21. Hartner A, Porst M, Gauer S *et al.* Glomerular osteopontin expression and macrophage infiltration in glomerulosclerosis of DOCA-salt rats. Am J Kidney Dis 2001; 38: 153–164
22. Hartner A, Schaefer L, Porst M *et al.* Role of fibrillin-1 in hypertensive and diabetic glomerular disease. Am J Physiol Renal Physiol 2006; 290: F1329–F1336
23. Villar-Martini VC, Carvalho JJ, Neves MF *et al.* Hypertension and kidney alterations in rat offspring from low protein pregnancies. J Hypertens Suppl 2009; 27: S47–S51
24. Zou J, Yaoita E, Watanabe Y *et al.* Upregulation of nestin, vimentin and desmin in rat podocytes in response to injury. Virchows Arch 2006; 448: 485–492
25. Greka A, Mundel P. Cell biology and pathology of podocytes. Annu Rev Physiol 2012; 74: 299–323
26. Hartner A, Cordasic N, Menendez-Castro C *et al.* Lack of {alpha}8-integrin aggravates podocyte injury in experimental diabetic nephropathy. Am J Physiol Ren Physiol 2010; 299: F1151–F1157
27. Menke AL, IJpenberg A, Fleming S *et al.* The wt1-heterozygous mouse; a model to study the development of glomerular sclerosis. J Pathol 2003; 200: 667–674
28. Vaughan MR, Quaggin SE. How do mesangial and endothelial cells form the glomerular tuft? J Am Soc Nephrol 2008; 19: 24–33
29. Kreidberg JA, Sariola H, Loring JM *et al.* WT-1 is required for early kidney development. Cell 1993; 74: 679–691
30. Wagner KD, Wagner N, Schedl A. The complex life of WT1. J Cell Sci 2003; 116: 1653–1658
31. Morrison AA, Viney RL, Saleem MA *et al.* New insights into the function of the Wilms tumor suppressor gene WT1 in podocytes. Am J Physiol Ren Physiol 2008; 295: F12–F17
32. Wells J, Rivera MN, Kim WJ *et al.* The predominant WT1 isoform (+KTS) encodes a DNA-binding protein targeting the planar cell polarity gene Scribble in renal podocytes. Mol Cancer Res 2010; 8: 975–985
33. Kretzler M, Koeppen-Hagemann I, Kriz W. Podocyte damage is a critical step in the development of glomerulosclerosis in the uninephrectomised-desoxycorticosterone hypertensive rat. Virchows Arch 1994; 425: 181–193
34. Kriz W, LeHir M. Pathways to nephron loss starting from glomerular diseases-insights from animal models. Kidney Int 2005; 67: 404–419

Received for publication: 30.3.2012; Accepted in revised form: 1.10.2012

Nephrol Dial Transplant (2013) 28: 1417–1425
doi: 10.1093/ndt/gfs596
Advance Access publication 16 January 2013

ORIGINAL ARTICLE

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

Istvan Arany[1],
Jeb Clark[1],
Dustin K. Reed[1]
and Luis A. Juncos[2,3]

[1]Division of Pediatric Nephrology, Department of Pediatrics, University of Mississippi Medical Center, Jackson, MS, USA, [2]Division of Nephrology, Department of Medicine, University of Mississippi Medical Center, Jackson, MS, USA and [3]Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, MS, USA

Correspondence and offprint requests to: Istvan Arany; E-mail: iarany@umc.edu

Keywords: cytochrome *c*, mitochondria, nicotine, p66shc, ROS

ABSTRACT

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 (H_2O_2)-

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 + H_2O_2-dependent ROS production, mitochondrial depolarization and injury in RPTCs. Additionally, Ch-NIC + H_2O_2-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 modification (promoter hypomethylation).

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.

INTRODUCTION

Smoking/tobacco use is a serious health issue worldwide with a recently recognized impact on kidney health [1]. Nicotine (NIC), a major alkaloid of tobacco smoke, links smoking/tobacco use to renal injury [2]. Epidemiologic and experimental data show that smoking/NIC augments progression of chronic kidney disease [1, 3–5] and also exacerbates acute renal ischemia/reperfusion injury (IR-AKI) [6]. Importantly, NIC replacement therapy causes abnormalities in renal hemodynamics similar to smoking [7–9] and is associated with increased patient mortality in intensive care units [10, 11], perhaps in part via enhanced renal toxicity.

Smoking increases renal oxidative stress, which is an important factor in the pathogenesis of renal vascular and epithelial injury [12, 13]. Animal studies have demonstrated that chronic nicotine (Ch-NIC) exposure increases renal oxidative stress [14, 15] by suppressing antioxidant responses in the kidney [16]. Our recent data showed that Ch-NIC may exacerbate IR-AKI-induced oxidative stress by increasing the production of reactive oxygen species (ROS) in proximal tubule cells [6]. While the major sources of intracellular ROS are the mitochondria, the nicotinamide adenine dinucleotide phosphate (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]. Indeed, Ch-NIC adversely affects mitochondrial function and mitochondrial ROS production [18, 19]; however, the mechanism by which it does so is unknown.

One of the key regulators of mitochondrial production of oxidative stress is the adaptor protein p66shc, a splice variant of the ShcA locus [20]. We have shown that it is an important regulator of mitochondrial ROS generation in proximal renal tubular cells subjected to oxidant injury [21]. One mechanism by which p66shc promotes oxidative stress is as follows: oxidative stress phosphorylates p66shc at its Ser36 residue that facilitates its translocation into the mitochondrial intermembrane space. After dephosphorylation, it binds cytochrome *c*, which in turn augments the production of H_2O_2 by diverting electrons from complex IV of the electron transport chain [22] resulting in mitochondrial depolarization by increasing permeability transition [21, 22]. Thus, the aim of this study was to assess whether Ch-NIC augments oxidative stress-dependent ROS generation and renal cell injury via Ser36 phosphorylation of p66shc and its subsequent binding to cytochrome *c*, in renal proximal tubular cells.

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 H_2O_2 [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 + H_2O_2 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.

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_2O_2 to the media) using the fluorescent '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_2O_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_2O_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 a 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 wellas 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_2O_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_2O_2 or both and firefly (p66shc-Luc) as well as Renilla luciferase activities were determined. Both Ch-NIC and H_2O_2 treatment increased the activity of the p66shc promoter (Figure 2A). In addition, Ch-NIC exacerbated H_2O_2-dependent activity of the promoter. In separate experiments, we found that this upregulation of the p66shc promoter by NIC and H_2O_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_2O_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_2O_2 as before. We found that PIF inhibited, while 5AZA increased NIC- and H_2O_2-dependent activation of the p66shc promoter (Figure 2B). These results suggest that Ch-NIC- and H_2O_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_2O_2 treatment than its corresponding vector-transfected counterpart [21]. As shown in Figure 3A, knockdown of p66shc significantly attenuated NIC + H_2O_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

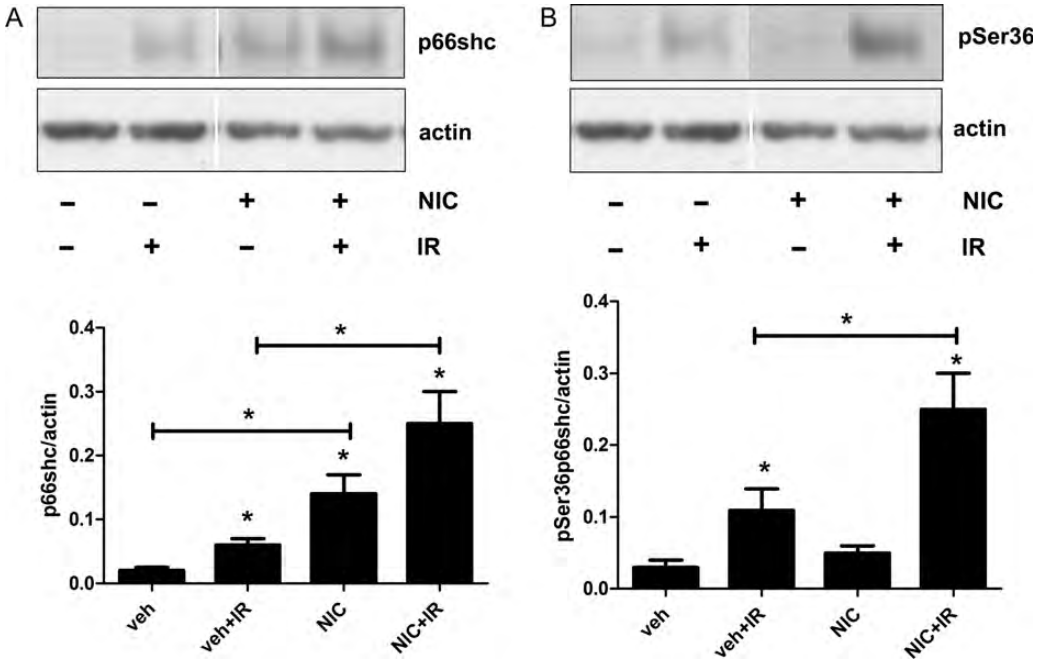

FIGURE 1: Effects of Ch-NIC on expression and Ser36 phosphorylation of p66shc in control mice and mice subjected to I/R-AKI. Kidney lysates were prepared and blotted with an anti-p66shc (**A**) and after stripping by an anti-pSer36-p66shc (**B**) antibody and finally with an anti-actin antibody after serial stripping. Representative examples are shown in the top panels, while the densitometric analysis of the results, normalized to actin, is shown in the bottom graphs. $N = 6$, *$P < 0.05$ compared with the vehicle control or as indicated.

H_2O_2-induced injury. Accordingly, TKPTS cells were transfected with increasing amounts of p66shc and then treated with H_2O_2 as before. As shown in Figure 3B, H_2O_2-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 andits 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 H_2O_2 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 + H_2O_2-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 + H_2O_2-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 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

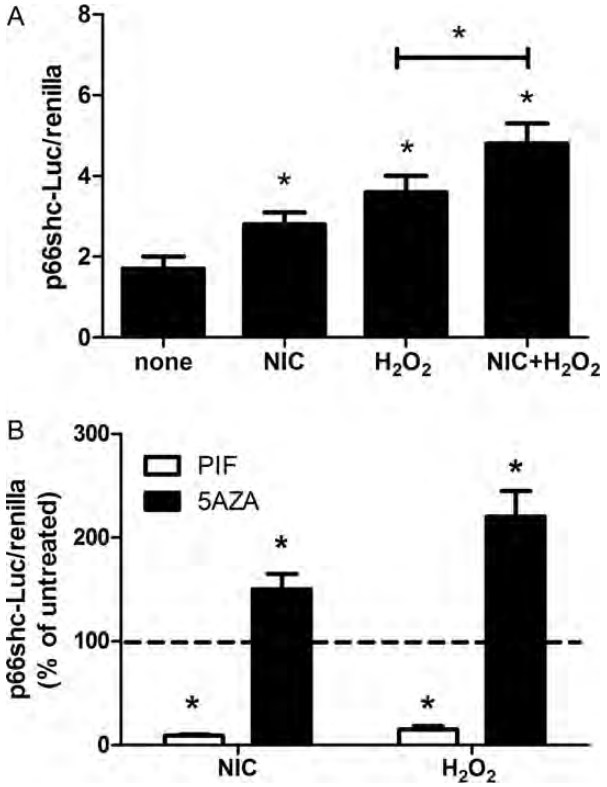

FIGURE 2: Ch-NIC increases the transcription of p66shc via p53- and epigenetic (DNA hypomethylation) mechanisms. (**A**) To assess transcription of p66shc in response to Ch-NIC and/or H_2O_2, TKPTS cells were transfected with a p66shc promoter luciferase-plasmid together with a Renilla luciferase plasmid. Firefly (p66shc-Luc) and Renilla luciferase activities were determined 24 h after treatment. The values were calculated as firefly/renilla ratios. *P < 0.05 compared with untreated. (**B**) To determine whether the p66shc promoter is upregulated by p53 and hypomethylation, after transfection with the p66shc-Luc/Renilla plasmids TKPTS cells were treated with either 50 μM PIF or 100 nM 5AZA followed by NIC or H_2O_2 for 24 h. Results were calculated as firefly/Renilla ratios and expressed as percentage of values obtained without PIF or 5AZA treatment. *P < 0.05 compared with the values without PIF or 5AZA.

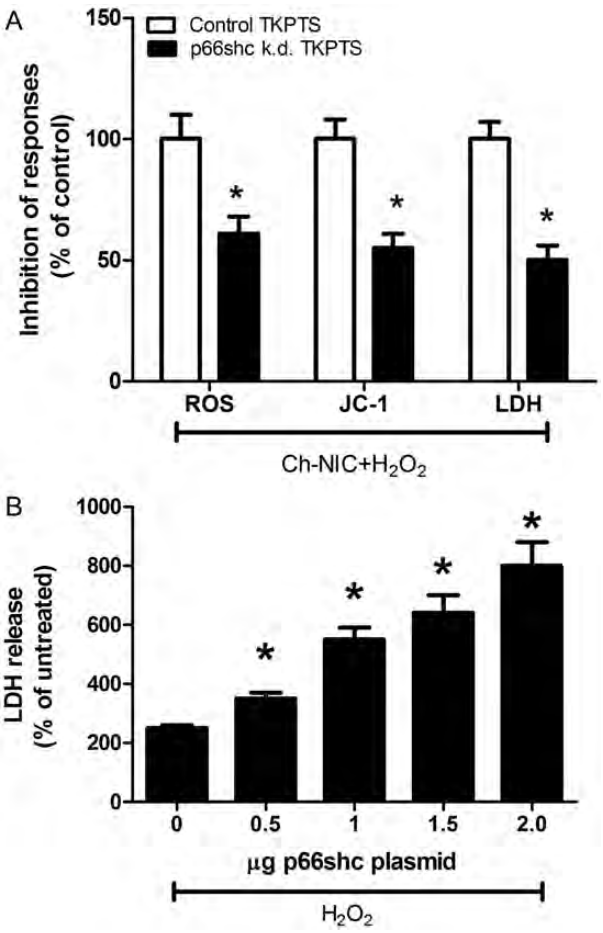

FIGURE 3: The role of p66shc in the adverse effects of Ch-NIC *in vitro*. (**A**) p66shc knockdown (p66shc k.d. TKPTS) cells were exposed to Ch-NIC for 24 h and then treated with H_2O_2. Immediately after H_2O_2 exposure, ROS production and mitochondrial depolarization (JC-1) were determined, although LDH release was measured 24 h later. The results were compared with control TKPTS cells that were treated similarly and expressed as percentage of responses from control cells. *P < 0.05 compared with untransfected cells. (**B**) To test whether rescuing p66shc levels—which mimics the effects of Ch-NIC—would restore H_2O_2-induced injury, TKPTS cells were transfected with increasing amounts of a w.t. p66shc plasmid and treated with H_2O_2. Twenty-four hours later, LDH release was determined to assess cellular injury. The results are expressed as percentage of released LDH shown in untreated cells. *P < 0.05 compared with untransfected cells.

augments H_2O_2-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 H_2O_2 [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 (H_2O_2 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

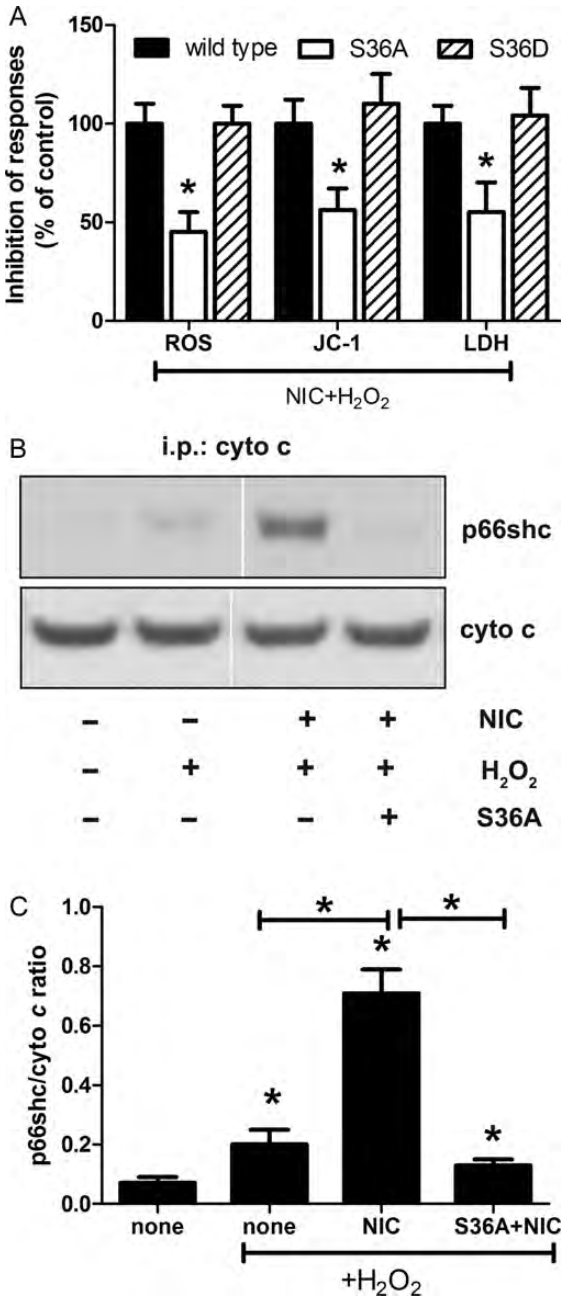

FIGURE 4: The Ser36 phosphorylation of p66shc is necessary for adverse effects of Ch-NIC and binding of p66shc to cytochrome C. (**A**) TKPTS cells were transfected with the Ser36 phosphorylation-deficient (S36A) or the Ser36 phosphomimetic (S36D) mutant plasmid and exposed to Ch-NIC and H_2O_2 as before and ROS production, mitochondrial depolarization as well as LDH release was determined and compared with vector-transfected (w.t.) cells. Results were compared with w.t. TKPTS cells that were treated similarly and expressed as percentage of responses of control cells. *P < 0.05 compared with w.t. cells. (**B**) To determine whether the Ser36 phosphorylation of p66shc is needed for p66shc to bind to cytochrome *c*, TKPTS cells were treated with Ch-NIC and H_2O_2 in the presence or absence of the S36A mutant. Cell lysates were immunoprecipitated with an anti-cytochrome *c* antibody and immunoblotted with an anti-p66shc antibody. The same blot was re-hybridized with an anti-cytochrome *c* antibody after stripping. A representative example is shown in (B), while the densitometric analyses of the p66shc/cytochrome *c* ratios are shown in **(C)**. *P < 0.05 compared with the untreated control or as indicated.

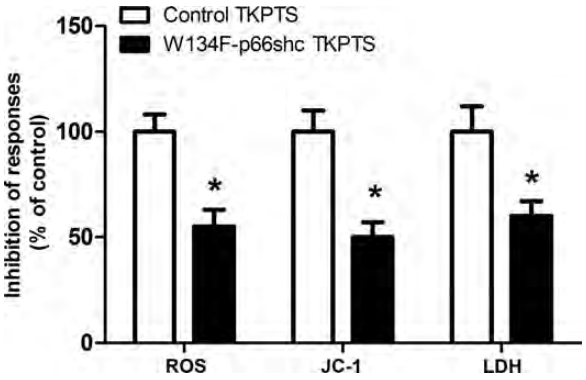

FIGURE 5: Binding of p66shc to cytochrome *c* is necessary for Ch-NIC-induced exacerbation of oxidant injury. Cells transfected with a p66shc mutant (W134F), which is impaired in cytochrome *c*-binding. ROS production, mitochondrial depolarization and cell injury were determined after treatment with Ch-NIC + H_2O_2 as described before. The results are expressed as percentage of untransfected (control) cells that were treated similarly. *P < 0.005.

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 methyltransferase 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 renal 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

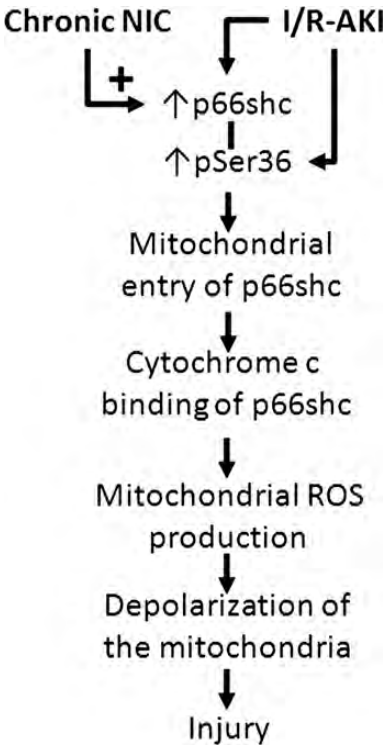

FIGURE 6: Proposed effects of Ch-NIC exposure on renal I/R injury. I/R-AKI upregulates the p66shc promoter through p53 and DNA hypomethylation then Ser36-phosphorylates it leading to increased translocation of p66shc to the mitochondria and ultimately increased cytochrome *c* binding. The result is augmentation of mitochondrial ROS production and mitochondrial depolarization as well as consequent injury. Ch-NIC exacerbates this process by augmenting the expression—but not Ser36 phosphorylation—of p66shc through p53- and DNA hypomethylation-dependent mechanisms.

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-phophorylated 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 + H_2O_2-mediated ROS production, mitochondrial depolarization and cellular 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 H_2O_2-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 H_2O_2-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 + H_2O_2-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.

ACKNOWLEDGEMENTS

The Authors thank Dr. Irani (Cardiovascular Institute, University of Pittsburgh) for the p66shc-Luc plasmid and Ms. Mardelle Susman (UTMB) for editing this manuscript. These studies were supported by an American Heart Association Greater Southeast Affiliate Grant-in-Aid (10GRNT3790019, I. Arany), an Intramural Research Support Program Award from the University of Mississippi Medical Center (I. Arany) and NIH grant DK073401 (L.A. Juncos).

CONFLICT OF INTEREST STATEMENT

None declared.

(See related article by Speeckaert *et al.* Chronic nicotine exposure and acute kidney injury: new concepts and experimental evidence. *Nephrol Dial Transplant* 2013; 28: 1329–1331.)

REFERENCES

1. Orth SR, Ritz E, Schrier RW. The renal risks of smoking. Kidney Int 1997; 51: 1669–1677
2. Jaimes EA, Tian RX, Raij L. Nicotine: the link between cigarette smoking and the progression of renal injury? Am J Physiol Heart Circ Physiol 2007; 292: H76–H82
3. Hua P, Feng W, Ji S *et al.* Nicotine worsens the severity of nephropathy in diabetic mice: implications for the progression of kidney disease in smokers. Am J Physiol Renal Physiol 2010; 299: F732–F739
4. Jaimes EA, Tian RX, Joshi MS *et al.* Nicotine augments glomerular injury in a rat model of acute nephritis. Am J Nephrol 2009; 29: 319–326
5. Orth SR, Viedt C, Ritz E. Adverse effects of smoking in the renal patient. Tohoku J Exp Med 2001; 194: 1–15
6. Arany I, Grifoni S, Clark JS *et al.* Chronic nicotine exposure exacerbates acute renal ischemic injury. Am J Physiol Renal Physiol 2011; 301: F125–F133
7. Halimi JM, Philippon C, Mimran A. Contrasting renal effects of nicotine in smokers and non-smokers. Nephrol Dial Transplant 1998; 13: 940–944
8. Benowitz NL, Porchet H, Sheiner L *et al.* Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. Clin Pharmacol Ther 1988; 44: 23–28
9. Ritz E, Benck U, Franek E *et al.* Effects of smoking on renal hemodynamics in healthy volunteers and in patients with glomerular disease. J Am Soc Nephrol 1998; 9: 1798–1804
10. Paciullo CA, Short MR, Steinke DT *et al.* Impact of nicotine replacement therapy on postoperative mortality following coronary artery bypass graft surgery. Ann Pharmacother 2009; 43: 1197–1202
11. Lee AH, Afessa B. The association of nicotine replacement therapy with mortality in a medical intensive care unit. Crit Care Med 2007; 35: 1517–1521
12. Halimi JM, Mimran A. Renal effects of smoking: potential mechanisms and perspectives. Nephrol Dial Transplant 2000; 15: 938–940
13. Orth SR. Smoking—a renal risk factor. Nephron 2000; 86: 12–26
14. Cigremis Y, Turkoz Y, Akgoz M *et al.* The effects of chronic exposure to ethanol and cigarette smoke on the level of reduced glutathione and malondialdehyde in rat kidney. Urol Res 2004; 32: 213–218
15. Muthukumaran S, Sudheer AR, Menon VP *et al.* Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicology 2008; 243: 207–215
16. Husain K, Scott BR, Reddy SK *et al.* Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. Alcohol 2001; 25: 89–97
17. Wardle EN. Cellular oxidative processes in relation to renal disease. Am J Nephrol 2005; 25: 13–22
18. Banzet N, Francois D, Polla BS. Tobacco smoke induces mitochondrial depolarization along with cell death: effects of antioxidants. Redox Rep 1999; 4: 229–236
19. Yang Z, Harrison CM, Chuang GC *et al.* The role of tobacco smoke induced mitochondrial damage in vascular dysfunction and atherosclerosis. Mutat Res 2007; 621: 61–74
20. Ravichandran KS. Signaling via Shc family adapter proteins. Oncogene 2001; 20: 6322–6330
21. Arany I, Faisal A, Clark JS *et al.* p66shc-mediated mitochondrial dysfunction in renal proximal tubule cells during oxidative injury. Am J Physiol Renal Physiol 2010; 298: F1214–F1221
22. Giorgio M, Migliaccio E, Orsini F *et al.* Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell 2005; 122: 221–233
23. Arany I, Faisal A, Nagamine Y *et al.* p66shc inhibits pro-survival epidermal growth factor receptor/ERK signaling during severe oxidative stress in mouse renal proximal tubule cells. J Biol Chem 2008; 283: 6110–6117
24. Arany I, Reed DK, Grifoni SC *et al.* A novel U-STAT3-dependent mechanism mediates the deleterious effects of chronic nicotine exposure on renal injury. Am J Physiol Renal Physiol 2012; 302: F722–F729
25. Kim CS, Jung SB, Naqvi A *et al.* p53 impairs endothelium-dependent vasomotor function through transcriptional upregulation of p66shc. Circ Res 2008; 103: 1441–1450
26. Trinei M, Giorgio M, Cicalese A *et al.* A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. Oncogene 2002; 21: 3872–3878
27. Ventura A, Luzi L, Pacini S *et al.* The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. J Biol Chem 2002; 277: 22370–22376
28. Arany I, Clark JS, Ember I *et al.* Epigenetic modifiers exert renal toxicity through induction of p66shc. Anticancer Res 2011; 31: 3267–3271

29. Orsini F, Migliaccio E, Moroni M *et al.* The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. J Biol Chem 2004; 279: 25689–25695
30. Hall AM, Unwin RJ. The not so 'mighty chondrion': emergence of renal diseases due to mitochondrial dysfunction. Nephron Physiol 2007; 105: p1–10
31. Jassem W, Fuggle SV, Rela M *et al.* The role of mitochondria in ischemia/reperfusion injury. Transplantation 2002; 73: 493–499
32. Feldkamp T, Kribben A, Weinberg JM. Assessment of mitochondrial membrane potential in proximal tubules after hypoxia-reoxygenation. Am J Physiol Renal Physiol 2005; 288: F1092–F1102
33. Zhou X, Sheng Y, Yang R *et al.* Nicotine promotes cardiomyocyte apoptosis via oxidative stress and altered apoptosis-related gene expression. Cardiology 2010; 115: 243–250
34. Wong LS, Green HM, Feugate JE *et al.* Effects of 'second-hand' smoke on structure and function of fibroblasts, cells that are critical for tissue repair and remodeling. BMC Cell Biol 2004; 5: 13
35. Berger F, Gage FH, Vijayaraghavan S. Nicotinic receptor-induced apoptotic cell death of hippocampal progenitor cells. J Neurosci 1998; 18: 6871–6881
36. Satta R, Maloku E, Zhubi A *et al.* Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. Proc Natl Acad Sci USA 2008; 105: 16356–16361
37. Migliaccio E, Giorgio M, Mele S *et al.* The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 1999; 402: 309–313
38. Menini S, Iacobini C, Ricci C *et al.* Ablation of the gene encoding p66Shc protects mice against AGE-induced glomerulopathy by preventing oxidant-dependent tissue injury and further AGE accumulation. Diabetologia 2007; 50: 1997–2007
39. Graiani G, Lagrasta C, Migliaccio E *et al.* Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. Hypertension 2005; 46: 433–440
40. Menini S, Amadio L, Oddi G *et al.* Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. Diabetes 2006; 55: 1642–1650
41. Pacini S, Pellegrini M, Migliaccio E *et al.* p66SHC promotes apoptosis and antagonizes mitogenic signaling in T cells. Mol Cell Biol 2004; 24: 1747–1757
42. Spescha RD, Shi Y, Wegener S *et al.*. Deletion of the ageing gene p66Shc reduces early stroke size following ischaemia/reperfusion brain injury. Eur Heart J 2012
43. Ejerblad E, Fored CM, Lindblad P *et al.* Association between smoking and chronic renal failure in a nationwide population-based case-control study. J Am Soc Nephrol 2004; 15: 2178–2185

Received for publication: 18.10.2012; Accepted in revised form: 14.11.2012

Nephrol Dial Transplant (2013) 28: 1425–1433
doi: 10.1093/ndt/gfs564
Advance Access publication 20 December 2012

ORIGINAL ARTICLE

Recovery of urinary nanovesicles from ultracentrifugation supernatants

Luca Musante,
Mayank Saraswat,
Alessandra Ravidà,
Barry Byrne
and Harry Holthofer

Centre for BioAnalytical Sciences (CBAS), Dublin City University, Dublin, Ireland

Correspondence and offprint requests to: Harry Holthofer; E-mail: harry.holthofer@dcu.ie

Keywords: ammonium sulphate precipitation, exosomes, ultracentrifugation, vesicle recovery

ABSTRACT

Background. Urinary vesicles represent a newly established source of biological material, widely considered to faithfully 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