

**Sestrin2 and BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein3) regulate autophagy and mitophagy in renal tubular cells in acute kidney injury**

by

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## 【Backgrounds】

Ischemia is the leading cause of acute kidney injury (AKI) in the adult population. Proximal renal tubular cells have high rates of ATP consumption and are very sensitive to hypoxia. Mitochondrial damage is one of the most important factors in determining the survival of these cells.

Autophagy is one of the cellular processes that protect cells from genotoxic stress, oxidative stress, accumulation of misfolded proteins, and nutrient deprivation. Mitophagy also serves to eliminate the subset of mitochondria producing the most reactive oxygen species, and episodic removal of mitochondria will reduce the oxidative burden.

Sestrin2 expression is regulated mainly by p53 but also by HIF-1 $\alpha$  (2). Activation of p53 and HIF-1 $\alpha$  has been reported in several models of AKI. Overexpression of Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (BNIP3) has been reported to induce mitophagy.

## 【Purpose】

The aim of this study was to investigate the roles of autophagy and mitophagy in AKI in vivo and in vitro, and to examine sestrin2- and BNIP3-mediated signaling in renal tubular cells.

## 【Material and Methods】

**Induction of AKI.** To induce kidney injury, the left renal artery was occluded for 60 min. Rats were sacrificed at 0, 3, 6, 12, 24, 48, and 72 h after surgery. The left kidney was rapidly removed and processed for histological evaluation, protein extraction, and RNA extraction.

**Cell culture, plasmids, and siRNA.** NRK-52E cells (renal tubular cells from adult rat) were grown in Dulbecco's modified Eagle's medium (DMEM). For the hypoxia experiments, NRK-52E cells were placed in a hypoxic chamber. For the hydrogen peroxide experiments, hydrogen peroxide was added to the NRK-52E cells. NRK-52E cells were transfected with Small interfering RNAs (siRNAs) by lipofection. Western blot analyses were performed to confirm the efficiency of sestrin2 and BNIP3 knockdown.

**Isolation and histological examination of kidney tissue.** After the ischemic event, the rat kidneys were perfused in situ with sterile phosphate-buffered saline (PBS) and the left kidney was then rapidly excised. Thin sections were subjected to periodic acid-Schiff (PAS) staining. Immunohistochemical staining was performed using antibodies specific to sestrin2, LAMP1 and BNIP3.

**Western blot analysis.** Protein extracts of total renal tissue or NRK-52E cells were prepared and denatured. The membranes were probed with the appropriate primary antibodies (anti-LC3, or anti-sestrin2, anti-BNIP3, anti-LAMP1, anti-actin). The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG or HRP-donkey anti-rabbit IgG, and visualized using the Amersham ECL system.

**Real-time quantitative PCR.** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA extracted from kidneys was carried out. Total RNA was isolated from renal tissues. Samples of total RNA were reverse transcribed, and real-time qPCR was performed to quantify changes in *sestrin2*, *bnip3*, and *dram1* gene

expression.

**Transient transfection and luciferase assay.** NRK-52E cells were transfected with plasmid DNA by electroporation. Luciferase activity was measured 48 h after transfection. Normalization was achieved by cotransfecting cells with a  $\beta$ -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.

**Scanning laser confocal immunofluorescence microscopy and electron microscopy.** For confocal microscopy, NRK-52E cells were fixed and processed for imaging. For electron microscopy, cells were fixed. The samples were embedded in Epo-Araldite resin and ultrathin sections were cut.

**Caspase3 assay and TUNEL assay.** Caspase3 Fluorometric Protease Assay Kit was used for the measurement of caspase3 activity. The enzymatic activity was measured colorimetrically. Apoptosis TUNEL Kit II was used for the staining for TUNEL positive cells.

## **【Results】**

### **Sestrin2 and BNIP3 gene expression after ischemic AKI in vivo**

Sestrin2 mRNA levels were significantly increased between 3 and 48 h post-ischemia, and BNIP3 mRNA levels were dramatically elevated between 6 and 24 h after I/R injury.

### **Sestrin2 and BNIP3 protein expression after ischemic AKI in vivo**

Sestrin2 protein expression was increased markedly between 3 and 72 h after I/R. Sestrin2 levels were increased after I/R. BNIP3 protein levels were also increased between 12 and 48 h after I/R. BNIP3 levels were increased following I/R.

#### **Immunohistochemical examination of sestrin2 and BNIP3 expression in ischemic AKI**

Sestrin2 and BNIP3 expression was observed in cortical renal tubules at 12 h after I/R, and both proteins were localized mainly to the cytoplasm. In contrast, only low levels of sestrin2 and BNIP3 were detected in the cortical renal tubules of control rats. To confirm the specificity of the anti-sestrin2 and anti-BNIP3 antibodies, immunostaining was also performed in the presence of antigen-specific blocking peptides, which resulted in diminished cytoplasmic staining in cortical cells of the I/R-injured kidney. These results demonstrate that sestrin2 and BNIP3 were expressed mainly in the proximal tubules of the renal cortex 12 h after I/R.

#### **Increased sestrin2 promoter activity, and mRNA and protein expression in H<sub>2</sub>O<sub>2</sub>-treated NRK-52E cells in vitro**

Sestrin2 promoter activity was significantly increased by hypoxia and H<sub>2</sub>O<sub>2</sub>. Similarly, Sestrin2 mRNA levels were significantly increased under the same conditions. H<sub>2</sub>O<sub>2</sub> treatment increased sestrin2 mRNA expression. Consistent with the effects on mRNA expression, hypoxia induced a small but significant increase in sestrin2. H<sub>2</sub>O<sub>2</sub> treatment caused a marked increase in sestrin2 expression. CoCl<sub>2</sub>, well known activator of HIF-1 $\alpha$ , dose-dependently stimulated sestrin2 promoter activity, increased mRNA, and protein expression.

### **Increased BNIP3 promoter activity, and mRNA and protein expression in NRK-52E cells exposed to hypoxia or H<sub>2</sub>O<sub>2</sub>**

BNIP3 promoter activity and mRNA expression in NRK-52E cells were increased significantly by both hypoxia and H<sub>2</sub>O<sub>2</sub>, with hypoxia having a greater effect. Hypoxic treatment increased BNIP3 mRNA expression. BNIP3 proteins levels were increased by cell exposure to hypoxia and H<sub>2</sub>O<sub>2</sub>. Consistent with the effects on mRNA expression, H<sub>2</sub>O<sub>2</sub> induced a small but significant increase in BNIP3. Hypoxia caused a marked increase in BNIP3 expression. CoCl<sub>2</sub> dose-dependently stimulated BNIP3 promoter activity, increased mRNA, and protein expression in NRK-52E cells. Hypoxic conditions increased BNIP3 expression in the cytoplasm of NRK-52E cells.

### **Time courses of LC3-II and LAMP1 accumulation in oxidative stress in NRK-52E cells**

Western blot analysis showed that LC3-II, a marker of autophagy induction, expression was increased between 4 h and 8 h after oxidative stress and LAMP1, a marker of autophagic flux, increased between 8 h and 12 h. In the study 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. LAMP1 expression therefore followed LC3-II expression.

### **Modulation of autophagy in NRK-LC3 cells by manipulation of sestrin2 levels**

Western blot analysis of cell extracts showed that expression of the LC3-II was

markedly increased in cells overexpressing sestrin2. Many GFP-positive autophagosomes were visible in the sestrin2-overexpressing cells by confocal microscopy. Autophagosome formation was observed in electron micrographs of sestrin2-overexpressing cells and cells incubated with H<sub>2</sub>O<sub>2</sub>. Sestrin2 expression induced autophagy in NRK-LC3 cells. In contrast, NRK-LC3 cells transfected with sestrin2 siRNA and then incubated with H<sub>2</sub>O<sub>2</sub> showed significantly reduced LC3-II expression and numbers of GFP-positive autophagosomes following exposure to H<sub>2</sub>O<sub>2</sub>.

#### **Modulation of autophagy and mitophagy in NRK-LC3 cells by manipulation of BNIP3 levels**

Western blots of cell extracts showed that the expression of LC3-II was markedly increased in BNIP3-overexpressing cells. Many GFP-positive autophagosomes in BNIP3-overexpressing cells. 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. Moreover, mitophagy was evident in BNIP3-overexpressing cells examined by electron microscopy. BNIP3-overexpressing cells induced autophagosomal encapsulation of mitochondria, which confirmed that overexpression of BNIP3 induced mitophagy. We transfected control siRNA or BNIP3-targeting siRNA into NRK-LC3

cells and incubated the cells under hypoxic conditions. The number of GFP-positive autophagosomes was decreased in cells expressing BNIP3 siRNA. BNIP3-targeting siRNA into NRK-LC3 cells reduced LC3-II protein level both normoxia and hypoxia. Exposure of these cells to hypoxia induced autophagosomal encapsulation of mitochondria, which confirmed that hypoxia induced mitophagy.

#### **Modulation of H<sub>2</sub>O<sub>2</sub>-induced apoptosis in NRK-52E cells by overexpression of sestrin2 and BNIP3**

Elevated levels of cleaved caspase3 in NRK-52E cells exposed to 200  $\mu$ M or 400  $\mu$ M H<sub>2</sub>O<sub>2</sub>, but this was reduced in cells overexpressing sestrin2. In contrast to this effect, overexpression of BNIP3 slightly augmented the level of oxidative stress-induced cleaved caspase3. Treatment of NRK-52E cells with 200  $\mu$ M or 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> increased caspase3 activity, as expected, and this increase was significantly reduced by overexpression of sestrin2. In contrast, overexpression of BNIP3 further increased 200  $\mu$ M H<sub>2</sub>O<sub>2</sub>-stimulated caspase3 activity under our experimental conditions.

Apoptosis was significantly reduced by transfection with sestrin2 overexpression, and slightly reduced by BNIP3 overexpression.

#### **Modulation of hypoxia and H<sub>2</sub>O<sub>2</sub>-induced LDH release in NRK-52E cells by inhibition of sestrin2 and BNIP3 using siRNA**

We transfected NRK-52E cells with control siRNA or siRNA specific for BNIP3 and sestrin2, and measured LDH release following exposure of cells to hypoxic or oxidative stress. Under hypoxic conditions, LDH release was significantly increased by BNIP3 siRNA and



slightly, but not significantly, increased by sestrin2 siRNA. Under oxidative stress, LDH release was significantly increased by transfection with sestrin2 siRNA. 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. Endogenous BNIP3 and sestrin2 play protective roles during hypoxia and oxidative stress, respectively.

#### **【Conclusion】**

Our study has produced 2 novel findings. First, sestrin2 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 2 independent pathways, the p53–sestrin2 and HIF-1 $\alpha$ –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.