Nicotine Exposure Augments Renal Toxicity of 5-aza-cytidine Through p66shc: Prevention by Resveratrol

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Abstract. *Background/Aim: We have shown that either chronic nicotine (NIC) exposure or 5-aza-cytidine (AZA) augments oxidative stress-dependent injury through stimulating p66shc in renal cells. Hence, NIC could exacerbate adverse effects of AZA while antioxidants such as resveratrol (RES) could prevent it. Materials and Methods: Renal proximal tubule cells (NRK52E) were treated with 20 μM RES prior to 200 μM NIC plus 100 nM AZA and cell injury (LDH release) was determined. Reporter luciferase assays determined p66shc activation and RES-induced antioxidant responses. Genetic manipulations identified the mechanism of RES action. Results: NIC exacerbated AZA-dependent injury via augmenting p66shc transcription. While RES suppressed NIC+AZA-mediated injury, –surprisingly– it further enhanced activity of the p66shc promoter. RES protected cells via the cytoplasmic p66shc/Nrf2/heme oxygenase-1 (HO-1) axis. Conclusion: RES can protect the kidney from adverse effects of NIC in patients undergoing anticancer therapy.*

Studies demonstrated that nephrotoxicity is a frequent side-effect of various anticancer agents that hampers their clinical use (1). Previously we reported that the anticancer agent 5-aza-cytidine (AZA) elicits oxidative stress-mediated toxicity in cultured renal proximal tubule cells *via* transcriptional upregulation of the pro-oxidant p66shc gene and consequent increase in mitochondrial reactive oxygen species (ROS) release (2). Studies revealed that smoking augments severity and progression of renal diseases in humans (3) and in various animal models (4-6). However, there are no data available on whether smoking could affect renal toxicity of anticancer agents.

Smoking exerts its adverse effects *via* nicotine (NIC)-induced oxidative stress (7). We reported that NIC transcriptionally activates and Serine 36 phosphorylates p66shc that results in its mitochondrial translocation and therein cytochrome *c* binding and consequent excess ROS production (8) similar to AZA (2). Hence, it is plausible that NIC exposure exacerbates renal toxicity of AZA by further augmenting p66shc expression and resultant ROS production.

Resveratrol (RES) is a powerful dietary antioxidant (9) that has been shown to protect the kidney from cigarette smoke-associated injury in rats (10). RES exerts antioxidant effect – at least partly – through activation of the Nrf2/HO-1 axis (11, 12). Whether beneficial effect of RES on NIC+AZA-exposed renal cells are due to activation of Nrf2/HO-1 and/or down-regulation of p66shc is unknown.

Accordingly, we hypothesized that (i) NIC exposure additively augments AZA-dependent induction of p66shc transcription, thus, exacerbates AZA-associated injury and (ii) RES attenuates NIC+AZA-mediated injury by suppressing p66shc transcription and/or activation of HO-1 in cultured renal proximal tubule cells.

Materials and Methods

Cell culture and treatment. The rat proximal tubule cell line (NRK52E) was maintained in 5% CO_2 at 37°C in DMEM with 10% FBS as recommended. A set of cultures was treated with 200 μM nicotine (NIC; Sigma-Aldrich, St. Louis, MO, USA) or 100 nM 5-aza-cytidine (AZA; Sigma-Aldrich) as described earlier in mouse proximal tubule cells (2). Some cultures were co-treated with NIC+AZA. Resveratrol (RES; Selleckchem, Houston, TX, USA) was applied in 20 μM concentration overnight prior to NIC+AZA treatment.

Determination of cell injury. Extent of cell injury was determined by LDH release using the fluorescent "Cytotox-One Homogeneous Membrane Integrity" kit (Promega, Madison, WI) as described earlier (13). Briefly, cells grown in 96-well-plates were transfected and treated as described in the appropriate section and 24 h later LDH content of the supernatants were determined and compared to total

LDH content of untreated cell lysate. Values were calculated as % of total LDH content and expressed as % of control (untreated) values.

Reporter luciferase assay. NRK52E cells were transfected with the following reporter luciferase plasmids: p66shc promoter (14), antioxidant response element (ARE; Qiagen, Germantown, MD, USA) and a HO-1-promoter (15) together with a Renilla luciferase (Promega, Madison, WI, USA) using Lipofectamine 3000 reagent (Life Technologies, Grand Island, NY, USA) as recommended by the manufacturer. 24 h after treatment firefly and renilla luciferase activities were determined using the Dual Luciferase assay kit (Promega, Madison, WI, USA) as recommended by the manufacturer. Luciferase activities were calculated as ratios of the firefly and renilla activities and expressed as a percentage of the control (untreated) values.

Genetic manipulation of cellular targets. To knockdown p66shc, NRK52E cells were transfected with a short-hairpin-p66shc (shp66shc) plasmid (16). To prevent Serine36 phosphorylation or mitochondrial cytochrome *c* binding of p66shc, cells were transfected with a Serine-36 phosphorylation-deficient mutant p66shc (S36A) or a cytochrome c-binding-deficient mutant p66shc (W134F) as described elsewhere (16). Nrf2 expression was knocked down by a Nrf2 siRNA (Santa Cruz, Ja Jolla, CA) using Lipofectamine 3000 (Life Technologies, Grand Island, NY, USA) as previously described (17). HO-1 expression was knocked down by an HO-1 siRNA (Santa Cruz, La Jolla, CA) using Lipofectamine 3000 (Life Technologies, Grand Island, NY, USA).

Statistical analysis. Continuous variables are expressed as means and standard deviations (S.D.). One-way ANOVA with Holm-Sidak post-hoc test was used to evaluate differences between groups. Differences between means were considered significant if $p < 0.05$. All analyses were performed using the GraphPad InStat3 (La Jolla, CA, USA) software package.

Results

Nicotine treatment augments AZA-dependent cytotoxicity via p66shc and related oxidative stress in renal proximal tubule cells. NRK52E cells were treated with either 200 μM NIC or 100 nM AZA or with their combination and LDH release was determined 24 h later. Figure 1A shows that both AZA and NIC increased LDH release similar to that found in a mouse proximal tubule cell line (8, 18). Importantly, combination of NIC and AZA was additive. Previously we demonstrated that both NIC (8) and AZA (2) exerts cytotoxicity *via* p66shc. Hence, it is not surprising that knockdown of p66shc (shp66shc) attenuated NIC+AZA-associated increase in LDH release (Figure 1A). Since p66shc exerts its cytotoxic effect *via* increasing oxidative stress (2, 8), we determined whether NIC+AZA will do the same. Accordingly, cells were pre-treated with the antioxidant N-acetyl-cysteine (NAC; 100 μM) and NIC+AZA-mediated LDH release was determined. Figure 1A shows that NAC attenuated NIC+AZA-mediated injury. In addition, activity of the p66shc promoter was determined in a reporter luciferase assay, which revealed that NIC+AZA additively induces the p66shc promoter (Figure 1B).

Resveratrol inhibits NIC+AZA-mediated LDH release while augments p66shc transcription. NRK52E cells were pretreated with 20 μM RES overnight prior to treatment with 200 μM NIC+100 nM AZA and LDH release was determined. Figure 1A shows that RES pretreatment almost completely abolished NIC+AZA-mediated cytotoxicity. Since NIC+AZA-dependent cytotoxicity is mediated through p66shc (Figure 1) we tested whether RES inhibits NIC+AZA-mediated induction of the p66shc promoter. Accordingly, NRK52E cells were transfected with a p66shc promoter luciferase plasmid and pretreated with 20 μM RES followed by treatment with 200 μM NIC+100 nM AZA, as described. Figure 1B shows that –surprisingly– RES did not inhibit, but rather augmented NIC+AZA-dependent activation of the p66shc promoter, even though LDH release was inhibited under this condition (Figure 1A). In fact, RES itself modestly but significantly activated the p66shc promoter (Figure 1B) without increasing cellular toxicity (Figure 1A).

Resveratrol activates the antioxidant response element (ARE) via pSer36p66shc and Nrf2. To test whether RES induces antioxidant responses, activity of the antioxidant response element (ARE) was tested in NRK52E cells 24 h after treatment with 20 μM RES. Figure 2A shows that RES significantly activated the ARE reporter. We also showed that knockdown of Nrf2 (siNrf2) –a transcription factor that binds and hence, activates the ARE (19)– inhibited RES-dependent induction of the ARE (Figure 2A). Interestingly, inhibition of Ser36 phosphorylation of p66shc (S36A) also attenuates RES-dependent activation of the ARE (Figure 2A) while inhibition of mitochondrial cytochrome *c* binding of p66shc (W134F) does not.

Resveratrol induces the HO-1 promoter via pSer36p66shc and Nrf2. The activated Nrf2 –*via* the ARE– induces the HO-1 promoter (15). Accordingly, NRK52 cells were transfected with a HO-1 promoter luciferase reporter together with a renilla luciferase as described in Materials and Methods. Figure 2B shows that RES treatment significantly induced it, the extent of which is attenuated by knockdown of Nrf2 (siNrf2) or inhibition of Ser36 phosphorylation of p66shc (S36A) but not by inhibition of its mitochondrial cytochrome *c* binding (W134F).

Protective effect of resveratrol requires pSer36p66shc, Nrf2 and HO-1. In the next set of experiments, we determined whether RES mediates its protective effects *via* the pSer36p66shc/Nrf2/HO-1 pathway. Figure 3A shows that knockdown of HO-1 (siHO-1) or Nrf2 (siNrf2) completely abolished protective effects of RES on NIC+AZA-mediated cytotoxicity. In addition, inhibition of Ser36 phosphorylation of p66shc (S36A) but not its (mitochondrial) cytochrome *c* binding (W134) also significantly attenuated beneficial effects of RES.
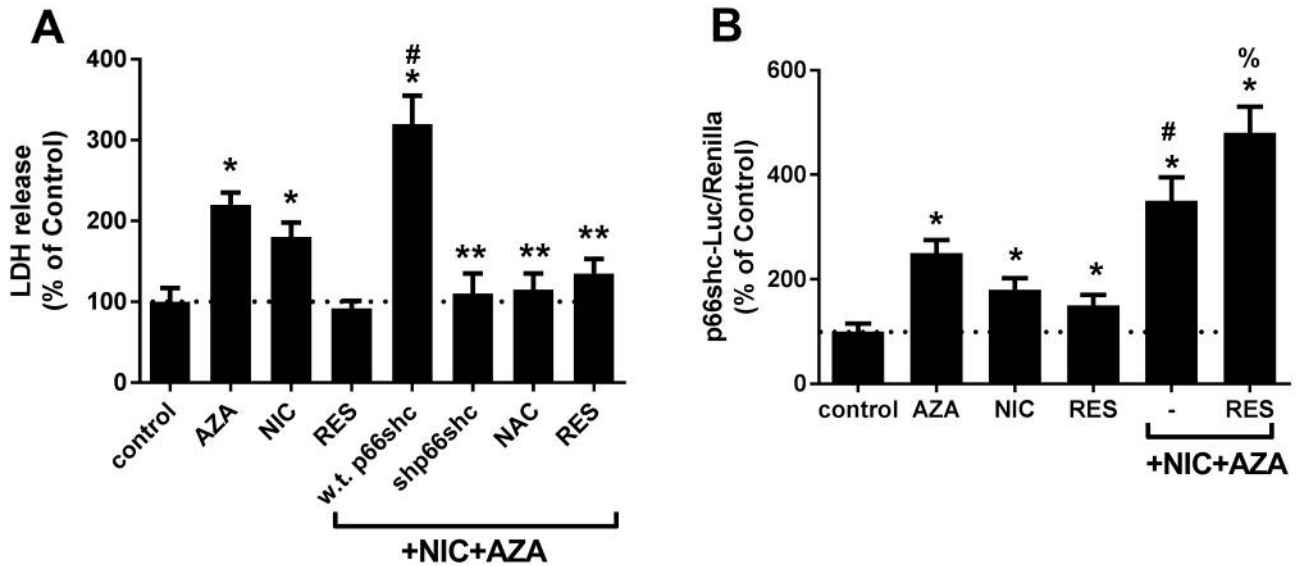
Figure 1. *Nicotine exposure exacerbates toxicity of AZA via p66shc-dependent oxidative stress, which is alleviated with resveratrol. (A) NRK52E cells were treated with either 100 nM AZA or 200 µM NIC or with their combination and LDH release was determined 24 h later. In some experiments p66shc was knocked-down by transfection of a short-hairpin p66shc construct (shp66shc). Oxidative stress was prevented by pre-treatment with 100 µM NAC. A set of cells were pretreated with 20 µM RES overnight prior to treatment with NIC+AZA. N=3; *p<0.05 compared to control (untreated) cells; #p<0.05 compared to either AZA or NIC alone; **p<0.05 compared to NIC+AZA in wild-type p66shc (w.t.p66shc) cells. (B) NRK52E cells were co-transfected with a p66shc promoter luciferase plasmid and a renilla plasmid and treated with either 100 nM AZA or 200 µM NIC or with their combination. A set of cells were pretreated with 20 µM RES overnight prior to treatment with NIC+AZA. 24 h later luciferase activities were determined. Values were calculated as firefly (p66shc) and renilla ratios and expressed as % of control values. N=3; *p<0.05 compared to control (untreated) cells; #p<0.05 compared to either AZA or NIC; %p<0.05 compared to NIC+AZA values.*

Discussion

Studies have suggested that smoking –primarily *via* NIC– worsens prognosis in cancer patients by interfering with cancer therapies (20) and potentially increasing their toxicity (21). However, effect of smoking on renal toxicity of anticancer agents has not been thoroughly evaluated. Several studies –including ours– have shown that NIC exposure/smoking exacerbates severity of acute (4) and chronic (6) kidney injury in experimental models and in the renal patient (3). Therefore, it is highly plausible that smoking/NIC exposure can enhance renal toxicity of anticancer agents. Previously we reported that the anticancer agent AZA increases oxidative stress-related injury in renal proximal tubule cells (2, 18). It is that AZA transcriptionally activates the p66shc promoter and phosphorylates Ser36 residue of the p66shc protein, which facilitates its mitochondrial translocation and binding to mitochondrial cytochrome *c* (2). The result is enhanced mitochondrial ROS production and consequent mitochondrial depolarization-dependent injury (2). We revealed similar mechanism of injury by NIC treatment in renal proximal tubule cells (8). In this study, we determined that AZA-dependent toxicity is exacerbated by NIC, which is p66shc- and oxidative stress-

dependent (Figure 1A). Based on our previous studies it can be substantiated that this oxidative stress is in fact, due to activation of p66shc. We also proved that adverse effect of NIC is due to augmented transcription of the p66shc gene (Figure 1B). Further studies are needed to determine the mechanism of this activation, which could involve p53 and epigenetic changes in the p66shc promoter (2, 8).

RES is a powerful antioxidant, protective effects of which is –at least partly– due to Nrf2-dependent induction of HO-1 (22). Indeed, RES activates the ARE *via* Nrf2 (Figure 2A), which induces the HO-1 promoter (Figure 2B) that leads to protection against NIC+AZA-mediated cytotoxicity (Figure 3A) in renal proximal tubule cells. However, besides Nrf2, p66shc also participates in RES-afforded cytoprotection (Figure 3A) even though p66shc also plays a role in NIC+AZA-mediated cytotoxicity (Figure 1A).

p66sch is known as a pro-oxidant enzyme that links oxidative stress to renal injury (2, 8, 23, 24). Interestingly, lately some studies –including ours (17)– revealed that under certain circumstances p66shc can serve as an antioxidant (25). It is that the Ser36 phsophorylated p66shc does not translocate to the mitochondria – as during its pro-oxidant function – but rather activates the ARE and hence, a set of antioxidant genes that harbors the ARE in their
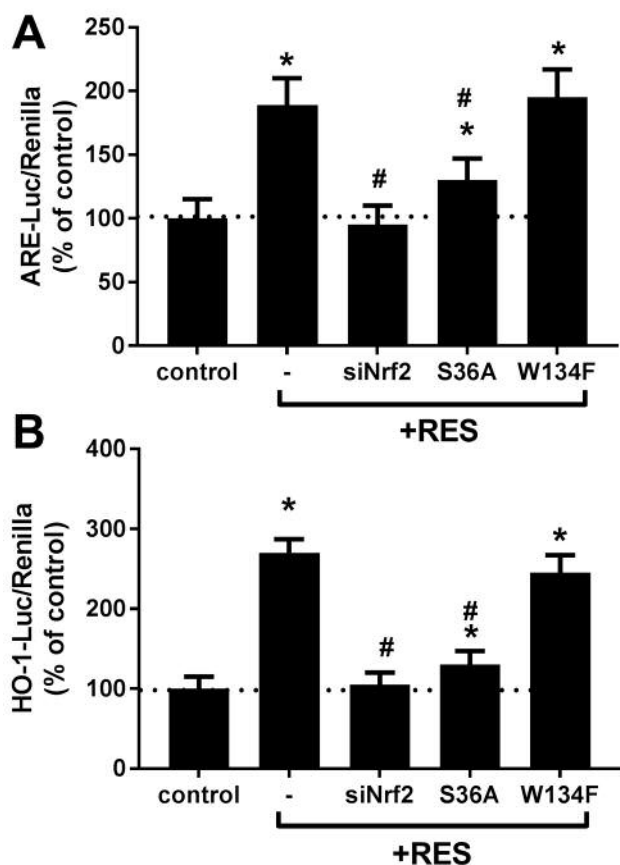
Figure 2. *Resveratrol activates the antioxidant response element (ARE) and the HO-1 promoter via Nrf2 and pSer36p66shc. (A) NRK52 cells were co-transfected with and ARE luciferase and renilla plasmid and treated with 20 μM RES. 24 h later luciferase activities were determined. Some sets were also co-transfected with either a Nrf2 siRNA (siNrf2) to knockdown Nrf2, S36Ap66shc (S36A) to inhibit Ser36 phosphorylation or W134Fp66shc (W134F) to inhibit mitochondrial cytochrome c binding of p66shc. Values were calculated and expressed as in Figure 2B. N=3; *p<0.05 compared to (untreated) control. #p<0.05 compared to RES treatment. Dotted line represents control value. (B) NRK52E cells were co-transfected with an HO-1 promoter luciferase and renilla plasmid and treated with 20 μM RES. Some sets were also co-transfected with either a Nrf2 siRNA (siNrf2), S36Ap66shc (S36A) or W134Fp66shc (W134F) plasmid as described in (A). Vales were calculated and expressed as described above. N=3; *p<0.05 compared to (untreated) control. #p<0.05 compared to RES treatment. Dotted line represents control value.*

Figure 3. *Protection by resveratol is afforded by pS36p66shc mediated activation of Nrf2 and consequent HO-1 induction. (A) NRK52 cells were transfected with either an HO-1 siRNA (siHO-1), Nrf2 siRNA (siNrf2), Ser36 (S36A)- and mitochondrial cytochrome c binding-deficient (W134F) p66shc plasmid and treated with RES+NIC+AZA as described above. 24 h later LDH release was determined. N=3; *p<0.05 compared to control. Lower dotted line represents control (untreated) value. Upper dotted line depicts NIC+AZA-treatment value. (B) Summary of our findings. NIC+AZA augments p66shc transcription and its Ser36 phosphorylation that results in mitochondrial translocation and –via binding to cytochrome c– ROS release, which injures the cells. RES treatment also augments p66shc transcription and its Ser36 phosphorylation, which is retained in the cytosol and activates the HO-1 promoter via Nrf2. The activated HO-1 exerts antioxidant/protective effects.*

promoter. This phenomenon has been described in erythroleukemia cells upon hemin treatment (25) and by us in renal proximal tubule cells upon coenzyme Q10 treatment (17). Some studies demonstrated that RES protects keratinocytes (26) and prostate cells (27) from injury *via* activating (Ser36 phosphorylating) p66shc. However, the mechanism of this protective effect has not been elucidated. We believe that those results with RES suggest a similar mechanism to that described with hemin and coenzyme
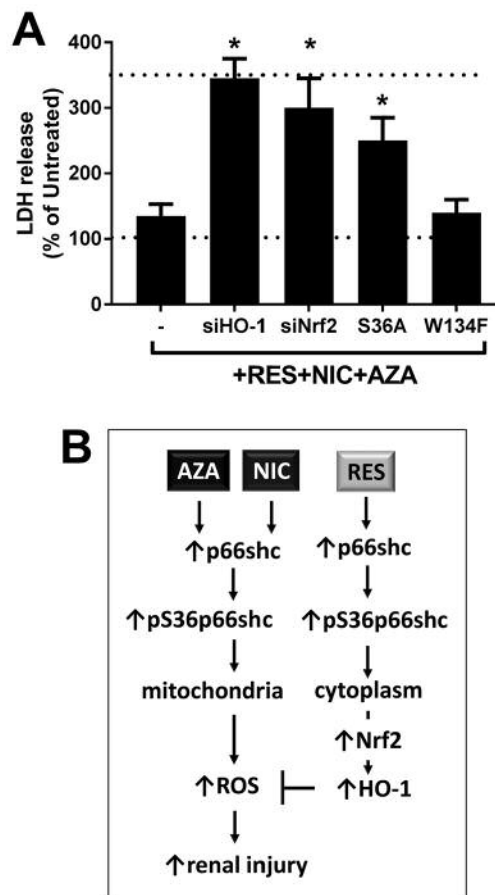
Q10. Here, we showd that RES protects renal proximal tubule cells against NIC+AZA-mediated oxidative stress (Figure 1A), which requires Ser36 phosphorylation (but not mitochondrial localization) of p66shc (Figure 3A). While RES Ser36 phosphorylates p66shc (Figure 2) it does not translocate and bind to mitochondrial cytochrome *c* (see S36A and W134F data): instead, it activates the ARE and HO-1 promoter (Figure 2) resulting in cytoprotection (Figure 3).

Our results rationalize that renal toxicity of AZA is more severe in smokers than in non-smokers. It is highly plausible that renotoxicity-enhancing effects of smoking might be applicable to other anticancer agents that activate p66shc. Previously we described that besides AZA, trichostatin A (2), taxol and dichloro-acetic acid (28) also exert their renal toxicity *via* activation of p66shc. While the best prevention would be smoking cessation, some long-term smokers refuse or can't quit. Hence, appropriate therapies might be needed. Our data offer therapeutic means to alleviate adverse effects of smoking on renotoxic effects of cancer therapies.

Acknowledgements

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