Sestrin-2 and BNIP3 regulate autophagy and mitophagy in renal tubular cells in acute kidney injury

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Ishihara M, Urushido M, Hamada K, Matsumoto T, Shimamura Y, Ogata K, Inoue K, Taniguchi Y, Horino T, Fujieda M, Fujimoto S, Terada Y. Sestrin-2 and BNIP3 regulate autophagy and mitophagy in renal tubular cells in acute kidney injury. Am J Physiol Renal Physiol 305: F495–F509, 2013. First published May 22, 2013; doi:10.1152/ajprenal.00642.2012.—Autophagy is a cellular recycling process induced in response to many types of stress. However, little is known of the signaling pathways that regulate autophagy during acute kidney injury (AKI). Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP)3 and sestrin-2 are the target proteins of hypoxia-inducible factor (HIF)-1α and p53, respectively. The aim of this study was to investigate the roles of BNIP3 and sestrin-2 in oxidative stress–induced autophagy during AKI. We used rat ischemia-reperfusion injury and cultured renal tubular (NRK-52E) cells as in vivo and in vitro models of AKI, respectively. Renal ischemia-reperfusion injury upregulated the expression of BNIP3 and sestrin-2 in the proximal tubules, as measured by immunohistochemical staining and Western blot analysis. In vitro, NRK-52E cells exposed to hypoxia showed increased expression of BNIP3 mRNA and protein in a HIF-1α-dependent manner. In contrast, sestrin-2 mRNA and protein expression were upregulated in a p53-dependent manner after exposure to oxidative stress (exogenous H2O2). NRK-52E cells stably transfected with a fusion protein between green fluorescent protein and light chain 3 were used to investigate autophagy. Overexpression of BNIP3 or sestrin-2 in these cells induced light chain 3 expression and formation of autophagosomes. Interestingly, BNIP3-induced autophagosomes were mainly localized to the mitochondria, suggesting that this protein selectively induces mitophagy. These observations demonstrate that autophagy is induced in renal tubules by at least two independent pathways involving p53-sestrin-2 and HIF-1α-BNIP3, which may be activated by different types of stress to protect the renal tubules during AKI.

Bcl-2/adenovirus E1B 19 kDa-interacting protein-3; acute kidney injury; autophagy; mitophagy; sestrin-2

ISCHEMIA is the leading cause of acute kidney injury (AKI) in the adult population. Prominent morphological features of ischemic AKI include effacement and loss of the proximal tubule brush border, patchy loss of tubular cells, focal areas of proximal tubular dilation, and increased apoptosis (9). The mechanisms that dictate the survival or death of renal cells under oxidative stress must be more completely understood before novel therapeutic strategies for the treatment of ischemic AKI can be explored. Proximal renal tubular cells have high rates of ATP consumption and are very sensitive to hypoxia; thus, mitochondrial damage is one of the most important factors in determining the survival of these cells (1, 43). Autophagy is one of the cellular processes that protect cells from genotoxic stress, oxidative stress, accumulation of misfolded proteins, and nutrient deprivation. We (20) have previously reported results from a study of autophagy in a mouse model of AKI. Autophagy plays roles in the pathogenesis of many diseases, and, in kidney disease, both beneficial and detrimental effects of autophagy have been reported (19–22). Our understanding of autophagy has expanded greatly in recent years, largely due to the identification of the many genes involved and to the development of improved methods to monitor the process, such as green fluorescent protein (GFP)-light chain 3 (LC3) to visualize autophagosomes in vivo (28). A number of groups (17, 25, 35) have demonstrated a close connection between autophagy and mitochondrial turnover. Removal of mitochondria that contain damaged components is accomplished via autophagy (mitophagy). Mitophagy also serves to eliminate the subset of mitochondria producing the most ROS, and episodic removal of mitochondria will reduce the oxidative burden. However, to the best of our knowledge, there have been no investigations of the mitophagy process in renal tubular cells.

Several novel proteins have been reported to regulate autophagy and mitophagy, including sestrin-2 and Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP)3. Sestrin-2 expression is regulated mainly by p53 but also by hypoxia-inducible factor (HIF)-1α. Activation of p53 and HIF-1α has been reported in several models of AKI; however, the involvement of p53 signaling in autophagy in renal cells remains unclear. Although sestrin-2 has been reported to induce autophagy in osteosarcoma cells (26), little is known of its role in AKI. BNIP3 contains a single Bcl-2 homology 3 domain and is localized primarily in the mitochondria (6, 11, 24, 48). Overexpression of BNIP3 has been reported to induce mitophagy by triggering mitochondrial depolarization (13, 17, 42). However, little is known of the function of BNIP3 and mitophagy in renal tubular cells. The aim of this study was to investigate the roles of autophagy and mitophagy in AKI in vivo and in vitro and to examine sestrin-2- and BNIP3-mediated signaling in renal tubular cells. Our data demonstrate that autophagy and mitophagy are induced in renal tubules in AKI by at least two independent pathways, p53-sestrin-2 and HIF-1α-BNIP3 pathways, and further suggest that these two pathways may function to protect renal tubules from different types of stress.
MATERIALS AND METHODS

**Induction of AKI.** Male Sprague-Dawley rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing 150–200 g were anesthetized by an intraperitoneal injection with pentobarbital sodium (30 mg/kg). To induce kidney injury, the left renal artery was occluded with Sugita aneurysm clips (Mizuho Ikakogyo, Tokyo, Japan) for 60 min. The clamps were then removed, and the incisions were closed. Rats were euthanized at 0, 3, 6, 12, 24, 48, and 72 h after surgery (n = 5 rats/group). The left kidney was then rapidly excised, frozen in liquid nitrogen, and homogenized in SDS sample buffer as previously described (41). Proteins were separated on 7.5% or 10–20% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (wt/vol) fat-free milk in PBS and probed with the appropriate primary antibodies against LC3 (MBL, Nagoya, Japan), Bnip3 (Santa Cruz Biotechnology, Santa Cruz, CA), and Actin (Sigma-Aldrich, St. Louis, MO). The proteins were visualized using an ECL detection system (GE Healthcare, Little Chalfont, UK). Immunoblot analysis was performed for sestrin-2 and BNIP3 blots. Data are means ± SE; n = 5. *P < 0.05 vs. control rats.

**Cell culture, plasmids, and small interfering RNA.** NRK-52E cells (renal tubular cells from an adult rat), originally purchased from American Type Culture Collection (Manassas, VA), were grown in DMEM (GIBCO) supplemented with 50 IU/ml penicillin and 10% heat-inactivated FCS (GIBCO) (38). Cells were cultured at 37°C in a 20% O2-5% CO2 atmosphere (referred to as normoxic conditions). For hypoxia experiments, NRK-52E cells were placed in a hypoxic chamber (AnaeroPack, Mitsubishi Gas Chemical) under 0% O2-5% CO2 atmosphere and were maintained at 37°C for 2, 4, or 6 h. For H2O2 experiments, 200 and 400 μM H2O2 was added to NRK-52E cells for the indicated times. Expression vectors encoding wild-type human sestrin-2, BNIP3, and HIF-1α were obtained from Addgene (Rockville, MD) and transfected into NRK-52E cells by electroporation, as previously described (38). Small interfering (si)RNAs specific for sestrin-2 and BNIP3 or control scrambled siRNAs were purchased from Ambion. NRK-52E cells were transfected with siRNAs by lipofection, as previously described. Western blot analyses were performed to confirm the efficiency of sestrin-2 and BNIP3 knockdown. All other chemicals were purchased from Funakoshi (Tokyo, Japan).

**Isolation and histological examination of kidney tissue.** Rats were anesthetized with pentobarbital at the indicated times after the ischemic event. The kidneys were perfused in situ with sterile PBS, and the left kidney was then rapidly excised, frozen in liquid nitrogen, and homogenized in SDS sample buffer, as previously described (23). For immunohistochemical experiments, kidneys were fixed in formalin overnight, dehydrated, and embedded in paraffin. Thin sections were cut and subjected to periodic acid-Schiff staining as previously described. Immunohistochemical staining was performed using streptavidin-biotin techniques using antibodies specific to sestrin-2 (cs-292558, Santa Cruz Biotechnology), lysosomal-associated membrane protein (LAMP)1 (cs-35684, Santa Cruz Biotechnology), and BNIP3 (cs-292463, Santa Cruz Biotechnology), as previously described (23, 38). The sequences of anti-sestrin-2- and anti-BNIP3-blocking peptides were CGEEWSQDLHSSGRDLRYS and CGEEWSQDLHSSGRDLRYS, respectively (Santa Cruz Biotechnology). Histology sections or immunoblot membranes were preincubated with blocking peptides (10 μg/ml) before the addition of the antibodies.

**Western blot analysis.** Protein extracts of total renal tissue or NRK-52E cells (50 μg samples) were prepared and denatured by heating at 100°C for 5 min in SDS sample buffer as previously described (41). Proteins were separated on 7.5% or 10–20% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (wt/vol) fat-free milk in PBS and probed with the appropriate primary antibodies against LC3 (MBL, Nagoya, Japan), Bnip3 (Santa Cruz Biotechnology, Santa Cruz, CA), and Actin (Sigma-Aldrich, St. Louis, MO). The proteins were visualized using an ECL detection system (GE Healthcare, Little Chalfont, UK). Immunoblot analysis was performed for sestrin-2 and BNIP3 blots. Data are means ± SE; n = 6. *P < 0.05 vs. control (0 h) by ANOVA.
Nagoya, Japan) or anti-sestrin-2, anti-BNIP3, anti-LAMP1, and anti-actin (H-300, cs-292558, sc-292463, sc-285412, and sc-10731, Santa Cruz Biotechnology). Primary antibodies were detected with horse-radish peroxidase-conjugated rabbit anti-goat IgG or horseradish peroxidase-conjugated donkey anti-rabbit IgG and visualized using an Amersham ECL system (Amersham, Arlington Heights, IL).

**Real-time quantitative PCR.** RT-PCR analysis of RNA extracted from kidneys was carried out as previously described (39). In brief, total RNA was isolated from renal tissues using TRI-REAGENT (Life Technologies, Gaithersburg, MD). Samples of total RNA (1 μg) were reverse transcribed, and real-time quantitative PCR was performed to quantify changes in Sestrin2, Bnip3, and DNA damage-regulated autophagy modulator 1 (Dram1) gene expression using the ABI LightCycler real-time PCR system (ABI, Los Angeles CA). RT-PCR of GAPDH served as a positive control. A three-step PCR was performed for 35 cycles. Samples were denatured at 94°C for 30 s, annealed at 58°C for 30 s, and extended at 72°C for 30 s. The primers used were obtained from ABI.

**Transient transfection and luciferase assay.** The sestrin-2 promoter (−2.5 kb) luciferase plasmid and BNIP3 promoter (−3.7 kb) luciferase plasmid were obtained from Addgene. NRK-52E cells were transfected with plasmid DNA (10 μg) by electroporation, as previously described (34). Luciferase activity was measured 48 h after transfection. Normalization was achieved by cotransf ecting cells with a β-galactosidase reporter construct. We established NRK-52E cells stably transfected with an LC3-GFP fusion protein as a marker of autophagy. In these cells, autophagy is indicated by the formation of GFP-positive autophagosomes. For some experiments, NRK-52E cells were cotransfected with a mitochondrial-targeted red fluorescent protein (mitoDsRed, Clontech).

**Scanning laser confocal immunofluorescence microscopy and electron microscopy.** For confocal microscopy, NRK-52E cells were fixed with 2% paraformaldehyde in PBS for 1 h and processed for imaging as previously described (38). For electron microscopy, cells were fixed in PBS containing 2% paraformaldehyde and 0.1% glutaraldehyde for 2 h. Samples were embedded in Epo-Araldite resin. 

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**Fig. 3. Immunohistochemical analysis of sestrin-2 and BNIP3 expression after I/R AKI.** A and B: immunohistochemical analysis of sestrin-2 expression in the renal cortex of a control kidney (A) or a kidney at 12 h after I/R injury (B). Magnification: ×100. C: high-power view of the renal cortex stained with anti-sestrin-2 antibody. Magnification: ×600. The arrows indicate sestrin-2-positive cells. D: immunohistochemical analysis of sestrin-2 expression in the renal cortex 12 h after I/R injury. Staining was performed in the presence of an anti-sestrin-2-blocking peptide. Magnification: ×100. E and F: immunohistochemical analysis of BNIP3 expression in the renal cortex of a control kidney (E) or a kidney at 12 h after I/R injury (F). Magnification: ×100. G: high-power view of the renal cortex stained with an anti-BNIP3 antibody. Magnification: ×600. The arrows indicate BNIP3-positive cells. H: immunohistochemical analysis of BNIP3 expression in the renal cortex at 12 h after I/R injury. Staining was performed in the presence of an anti-BNIP3-blocking peptide.
(Canemco), and ultrathin sections were cut under a Reichert ultramicroscope; these sections were counterstained with 0.3% lead citrate and examined under a Philips EM420 electron microscope.

**Caspase-3 assay and TUNEL assay.** A Caspase-3 Fluorometric Protease Assay Kit (MBL) was used for the measurement of caspase-3 activity, as previously described (39). In brief, cell lysates were incubated with reaction buffer and 50 mM caspase-3 substrate for 2 h at 37°C, and the enzymatic activity was measured colorimetrically. The Apoptosis TUNEL Kit II (MBL) was used for the staining for TUNEL-positive cells, as previously described (39).

**Statistics.** Results are presented as means ± SE. Differences between the groups were tested by two-way ANOVA followed by Scheffé’s test for multiple comparisons. Two groups were compared using unpaired t-tests. P values of <0.05 were considered statistically significant.

**RESULTS**

**Sestrin-2 and BNIP3 gene expression after ischemic AKI in vivo.** To examine the effects of ischemia-reperfusion (I/R) injury on the expression of sestrin-2, BNIP3, and DRAM1, we conducted real-time quantitative PCR analysis on rat kidney tissues. To induce I/R injury, the left renal artery was clamped for 60 min, and the kidney was excised from animals euthanized at 3, 6, 12, 24, 48, and 72 h after reperfusion. Kidneys from sham-operated rats were used as controls (0 h). Quantitative PCR analysis revealed dramatic changes in sestrin-2 and BNIP3 mRNA levels after I/R, but there was no effect on DRAM1 gene expression under our experimental conditions. Sestrin-2 mRNA levels were significantly increased compared with those in the controls between 3 and 48 h postischemia, and BNIP3 mRNA levels were dramatically elevated between 6 and 24 h after I/R injury (Fig. 1).

**Sestrin-2 and BNIP3 protein expression after ischemic AKI in vivo.** Western blot analyses were performed on kidney extracts to determine if I/R induced changes in the expression of sestrin-2 and BNIP3 protein. Consistent with the quantitative PCR results, sestrin-2 protein expression was markedly increased between 3 and 72 h after I/R compared with control rats (Fig. 2A). Quantitative densitometry of the blots revealed that sestrin-2 levels were increased 5.6-, 6.6-, 8.9-, 7.7-, 6.3-, and 5.4-fold at 3, 6, 12, 24, 48, and 72 h after I/R, respectively (Fig. 2B). BNIP3 protein levels were also increased compared with control rats between 12 and 48 h after I/R (Fig. 2A).

![Fig. 4. Analysis of sestrin-2 promoter activity, mRNA levels, and protein expression in NRK-52E cells subjected to oxidative stress. NRK-52E cells were exposed to hypoxia (2 or 4 h) or H2O2 (400 µM). A: sestrin-2 promoter activity was measured by luciferase assays. B: sestrin-2 mRNA levels were measured by PCR. C: sestrin-2 protein expression was measured by Western blot analysis. D: densitometric analysis of sestrin-2 protein expression after exposure to hypoxia (4 h) and H2O2 (400 µM). Cells were incubated with CoCl2 (250 or 500 µM) for 6 h. E: sestrin-2 promoter activity was measured by luciferase assays. F: sestrin-2 mRNA levels were measured by RT-PCR. G: sestrin-2 protein expression was measured by Western blot analysis. Data are means ± SE; n = 5. *P < 0.05 vs. control-treated cells.](http://ajprenal.physiology.org/2012/F498.png)
BNIP3 was only weakly expressed in control and sham-operated kidneys. BNIP3 levels were increased by 3.1-, 2.7-, and 1.8-fold at 12, 24, and 48 h after I/R, respectively, compared with control animals (Fig. 2B).

**Immunohistochemical examination of sestrin-2 and BNIP3 expression in ischemic AKI.** Next, we performed immunohistochemical experiments of sestrin-2 and BNIP3 expression in I/R AKI (Fig. 3). Under low-power microscopy, sestrin-2 and BNIP3 expression were observed in cortical renal tubules at 12 h after I/R (Fig. 3, B and F), and high-power views indicated that both proteins were localized mainly to the cytoplasm (Fig. 3, C and G). In contrast, only low levels of sestrin-2 and BNIP3 were detected in the cortical renal tubules of control rats (Fig. 3, A and E). To confirm the specificity of the anti-sestrin-2 and anti-BNIP3 antibodies, immunostaining was also performed in the presence of antigen-specific blocking peptides (Santa Cruz Biotechnology), which resulted in diminished cytoplasmic staining in cortical cells of I/R-injured kidneys (Fig. 3, D and H). These results demonstrate that sestrin-2 and BNIP3 were expressed mainly in the proximal tubules of the renal cortex 12 h after I/R.
Increased sestrin-2 promoter activity and mRNA and protein expression in H₂O₂-treated NRK-52E cells in vitro. To determine if sestrin-2 expression is induced by oxidative stress in renal cells, we examined sestrin-2 promoter activity, mRNA levels, and protein expression in cultured NRK-52E cells exposed to hypoxia and H₂O₂. We found that sestrin-2 promoter activity was significantly increased by hypoxia and H₂O₂ compared with control normoxia cells (Fig. 4A). Similarly, sestrin-2 mRNA levels were significantly increased under the same conditions. Compared with control cells, H₂O₂ treatment increased sestrin-2 mRNA expression by 17.5-fold (Fig. 4B). Consistent with the effects on mRNA expression, hypoxia induced a small but significant increase in sestrin-2 (Fig. 4C and D). However, H₂O₂ treatment caused a marked increase in sestrin-2 expression (8.9-fold; Fig. 4C and D). To examine the signaling pathway of sestrin-2 induction by hypoxia and oxidative stress, we used CoCl₂, a well-known activator of HIF-1α. As shown in Fig. 4, E–G, CoCl₂ dose dependently stimulated sestrin-2 promoter activity up to 6.1-fold, increased mRNA expression up to 6.2-fold, and increased protein expression up to 5.3-fold in NRK-52E cells.

Increased BNIP3 promoter activity and mRNA and protein expression in NRK-52E cells exposed to hypoxia or H₂O₂. BNIP3 promoter activity and mRNA expression in NRK-52E cells were increased significantly by both hypoxia and H₂O₂, with hypoxia having a greater effect (Fig. 5A and B). Compared with control cells, hypoxic treatment increased BNIP3 mRNA expression by 8.3-fold (Fig. 5B). BNIP3 protein levels were increased by cell exposure to hypoxia and H₂O₂ (Fig. 5, C and D). Consistent with the effects on mRNA expression, H₂O₂ induced a small but significant increase in BNIP3 (Fig. 5, C and D). However, hypoxia caused a marked increase in BNIP3 expression (12.6-fold; Fig. 5, C and D). To examine the signaling pathway of BNIP3 induction by hypoxia and oxidative stress, we used CoCl₂, a well-known activator of HIF-1α. As shown in Fig. 5, E–G, CoCl₂ dose dependently stimulated BNIP3 promoter activity up to 5.3-fold, increased mRNA expression up to 7.9-fold, and increased protein expression up to 9.7-fold in NRK-52E cells. We also observed by confocal microscopy that the hypoxic conditions increased BNIP3 expression in the cytoplasm of NRK-52E cells (Fig. 6).

Time courses of LC3-II and LAMP1 accumulation in oxidative stress in NRK-52E cells. To monitor autophagic flux in renal tubular cells during oxidative stress, we examined the time course of LC3-II and LAMP1 accumulation in cells incubated with H₂O₂. We used LC3-II and LAMP1 as markers of autophagy induction and autophagic flux. As shown in Fig. 7A, Western blot analysis showed that LC3-II expression was increased between 4 and 8 h after oxidative stress and that LAMP1 increased between 8 and 12 h. In the experiment examining oxidative stress by confocal microscopy, only LC3-II punctae were detected at 4 h, colocalized LC3-II and LAMP1 punctae were seen at 8 h, and only LAMP1 punctae were detected at 12 h (Fig. 7B). LAMP1 expression therefore followed LC3-II expression.

![Fig. 6. Analysis of BNIP3 protein expression in NRK-52E cells exposed to hypoxia by confocal microscopy. NRK-52E cells were incubated under normoxic (A) or hypoxic (B) conditions and examined by confocal microscopy for BNIP3 expression. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Original magnification: ×400.](http://ajprenal.physiology.org/DownloadedFrom/)
Modulation of autophagy in NRK-LC3 cells by manipulation of sestrin-2 levels. To examine the functional role of sestrin-2 in autophagy, we transiently transfected NRK-LC3 cells (NRK-52E cells stably expressing GFP-LC3) with control or sestrin-2 expression vectors. Western blot analysis of cell extracts showed that expression of the autophagy marker LC3-II was markedly increased in cells that overexpressed sestrin-2 compared with control cells (Fig. 8A). In addition, many GFP-positive autophagosomes were visible in sestrin-2-overexpressing cells by confocal microscopy (Fig. 8B). Furthermore, autophagosome formation was observed in electron micrographs of sestrin-2-overexpressing cells and cells incu-
bated with H₂O₂ (Fig. 8C). These data clearly show that sestrin-2 expression induced autophagy in NRK-LC3 cells. In contrast, NRK-LC3 cells transfected with sestrin-2 siRNA and then incubated with H₂O₂ showed significantly reduced LC3-II expression and numbers of GFP-positive autophagosomes compared with control cells after exposure to H₂O₂ (Fig. 8, D and E).

Modulation of autophagy and mitophagy in NRK-LC3 cells by manipulation of BNIP3 levels. We examined the functional role of BNIP3 by performing similar experiments with BNIP3-overexpressing or BNIP3-silenced NRK-LC3 cells. Western blots of cell extracts showed that the expression of LC3-II was markedly increased in BNIP3-overexpressing cells (Fig. 9A). Similar to sestrin-2-overexpressing cells, we found many GFP-positive autophagosomes in BNIP3-overexpressing cells (Fig. 9B). Thus, BNIP3 induced autophagy in NRK-LC3 cells. To examine the intracellular localization of BNIP3-induced autophagosomes, NRK-LC3 cells were cotransfected with a BNIP3 expression vector and a mitochondrial-targeted red fluorescent protein (mitoDsRed). By confocal microscopy, many GFP and mitoDsRed double-positive foci were visible, indicative of colocalization of mitochondria and autophagosomes (Fig. 9C). Moreover, mitophagy was evident in BNIP3-overexpressing cells examined by electron microscopy. As shown in Fig. 9D, BNIP3-overexpressing cells induced autophagosomal encapsulation of mitochondria, which confirmed that overexpression of BNIP3 induced mitophagy. We next transfected control siRNA or BNIP3-targeting siRNA into NRK-LC3 cells and incubated the cells under hypoxic conditions. We found that the number of GFP-positive autophagosomes was decreased in cells expressing BNIP3 siRNA compared with control siRNA (Fig. 10, A–C). Furthermore, BNIP3-targeting siRNA into NRK-LC3 cells reduced LC3-II protein levels in both normoxia and hypoxia (Fig. 10D). As shown in Fig. 10E, exposure of these cells to hypoxia induced autophagosomal encapsulation of mitochondria, which confirmed that hypoxia induced mitophagy.

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**Fig. 8.** Autophagy in NRK-LC3 cells is increased by overexpression of sestrin-2 and suppressed by sestrin-2 small interfering (si)RNA. A: Western blot analysis of LC3-II in control or sestrin-2-overexpressing NRK-52E cells. B: confocal microscopy of GFP-positive autophagosomes in NRK-LC3 cells transfected with control or sestrin-2 expression vectors. C: electron microscopy of autophagosomes in cells that overexpressed sestrin-2 (left) or were incubated with 400 μM H₂O₂ (right). D: Western blot analysis of LC3-II in control or sestrin-2 siRNA-transfected NRK-52E cells incubated with 400 μM H₂O₂. E: confocal microscopy of GFP-positive autophagosomes in sestrin-2 siRNA-transfected NRK-LC3 cells incubated with 400 μM H₂O₂.
Modulation of H2O2-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 \mu M H2O2, but this was reduced in cells that overexpressed sestrin-2 (Fig. 11A). In contrast to this effect, overexpression of BNIP3 slightly augmented the level of oxidative stress-induced cleaved caspase-3 (Fig. 11B). To confirm these findings, we measured caspase-3 activity in NRK-52E cells that overexpressed sestrin-2 or BNIP3. As shown in Fig. 11C, treatment of NRK-52E cells with 200 or 400 \mu M H2O2 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 \mu M H2O2-stimulated caspase-3 activity under our experimental conditions (Fig. 11D).

We also used TUNEL staining to evaluate apoptosis in NRK-52E cells under oxidative stress (400 \mu M H2O2) 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 H2O2-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. 13A). On the other hand, under oxidative stress, LDH release was significantly increased by transfection with sestrin-2 siRNA (Fig. 13B). 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.
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.
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 H₂O₂ 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 mammalian 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

![Image](https://example.com/image.png)
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. H2O2 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 H2O2-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. 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 H2O2 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.
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 H2O2 (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 mitochondrially encoded 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. 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.

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DISCLOSURES

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