depolarization (JC-1) and cell injury (LDH release). To further prove the role of p66shc in renal cell injury, we next tested whether returning p66shc would restore...
H₂O₂-induced injury. Accordingly, TKPTS cells were transfected with increasing amounts of p66shc and then treated with H₂O₂ as before. As shown in Figure 3B, H₂O₂-dependent injury incrementally increased in proportion to increasing amounts of p66shc, implying that the extent of oxidative injury depends on the levels of p66shc. These results suggest that a Ch-NIC-induced increase in p66shc (as seen in vivo) may be responsible for Ch-NIC-induced exacerbation of IR-AKI.

**Ch-NIC-induced exacerbation of renal cell injury is dependent on Ser36 phosphorylation of p66shc and its subsequent binding to mitochondrial cytochrome c**

Because Ser36 phosphorylation of p66shc determines its mitochondrial translocation [29], this may be responsible for the deleterious effects of p66shc on injury. Thus, we tested whether the Ser36-phosphorylated p66shc mediates the adverse effects of Ch-NIC on renal cell injury. For this, TKPTS cells were transfected with mutant p66shc plasmids in which the Ser36 residue was either mutated to alanine (S36A) or aspartic acid (S36D); the S36A mutation impairs Ser36 phosphorylation, while the S36D mutation is phosphomimetic [23]. Transfected and wild-type (w.t.) cells were treated with Ch-NIC followed by H₂O₂ as before and ROS production, mitochondrial depolarization as well as cell injury was determined. As shown in Figure 4A, the S36A mutation attenuated Ch-NIC + H₂O₂-induced ROS production, mitochondrial depolarization and LDH release; whereas the phosphomimetic mutation (S36D) did not. Not only did the S36A mutation inhibit injury, it also inhibited the binding of p66shc to cytochrome c (Figure 4B and C). It is important to note that Ser36 phosphorylation is required only for mitochondrial translocation but not cytochrome c binding [22, 23, 29].

Finally, we determined whether the binding of p66shc to cytochrome c is needed to inflict adverse effects of Ch-NIC on cell injury. To test this, we transfected cells with a p66shc mutant that is impaired in cytochrome c-binding (W134F) [21], and found that Ch-NIC + H₂O₂-dependent ROS production, mitochondrial depolarization and LDH release were attenuated in these cells (Figure 5). Taken together, these results suggest that phosphorylation of the Ser36 residue of p66shc, and its subsequent binding to cytochrome c is vital in mediating the adverse effects of Ch-NIC.

**DISCUSSION**

We previously reported that Ch-NIC exacerbates oxidative stress and I/R-AKI in vivo as well as mitochondrial ROS release and consequent injury in cultured RPTCs in vitro [6]. We have also reported that I/R-AKI or oxidant stress is associated with increases in Ser36 phosphorylation of p66shc in vivo and in vitro, respectively [21, 23], which is responsible for increased mitochondrial ROS production and consequent injury. Thus, our current study is an extension of these previous ones; our goal was to elaborate on the role of p66shc in Ch-NIC-induced exacerbation of I/R-AKI. We provide several new pieces of evidence that support our contention that p66shc is a key determinant of Ch-NIC-induced adverse effects in renal cells: (i) Ch-NIC augments IR-AKI-dependent serine36 phosphorylation as well as basal and IR-AKI-dependent renal expression of p66shc in vivo (Figure 1); (ii) Ch-NIC...
augments H₂O₂-dependent binding of p66shc to cytochrome c (Figure 4B and C); (iii) knockdown of p66shc (Figure 3A), mutation of its Ser36 phosphorylation (S36A) (Figure 4A) or W134 cytochrome c-binding (W134F) (Figure 5) site attenuates adverse effects of Ch-NIC on ROS production, mitochondrial depolarization and cell injury in vitro and (iv) Ch-NIC exerts its adverse effects through increasing the activity of the p66shc promoter via p53- and epigenetic (promoter hypomethylation)-dependent mechanisms (Figure 2B), hence, the more p66shc the more injury (Figure 3B).

The major sources of intracellular ROS are the mitochondria, the NADPH- and xanthine-oxidase systems [17]. Our previous study suggests that a major part of Ch-NIC- and oxidant injury-induced intracellular ROS originates in the mitochondria [6]. Excess mitochondrial ROS production is deleterious to the ischemic kidney [30, 31], especially the proximal tubules [32] via induction of permeability transition and the consequent depolarization of the mitochondria that results in the release of pro-apoptotic factors. Studies by others suggest that the adaptor protein p66shc is involved in this process [22]. That is (oxidative), stress-dependent Ser36 phosphorylation of p66shc facilitates its mitochondrial translocation, where, after dephosphorylation, binds to cytochrome c [22] that diverts electrons from complex IV of the electron transport chain and enhances mitochondrial production of H₂O₂ [22]. The result is increased ROS production, mitochondrial depolarization and consequent injury [22]. Cultured renal proximal tubule cells follow this mechanism of injury in response to oxidative stress (H₂O₂ treatment) [21]. Our present study is an extension of those previous findings: we investigated the role of p66shc, its Ser36 phosphorylation and cytochrome c binding in adverse effects of Ch-NIC on
oxidative stress in vitro. We found that Ch-NIC augments basal and IR-AKI-dependent expression of p66shc (Figure 1A) most likely through upregulation of the p66shc promoter as evidenced in vitro (Figure 2A). Moreover, the adverse effects of Ch-NIC on H_2O_2-mediated increase in ROS production, mitochondrial depolarization and consequent injury depends on the p66shc levels (Figure 3A and B), its Ser36 phosphorylation (Figure 4A) and cytochrome c binding (Figure 4B).

The mechanisms by which Ch-NIC or IR-AKI/H_2O_2 upregulate the activity of the p66shc promoter are not well understood. We and others have shown that the activity of the p66shc promoter is activated by p53 [25, 26] and hypomethylation [27, 28]. It is important to note that smoking/NIC increases the expression of p53 mRNA in cardiomyocytes [33], fibroblasts [34] and certain regions of the brain [35], and inhibits the activity of DNA methyltransferase 1 [36]. We also noted that the expression of p53 is higher in the Ch-NIC-exposed kidneys (data not shown) and that the DNA methylase inhibitor 5AZA increases basal and H_2O_2-dependent activation of the p66shc promoter [28]. In this protocol, we demonstrated that Ch-NIC augments basal and oxidant-dependent activity of the p66shc promoter (Figure 2A), which is p53- and DNA hypomethylation-dependent; the p53 inhibitor PIF attenuated while the DNA methylase inhibitor 5AZA increased Ch-NIC- or H_2O_2-induced activity of the p66shc promoter (Figure 2B). Altogether, Ch-NIC promotes transcription of p66shc through p53-dependent and epigenetic (promoter DNA-hypomethylation) mechanisms, which attributes to its adverse effects.

The finding that Ch-NIC augments basal and H_2O_2-induced p66shc raised the possibility that it may play a role in Ch-NIC-induced exacerbation of oxidant injury. If this is the case, then ROS production, mitochondrial depolarization, as well as injury, can be mitigated by any maneuver that interrupts the p66shc system, i.e. knockdown of p66shc or by mutating either its Ser36 residue or cytochrome c-binding (W134) site [21]. We found that the TKPTS cells with p66shc knockdown were less susceptible to NIC+H_2O_2-mediated ROS
production, mitochondrial depolarization and cellular injury. Moreover, overexpression of p66shc increased injury—in a dose-dependent manner—in response to oxidative stress (Figure 3B). Thus taken together, these experiments suggest that the severity of oxidative injury is determined in part by the level of p66shc. Indeed, p66shc knockout mice show less oxidative stress and prolonged lifetime [37].

Oxidant injury causes serine36 phosphorylation of p66shc and may increase susceptibility to oxidative stress and cell death [21–23, 37, 38]. Hence, phosphorylation of p66shc is thought to be involved in the pathogenesis of diseases associated with oxidative damage [22, 37, 38]. Therefore, we determined the levels of pSer36–p66shc in kidneys from mice that were exposed to Ch-NIC then underwent I/R-AKI. We found that Ser36-phosphorylated p66shc is elevated in the ischemic kidney (Figure 1B) and Ch-NIC exposure exacerbated this increase (Figure 1B). To demonstrate that Ser36-phosphorylated p66shc mediates the adverse effects of Ch-NIC, TKPTS cells were transfected with mutant p66shc plasmids in which the Ser36 residue was either mutated to alanine (S36A) or aspartic acid (S36D): the S36A mutation impairs Ser36 phosphorylation, while the S36D is a phosphomimetic mutation [23]. As with the p66shc knockdowns, preventing Ser36 phosphorylation of p66shc, using the S36A mutation, attenuated NIC + H2O2-mediated ROS production, mitochondrial depolarization and cell injury (Figure 4A). Moreover, substitution of Ser36 for the phosphomimetic aspartic acid (S36D) did not attenuate Ch-NIC-induced adverse effects, suggesting that serine phosphorylation of the Ser36 residue of p66shc is essential for mediating the adverse effects of Ch-NIC. The role of Ser36-phosphorylated p66shc is widely recognized in the pathogenesis of a variety of diseases including but not restricted to cardiovascular diseases [39], type 2 diabetes [40], impaired mitogenic signaling in T cells [41] and stroke [42]. Also, the role of p66shc in smoking-associated increase in cardiovascular oxidative stress is proposed [39] but has never been studied. Thus, our studies fill in the gap: we provide evidence that the Ser36-phosphorylated p66shc is essential for Ch-NIC-mediated increase in ROS production and mitochondrial dysfunction in renal proximal tubule cells.

Ser36-phosphorylated p66shc facilitates renal injury because it is able to readily translocate into the mitochondrial intermembrane space where it is dephosphorylated and binds to cytochrome c [21, 22] resulting in increased ROS production, mitochondrial depolarization and consequent injury [21, 22]. This mechanism can be tested by using the S36A mutant, because it decreases Ser36-phosphorylated p66shc, which in turn leads to a decrease in available p66shc in the mitochondria and thus, decreased binding of p66shc to cytochrome c. We previously reported that increased binding of p66shc to cytochrome c plays a role in H2O2-dependent proximal tubule injury in vitro and suggested its existence in the ischemic kidney [21, 23]. Our current study expanded on our previous ones; we established that Ch-NIC-induced exacerbation of renal injury is dependent on increased Ser36 phosphorylation of p66shc (Figure 4A), and thus likely depends on the translocation of p66shc to the mitochondria and its subsequent binding to cytochrome c. Indeed, Ch-NIC-exacerbated H2O2-induced binding of p66shc to cytochrome c (Figure 4B and C); this process was attenuated in the S36A mutant TKPTS cells (Figure 4B and C). Moreover, this interaction is further supported by the experiments in which cytochrome c binding of p66shc was impaired via mutation of the W154–W154F. Indeed, in the presence of the W134F mutant NIC + H2O2-mediated ROS production, mitochondrial depolarization and LDH release were significantly attenuated (Figure 5). Thus, our results provide strong evidence that Ser36 phosphorylation of p66shc is necessary for Ch-NIC to increase p66shc binding to cytochrome c and augment renal cellular injury.

In conclusion, smoking/Ch-NIC exposure elevates oxidative stress/ROS production in various organs including the kidneys [2–6, 43]. The mitochondria is an important target of cigarette smoke [18, 19], but the mechanism by which it increases mitochondrial ROS production is unclear. Based on our results, we propose that Ch-NIC exposure augments the expression of p66shc at the level of transcription and this effect is p53– and promoter hypomethylation-dependent. Increased p66shc, in turn, undergoes Ser36 phosphorylation by oxidative stress, resulting in augmented cytochrome c binding and consequent amplification of mitochondrial ROS production and depolarization, as well as injury (Figure 6). The above-described mechanism may be used in a clinical setting.
to prevent/ameliorate oxidative stress-associated renal injury in chronic smokers.

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CONFLICT OF INTEREST STATEMENT

None declared.


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Recovery of urinary nanovesicles from ultracentrifugation supernatants

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represent pathological events in the kidneys and the urogenital epithelium. The majority of currently applied isolation protocols involve cumbersome centrifugation steps to enrich vesicles from urine. To date, the efficiency of these approaches