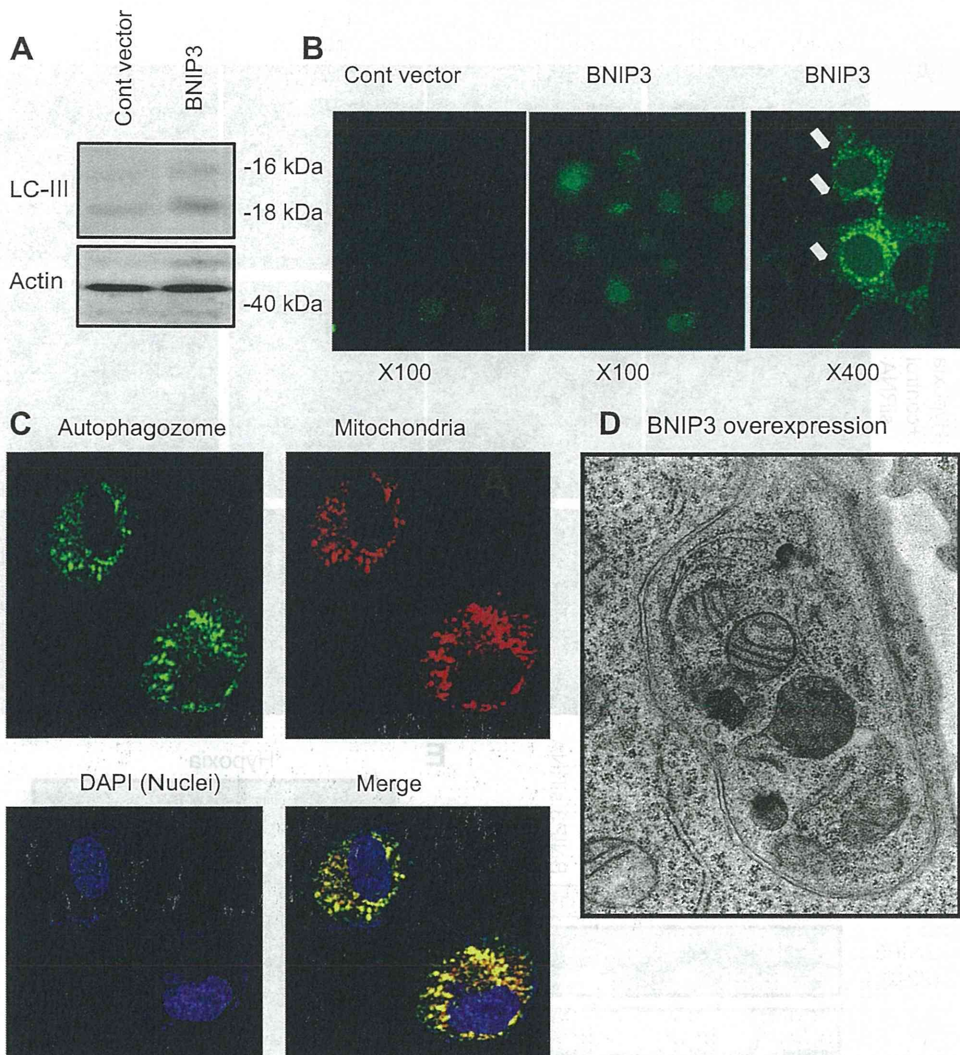


Fig. 9. Autophagy and mitophagy in NRK-LC3 cells are increased by overexpression of BNIP3. *A*: Western blot analysis of LC-3-II in control or BNIP3-overexpressing cells NRK-52E cells. *B*: confocal microscopy of GFP-positive autophagosomes in NRK-LC3 cells transfected with control or BNIP3 expression vectors. *C*: confocal microscopy of GFP and mitochondria-targeted red fluorescent protein (mitoDsRed) double-positive foci, indicative of colocalization of mitochondria and autophagosomes, in NRK-LC3 cells transfected with BNIP3 and mitoDsRed vectors. *D*: electron microscopy of BNIP3-overexpressing NRK-52E cells incubated in normoxia. Mitochondria encapsulated in autophagosomes, indicative of mitophagy, are evident.

Modulation of H_2O_2-induced apoptosis in NRK-52E cells by overexpression of sestrin-2 and BNIP3. To evaluate the relationship between sestrin-2 and apoptosis, we exposed sestrin-2-transfected NRK-52E cells to oxidative stress and analyzed the expression of cleaved caspase-3, a marker of apoptosis, by Western blot analysis. We found elevated levels of cleaved caspase-3 in NRK-52E cells exposed to 200 or 400 μM H_2O_2, but this was reduced in cells that overexpressed sestrin-2 (Fig. 11*A*). In contrast to this effect, overexpression of BNIP3 slightly augmented the level of oxidative stress-induced cleaved caspase-3 (Fig. 11*B*). To confirm these findings, we measured caspase-3 activity in NRK-52E cells that overexpressed sestrin-2 or BNIP3. As shown in Fig. 11*C*, treatment of NRK-52E cells with 200 or 400 μM H_2O_2 increased caspase-3 activity, as expected, and this increase was significantly reduced by overexpression of sestrin-2. In contrast, overexpression of BNIP3 further increased 200 μM H_2O_2-stimulated caspase-3 activity under our experimental conditions (Fig. 11*D*).

We also used TUNEL staining to evaluate apoptosis in NRK-52E cells under oxidative stress (400 μM H_2O_2) and found that apoptosis was significantly reduced by transfection with sestrin-2 overexpression and slightly reduced by BNIP3 overexpression (Fig. 12). These data are in accordance with the results of both the caspase-3 assay and Western blot analysis of cleaved caspase-3.

Modulation of hypoxia and H_2O_2-induced LDH release in NRK-52E cells by inhibition of sestrin-2 and BNIP3 using siRNA. We transfected NRK-52E cells with control siRNA or siRNA specific for BNIP3 and sestrin-2 and measured LDH release after the exposure of cells to hypoxic or oxidative stress. Our results showed that under hypoxic conditions, LDH release was significantly increased by BNIP3 siRNA and slightly, but not significantly, increased by sestrin-2 siRNA (Fig. 13*A*). On the other hand, under oxidative stress, LDH release was significantly increased by transfection with sestrin-2 siRNA (Fig. 13*B*). Moreover, in both experiments, we evaluated LDH release in the presence or absence of the autophagy inhibitor 3MA and the lysosomal inhibitor E64d/pepstatin A. 3MA and E64d/pepstatin A significantly increased the amount of LDH released under both hypoxic and oxidative stress conditions (Fig. 13, *C* and *D*). These data demonstrated that autophagy plays a protective role and increases cell viability under oxidative stress. Endogenous BNIP3 and sestrin-2 play protective roles during hypoxia and oxidative stress, respectively.

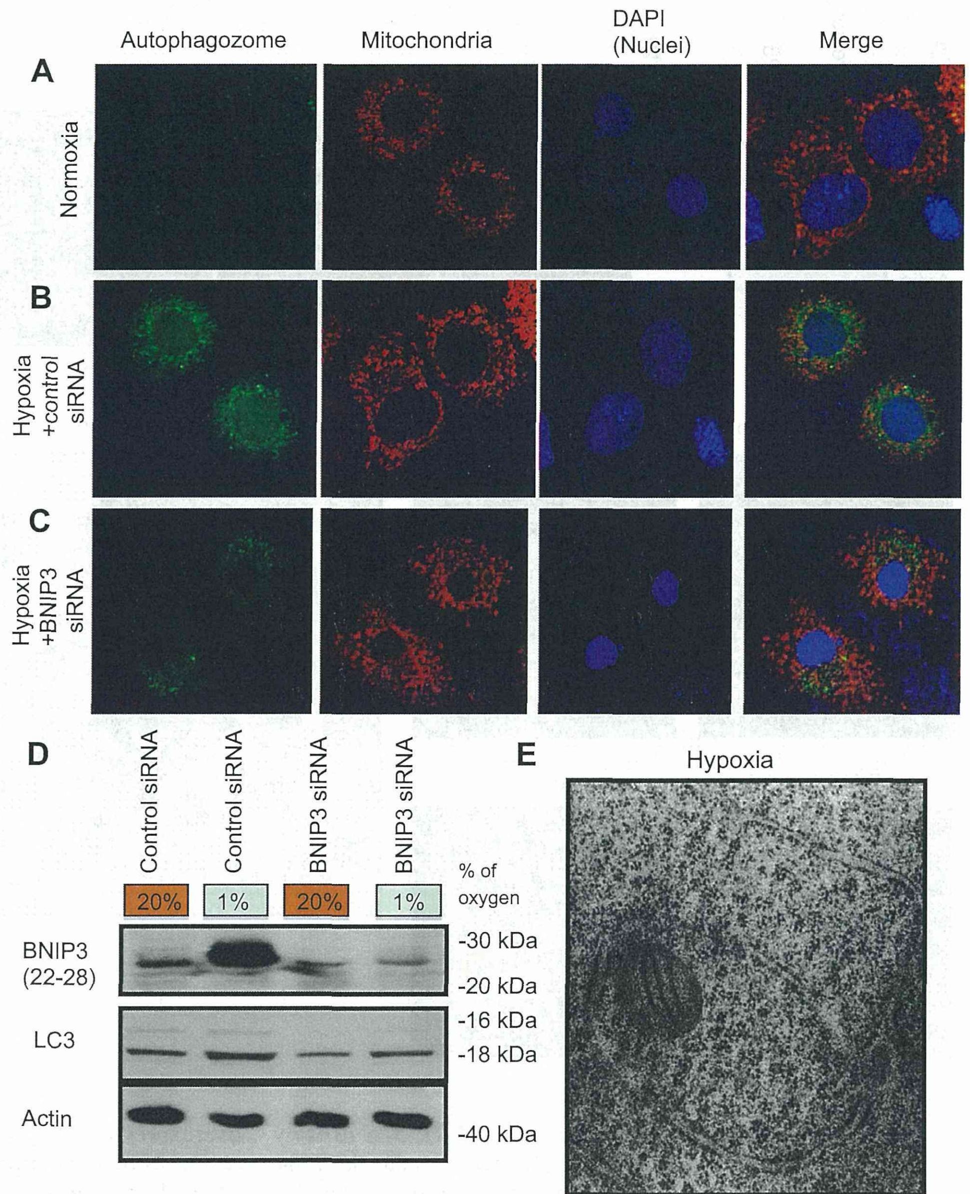


Fig. 10. Autophagy and mitophagy in NRK-LC3 cells are suppressed by BNIP3 siRNA. *A–C*: reduction in hypoxia-induced GFP-positive autophagosomes in BNIP3 siRNA-transfected NRK-LC3 cells. *A*: normoxia. *B*: hypoxia with control siRNA transfection. *C*: hypoxia with BNIP3 siRNA transfection. *D*: Western blot analysis of LC-3-II in control or BNIP3 siRNA-transfected NRK-52E cells. *E*: electron microscopy of NRK-52E cells incubated under hypoxic conditions. Mitochondria encapsulated in autophagosomes, indicative of mitophagy, are evident.

DISCUSSION

In this study, we demonstrated that autophagy is induced in renal tubules during AKI by at least two independent pathways: p53-sestrin-2 and HIF-1α-BNIP3 pathways. Mitophagy is also observed in renal tubular cells that overexpress BNIP3 and in wild-type cells under hypoxic conditions. Our findings therefore suggest that the two autophagy-promoting pathways may be induced under different stress conditions to protect renal tubules in AKI.

This is the first study to demonstrate that autophagy and mitophagy are induced, at least partially, by two signaling pathways in renal tubular cells after oxidative stress. We (20) previously using GFP-LC3 transgenic mice to investigate autophagy in kidney tissues during cisplatin nephrotoxicity and demonstrated that autophagy mainly occurred in the proximal tubules. Despite some controversies, most pharmacological, genetic, and knockout studies have supported a renoprotective role for autophagy in renal tubular cells in AKI. Several reports (37, 47) have suggested that autophagy is induced as part of an adaptive response that suppresses apoptosis and prolongs survival of renal tubular epithelial cells. Consistent with this, renal I/R injury is exacerbated by inhibition of autophagy by chemical inhibitors or conditional gene knockout. The authors of this study concluded that autophagy is a protective mechanism for cell survival (21, 22). A number of groups (17, 25, 35) have recently demonstrated a close connection between autophagy and mitochondrial turnover. Mitochondrial quality control is the process whereby mitochondria undergo successive rounds of fusion and fission with a dynamic exchange of components to segregate functional and damaged elements (16). Removal of a damaged mitochondrion is accomplished via mitophagy (12). Mitophagy also serves to eliminate the subset of mitochondria producing the most ROS, and episodic removal of mitochondria reduces the oxidative burden (25, 27, 44). However, the precise signal transduction pathways that induce autophagy and mitophagy in AKI remain unclear.

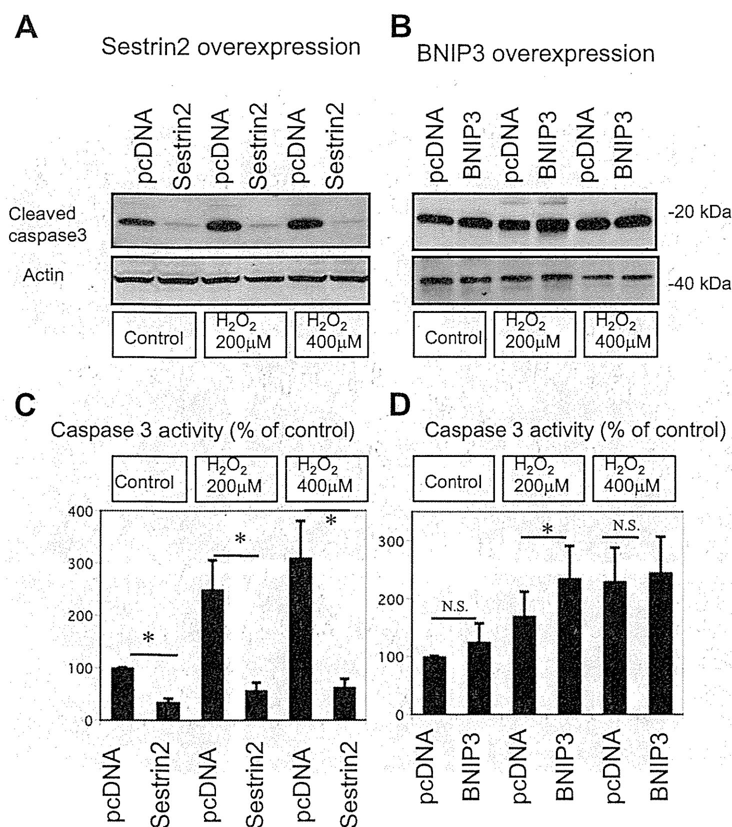
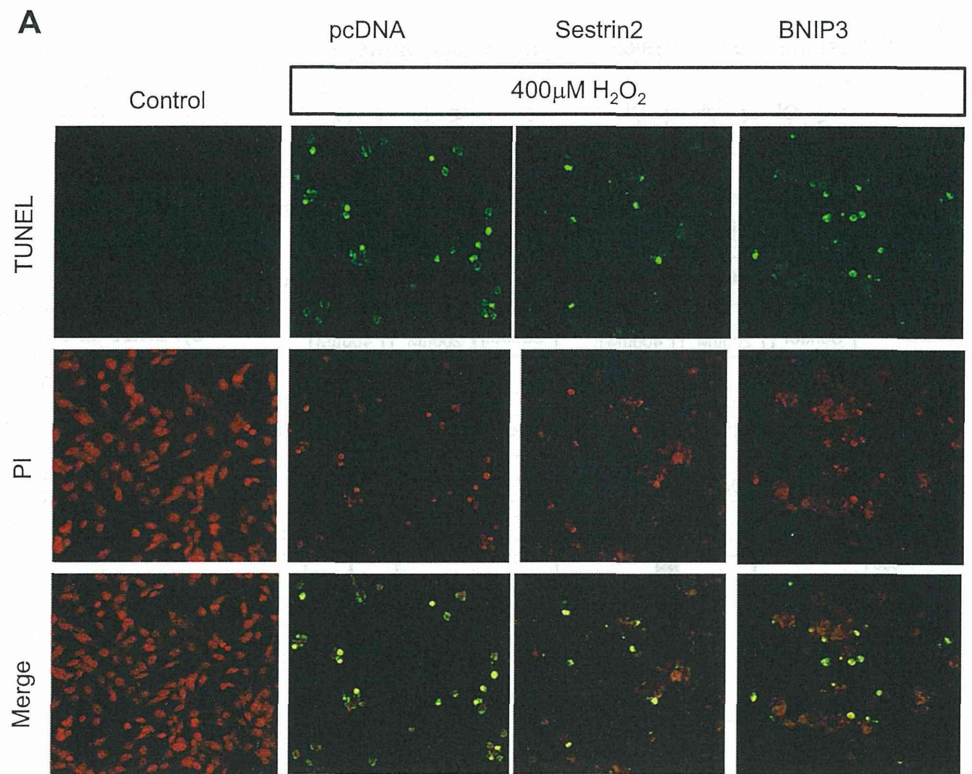


Fig. 11. Apoptosis is reduced by sestrin-2 overexpression and increased by BNIP3 in NRK-52E cells. *A*: Western blot analysis of cleaved caspase-3 after incubation of control or sestrin-2-overexpressing NRK-52E cells with 200 or 400 μM H_2O_2. B: BNIP3-overexpressing NRK-52E cells exhibited a slightly augmented H_2O_2-induced increase in cleaved caspase-3. *C*: attenuation of the H_2O_2-induced increase in cleaved caspase-3 in sestrin-2-overexpressing NRK-52E cells. *D*: slight increment of H_2O_2-induced caspase3 activity was observed at 200 μM H_2O_2 in NRK-52E cells that overexpressed BNIP3. Data are means ± SE; $n = 6$. NS, not significant. $^*P < 0.05$ vs. pcDNA-transfected cells.

For our study, we analyzed sestrin-2 and BNIP3 mRNA and protein levels and detected striking changes in sestrin-2 and BNIP3 expression after I/R under our experimental conditions. In vitro expression of sestrin-2 and BNIP3 proteins was also dramatically affected by H_2O_2 and hypoxia, respectively. We further noted that overexpression of BNIP3 caused mitophagy in NRK-LC3 cells, as visualized by confocal microscopy and electron microscopy. Mitochondria loaded with a fluorescent marker partially colocalized with LC3-positive autophagosomes under hypoxic conditions and in cells that overexpressed BNIP3. Furthermore, mitochondria encapsulated in autophagosomes were observed by electron microscopy under the same conditions. To the best of our knowledge, this is the first study to demonstrate mitophagy in renal tubular cells. Several recent studies (27, 33, 36) have suggested that mitochondrial clearance in many cell types, including reticulocytes, is partly dependent on autophagy. This concept first emerged from studies with cells and mice deficient in Nix (member of the BNIP3 family and known as BNIP3L) that is present on the mitochondrial outer membrane (10, 29). Nix seems to function selectively in mitochondrial clearance during erythroid differentiation (29). One proposed mechanism for the selective degradation of mitochondria is through BNIP3 family-mediated mitochondrial recognition (23). The results of our present study are in accordance with these observations in reticulocytes (27, 29, 33, 36). Further studies are required to more precisely delineate the role of mitophagy and its molecular mechanisms in the pathophysiology of AKI.

We examined the regulation and functional roles of sestrin-2 in AKI in vitro and in vivo. Sestrin-2 was identified in 2002 as the hypoxia response gene Hi95 (hypoxia-induced gene no. 95) in a human glioma cell line (3). Sestrin-2 is regulated by both p53 and HIF-1α (2). A study (2) of sestrin-2 knockout mice has revealed the critical role of sestrin-2 in p53 and mammalial target of rapamycin (mTOR) signaling (2). Accumulation of ROS leads to p53 activation and transactivation of various p53 targets (32). Recently, several mechanisms have been reported that connect p53 and autophagy, including activation of sestrin-2, transactivation of 5′-AMP-activated protein kinase (AMPK), and activation of DRAM1 (8, 15, 19). We examined the regulation of sestrin-2 and DRAM1 mRNA expression in our in vivo experiments. We found that sestrin-2 transcription was markedly upregulated in the rat I/R AKI model, whereas there was no significant change in DRAM1 expression under our experimental conditions. Recent studies (2, 34) have revealed that sestrin-2 interacts with AMPK to regulate target of rapamycin complex activity (2, 34). Thus, we focused on sestrin-2 as a p53-related gene that may induce autophagy in AKI. We also examined autophagy induced by overexpression of sestrin-2 in NRK-LC3 cells using confocal microscopy and electron microscopy. LC3-positive autophagosomes were observed in NRK-LC3 cells exposed to hypoxia and in cells that overexpressed sestrin-2. The formation of LC3-positive autophagosomes in response to hypoxia was partially inhibited by siRNA-mediated silencing of sestrin-2. To the best of our knowledge, this is the first demonstration of sestrin-2-induced autophagy in renal tubular cells. Furthermore, we demonstrated that sestrin-2-induced autophagy is at least partially mediated by p53. Thus, the p53-sestrin-2 pathway signals for oxidative stress-induced autophagy in renal tubular cells. We showed that apoptosis of NRK-52E cells incubated under hypoxic conditions was significantly reduced by overexpression of



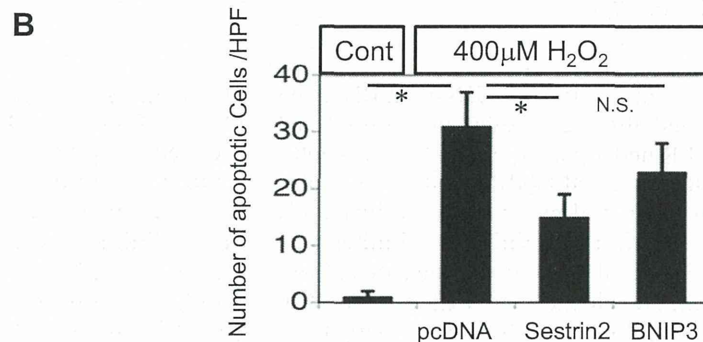


Fig. 12. TUNEL assay to evaluate apoptosis in NRK-52E cells exposed to oxidative stress. *A*: TUNEL assay to evaluate apoptosis (green) in NRK-52E cells exposed to 400 μM H_2O_2 to induce oxidative stress. Nuclei were stained with propidium iodide (PI; red). The number of apoptotic cells was reduced by transfection with the sestrin-2 expression vector and, to a lesser extent, with the BNIP3 expression vector. *B*: quantitative analysis demonstrating that, under our experimental conditions of oxidative stress, the number of apoptotic cells was significantly reduced by transfection with the sestrin-2 expression vector and modestly but not significantly reduced by transfection with the BNIP3 expression vector. Data are means ± SE; $n = 6$. *$P < 0.05$ vs. pcDNA-transfected cells.

sestrin-2 by caspase-3 activity and TUNEL assay. Our recent report (20) demonstrated that autophagy occurs before apoptosis in renal tubular cells during AKI. However, the role played by autophagy under apoptotic conditions remains controversial. Recently, Yang et al. (47) and Periyasamy-Thandavan et al. (30) reported that inhibition of cisplatin-induced autophagy enhanced apoptosis. Their results suggested a protective role for autophagy in cisplatin-induced tubular cell injury (30). In contrast, several studies (7, 45) have also demonstrated that autophagy may contribute to tubular cell death during AKI, suggesting that some discrepancies exist in previous reports examining the relationship between autophagy and apoptosis. In the present study, we demonstrated that the two autophagy-promoting pathways have different effects on apoptosis. There may be cross-talk between the p53-sestrin-2 and HIF-1α-BNIP3 signaling pathways. H_2O_2 induces autophagic cell death via BNIP3-mediated suppression of the mTOR pathway in glioma cells (4) and activates BNIP3 through HIF-1α in neural cells (49). In macrophages, hypoxia upregulates sestrin-2 through HIF-1α and other mechanisms (14). These reports therefore suggest that cross-talk between the H_2O_2-p53-sestrin-2 and hypoxia-HIF-1α-BNIP3 pathways may vary depending on the cell type and experimental conditions. Thus, it is difficult to clearly identify the differences between hypoxia- and oxidative stress-induced signaling in our experiments.

The COOH-terminal domain of BNIP3 is known to be critical for mitochondrial targeting and the proapoptotic function of BNIP3 in cardiac myocytes (31). Our results showing that overexpression of BNIP3 increased apoptosis is in accordance with these previous reports. Therefore, our findings suggest that the two autophagy-promoting signaling pathways may regulate apoptosis in different ways. Our results suggest a complex interaction between autophagy and apoptosis, with the two autophagy-related proteins, sestrin-2 and BNIP3, playing different roles in apoptosis regulation. Recent reports have also suggested that the interaction between autophagy and apoptosis is not straightforward. First, the role of mTOR, the key molecule of autophagy, in apoptosis is controversial. In various models, mTOR inhibition can either sensitize cells to

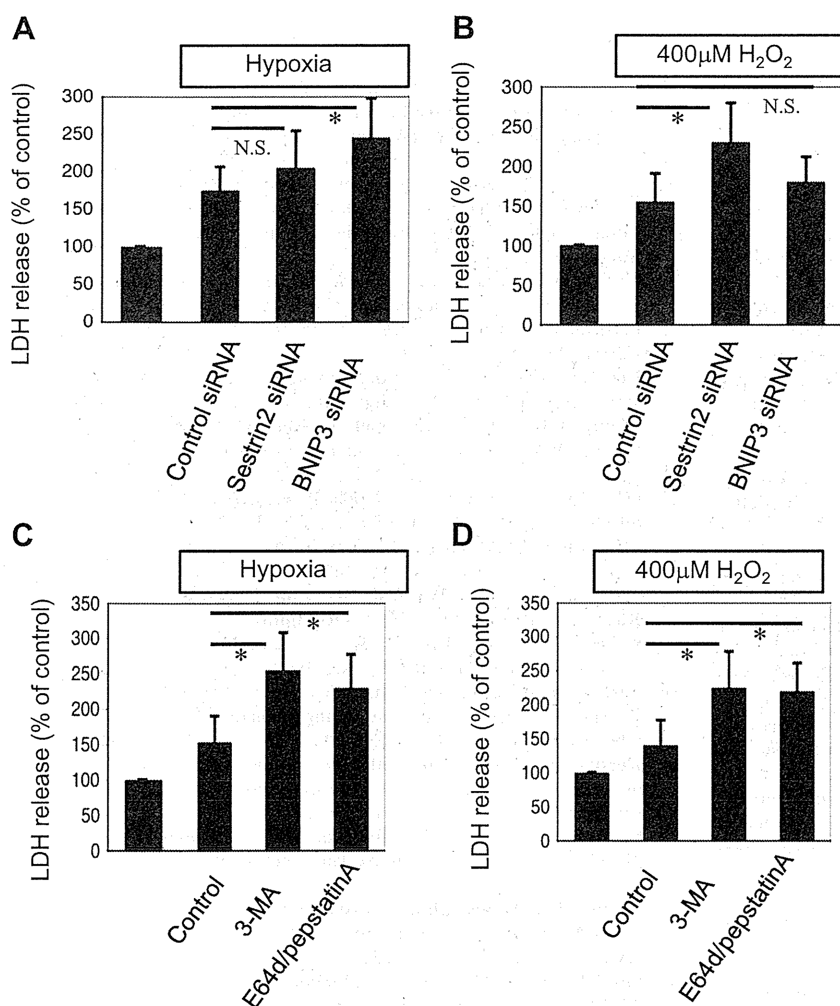


Fig. 13. Modulation of H_2O_2-induced LDH release in NRK-52E cells by siRNA-mediated knockdown of sestrin-2 and BNIP3. *A*: results of the LDH release assay to evaluate the viability of NRK-52E cells exposed to hypoxia for 2 h. LDH release was increased by transfection with BNIP3 siRNA compared with control siRNA. *B*: results of the LDH release assay to evaluate the viability of NRK-52E cells incubated with 400 μM H_2O_2. LDH release was increased by transfection with sestrin-2 siRNA compared with control siRNA. Data are means ± SE; $n = 6$. $^*P < 0.05$ vs. control siRNA-transfected cells. *C*: viability of NRK-52E cells exposed to hypoxia for 2 h in the presence or absence of the autophagy inhibitor 3MA and lysosomal inhibitor E64d/pepstatin A. *D*: viability of NRK-52E cells exposed to 400 μM H_2O_2 for 2 h in the presence or absence of 3MA and E64d/pepstatin A. 3MA and E64d/pepstatin A significantly increased LDH release under both hypoxic and oxidative conditions (*C* and *D*). Data are means ± SE; $n = 6$. $^*P < 0.05$ vs. control cells.

apoptosis (5, 18) or reduce apoptosis (46). Second, the role of sestrin-2 as a pro- or antiapoptotic protein is also controversial. Sestrin-2 expression inhibits cell growth and proliferation in response to genotoxic stress (2, 3) and protects MEF-7 cells against ischemia, low glucose, and H_2O_2 (2, 3). There are several reports concerning BNIP3, apoptosis, and cell death. In cardiomyocytes, localization of BNIP3 to the mitochondria causes cytochrome *c* release, which results in caspase activation and subsequent apoptosis (27). In neurons, BNIP3 has been implicated in the release of endonuclease G, but not cytochrome *c*, from the mitochondria (28), which results in caspase-independent cell death. In epithelium-derived cells, overexpression of BNIP3 fails to induce the release of mitochondrial proteins and fails to activate caspases (29, 30). The mechanisms that regulate BNIP3 at the mitochondria are not well understood; however, several lines of evidence give some insight into how BNIP3 might regulate mitochondrial function. In murine fibroblasts lacking Bax and Bak, BNIP3 fails to induce mitochondrial dysfunction and cell death (31). Thus, our data showing that the two autophagy-promoting signaling pathways regulate apoptosis in different ways may be a consequence of differing cell types or experimental conditions. Thus, our data showing that the two autophagy-promoting signaling pathways regulate apoptosis in different ways may be due to cell types or experimental conditions. Further research is needed to gain insights into the molecular mechanisms that connect autophagy and apoptosis in AKI.

In summary, our study produced two novel findings. First, sestrin-2 and BNIP3 are upregulated in proximal tubular cells during I/R AKI in vivo. Second, autophagy and mitophagy are induced in renal tubules in AKI by at least two independent pathways, the p53-sestrin-2 and HIF-1α-BNIP3 pathways, which may regulate autophagy and mitophagy, respectively. Further studies are necessary to gain a more precise understanding of the molecular mechanisms that protect renal cells against oxidative stress after I/R injury.

ACKNOWLEDGMENTS

The authors thank Reiko Matsumoto, Sekie Saito, and Akiko Takano for technical assistance.

GRANTS

This work was supported by the Kidney Foundation of Japan and the Kochi Organization for Medical Reformation and Renewal (to Y. Terada) as well as a grant from the Ministry of Education, Science, Culture and Sports of Japan (to Y. Terada, Y. Shimamura, K. Inoue, and S. Fujimoto).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.I. and Y. Terada conception and design of research; M.I., K.I., and Y. Taniguchi performed experiments; M.I., M.U., and Y.S. analyzed data; M.I., T.M., K.O., and T.H. prepared figures; K.H., T.H., M.F., S.F., and Y. Terada interpreted results of experiments; S.F. drafted manuscript.

REFERENCES

1. **Bonventre JV.** Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure. *J Am Soc Nephrol* 14: S55–S61, 2003.
2. **Budanov AV, Karin M.** p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* 134: 451–460, 2008.
3. **Budanov AV, Shoshani T, Faerman A, Zelin E, Kamer I, Kalinski H, Gorodin S, Fishman A, Chajut A, Einat P, Skaliter R, Gudkov AV, Chumakov PM, Feinstein E.** Identification of a novel stress-responsive gene Hi95 involved in regulation of cell viability. *Oncogene* 21: 6017–6031, 2002.
4. **Byun YJ, Kim SK, Kim YM, Chae GT, Jeong SW, Lee SB.** Hydrogen peroxide induces autophagic cell death in C6 glioma cells via BNIP3-mediated suppression of the mTOR pathway. *Neurosci Lett* 461: 131–135, 2009.
5. **Castedo M, Ferri KF, Kroemer G.** Mammalian target of rapamycin (mTOR): pro-and anti-apoptotic. *Cell Death Differ* 9: 99–100, 2002.
6. **Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, Saxena S, Gietz RD, Greenberg AH.** The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* 186: 1975–1983, 1997.
7. **Chien CT, Shyue SK, Lai MK.** Bcl-x_L augmentation potentially reduces ischemia/reperfusion induced proximal and distal tubular apoptosis and autophagy. *Transplantation* 84: 1183–1190, 2007.
8. **Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM.** DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* 126: 121–134, 2006.
9. **Devarajan P.** Update on mechanisms of ischemic acute kidney injury. *J Am Soc Nephrol* 17: 1503–1520, 2006.
10. **Diwan A, Koesters AG, Odley AM, Pushkaran S, Baines CP, Spike BT, Daria D, Jegga AG, Geiger H, Aronow BJ, Molkentin JD, Macleod KF, Kalfa TA, Dorn 2nd G.** Unrestrained erythroblast development in Nix−/− mice reveals a mechanism for apoptotic modulation of erythropoiesis. *Proc Natl Acad Sci USA* 104: 6794–6799, 2007.
11. **Diwan A, Krenz M, Syed FM, Wansapura J, Ren X, Koesters AG, Li H, Kirshenbaum LA, Hahn HS, Robbins J, Jones WK, Dorn GW.** Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. *J Clin Invest* 117: 2825–2833, 2007.
12. **Dutta D, Calvani R, Bernabei R, Leeuwenburgh C, Marzetti E.** Contribution of impaired mitochondrial autophagy to cardiac aging mechanisms and therapeutic opportunities. *Circ Res* 110: 1125–1138, 2012.
13. **Elmore SP, Qian T, Grissom SF, Lemasters JJ.** The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 15: 2286–2287, 2001.
14. **Essler S, Dehne N, Brüne B.** Role of sestrin2 in peroxide signaling in macrophages. *FEBS Lett* 583: 3531–3535, 2009.
15. **Feng Z, Zhang H, Levine AJ, Jin S.** The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci USA* 102: 8204–8209, 2005.
16. **Fischer F, Hamann A, Osiewacz HD.** Mitochondrial quality control: an integrated network of pathways. *Trends Biochem Sci* 37: 284–292, 2012.
17. **Gottlieb RA, Carreira RS.** Autophagy in health and disease. Mytophagy as a way of life. *Am J Physiol Cell Physiol* 299: C203–C210, 2010.
18. **Gu L, Zhou C, Liu H, Gao J, Li Q, Mu D, Ma Z.** Rapamycin sensitizes T-ALL cells to dexamethasone-induced apoptosis. *J Exp Clin Cancer Res* 29: 150, 2010.
19. **Huber TB, Edelstein CL, Hartleben B, Inoki K, Jiang M, Koya D, Kume S, Lieberthal W, Pallet N, Quiroga A, Ravichandran K, Susztak K, Yoshida S, Dong Z.** Emerging role of autophagy in kidney function, diseases and aging. *Autophagy* 8: 1–12, 2012.
20. **Inoue K, Kuwana H, Shimamura Y, Ogata K, Taniguchi Y, Kagawa T, Horino T, Takao T, Morita T, Sasaki S, Mizushima N, Terada Y.** Cisplatin-induced macroautophagy occurs prior to apoptosis in proximal tubules in vivo. *Clin Exp Nephrol* 14: 112–122, 2010.
21. **Jiang M, Liu K, Luo J, Dong Z.** Autophagy is a renoprotective mechanism during in vitro hypoxia and in vivo ischemia-reperfusion injury. *Am J Pathol* 176: 1181–1192, 2010.
22. **Kimura T, Takabatake Y, Takahashi A, Kaimori J, Matsui I, Namba T, Kitamura H, Niimura F, Matsusaka T, Soga T, Rakugi H, Isaka Y.** Autophagy protects the proximal tubule from degeneration and acute ischemic injury. *J Am Soc Nephrol* 22: 902–913, 2011.
23. **Kobayashi T, Terada Y, Kuwana H, Tanaka H, Okado T, Kuwahara M, Tohda S, Sakano S, Sasaki S.** Expression and function of the Delta-1/Notch-2/Hes-1 pathway during experimental acute kidney injury. *Kidney Int* 73: 1240–1250, 2008.
24. **Kubli DA, Quinsay MN, Huang C, Lee Y, Gustafsson AB.** Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 295: H2025–H2031, 2008.
25. **Lee J, Giordano S, Zhang J.** Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem J* 441: 523–540, 2012.
26. **Maiuri MC, Malik SA, Morselli E, Kepp O, Criollo A, Mouchel PL, Carnuccio R, Kroemer G.** Stimulation of autophagy by the p53 target gene sestrin2. *Cell Cycle* 8: 1571–1576, 2009.
27. **Mizushima N, Levine B.** Autophagy in mammalian development and differentiation. *Nature Cell Biol* 12: 823–830, 2010.
28. **Mizushima N, Yoshimori T, Levine B.** Methods in mammalian autophagy research. *Cell* 140: 313–326, 2010.
29. **Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, Rogov V, Löhr F, Popovic D, Occhipinti A, Reichert AS, Terzic J, Dötsch V, Ney PA, Dikic I.** Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep* 11: 45–51, 2009.
30. **Periyasamy-Thandavan S, Jiang M, Wei Q, Smith R, Yin XM, Dong Z.** Autophagy is cytoprotective during cisplatin injury of renal proximal tubular cells. *Kidney Int* 74: 631–640, 2008.
31. **Regula KM, Ens K, Kirshenbaum LA.** Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res* 91: 226–231, 2002.
32. **Sablina AA, Budanov AV, Ilyinskaya GV, Agapova LS, Kravchenko JE, Chumakov PM.** The antioxidant function of the p53 tumor suppressor. *Nat Med* 11: 1306–1313, 2005.
33. **Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, Wang J.** Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 454: 232–235, 2008.
34. **Sanli T, Linher-Melville K, Tsakiridis T, Singh G.** Sestrin2 modulates AMPK subunit expression and its response to ionizing radiation in breast cancer cells. *PloS One* 7: e32035, 2012.
35. **Sansanwal P, Yen B, Gahl WA, Ma Y, Ying L, Wong LJ, Sarwal MM.** Mitochondrial autophagy promotes cellular injury in nephropathic cystinosis. *J Am Soc Nephrol* 21: 272–283, 2010.
36. **Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, Kundu M, Opferman JT, Cleveland JL, Miller JL, Ney PA.** NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci USA* 104: 19500–19505, 2007.
37. **Suzuki C, Isaka Y, Takabatake Y, Tanaka H, Koike M, Shibata M, Uchiyama Y, Takahara S, Imai E.** Participation of autophagy in renal ischemia/reperfusion injury. *Biochem Biophys Res Commun* 368: 100–106, 2008.
38. **Tanaka H, Terada Y, Kobayashi T, Okado T, Inoshita S, Kuwahara M, Seth A, Sato Y, Sasaki S.** Expression and function of Ets-1 during experimental acute renal failure in rats. *J Am Soc Nephrol* 15: 3083–3092, 2004.
39. **Terada Y, Inoshita S, Hanada S, Shimamura H, Kuwahara M, Ogawa W, Kasuga M, Sasaki S, Marumo F.** Hyperosmolality activates Akt and regulates apoptosis in renal tubular cells. *Kidney Int* 60: 553–567, 2001.
40. **Terada Y, Tanaka H, Okado T, Shimamura H, Inoshita S, Kuwahara M, Sasaki S.** Expression and function of the developmental gene Wnt-4 during experimental acute renal failure in rats. *J Am Soc Nephrol* 14: 1223–1233, 2003.
41. **Terada Y, Tomita K, Homma MK, Nonoguchi H, Yang T, Yamada T, Yuasa Y, Krebs EG, Sasaki S, Marumo F.** Sequential activation of Raf-1 kinase, mitogen-activated protein (MAP) kinase kinase, MAP kinase, and S6 kinase by hyperosmolality in renal cells. *J Biol Chem* 269: 31296–31301, 1994.
42. **Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, Shirihai OS.** Fission and selective fusion govern

mitochondrial segregation and elimination by autophagy. *EMBO J* 27: 433–446, 2008.
43. **Wallin A, Zhang G, Jones TW, Jaken S, Stevens JL.** Mechanism of the nephrogenic repair response. Studies on proliferation and vimentin expression after 35*S*-1,2-dichlorovinyl-L-cysteine nephrotoxicity in vivo and in cultured proximal tubule epithelial cells. *Lab Invest* 66: 474–484, 1992.
44. **Wang K, Klionsky DJ.** Mitochondria removal by autophagy. *Autophagy* 7: 297–300, 2011.
45. **Wu HH, Hsiao TY, Chien CT, Lai MK.** Ischemic conditioning by short periods of reperfusion attenuates renal ischemia/reperfusion induced apoptosis and autophagy in the rat. *J Biomed Sci* 16: 19–22, 2009.
46. **Xi H, Kurtoglu M, Liu H, Wangpaichitr M, You M, Liu X, Savaraj N, Lampidis TJ.** 2-Deoxy-D-glucose activates autophagy via endoplasmic reticulum stress rather than ATP depletion. *Cancer Chemother Pharmacol* 67: 899–910, 2011.
47. **Yang C, Kaushal V, Shah SV, Kaushal GP.** Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells. *Am J Physiol Renal Physiol* 294: F777–F787, 2008.
48. **Yasuda M, Theodorakis P, Subramanian T, Chinnadurai G.** Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence. *J Biol Chem* 273: 12415–12421, 1998.
49. **Zhang S, Zhang Z, Sandhu G, Ma X, Yang X, Geiger J, Kong J.** Evidence of oxidative stress-induced BNIP3 expression in amyloid beta neurotoxicity. *Brain Res* 1138: 221–230, 2007.

5-Aminolevulinic Acid Protects against Cisplatin-Induced Nephrotoxicity without Compromising the Anticancer Efficiency of Cisplatin in Rats In Vitro and In Vivo

Yoshio Terada[1*], Keiji Inoue[2], Tatsuki Matsumoto[1], Masayuki Ishihara[1], Kazu Hamada[1], Yoshiko Shimamura[1], Koji Ogata[1], Kosuke Inoue[1], Yoshinori Taniguchi[1], Taro Horino[1], Takashi Karashima[2], Kenji Tamura[2], Hideo Fukuhara[2], Shimpei Fujimoto[1], Masayuki Tsuda[3], Taro Shuin[2]

1 Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Japan, **2** Department of Urology, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Japan, **3** Institute for Laboratory Animal Research, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku, Japan

Abstract

Background/Aims: Nephrotoxicity is a frequent and major limitation in cisplatin (CDDP)-based chemotherapy. 5-Aminolevulinic acid (ALA) is widely distributed in animal cells, and it is a precursor of tetrapyrole compounds such as heme that is fundamentally important in aerobic energy metabolism. The aim of this study is to evaluate the protective role of ALA in CDDP-induced acute kidney injury (AKI).

Method: We used CDDP-induced AKI rat model and cultured renal tubular cells (NRK-52E). We divided four groups of rats: control, CDDP only, CDDP + ALA(post);(ALA 10 mg/kg + Fe in drinking water) after CDDP, CDDP + ALA(pre & post).

Result: CDDP increased Cr up to 6.5 mg/dl, BUN up to 230 mg/dl, and ALA significantly reduced these changes. ALA ameliorates CDDP-induced morphological renal damages, and reduced tubular apoptosis evaluated by TUNEL staining and cleaved caspase 3. Protein and mRNA levels of ATP5α, complex(COX) IV, UCP2, PGC-1α in renal tissue were significantly decreased by CDDP, and ALA ameliorates reduction of these enzymes. In contrast, Heme Oxigenase (HO)-1 level is induced by CDDP treatment, and ALA treatment further up-regulates HO-1 levels. In NRK-52E cells, the CDDP-induced reduction of protein and mRNA levels of mitochondrial enzymes was significantly recovered by ALA + Fe. CDDP-induced apoptosis were ameliorated by ALA + Fe treatment. Furthermore, we evaluated the size of transplantated bladder carcinoma to the rat skin, and ALA did not change the anti cancer effects of CDDP.

Conclusion: These data suggested that the protective role of ALA in cisplatin-induced AKI is via protection of mitochondrial viability and prevents tubular apoptosis. Also there are no significant effects of ALA on anticancer efficiency of CDDP in rats. Thus, ALA has the potential to prevent CDDP nephrotoxicity without compromising its anticancer efficacy.

Citation: Terada Y, Inoue K, Matsumoto T, Ishihara M, Hamada K, et al. (2013) 5-Aminolevulinic Acid Protects against Cisplatin-Induced Nephrotoxicity without Compromising the Anticancer Efficiency of Cisplatin in Rats In Vitro and In Vivo. PLoS ONE 8(12): e80850. doi:10.1371/journal.pone.0080850

Editor: Jeff M. Sands, Emory University, United States of America

Received August 7, 2013; **Accepted** October 13, 2013; **Published** December 6, 2013

Copyright: © 2013 Terada et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by The Kidney Foundation Japan and the Kochi organization for medical reformation and renewal (to YT); grants from the Ministry of Education, Science, Culture and Sports of Japan (to YT, KI, YS, KI, SF, TS); and a grant-in-aid for Progressive Renal Diseases Research, Research on Rare and Intractable Disease, from the Ministry of Health, Labour and Welfare of Japan (to YT). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: terada@kochi-u.ac.jp

These authors contributed equally to this work.

Introduction

Cisplatin is one of the most effective and potent anticancer drugs in the treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder, and testicular cancers [1]. The major constraint to cisplatin-based chemotherapy is the frequent development of nephrotoxicity [2]. The antineoplastic effect of cisplatin is dose dependent, yet the risk of nephrotoxicity often precludes the use of higher doses to maximize the therapeutic effect. Cisplatin induces apoptosis of renal proximal tubule cells (LLC-PK1) in vitro by means of mitochondria-dependent and -independent pathways [3], partly through the activation of caspase-3 [4]. Oxidant stress also appears to contribute to the cisplatin-induced apoptosis of renal tubular cells, both in vitro and in vivo [5]. Several studies, including ours, suggest that caspase inhibitors and knockout of apoptosis-related genes attenuate cisplatin-induced acute kidney injury (AKI) in rats [6,7]. Mitochondria have a variety of important intracellular functions, including ATP production, synthesis of reactive oxygen species, and regulation of the cell death pathway. Recent studies, including ours, have demonstrated that mitochondrial function is one of the key factors protecting cells from oxidative stress in AKI [8,9].

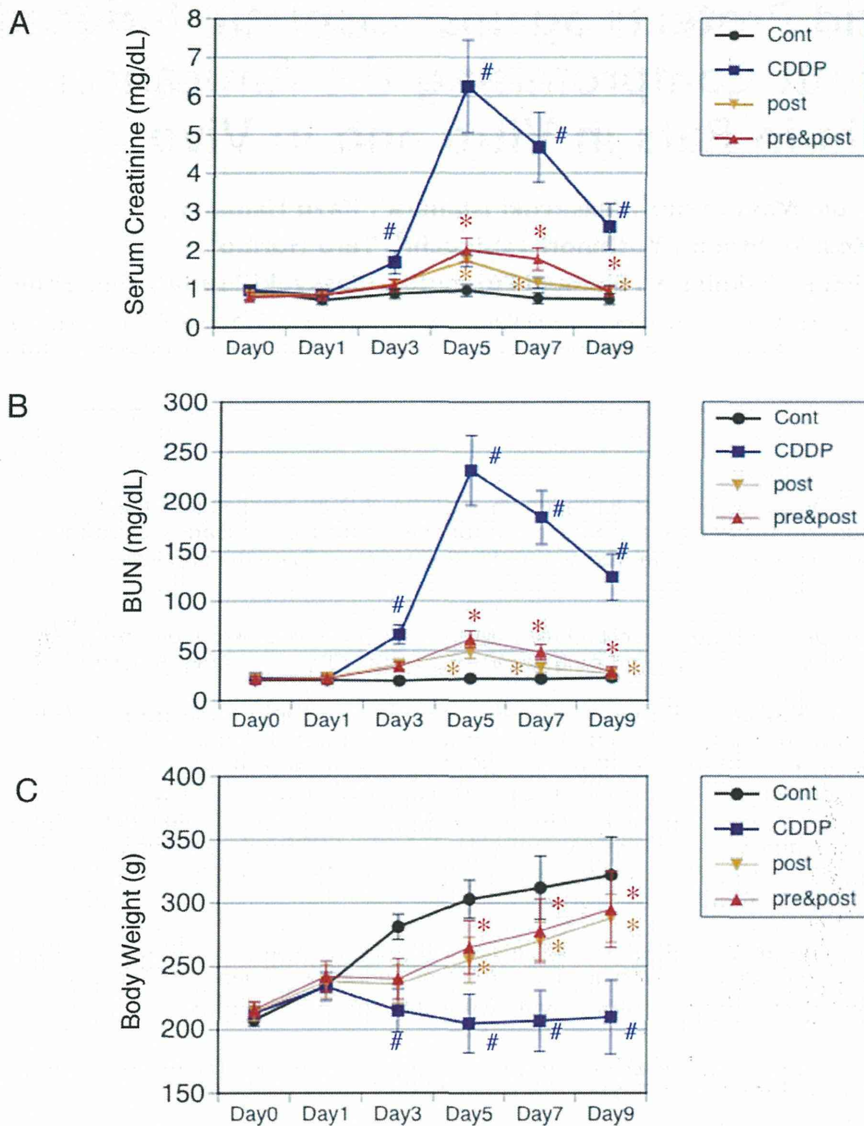


Figure 1. Blood urea nitrogen (BUN) and serum creatinine (Cre) levels in ALA treated rats after cisplatin injection. Rats were divided into four subgroups: 1) a control (saline) group, 2) a cisplatin group, 3) an ALA–treated post cisplatin-injection group, 4) an ALA–treated pre & post cisplatin-injection group (n = 8 for each group). Serum creatinine (A) and blood urea nitrogen (B), and body weight (C) were measured at the indicated times. Data are mean ± SEM of 8 rats per group. Statistically significant differences (*$p<0.05$ v.s. CDDP, #$p<0.05$ v.s. control) are indicated.
doi:10.1371/journal.pone.0080850.g001

Changes in mitochondrial structure and membrane potential were reported in the proximal tubules during AKI [8,9].

5-Aminolevulinic acid (ALA) is the naturally occurring metabolic precursor of an endogenously synthesized photosensitizer, protoporphyrin IX (PpIX) [10–12]. ALA is widely distributed in animal cells, and it is a precursor of tetrapyrole compounds such as heme, which is fundamentally important in aerobic energy metabolism [13]. Here, we explored the relevance of ALA in protecting renal tubular cells in cisplatin-treated rats through the attenuation of mitochondrial enzymes and the apoptotic pathway. Thus, ALA has the potential to prevent cisplatin nephrotoxicity without compromising the anticancer efficacy of cisplatin. This study was conducted to determine whether ALA affects the course of cisplatin-induced AKI. To achieve this, we examined differences in the renal function, histology, changes of mitochondrial enzymes, and tubular cell apoptosis in cisplatin-induced AKI. Our data demonstrated that ALA has the potential to prevent cisplatin nephrotoxicity without compromising the anticancer efficacy of cisplatin.

Materials and Methods

Induction of cisplatin-induced AKI

Male Sprague-Dawley rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing 150–200 g were anesthetized by intraperitoneal injection with sodium pentobarbital (30 mg/kg). Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline at a concentration of 1 mg/mL. The rats were given a single intraperitoneal injection of either a vehicle (saline) or cisplatin (8 mg/kg body weight). 5-ALA 10 mg/kg + Fe (sodium ferrous citrate, 15.7 mg/kg) dissolved in drinking water were administered to rats. 5-ALA (COSMO BIO co., ltd. Tokyo, Japan), and sodium ferrous citrate (kindly provided by SBI Pharmaceuticals Co., Ltd., Tokyo, Japan) were prepared. 5-ALA and Fe were purchased form

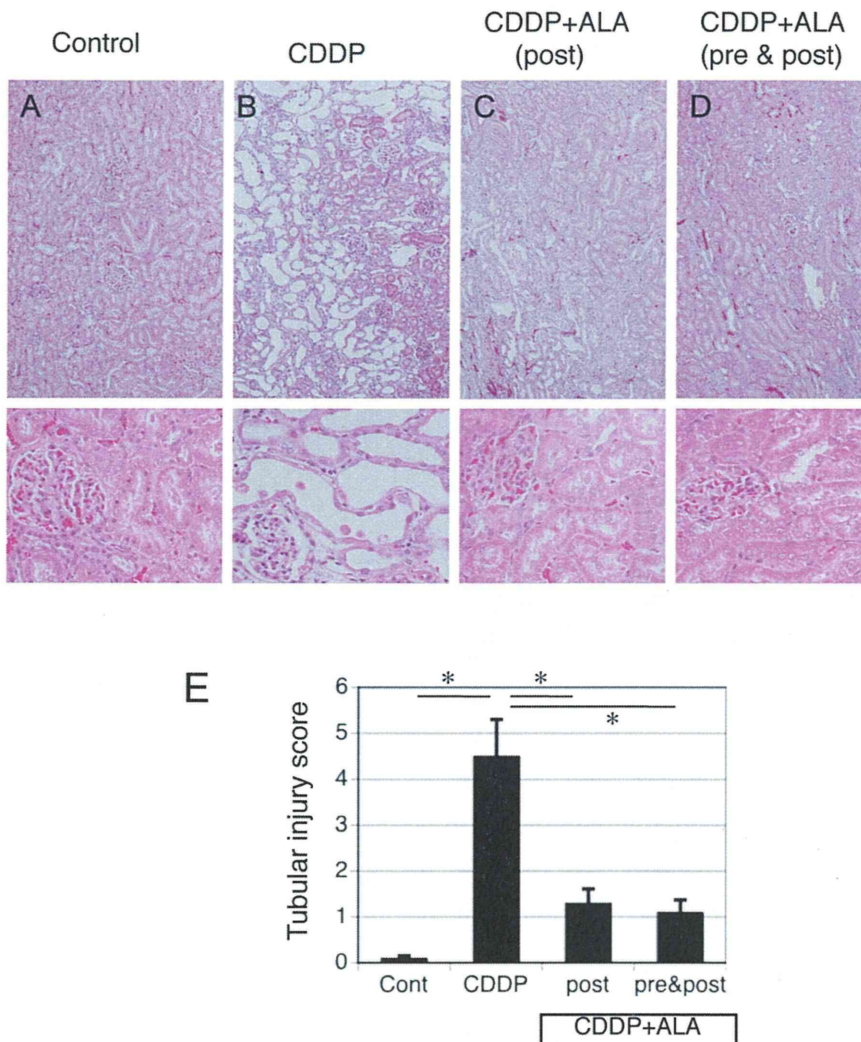


Figure 2. Renal histopathology and scores for characteristic histological signs of renal injury in ALA treated rats with cisplatin-induced AKI. A, B) Kidneys were removed 5 days after injection with cisplatin (8 mg/kg) or control. The cisplatin-treated rats exhibited acute structural damage characterized by tubular necrosis, swelling and tubular dilation, extensive epithelial vacuolization, and hyaline casts in renal tubules. C, D) Kidneys of ALA treatment (both post and pre & post) show a very slight loss of tubular epithelial cells and very low levels of intratubular debris and cast formation (Magnification, X50 upper, X200 lower figures) E) ALA reduced the renal injury score in cisplatin-treated rats. The semiquantitative histological injury score was significantly higher in cisplatin-treated rats than in controls. Data are the mean SEM of 6 rats per group. Statistically significant differences (* $p<0.05$) are indicated.
doi:10.1371/journal.pone.0080850.g002

Sigma-Aldrich (St. Louis, MO, USA). The animals were divided into 4 subgroups: (1) a control (saline) group, (2) a cisplatin group, (3) an ALA-treated post-cisplatin-injection group (post), and (4) an ALA-treated pre- and post-cisplatin-injection group (pre & post) (n = 8 for each group). The blood was obtained via tail vein at 1, 3, 5, 7, and 9 days after cisplatin injection. The rats were killed at 5 and 9 days after surgery (Figure S1). The left kidney was rapidly removed and processed for histological evaluation, protein extraction, and RNA extraction at day 5 and 9 as previously described [14,15]. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Kochi (#20–027), and experiments were conducted in accordance with institutional guidelines. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Effects of ALA on the antitumorigenic effects of cisplatin

Male F344/NJcl-rnu/rnu rats (immunodeficiency rats) (Saitama Experimental Animal Supply) weighing 150–200 g were anesthetized by intraperitoneal injection with sodium pentobarbital (30 mg/kg). 253J-BV (a bladder carcinoma cell line) cells (2×10^7) originally purchased from American Type Culture Collection (Manassas, VA, USA) were subcutaneously injected into the skin of the back. The rats were given a single intraperitoneal injection of either a vehicle (saline) or cisplatin (8 mg/kg body weight). 5-ALA 10 mg/kg + Fe (sodium ferrous citrate, 15.7 mg/kg) dissolved in drinking water were administered to rats. The animals were divided into 4 subgroups: (1) a control (saline) group, (2) a cisplatin group, (3) an ALA-treated post-cisplatin-injection group (post), and (4) an ALA-treated pre- and post-cisplatin-injection group (pre & post) (n = 5 for each group). The volume of the carcinoma was measured at 1, 3, 5, 7, and 9 days after surgery.