Epigenetic Modifiers Exert Renal Toxicity through Induction of p66shc

ISTVAN ARANY[1], JEB S. CLARK[1], ISTVAN EMBER[2] and LUIS A. JUNCOS[3,4]

[1]Department of Pediatrics, Division of Pediatric Nephrology, [3]Department of Medicine, Division of Nephrology, and [4]Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, MS, U.S.A.; [2]Institute of Public Health, University of Pecs, Pecs, Hungary

Abstract. *Background/Aims: Trichostatin A (TSA) and 5-azacytidine (5AZA) induce reactive oxygen species (ROS)-mediated injury in renal proximal tubule cells. Since TSA and 5AZA are activators of p66shc, we questioned whether p66shc may mediate renal toxicity of TSA- and 5AZA. Materials and Methods: Renal proximal tubule cells were treated with either TSA or 5AZA for 24 hours followed by treatment with 200 μM H_2O_2. Expression of p66shc and activity of its promoter, as well as its mitochondrial and cytochrome c binding were determined. Impact of knockdown of p66shc and mutation of its cytochrome c-binding site on ROS production and cell injury was studied. Results: TSA, and 5AZA increased expression of p66shc through induction of its promoter and also increased its mitochondrial/cytochrome c binding. Knockdown or mutation of the cytochrome c binding site of p66shc attenuated ROS production and cell injury. Conclusion: Therapeutic means that interfere with induction of p66shc may ameliorate renal toxicity of those epigenetic modifiers.*

Epigenetic drugs, such as inhibitors of DNA methyltransferases and histone deacetylases, restore activities of genes that are involved in normal cell function in cancer cells (1) but also exhibit renal toxicity that limits their effectiveness (2-4), which may be due to increased production of reactive oxygen species (ROS) (5, 6). We have reported that the histone deacetylase inhibitor trichostatin A (TSA) and the DNA methyltransferase inhibitor 5-azacytidine (5AZA) injure renal proximal tubules and also exacerbate oxidative stress-induced injury through increasing mitochondrial ROS production (7).

The p66shc adaptor protein is a known mediator of cell injury (8). We demonstrated that p66shc, through binding to the mitochondria and within it cytochrome c, is an important mediator of oxidative stress-induced injury in cultured renal proximal tubule cells and probably also in the ischemic kidney (9, 10). Since both TSA and 5AZA have been shown to modify the p66shc gene (11), it is plausible that p66shc, at least partly, mediates renal toxicity of these epigenetic modifiers.

Accordingly, the aim of the present study was to evaluate the role of p66shc in ROS production and cell injury of renal proximal tubule cells after treatment with TSA and 5AZA alone or in combination with oxidative stress.

Materials and Methods

Cell culture and treatment. The immortalized mouse proximal tubule cell line (TKPTS) was a gift from Dr. Bello-Reuss (12). Cells were grown in 5% CO_2 at 37˚C as described elsewhere (10). Oxidative stress was induced by treatment of semi-confluent cells with 200 μM H_2O_2 for 24 hours as described elsewhere (13). Some cultures were pretreated with TSA (50 nM, Sigma-Aldrich, St. Louis, MO, USA) or 5AZA (100 nM; Sigma–Aldrich) 24 hours prior to treatment with H_2O_2. The p66shc knockdown variant of TKPTS cells was developed by transfecting a p66shc shRNA construct (10), while control cells were transfected with the appropriate vector alone (10). These cells were maintained in a similar manner to the parental TKPTS cells.

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). Briefly, 24 hours after treatment, an aliquot of the growth medium was removed and saved. The monolayer was lysed according to the manufacturer's recommendation and lactate dehydrogenase (LDH) content was determined by a fluorescent substrate both in the medium and cell lysate. LDH release was calculated as the percentage of LDH in the medium compared to the total LDH content (medium+lysate).

Correspondence to: Istvan Arany, Department of Pediatrics, Division of Pediatric Nephrology, University of Mississippi Medical Center, Jackson, MS, U.S.A. Tel: +1 6018159464, Fax: +1 6019845981, e-mail: iarany@umc.edu

Key Words: Epigenetic modifiers, kidney, toxicity, ROS, p66shc, mitochondria.

Determination of ROS production. Intracellular generation of ROS was determined by fluorescent oxidant-sensitive 2',7'-dichlorofluorescein-diacetate dye (DCFDA; Invitrogen, Grand Island, NY, USA) in a 96-well-plate as described elsewhere (9). ROS production was calculated as the increase in fluorescence/30 minutes/0.5×10^6 cells and expressed as a percentage of that of corresponding untreated cells. The linear phase of the curve (between 60 and 90 minutes after adding of H_2O_2) was used for calculation.

Isolation of mitochondria. The Mitochondria Isolation Kit for Cultured Cells (Pierce; Rockford, IL, USA) was used as suggested by the manufacturer. Protein content was determined by a BioRad Protein Assay (BioRad; Hercules, CA, USA).

Western blotting and immunoprecipitation. Cell and mitochondrial lysates were separated by sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS/PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad) and hybridized with the appropriate primary and secondary antibodies. Bands were visualized by an enhanced chemiluminescence system (Pierce) and analyzed by densitometry (UnScan-It; Silk Scientific Corp., Orem, UT, USA). For immunoprecipitation, 500 μg of cell lysates were incubated with the appropriate primary antibody overnight at 4˚C using the Catch and Release v2.0 reversible immunoprecipitation system (Millipore; Charlottesville, VA, USA). Immunoprecipitated proteins were resolved by SDS/PAGE.

Plasmid transfection and measurement of reporter luciferase activity. The p66shc-Luc plasmid containing the -1096 to +44 base-pair region (relative to the ATG codon) of the human p66shc gene promoter was a gift from Dr. Irani (Cardiovascular Institute, University of Pittsburgh, PA, USA) (14). The W134F mutant of the p66shc gene which is impaired in its cytochrome *c* binding was constructed as described elsewhere (9). The Renilla luciferase plasmid was purchased from Promega (Madison, WI, USA). Transient plasmid transfection was carried out by using the Xfect™ reagent (Clontech, Mountain View, CA, USA) as recommended by the manufacturer.

Firefly and Renilla luciferase activities were determined 24 hours after treatment by the Dual Luciferase Assay System (Promega) in a Modulus luminometer (Turner Biosystem, Sunnyvale, CA, USA) as recommended by the manufacturer. p66shc-Luc activity was normalized to the internal Renilla-Luc activity.

Statistical analysis. Continuous variables are expressed as mean and standard deviation (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

TSA and 5AZA up regulate expression of p66shc in mouse renal proximal tubule cells. Cells were treated with 50 nM TSA or 100 nM 5AZA for different times as indicated in Figure 1A and B, cell lysates were subjected to Western blotting. As can be seen, both TSA and 5AZA significantly increased expression of the p66shc protein as early as 6 hours after the treatment, while levels of the p52shc and p46shc isoforms were unchanged. In the next step, the p66shc-Luc plasmid together with a Renilla-Luc plasmid were transiently transfected into TKPTS cells. Cells were treated with TSA or 5AZA and 24 hours later firefly (p66shc) and Renilla luciferase activities were determined. Results in Figure 1C indicate that both TSA and 5AZA significantly increased activity of the p66shc promoter.

Knockdown of p66shc attenuates TSA and 5AZA-mediated increase in ROS production and injury in renal proximal tubule cells. p66shc knockdown version of TKPTS cells (10) were treated with TSA or 5AZA for 24 hours and ROS production was determined after adding 200 μM H_2O_2. As can be seen in Figure 2A, knockdown of p66shc significantly attenuated ROPS production by TSA and 5AZA, and by H_2O_2 in the presence of TSA and 5AZA. Not surprisingly, the extent of cell injury, as evidenced by LDH release, was also significantly attenuated in the p66shc knockdown cells (Figure 2B) similarly to ROS production.

TSA and 5AZA increase mitochondrial and cytochrome c binding of p66shc. TKPTS cells were treated with TSA or 5AZA for overnight and mitochondria were isolated and subjected to Western blotting. Figure 3A shows that both TSA and 5AZA significantly increased the amount of mitochondrial-bound p66shc. In addition, lysates from TKPTS cells that were treated with TSA or 5AZA were immunoprecipitated with an anti-cytochrome *c* antibody then subjected to SDS/PAGE and Western blotting. The blot was first hybridized with an anti-p66shc antibody after stripping with an anti-cytochrome *c* antibody. As can be seen in Figure 3B both TSA and 5AZA treatment significantly increased cytochrome *c* binding of p66shc.

Mutation of the cytochrome c binding site of p66shc attenuates TSA- and 5AZA-mediated injury in renal proximal tubule cells. TKPTS cells were transiently transfected with the W134F mutant of p66shc then treated with TSA or 5AZA prior to treatment with 200 μM H_2O_2 and LDH release was determined 24 hours later. Figure 3C shows that mutation of the cytochrome c binding site of p66shc attenuated TSA/5AZA- as well as TSA/5AZA+H_2O_2-induced injury.

Discussion

Various stress stimuli increase binding of p66shc to mitochondrial cytochrome *c*, which facilitates ROS production through the mitochondrial electron transport chain (15, 16). Earlier, we demonstrated that oxidative stress (H_2O_2 treatment) *in vitro* or ischemia/reperfusion injury *in vivo* increases mitochondrial, and within it cytochrome *c*, binding of p66shc in renal proximal tubule cells or in the

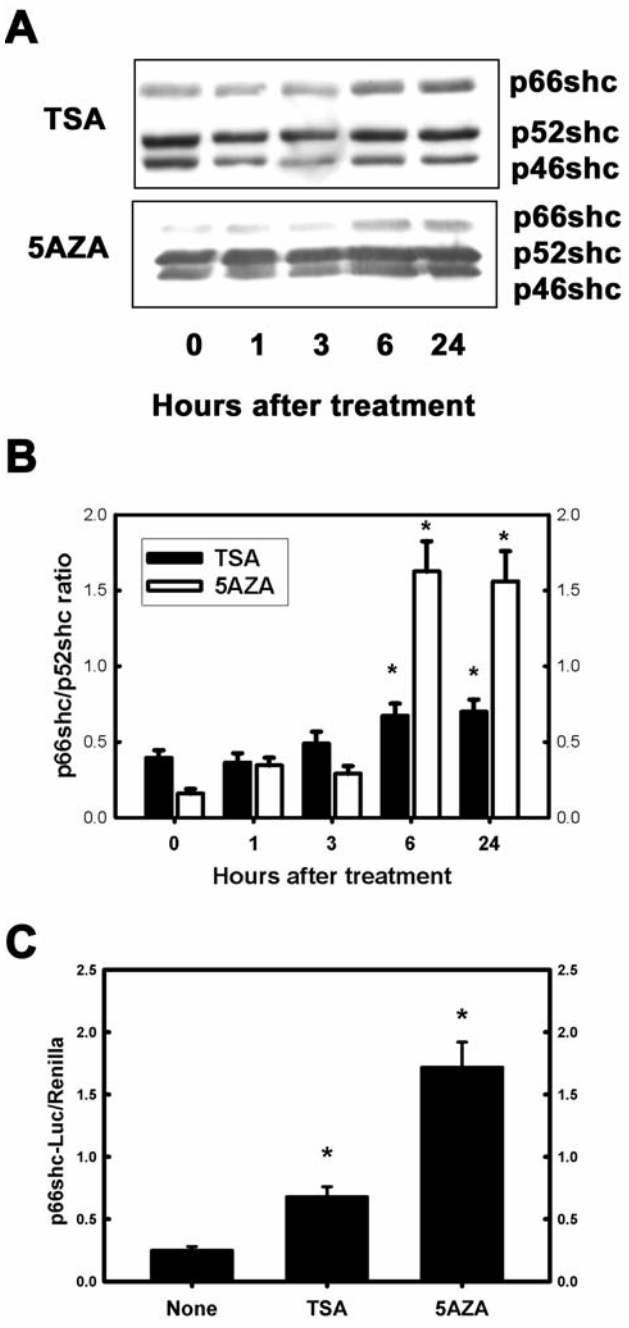

Figure 1. *Epigenetic modifiers increase p66shc expression through induction of its promoter. A: TKPTS cells were treated with either 50 nM trichostatin A (TSA) or 100 nM 5-aza-cytidine (5AZA) for 1, 3, 6 and 24 hours. Cell lysates were prepared and expression of p66shc was determined by Western blotting. The blot is representative of three independent experiments. B: Densitometry of blots from (A). Data are means±S.D. (n=3) *p<0.05 compared to the appropriate control (0 hour). C: TKPTS cells were transiently transfected with p66shc-Luc promoter construct together with a Renilla luciferase plasmid and treated with either 50 nM TSA or 100 nM 5AZA for 24 hours. Luciferase activities were determined and expressed as p66shc-Luc/Renilla ratios. Data are shown are means±S.D. (n=3) *p<0.05 compared to untreated control (none).*

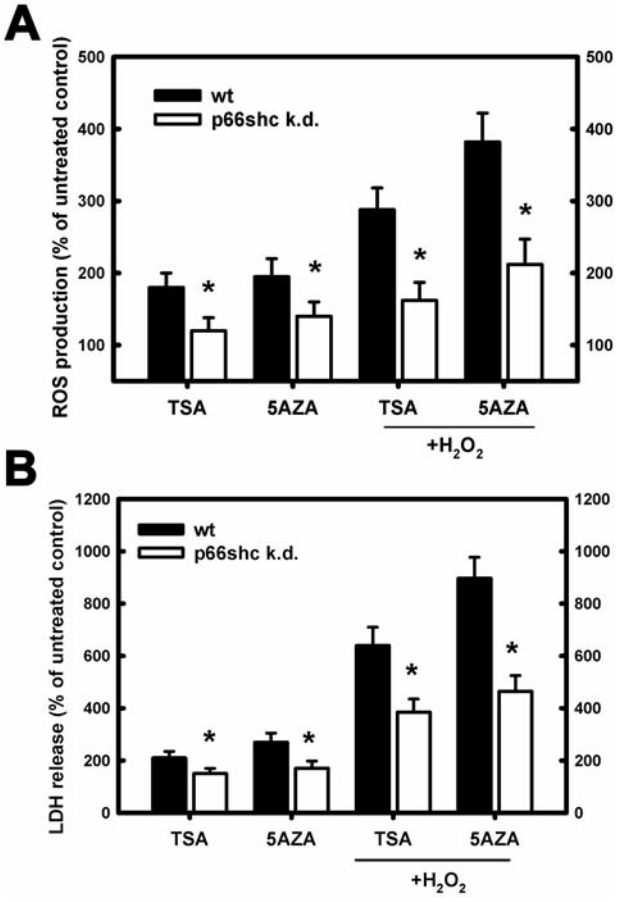

Figure 2. *Knockdown of p66shc attenuates trichostatin A (TSA) or 5-aza-cytidine (5AZA)-induced reactive oxygen species (ROS) production and cell injury. A: Wild type (wt=vector control) and p66shc knockdown (p66shc k.d.) cells were pre-treated with either 50 nM TSA or 100 nM 5AZA for 24 hours. Cells were collected and loaded with the fluorescent dye DCFDA. ROS production was determined in the presence and absence of 200 μM H_2O_2 as described in the Materials and Methods. Values are expressed as a percentage that of the untreated controls (means±S.D., n=3) *p<0.05 compared to wt control. B: Wild type (wt=vector control) and p66shc knockdown (p66shc k.d.) cells were treated with TSA or 5AZA for 24 hours as in (A) then treated with 200 μM H_2O_2 for 24 hours. Lactate dehydrogenase (LDH) release was determined as described in the Materials and Methods. Values are expressed as a percentage that of the untreated controls (means±S.D., n=3) p*<0.05 compared to wt control.*

kidney, respectively (9). 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 (9).

Ventura *et al*. reported that activity of the p66shc promoter is up regulated through epigenetic modifiers such as TSA or 5AZA (11). Our current results confirmed this observation in mouse renal proximal tubule cells: TSA and 5AZA treatments elevate protein levels of p66shc (Figure 1A and B) *via* inducing the p66shc promoter (Figure 1C). To reveal

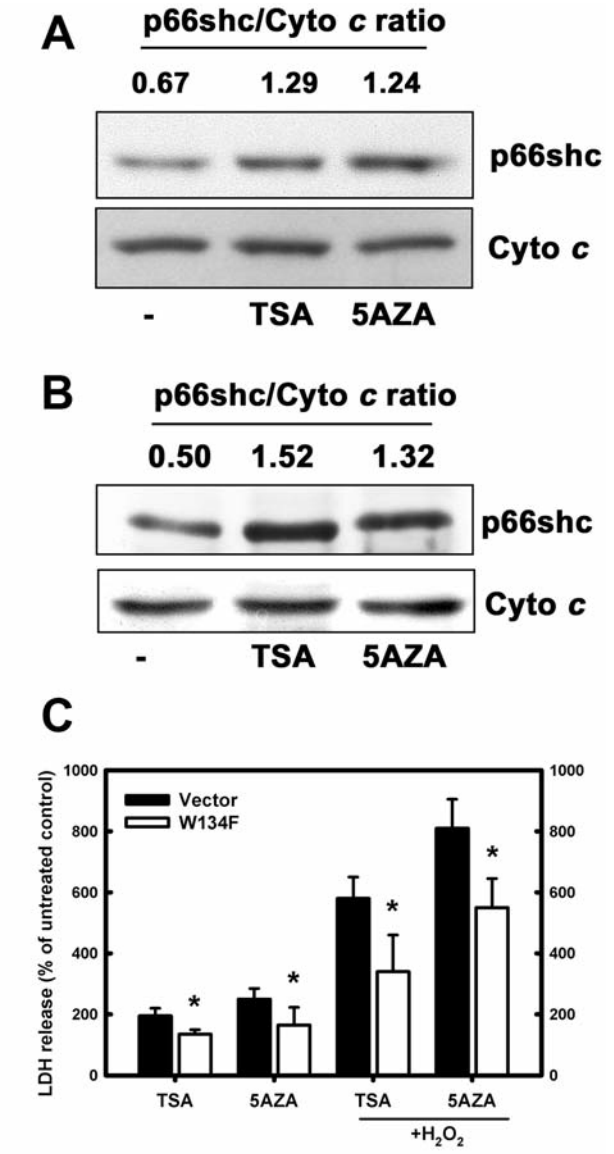

Figure 3. *Epigenetic modifiers increase mitochondrial, as well as cytochrome c, binding of p66shc. A: TKPTS cells were treated with either 50 nM TSA or 100 nM 5AZA overnight and mitochondria were then isolated. Mitochondrial lysates were subjected to SDS/PAGE and Western blotting. Blots were hybridized with an anti-p66shc antibody after stripping with an anti-cytochrome c antibody. Results shown are representative of three independent experiments. Ratios of p66shc/cytochrome c were calculated by densitometry. B: TKPTS cells were treated with either 50 nM TSA or 100 nM 5AZA overnight and cell lysates were prepared. Lysates were immunoprecipitated with an anti-cytochrome c antibody then subjected to SDS/PAGE and Western blotting. Blots were hybridized with an anti-p66shc antibody after stripping with an anti-cytochrome c antibody. Results shown are representative of three independent experiments. Ratios of p66shc/cytochrome c are calculated upon densitometry. C: TKPTS cells were transiently transfected with the W134F mutant of p66shc then pretreated with either 50 nM TSA or 100 nM 5AZA for 24 hours prior to treatment with 200 μM H_2O_2 for 24 hours. LDH release was determined as described in the Materials and Methods. Values are expressed as a percentage that of the untreated controls (means±S.D., n=3) p*<0.05 compared to the vector control.*

the mechanism of this induction needs further studies. In addition, these agents also increase mitochondrial, and within it cytochrome *c*, binding of p66shc (Figure 3A and B). We also showed that mutation of the cytochrome *c* binding site of p66shc (W134F) attenuated TSA/5AZA- as well as TSA/5AZA plus oxidative stress-induced injury (Figure 3C). Since the cytochrome *c*-bound p66shc exacerbates ROS production and consequent injury (9), the observed attenuation of LDH release in W134F mutant cells (Figure 3C) is probably due to the reduced ROS production. This is supported by the fact that knockdown of p66shc attenuated ROS production and LDH release induced by TSA and 5AZA, as well as by TSA/5AZA+H_2O_2 (Figure 2A and B).

These results may contradict previous observations that pretreatment with TSA attenuates cisplatin-induced nephrotoxicity *in vitro* (17). However, in those experiments we employed TSA for a short period of time (4 hours) as opposed to 24 hours here. It has been demonstrated by others that protective or deleterious effects of TSA are dependent on timing and/or dosage of TSA treatment (18, 19). As we showed in this project, short treatment, as opposed to that of at least 6 hours or more, with TSA did not enhance p66shc expression (Figure 1A and B). Thus, the discrepancy is most likely due to timing/induction of p66shc.

Cancer patients often demonstrate generalized elevated oxidative stress (20), which can be further exacerbated by treatment with epigenetic modifiers (5, 6) that could mean an increased risk for kidney injury. Furthermore, our results also imply that responses to oxidative stress, such as acute renal ischemia, may be more severe in cancer patients undergoing TSA or 5AZA treatment than those without treatment. We propose that this phenomenon might be due to the epigenetic modifier-induced increase in expression and consequent mitochondrial/cytochrome *c* binding of p66shc. Thus, additional means that intervene with activation of p66shc might help preventing renal toxicity of such epigenetic drugs.

Conclusion

Epigenetic modifiers such as TSA and 5AZA increase expression and mitochondrial cytochrome *c* binding of p66shc in renal proximal tubule cells. The result is increased mitochondrial ROS production and consequent injury, which exacerbates additional oxidative stress such as acute renal ischemia. Thus, renal toxicity of epigenetic modifiers can be modified by preventing renal induction of p66shc.

Acknowledgements

The Authors thank Dr. Irani (Cardiovascular Institute, University of Pittsburg) for providing the p66shc-Luc reporter plasmid. These studies were supported by an American Heart Association Midwest Affiliate Grant-in-Aid (10GRNT3790019, I.A.) and an Intramural

Research Support Program Award from the University of Mississippi Medical Center (I.A.), as well as an NIH grant (DK073401, LAJ).

References

1 Sigalotti L, Fratta E, Coral S, Cortini E, Covre A, Nicolay HJ, Anzalone L, Pezzani L, Di Giacomo AM, Fonsatti E, Colizzi F, Altomonte M, Calabro L and Maio M: Epigenetic drugs as pleiotropic agents in cancer treatment: biomolecular aspects and clinical applications. J Cell Physiol *212*: 330-344, 2007.

2 Peterson BA, Collins AJ, Vogelzang NJ and Bloomfield CD: 5-Azacytidine and renal tubular dysfunction. Blood *57*: 182-185, 1981.

3 Peterson BA, Bloomfield CD, Gottlieb AJ, Coleman M and Greenberg MS: 5-Azacytidine and zorubicin for patients with previously treated acute nonlymphocytic leukemia: a Cancer and Leukemia Group B pilot study. Cancer Treat Rep *66*: 563-566, 1982.

4 Kintzel PE: Anticancer drug-induced kidney disorders. Drug Saf *24*: 19-38, 2001.

5 Gao S, Mobley A, Miller C, Boklan J and Chandra J: Potentiation of reactive oxygen species is a marker for synergistic cytotoxicity of MS-275 and 5-azacytidine in leukemic cells. Leuk Res *32*: 771-780, 2008.

6 Xu WS, Parmigiani RB and Marks PA: Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*: 5541-5552, 2007.

7 Nadasi E, Clark JS, Szanyi I, Varjas T, Ember I, Baliga R and Arany I: Epigenetic modifiers exacerbate oxidative stress in renal proximal tubule cells. Anticancer Res *29*: 2295-2299, 2009.

8 Pellegrini M, Pacini S and Baldari CT: p66SHC: the apoptotic side of Shc proteins. Apoptosis *10*: 13-18, 2005.

9 Arany I, Faisal A, Clark JS, Vera T, Baliga R and Nagamine Y: p66SHC-mediated mitochondrial dysfunction in renal proximal tubule cells during oxidative injury. Am J Physiol Renal Physiol *298*: F1214-F1221, 2010.

10 Arany I, Faisal A, Nagamine Y and Safirstein RL: p66shc inhibits pro-survival epidermal growth factor receptor/ERK signaling during severe oxidative stress in mouse renal proximal tubule cells. J Biol Chem *283*: 6110-6117, 2008.

11 Ventura A, Luzi L, Pacini S, Baldari CT and Pelicci PG: The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. J Biol Chem *277*: 22370-22376, 2002.

12 Ernest S and Bello-Reuss E: Expression and function of P-glycoprotein in a mouse kidney cell line. Am J Physiol *269*: C323-333, 1995.

13 Arany I, Megyesi JK, Kaneto H, Tanaka S and Safirstein RL: Activation of ERK or inhibition of JNK ameliorates H_2O_2 cytotoxicity in mouse renal proximal tubule cells. Kidney Int *65*: 1231-1239, 2004.

14 Kim CS, Jung SB, Naqvi A, Hoffman TA, DeRicco J, Yamamori T, Cole MP, Jeon BH and Irani K: p53 impairs endothelium-dependent vasomotor function through transcriptional up regulation of p66shc. Circ Res *103*: 1441-1450, 2008.

15 Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F and Pelicci PG: Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell *122*: 221-233, 2005.

16 Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, Martin-Padura I, Pelliccia G, Trinei M, Bono M, Puri C, Tacchetti C, Ferrini M, Mannucci R, Nicoletti I, Lanfrancone L, Giorgio M and Pelicci PG: 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 *279*: 25689-25695, 2004.

17 Arany I, Herbert J, Herbert Z and Safirstein RL: Restoration of CREB function ameliorates cisplatin cytotoxicity in renal tubular cells. Am J Physiol Renal Physiol *294*: F577-581, 2008.

18 Dong G, Luo J, Kumar V and Dong Z: Inhibitors of histone deacetylases suppress cisplatin-induced p53 activation and apoptosis in renal tubular cells. Am J Physiol Renal Physiol *298*: F293-300, 2010.

19 Dong G, Wang L, Wang CY, Yang T, Kumar MV and Dong Z: Induction of apoptosis in renal tubular cells by histone deacetylase inhibitors, a family of anticancer agents. J Pharmacol Exp Ther *325*: 978-984, 2008.

20 Droge W: Free Radicals in the Physiological Control of Cell Function. Physiol Rev *82*: 47-95, 2002.

Received June 16, 2011
Revised August 10, 2011
Accepted August 11, 2011