

Supplementary materials for**Erythropoietin Receptor- A Downstream Effector of Klotho-Induced Cytoprotection**

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Running title: Klotho and EpoR in H₂O₂ cytotoxicity

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MATERIALS AND METHODS

Human study

The study was approved by the Institutional Review Board at the University of Texas (UT) Southwestern Medical Center. The human kidney samples were dissected from normal kidney tissues adjacent to the tumor tissue whose kidneys were surgically removed due to kidney carcinoma in affiliated hospital with UT Southwestern. Recruited subjects gave informed consent.

Animal study

Transgenic mice over-expressing Klotho (*Tg-Kl*; EFmKL46 line)¹ and Klotho hypo-morphic mice (*Kl^{-/-}*)² were maintained at the Animal Research Center at the University of Texas Southwestern Medical Center. Wild type littermates were used as controls. Background of those genetically manipulated mice is 129sv. The age of mice ranged from 6 - 8 weeks. Normal Sprague-Dawley (SD) rats (220-250 gm body weight) were purchased from Harlan (Indianapolis, IN). Rats were intraperitoneally injected once with full extracellular domain of recombinant mouse Klotho protein (rMKI)¹ at a dose of 0.01 mg/Kg BW.³ One day after injection, rats were anesthetized for terminal organ harvest.¹ All animal work was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Nephron segments and collecting ducts were dissected using established methods.^{4, 5} Accuracy and specificity of dissection was verified by RT-PCR of segment-specific markers (**Figure 1**).

Generation of synthetic anti-EpoR Fab

The synthetic human Fab was isolated from a phage-displayed library (Library F constructed by Sidhu Laboratory) with diversity introduced into the three heavy-chain complementarity-determining regions (CDRs) and the third light chain CDR. Biopanning, phage ELISAs and Fab

protein purification were performed as described.^{6,7} Briefly, phage particles from the library were cycled through rounds of binding selection with an Fc fusion of the EpoR ectodomain (R&D Systems Inc. Minneapolis, MN) coated on 96-well Maxisorp Immunoplates (Fisher Scientific, Nepean, ON, Canada) as the capture target. After five rounds of selection, phage particles were produced from individual clones grown in a 96-well format and the culture supernatants were used in phage ELISAs to detect specific binding clones. Positive clones were subjected to DNA sequence analysis. Fab proteins were purified from bacterial cultures by affinity purification on a Protein A affinity column (GE Healthcare, Mississauga, ON, Canada). Using competitive phage ELISAs, we estimated the affinity of Fab-6 for the EpoR-ECD to be in the single-digit nanomolar range (data not shown).

Cell lines

BaF3 cell line, a murine interleukin 3 (IL-3)-dependent hematopoietic pro-B-cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), penicillin G (100 U/ml) and streptomycin (100 mg/ml), 2 mM L-glutamine (both Gibco-Invitrogen, Carlsbad, CA, USA) and filtered IL-3-containing supernatant (10%) from WEHI-3 cells (ATCC). BaF3-HA-EpoR cell line was generated by stably expressing HA-tagged murine full length EpoR via a retroviral vector.⁸ Normal rat kidney (NRK cells) polarized epithelial cells were purchased from ATCC and cultured at 37°C in a 95% air 5% CO₂ atmosphere. Cells were passed in high-glucose (450 mg/dl) DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). For transient transfections, NRK cells were grown to ~70% confluence and 1.0 µg of cDNA plasmid was introduced per 35-mm plate with Lipofectamine Plus (Invitrogen, Carlsbad, CA) following product instructions. Experiments were performed at 48 hrs post transfection.

Materials, antibodies, and plasmid constructs

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except otherwise noted. Culture media, Lipofectamine 2000 and Lipofectamine plus (Invitrogen, Carlsbad, CA); penicillin and streptomycin (Cambrex, East Rutherford, NJ); enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ); and nitrocellulose and polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Soluble recombinant murine Klotho protein containing the extracellular domain (amino acid number 31-982) (rMKI) with C-terminal V5 and 6xHis tags were purified from conditional medium by affinity column chromatography using anti-V5 antibody (Sigma-Aldrich, St. Louis, MO) as previous described (43). EPO (Epogen, 10,000 units/ml) was purchased from Amgen (Thousand Oaks, CA).

Antibodies used were: HA (Sigma-Aldrich, St. Louis, MO), Anti-EpoR M-20 (Santa Cruz, CA); anti-Klotho monoclonal antibody (KM2076) (gift from M. Kuro-o, Univ. Texas Southwestern Medical Center);³ Anti-EpoR monoclonal A82 (gift from Dr. S. Elliot, Amgen, Thousand Oaks CA); total and phospho-Erk, total and phospho-Jak2 (Millipore, Temecula, CA); Total Stat5 (Santa Cruz, CA); phospho-Stat5 (Cell Signaling Danvers, MA); neutrophil gelatinase-associated lipocalin (NGAL) (R&D Systems, Inc., Minneapolis, MN); β -actin (Sigma-Aldrich, St. Louis, MO). Mammalian expression plasmids used were: 1) pcDNA3.1(+)-HA Vector; 2) pcDNA3.1(+)-HA-EpoR vector as described in our earlier publication.⁸ Generation of the synthetic Fab and IgG is described above.

RNA interference

Small interfering RNA (siRNA) of EpoR duplexes were designed by using the web-based BLOCK-iT RNAi Designer software (Invitrogen, Carlsbad, CA). Knockdown was achieved by cotransfection of two siRNAi's per gene. NRK cells at ~80% confluence in serum-containing

DMEM without antibiotics were transfected with Lipofectamine 2000 following the manufacturer's instructions. The efficiency of EpoR mRNA and protein knockdown was evaluated by RT-qPCR and immunoblot respectively to optimize experimental conditions (dose of siRNA and time after transfection). The following siRNA oligonucleotides were used: rat EpoR-siRNA²²² sense 5'-CCG AAG AAC UUC UAU GCU UTT-3' and anti-Sense: 5'-AAG CAU AGA AGU UCU UCG GTT-3'; and scramble control²²² sense 5'- CCG AAG UUC AUC GUA ACU UTT-3" and anti-sense 5'-AAG UUA CGA UGA ACU UCG GTT-3"; rat EpoR-siRNA¹²⁵⁸ sense 5'-CCC UGC GAC UAU GGA UGA ATT-3" and anti-sense 5'-UUC AUC CAU AGU CGC AGG GTT-3'; and scramble control¹²⁵⁸ sense 5'-CCC AGC AUC GGU UAU GGA ATT-3' and anti-sense 5'-UUC CAU AAC CGA UGC UGG GTT-3'. EpoR-siNRA²²² and its control²²² were eventually selected as tool to knock down endogenous EpoR in NRK cells.

RNA extraction, RT-PCR and real time PCR

Total RNA was extracted using RNAeasy kit (Qiagen, Germantown, MD) from NRK cells or rodent kidney tissues as described.^{3,9} Complementary DNA (cDNA) was generated with oligo-dT primers using SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Primers 5'-GGA CCC TCT CAT CTT GAC-3' and 5'-TAG CAA CCA TTC ATC CAA TAC C-3' were used for detection of *EpoR* transcript. The primers and conditions of PCR for other rat genes: *NaPi-2a*, *NKCC2*, *AQP2*, *Klotho* and *β-actin* were described in our previous publications.^{4,5} PCR products were analyzed by 2% agarose ethidium bromide gel electrophoresis. Primers used for qPCR to detect EpoR transcripts were 5'-GGA CCC TCT CAT CTT GAC GC-3' and 5'-CTT GGG ATG CCA GGC CAG AT-3'; and to detect cyclophilin 5'-GTC TCT TTT CGC CGC TTG CT-3' and 5'-TCT GCT GTC TTT GGA ACT TTG TCT G-3' respectively with conditions described in the literature.⁹ Briefly, PCR was performed in an ABI Prism 7000 Sequence Detector (Applied BioSystems, Foster City, CA), with one cycle (95°C for 10 min) and then 40 cycles (95°C for 15 s and 60°C for 1 min) in triplicate for each

sample. PCR products were analyzed by gel electrophoresis (data not shown), and also were amplified using Big Dye Terminator 3.1 chemistry (Applied Biosystems Inc. ABI), and analyzed on ABI capillary instruments by DNA Sequencing Core Facility in UT Southwestern Medical Center.

LDH and TUNEL assays and immunocytochemistry

NRK cells were seeded in 12 well-plates and rendered quiescent overnight after 100% confluence. Next day, cells were treated with different concentration of H₂O₂ with or without rMKI Klotho. At designated time points, culture medium was collected and immediately centrifuged at 4°C (1400 rpm x 5 min) to remove cells and cellular debris. Supernatants were harvest for measurement of LDH release with LDH cytotoxicity detection kit (Clontech Laboratories Inc., Mountain View, CA) according to manufacturer's instruction. For TUNEL staining, NRK cells were seeded on glass cover slips in 12-well plates and treated as mentioned above. After removal of culture medium, cells were rinsed with pre-cold PBS thrice and fixed in 4% paraformaldehyde (PFA) for 20 minutes. Apoptotic cells were detected with *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. NRK cells were co-stained with DAPI and visualized with a Zeiss LSM-510 laser scanning microscope. For EpoR immunocytochemisry, NRK cells were fixed in 4% PFA in PBS for 10 min, permeabilized in Triton X-100 (0.1% in PBS, 3 min), and blocked by 1.5% BSA and 10% goat serum in PBS (1 h). Specimens were incubated with primary antibodies including phospho-Stat5 (1/50) and T-Stat5 (1/100) overnight at 4°C, followed by incubation with FITC-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 60 minutes. Images were visualized with a Zeiss LSM-510 confocal microscope (Carl Zeiss, Advanced Imaging Microscopy, Germany).

Kidney immunohistochemistry

Four μm sections of paraffin embedded kidneys were made and subjected to immunohistochemistry. Rodent kidneys were permeabilized in Triton X-100 (0.1% in PBS, 3 min), and antigens were retrieved, and blocked. Specimens were incubated with primary antibodies overnight at 4°C, followed by incubation with variable fluoresce-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 60 minutes. Primary antibodies used in this study were rat monoclonal antibody for Klotho (KM2076) (1:250),¹⁰ phospho-Erk (1/50), T-Erk (1/100), phospho-Jak2 (1/50), and T-Jak2 (1/100). Rhodamine-phalloidin (1:50) (Molecular Probes, Eugene, OR) or DAPI was applied for staining β -actin filaments and for staining nuclei respectively. Sections were visualized with a Zeiss LSM-510 laser scanning microscope (Carl Zeiss, Advanced Imaging Microscopy, Germany).

Immunoprecipitation and immunoblot

Immunoprecipitation of HA-EpoR protein from BaF-HA-EpoR cell lysates and immunoblotting was performed as described previously.^{3,4} Briefly, human kidney tissues, rodent kidney tissues, or NRK cells were homogenized in RIPA buffer [150 mM NaCl, 50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS] containing fresh phosphatase inhibitors and protease inhibitors and cleared by centrifugation (14,000xg, 4°C, 30 min), and protein content was determined by the method of Bradford. Sixty μg proteins were loaded onto SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in nonfat milk, PVDF membranes were probed with primary antibodies overnight at 4°C followed by secondary antibodies conjugated with horseradish peroxidase and signal was visualized by enhanced chemi-luminescence (Amersham Life Sciences). Protein abundance was quantified by densitometry using the Scion/NIH Image J software (Scion, Frederick, MD).

REFERENCES

1. Kurosu H, Yamamoto M, Clark JD, *et al.* Suppression of aging in mice by the hormone Klotho. *Science* 2005; **309**: 1829-1833.
2. Kuro-o M, Matsumura Y, Aizawa H, *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45-51.
3. Hu MC, Shi M, Zhang J, *et al.* Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. *Kidney Int* 2010; **78**: 1240-1251.
4. Hu MC, Shi M, Zhang J, *et al.* Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J* 2010; **24**: 3438-2450.
5. Fuster DG, Zhang J, Shi M, *et al.* Characterization of the sodium/hydrogen exchanger NHA2. *J Am Soc Nephrol* 2008; **19**: 1547-1556.
6. F.A. F, Sidhu SS. Making antibodies in bacteria. *In Making and using antibodies Edited by G C Howard & M R Kaser E: CRC Press* 2007: 157-180.
7. Fellouse FA, Esaki K, Birtalan S, *et al.* High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries. *J Mol Biol* 2007; **373**: 924-940.
8. Bulut GB, Sulahian R, Ma Y, *et al.* Ubiquitination regulates the internalization, endolysosomal sorting, and signaling of the erythropoietin receptor. *J Biol Chem* 2011; **286**: 6449-6457.
9. Hu MC, Shi M, Zhang J, *et al.* Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2011; **22**: 124-136.
10. Kato Y, Arakawa E, Kinoshita S, *et al.* Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. *Biochem Biophys Res Commun* 2000; **267**: 597-602.