The Role of p66shc in Taxol- and Dichloroacetic Acid-dependent Renal Toxicity

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Abstract. *Background/Aim: Taxol and dichloroacetic acid (DCA) are anticancer agents with potential renal toxicity. Previously, we have shown that the Ser36-phosphorylated p66shc adaptor protein mediates renal toxicity of selected anticancer modalities through increasing production of intracellular reactive oxygen species and consequent mitochondrial depolarization. Here, we analyzed whether p66shc plays a role in potential renal toxicity of Taxol and DCA. Materials and Methods: Cultured renal proximal tubule cells (TKPTS) were used. ROS production, mitochondrial depolarization (JC-1), cell injury [lactate dehydrogenase (LDH) release] and Ser36 phosphorylation of p66shc were determined after treatment with Taxol and DCA. Involvement of p66shc in adverse effects of these drugs was determined in p66shc knockdown, Ser36 phosphorylation (S36A) and cytochrome c-binding (W134F)- deficient cells. Results: Both Taxol and DCA increased ROS production, mitochondrial depolarization, injury and Ser36 phosphorylation of p66shc in TKPTS cells. We showed that ROS production is responsible for mitochondrial depolarization and consequent injury. Knockdown of p66shc, mutation of its Ser36 (S36A) or cytochrome c binding site (W134F) attenuated adverse effects of the two drugs. Conclusion: Taxol and DCA are potentially nephrotoxic owing their adverse effects on activation of p66shc. Manipulation of expression or activity of p66shc may provide a means of ameliorating nephrotoxicity of these agents.*

[#]This work is dedicated to the memory of the late Dr. Ember.

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The adaptor protein p66shc is a known mediator of oxidative stress-associated injury in a variety of cell types (1). Adverse effects of p66shc involve reactive oxygen species (ROS)-dependent phosphorylation of its serine36 residue (S36) and its translocation to the mitochondrial intermembrane space where, – after de-phosphorylation – it binds cytochrome *c* (2), thus, producing H_2O_2 within the mitochondria (3). The result is mitochondrial depolarization and consequent injury (4). We have already demonstrated that cisplatin (5, 6), trichostatin A and 5-aza-cytidine (7) phosphorylate the serine36 of p66shc and enhance mitochondrial cytochrome *c* binding, thereby augmenting ROS production, mitochondrial depolarization and consequent injury of renal proximal tubule cells, which may drive renal toxicity of these drugs.

Taxol and dichloroacetic acid (DCA) are important anticancer drugs (8, 9) that may induce nephrotoxicity (10). Since Taxol mediates Ser36 phosphorylation of p66shc in a variety of cell types (11), p66shc may mediate the nephrotoxicity of Taxol and perhaps of DCA.

Thus, the present study aimed to determine the role of p66shc in potential nephrotoxicity of Taxol and DCA.

Materials and Methods

Cell culture and treatment. The immortalized mouse proximal tubule cell line TKPTS was maintained in 5% CO_2 at 37˚C, as described elsewhere (12). Some cultures were pre-treated with Taxol (40 μM; Sigma-Aldrich, St. Louis, MO, USA) or DCA (100 μM; Sigma–Aldrich) as indicated. The p66shc knockdown variant of TKPTS was developed as described elsewhere (12).

Assessment of cell injury. The extent of cell injury was determined by the fluorescent CytoTox-One Homogenous Membrane Integrity assay kit (Promega, Madison, WI, USA) as described elsewhere (13).

Determination of ROS production. Intracellular generation of ROS was determined by fluorescent oxidant-sensitive 2',7'-dichlorofluorescein-diacetate dye (DCFDA; Life Technologies, Grand Island, NY, USA) in a 96-well-plate as described elsewhere (14). ROS production was

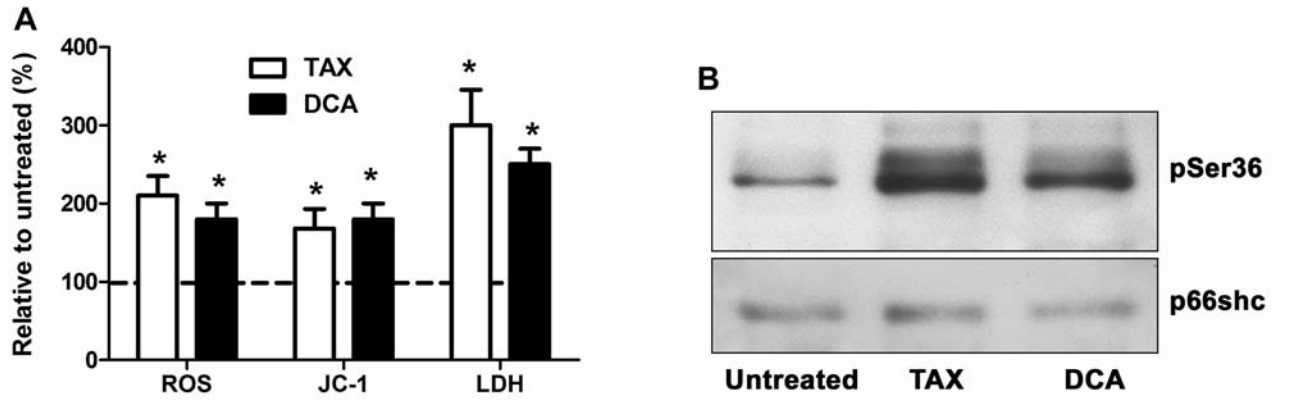

Figure 1. *Effects of taxol and dichloroacetic acid on reactive oxygen species (ROS) production, mitochondrial depolarization, cell injury and serine36 phosphorylation of p66shc in cultured renal proximal tubule cells. A: TKPTS cells were treated with 40 μM Taxol (TAX) or 100 μM dichloroacetic acid (DCA). ROS production and mitochondrial depolarization were determined immediately, while LDH release was determined 24 hours later. Bars represent the mean±S.D., n=3. *p<0.05 compared to untreated cells. B: TKPTS cells were treated with 40 μM TAX or 100 μM DCA for 60 minutes and serine36 phosphorylation of p66shc and the level of unphosphorylated p66shc were determined by western blotting. Data shown are representatives of three independent experiments.*

calculated as the increase in fluorescence/30 minutes/0.5×10^6 cells and expressed as a percentage of that of corresponding untreated cells.

Western blotting. Cell lysates were subjected to electrophoresis and western blotting as described elsewhere (13). Bands were visualized by an enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford, IL, USA) and analyzed by densitometry (UnScan-It; Silk Scientific Corp., Orem, UT, USA).

Plasmid transfection. The Ser36 phosphorylation- (S36A), and cytochrome c-binding- (W134F)-deficient mutants of p66shc were transiently transfected using the Lipofectamine 2000 reagent (Life Technologies) as recommended by the manufacturer.

Statistical analysis. Continuous variables are expressed as means and standard deviations (S.D.). Statistical differences between the treated and control groups were determined by Student's *t*-test. Differences between means were considered significant if $p<0.05$. All analyses were performed using the SigmaStat 3.5 (Systat, San Jose, CA, USA) software package.

Results

Taxol and DCA increase ROS production, mitochondrial depolarization and cell injury, as well as serine36 phosphorylation of p66shc in renal proximal tubule cells. TKPTS cells were treated with 40 μM Taxol or 100 μM DCA: ROS production and mitochondrial depolarization (JC-1) were determined immediately, while cell injury as LDH release was determined 24 hours later. Data in Figure 1A demonstrate that both Taxol and DCA significantly increased oxidative stress, mitochondrial depolarization and injury. In addition, TKPTS cells were treated with Taxol or DCA for 60 minutes and serine36 phosphorylation of p66shc was determined. Figure 1B shows that both agents significantly increased pSer36p66shc levels in TKPTS cells.

Manipulation of expression, serine36 phosphorylation, or cytochrome c binding of p66shc affects the influence of Taxol and DCA in renal proximal tubule cells. Since both Taxol and DCA increased serine36 phosphorylation of p66shc (Figure 1B), we evaluated whether p66shc, through its serine36 phosphorylated form, is involved in adverse effects of these agents. Firstly, p66shc-knockdown (kd) cells were treated with Taxol or DCA and ROS production was determined. Figure 2 shows that extent of Taxol/DCA-dependent ROS production was significantly lower in p66shc kd cells than in p66shc wild-type (wt) ones. Similar results were obtained when TKPTS cells were transiently transfected with the serine36 phosphorylation mutant (S36A) of p66shc. By transfecting the W134F p66shc mutant, Taxol/DCA-dependent binding of p66shc to mitochondrial cytochrome *c* was impaired, which also mitigated ROS production (Figure 2).

TAX/DCA-dependent increase in ROS production is responsible for mitochondrial depolarization and consequent injury. To determine whether ROS production is responsible for the observed mitochondrial depolarization and cell injury, TKPTS cells were pre-treated with the ROS scavenger N-acetylcysteine (NAC; 100 μM) one hour prior to treatment with Taxol or DCA, and either mitochondrial depolarization or LDH release was determined as described above. Figure 3 shows that scavenging of ROS significantly attenuated both mitochondrial depolarization and cell injury. To demonstrate whether mitochondrial depolarization plays a role in Taxol/DCA-dependent cell injury, TKPTS cells were pretreated with the mitochondrial polarity transition inhibitor cyclosporine A (CsA; 5 μM) one hour prior to treatment with Taxol or DCA, and LDH release was determined. Figure 3 demonstrates that CsA pre-treatment significantly attenuated LDH release.

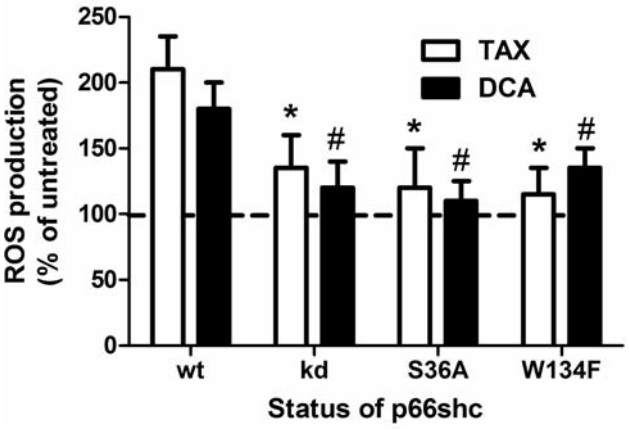

Figure 2. *Effects of p66shc knockdown and impairement in serine36 phosphorylation/cytochrome c binding of p66shc on Taxol (TAX)/dichloroacetic acid (DCA)-mediated reactive oxygen species (ROS) production. p66shc-knockdown (kd), serine 36 phosphorylation (S36A)- and cytochrome c binding (W134F)-deficient cells were treated with 40 μM TAX or 100 μM DCA and ROS production was determined and compared to that of wild-type p66shc cells. Data represent the mean±S.D., n=3. *p<0.05 compared to TAX-treated, #p<0.05 compared to DCA-treated, Untr.: untreated.*

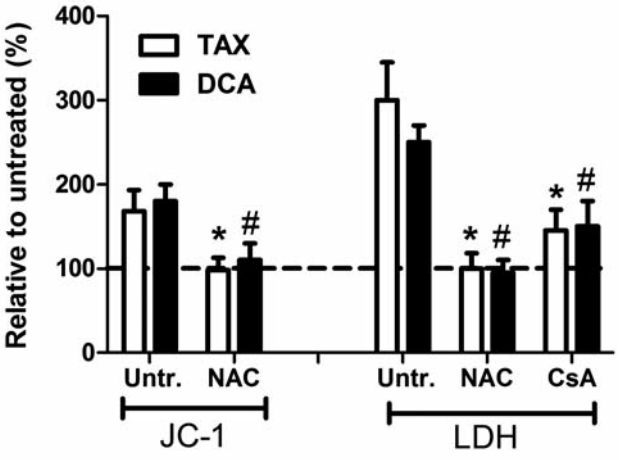

Figure 3. *Effects of reactive oxygen species (ROS) scavenging and inhibition of mitochondrial depolarization on Taxol (TAX)/dichloroacetic acid (DCA)-mediated cell injury. TKPTS cells were pre-treated with 100 μM N-acetylcysteine (NAC) for one hour prior to treatment with 40 μM TAX or 100 μM DCA and mitochondrial depolarization (JC) and cell injury [lactate dehydrogenease (LDH release)] were determined. Bars represent the mean±S.D., n=3. *p<0.05 compared to TAX-treated, #p<0.05 compared to DCA-treated. Another set of cells were pre-treated with 5 μM Cyclosporine A (CsA) for one hour prior to treatment with 40 μM TAX or 100 μM DCA and LDH release was determined. Data represent the mean±S.D., n=3. *p<0.05 compared to TAX-treated, #p<0.05 compared to DCA-treated.*

Discussion

Certain anticancer drugs can generate ROS that cause oxidative stress and apoptosis of cancer cells (15), however, this can also result in nephrotoxicity, which is a known side-effect of several anticancer drugs (16, 17).

Oxidative stress increases serine36 phosphorylation of the adaptor protein p66shc and consequently its binding to mitochondrial cytochrome *c*, which facilitates ROS production through the mitochondrial electron transport chain (3, 4). Earlier, we demonstrated that oxidative stress *in vitro*, and ischemia/reperfusion injury *in vivo* increase mitochondrial and cytochrome *c* binding of p66shc in renal proximal tubule cells and in the kidney, respectively (14). We also showed that this binding is important for excessive ROS production and consequent mitochondrial depolarization in oxidative stress-induced injury in renal proximal tubule cells (14). We also observed that this mechanism may be responsible for the nephrotoxicity of anticancer drugs, such as cisplatin (5, 6), and a group of epigenetic modifiers (7). Taxol is a potent anticancer drug (8) which is frequently used in combination with cisplatin (18); hence, it may increase the known nephrotoxicity of cisplatin (19). DCA is also a promising anticancer agent (9) with demonstrated nephrotoxicity in rats (10). In addition, some studies demonstrated impact of Taxol on p66shc phosphorylation in lung (11) and macrophage (20) cells *in vitro*. On the other hand, the impact of Taxol or DCA on p66shc phosphorylation in renal cells is unknown. Thus, our results presented in Figure 1B are novel: both Taxol and DCA increased serine36 phosphorylation of p66shc. In the light of the well-described role of pSer36p66shc in ROS production, mitochondrial depolarization and injury (3, 12, 13), the observed adverse effects of Taxol and DCA in renal cells (Figure 1A) might be due to the serine36-phosphorylated p66shc. Indeed, knocking-down expression of p66shc, mutation of its Ser36 residue (S36A), and preventing its binding to mitochondrial cytochrome *c* (W134F mutant) significantly attenuated Taxol/DCA-dependent ROS production (Figure 2). Since ROS are responsible for mitochondrial depolarization and consequent injury (Figure 3), we can safely assume that mutation of p66shc affects mitochondrial depolarization and injury similarly to ROS. These experiments have shown that adverse effects of Taxol/DCA on renal proximal tubule cells are p66shc-dependent.

The mitochondrial permeability transition pore is normally closed but various pathophysiological conditions can open it, leading to a decline in ATP synthesis and consequent necrosis, as suggested for renal I/R injury (21). The opening of this pore and consequent cell death can be prevented by either inhibitors of the mitochondrial permeability transition pore, such as cyclosporine A (22), or by antioxidants (23, 24). In this scenario, the opening of the pore is the consequence of elevated ROS production (25) as opposed to the scenario when the opening of the pore is the cause of excessive ROS release (26). Our studies demonstrated that

p66shc-dependent production of ROS by Taxol or DCA (Figure 2) is responsible for increased mitochondrial depolarization and consequent injury (Figure 3)

Conclusion

Taxol and DCA are potentially nephrotoxic owing their adverse effects to activation of p66shc. Manipulation of expression or activity of p66shc may provide a means of ameliorating the nephrotoxicity of these agents.

Acknowledgements

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