Potent Therapy and Transcriptional Profile of Combined Erythropoietin-Derived Peptide Cyclic Helix B Surface Peptide and Caspase-3 siRNA against Kidney Ischemia/Reperfusion Injury in Mice

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ABSTRACT

Cause-specific treatment and timely diagnosis are still not available for acute kidney injury (AKI) apart from supportive therapy and serum creatinine measurement. A novel erythropoietin-derived cyclic helix B surface peptide (CHBP) protects kidneys against AKI with different causes, but the underlying mechanism is not fully defined. Herein, we investigated the transcriptional profile of renoprotection induced by CHBP and its potential synergistic effects with siRNA targeting caspase-3, an executing enzyme of apoptosis and inflammation (CASP3siRNA), on ischemia/reperfusion (IR)-induced AKI. Utilizing a mouse model with 30-minute renal bilateral ischemia and 48-hour reperfusion, the renoprotection of CHBP or CASP3siRNA was demonstrated in renal function and structure, active caspase-3 and HMGB1 expression. Combined treatment of CHBP and CASP3siRNA further preserved kidney structure and reduced active caspase-3 and HMGB1. Furthermore, differentially expressed genes (DEGs) were identified with fold change >1.414 and $P < 0.05$. In IR kidneys, 281 DEGs induced by CHBP were mainly involved in promoting cell division and improving cellular function and metabolism (upregulated signal transducer and activator of transcription 5B and solute carrier family 22 member 7). The additional administration of CASP3siRNA caused 504 and 418 DEGs in IR + CHBP kidneys with or without negative control small-interfering RNA, with 37

genes in common. These DEGs were associated with modulated apoptosis and inflammation (upregulated BCL6, SLPI, and SERPINA3M) as well as immunity, injury, and microvascular homeostasis (upregulated complement factor H and GREM1 and downregulated ANGPTL2). This proof-of-effect study indicated the potent renoprotection of CASP3siRNA upon CHBP at the early stage of IR-induced AKI. Underlying genes, BCL6, SLPI, SERPINA3M, GREM1, and ANGPTL2, might be potential new biomarkers for clinical applications.

SIGNIFICANCE STATEMENT

It is imperative to explore new strategies of cause-specific treatment and timely diagnosis for acute kidney injury (AKI). CHBP and CASP3siRNA synergistically protected kidney structure after 48-hour ischemia/reperfusion-induced AKI with reduced injury mediators CASP3 and high mobility group box 1. CHBP upregulated cell division-, function-, and metabolism-related genes, whereas CASP3siRNA further regulated immune response- and tissue homeostasis-associated genes. Combined CHBP and CASP3siRNA might be a potent and specific treatment for AKI, and certain dysregulated genes secretory leukocyte peptidase inhibitor and SERPINA3M could facilitate timely diagnosis.

Introduction

Acute kidney injury (AKI) is a public health problem and has attracted much attention in recent years (Mehta et al., 2016). Worldwide, AKI affects about 2% of patients in hospital admissions, with a rate of mortality of about 12%, both of

which were increased to around 20% in the intensive care unit (Bouchard et al., 2015; Yang et al., 2015d). There is no specific treatment for AKI apart from passive support or renal replacement therapy, such as volume control or dialysis in clinic (Moore et al., 2018). It is urgent, therefore, to develop specific and effective treatment for AKI to reduce mortality and prevent its progression to chronic kidney disease (Mehta et al., 2015; Noble et al., 2020).

Renal ischemia/reperfusion (IR) injury is a major cause of AKI, characterized by apoptosis, inflammation, and immune response-associated damage (Bellomo et al., 2012; Dong et al., 2019). Recently, the innate repair mechanism in AKI has attracted great attention, which is highlighted by an innate

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repair receptor, a heterodimer of erythropoietin (EPO) receptor and β common receptor (EPOR/ β cR) (Brines and Cerami 2012). EPO, a natural ligand of EPOR/ β cR, is defective in tissue protection because of low affinity but has high affinity to a homodimer receptor (EPOR)₂ in erythropoiesis (Gobe et al., 2014; Wang et al., 2017; Shi et al., 2018). EPO-derived helix B surface peptide (HBSP) and cyclic HBSP [CHBP, more stable and potent than HBSP (Yang et al., 2014a)] only bind to EPOR/ β cR, and remain the tissue protective property but without erythropoiesis, so have promising potential for clinical application (Brines et al., 2008; Patel et al., 2012; Wu et al., 2013; Yang et al., 2013). In the IR kidney, CHBP reduces endoplasmic reticulum stress (Zhang et al., 2020) and increases autophagy (Yang et al., 2014a), leading to less apoptosis (Kaushal and Shah 2016). CHBP also ameliorated renal inflammation and reduced chronic deposition of extracellular matrix through inactivating forkhead box O 3a after IR (Yang et al., 2015a). Nevertheless, the exact underlying mechanism in the renoprotection of CHBP is incompletely understood.

Caspase-3, upregulated by IR in the kidney, is a major effector enzyme in the process of apoptosis as well as inflammation (Yang et al., 2011a; Li et al., 2019). Evidence suggests that downregulating the expression of active caspase-3 is presented by HBSP/CHBP treatment in IR kidneys (Yang et al., 2013, 2015b). The contributing role of caspase-3 in IR kidneys was further verified by small-interfering RNA (siRNA), showing that serum-stabilized siRNA targeting caspase-3 greatly reversed renal function and inflammation in a 2-week porcine kidney autotransplantation model (Yang et al., 2014b). It is also intriguing to discover whether there are synergistic effects on IR-induced AKI by combined administration of HBSP/CHBP and caspase-3 siRNA (CASP3siRNA).

In the present study, the effect of CHBP was explored by a single peritoneal injection as well as its cotreatment with CASP3siRNA injected via the tail vein in a 48-hour mouse renal IR model. To delineate the possible mechanisms of single/simultaneous administration, the modern technology of transcriptomic microarray analysis was also used to disclose a transcriptional overview in an array of genes and their biologic involvements.

Materials and Methods

CHBP. The sequence of CHBP was the same with HBSP, QEQ-LERALNSS, and it was thioether-cyclized (molecule weight 1416.7). The detailed structure of CHBP was described previously, which was designed and synthesized by Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China (Yang et al., 2014a).

Caspase-3 siRNA. CASP3siRNA, targeting murine caspase-3 mRNA (NCBI CoreNucleotide Accession No. BC038825), were synthesized (Life Technologies, Paisley, UK). The sequences of the CASP3siRNA (Ambion In Vivo, catalog number: 4457309) were sense 5'-CCUGGUUACUAUCCUGGAtt-3' and antisense 5'-UCCAGGAAUAGUACCAGGtg-3'. The negative control siRNA (NCsiRNA) was also provided by Life Technologies (Ambion In Vivo, catalog number: 4457289), with sequences of sense 5'-UAACGACGCGACGACGUAAtt-3' and antisense 5'-UUACGUCGUCGUCGUUAtt-3'. Both

CASP3siRNA and NCsiRNA were chemically modified by locked nuclei acid.

Renal IR Surgery. Male C57BL/6 mice, 8–12 weeks, were purchased from the Experimental Animal Center of Yangzhou University, China. All animal experiments were performed according to the guidelines of the Laboratory Animal Monitoring Committee of Jiangsu Province.

The renal IR surgical procedures were performed under general anesthesia by intraperitoneal injection of pentobarbital sodium at 75 mg/kg body weight (BW). Bilateral kidneys were exposed via dorsal incisions sequentially, and the renal pedicle was carefully isolated and occluded using a nontraumatic vascular clamp for 30 minutes. The efficacy of occlusion was confirmed by the color change of kidney surface, eventually, to dark red. Followed by removing the clamps, patched blanching appeared to the kidney surface and then normal pink, indicating blood reperfusion. Sham operation was performed in a similar manner, except with clamping of renal pedicles. Mice were randomly divided into seven groups ($n = 6$) as follows: 1) sham, 2) IR, 3) IR + CASP3siRNA, 4) IR + NCsiRNA, 5) IR + CHBP, 6) IR + CHBP + CASP3siRNA, and 7) IR + CHBP + NCsiRNA. Having six animals in each group was determined using power calculation according to the change of the key parameter in our previous IR time course model (Zhang et al., 2020) and CHBP intervention study (Yang et al., 2014a). The experimental design was shown in Fig. 1A. A total of 0.03 mg/kg BW of siRNA (dissolved in saline) was injected into the tail vein 2 hours before surgery. Twenty-four nanomoles per kilogram BW of CHBP (dissolved in saline) was given through intraperitoneal injection at 15 minutes after clamps were released.

Sample Collection. At 48 hours of renal IR injury, animals were anesthetized with pentobarbital sodium, followed by cardiac puncture for drawing whole blood. The serum sample from each animal was then obtained by centrifuging at 10,000 rpm for 15 minutes and stored individually at -80°C . Kidneys were removed and transversally cut at the midplane, following crosscutting from the middle. One quarter of each kidney was fixed in 10% neutral formalin for 24 hours, whereas two quarters were rapidly frozen in liquid nitrogen, and the fourth part was preserved in RNAlater (Life Technologies).

Biochemistry Analysis. The serum creatinine (SCr) level of each animal was determined using a QuantiChrom Creatinine Assay Kit separately (BioAssay Systems, Hayward). Briefly, 30 μl of standard or sample serum was transferred into a 96-well plate followed by adding in 200 μl working reagent per well, a mixture of reagent A and B. Absorbance at 510 nm was read immediately and 5 minutes later. Calculation was performed according to the manufacturer's instruction. The detection was performed three times independently.

Histologic Assessment. Hematoxylin & eosin (H&E) staining of kidney tissues was performed to observe and evaluate the degree of tubulointerstitial damage (TID) in the cortex using a scoring system by assessing tubular damage (degeneration and detachment from basement membrane), interstitial expansion (edema or inflammatory cell infiltration), and dilation of tubular lumina. Histologic changes were graded based on the percentage of damaged area involved: <5% area was scored 0, 5%–25% area was scored 1, 25%–50% area was scored 2, 50%–75% area was scored 3, and area exceeding 75% was scored 4. Kidney sections were blindly reviewed by two researchers independently. The scores from three compartments (tubular and interstitial areas, tubular lumina) of each kidney were obtained from 12 fields at 200 magnifications. The average scores per field of three compartments were then summed up for each kidney. The final score

ABBREVIATIONS: AEC, 3-amino-9-ethylcarbazole; AKI, acute kidney injury; Angptl2, Angiotensin-like 2; Bcl6, B cell leukemia/lymphoma 6; BW, body weight; CASP3siRNA, caspase-3 small-interfering RNA; CFH, complement factor H; CHBP, cyclic helix B surface peptide; DEG, differentially expressed gene; EPO, erythropoietin; EPOR/ β cR, erythropoietin receptor and β common receptor; FC, fold change; GO, Gene Ontology; GREM1, gremlin 1; HBSP, helix B surface peptide; H&E, hematoxylin & eosin; HMGB1, high mobility group box 1; IR, ischemia/reperfusion; ISEL, in situ end-labeling; LSD, least significant difference; NCsiRNA, negative control small-interfering RNA; qPCR, quantitative polymerase chain reaction; SCr, serum creatinine; siRNA, small-interfering RNA; SLC22A7, solute carrier family 22 member 7; SLPI, secretory leukocyte peptidase inhibitor; TEC, tubular epithelial cell; TID, tubulointerstitial damage.

Fig. 1. CHBP and/or CASP3siRNA preserved renal function and structure in IR kidneys. (A) Schematic diagram of mouse renal IR models with the treatment of CHBP and/or CASP3siRNA. Bilateral kidney pedicles were occluded for 30 minutes, followed by 48-hour reperfusion. CASP3siRNA or NCsiRNA was injected via the tail vein at a dose of 0.03 mg/kg BW 2 hours before surgery. CHBP was given through intraperitoneal cavity at 24 nmol/kg BW 15 minutes postreperfusion. (B) The level of SCr was demonstrated for each group. There were six animals in each group, and the detection was repeated for three times independently. The experiment was performed three times independently. (C) Representative photomicrographs of H&E staining in renal cortex were shown for each group. Scale bar, 100 μ m. (D) Semiquantitative analysis of TID score ($n = 6$). The sections were blindly scored by two researchers independently. Data were shown as means $\pm$ S.D. $n = 6$ animals per group; * $P < 0.05$; ** $P < 0.01$. Statistical comparisons were calculated by ANOVA followed by post hoc LSD test.

for the animal was then calculated by averaging the scores from left and right kidneys.

In Situ End-Labeling of Apoptotic Cells. Apoptotic cells were detected using a TUNEL Apoptosis Detection Kit (Millipore, MA) by in situ end-labeling (ISEL), as previously described (Wu et al., 2013). Paraffin-embedded kidney sections were dewaxed and digested by proteinase K at 20 µg/ml for 10 minutes at 37°C. The sections were then applied with equilibration buffer, terminal deoxynucleotidyl transferase, and anti-digoxigenin-peroxidase sequentially. The labeling of apoptotic cells was then revealed with 3-amino-9-ethylcarbazole (AEC, dark red color). Apoptotic cells were examined at 400 magnifications in up to 20 fields of tubulointerstitial areas in the cortex. The number of positively stained cells in each animal was calculated by averaging the average number per field from left and right kidneys. This was blindly reviewed by two researchers independently.

Immunostaining of Active Caspase-3 in Kidneys. Active 17 kDa subunit of caspase-3 was stained on kidney paraffin sections using the method described before (Yang et al., 2011b). Briefly, sections were dewaxed, and antigen retrieval was performed before incubation with a rabbit-anti-mouse 17 kDa caspase-3 antibody (1:100 dilution; R&D System, Abingdon, UK). For negative control, normal rabbit immunoglobulin G was applied at the same concentration of primary antibody. Seventeen kilodalton caspase-3+ cells were counted at 400 magnifications in up to 20 cortical fields of each kidney blindly by two researchers independently. The number of apoptotic cells for each animal was obtained by averaging the numbers from all fields in both kidneys.

Western Blot Analysis. Twenty-five micrograms of kidney homogenate was separated in reduced SDS-PAGE gels and electroblotted onto a polyvinylidene fluorid membrane. The membrane was then blocked in 5% (weight/volume) nonfat milk, followed by probing with an anti-full length caspase-3 antibody (CST, Danvers, MA) at 1:400 dilution, an anti-high mobility group box 1 (HMGB1) antibody (CST) at 1:1000, or an anti-β-actin antibody (Abcam, Cambridge, UK) at 1:8000 dilution for overnight at 4°C. The corresponding secondary antibody (Jackson ImmunoResearch Laboratories, West Grove) was then applied to the membrane for 2 hours at room temperature. Afterward, antibody binding was revealed using enhanced chemiluminescence substrate (Thermo Scientific, Waltham, MA) and a Molecular Imager Chemi Doc XRS + system (Bio-Rad, Berkeley). The experiment was performed three times independently.

Microarray Analysis. For the kidney stored in RNAlater, microarray analysis was performed to reveal the profile of whole genomic transcripts by Shanghai Biotechnology Corporation, China. The detection was done in four groups ($n = 3$): IR, IR + CHBP, IR + CHBP + CASP3siRNA, and IR + CHBP + NCsiRNA. The chosen kidney tissue samples from three animals in each group were nearest to the average level of the group in renal function and structure. The total 12 kidney samples were analyzed individually. The RNA integrity and quantity were monitored by the 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop One (Thermo Scientific), respectively. Two micrograms RNA with an Integrity Number of no less than eight was required for the genomic profile analysis. The Agilent Whole Mouse Genome Oligo Microarray was applied to interrogate about 41,174 transcripts targeting 34,000 well established annotated genes. The criteria of fold change (FC) > 1.414 (upregulated genes) or FC < -1.414 (downregulated genes) and $P < 0.05$ was used for sorting significant differentially expressed genes (DEGs). The cutoff value of FC was based on the fact that 0.5 cycle was the minimum number of polymerase chain reaction (PCR) cycle to distinguish the expressional differences between two samples.

Validation of Candidate DEGs by Quantitative PCR. Total RNAs were extracted by Trizol reagent from the kidney tissues of the same animals selected for microarray analysis. One microgram total RNA was used for reverse transcription in a 20-µl reaction system supplemented with 4 µl of 5x HiScript II qRT SuperMix and RNase-free water using a kit of HiScript II Q RT SuperMix for quantitative PCR (qPCR) (Vazyme, Nanjing, China). The temperature setting was

50°C for 15 minutes, followed by 85°C for 2 minutes. One microliter of cDNA product was amplified within an SYBR reaction system (Bioline, London, UK) containing 200 nM forward and reverse primers (Table 1, Biomix, Nantong, China) at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 55°C for 60 seconds. The level of β-actin mRNA was used as an endogenous control.

Gene Function Analysis. Functional enrichment analysis of significant DEGs identified between groups was performed using Gene Ontology (GO; <http://geneontology.org/>) (Harris et al., 2004). The resulting GO terms with P values less than 0.05 were considered significantly enriched.

Statistical Analysis. Data were expressed as mean ± S.D. The statistical analyses of the data were performed using IBM SPSS Statistics version 26.0 software. One-way ANOVA analysis was used to check the homogeneity of variance, and then post hoc least significant difference (LSD) test was used for multiple comparisons. The data of qPCR were analyzed using a two-tailed unpaired Student's t test between two groups. Statistical significance was defined as $P < 0.05$.

Results

Improved Kidney Function and Structure. At 48 hours, the SCr level raised by IR was significantly reduced by the treatment of CHBP (1.27 ± 0.09 vs. 0.78 ± 0.23 , $P < 0.01$), CASP3siRNA (1.27 ± 0.09 vs. 0.86 ± 0.12 , $P < 0.01$), or CHBP + CASP3siRNA (1.27 ± 0.09 vs. 0.88 ± 0.12 , $P < 0.01$, Fig. 1B). However, no significant difference was observed among these treatments. IR mice treated with CASP3siRNA or CHBP + CASP3siRNA demonstrated a significantly lower SCr level than those treated with NCsiRNA (0.86 ± 0.12 vs. 1.30 ± 0.71 , $P < 0.01$) or CHBP + NCsiRNA (0.88 ± 0.12 vs. 1.16 ± 0.20 , $P < 0.05$), respectively.

Representative images for H&E staining were chosen to show the damage in different parts of the cortex, such as the tubular area, the tubular lumen, and the interstitial area (Fig. 1C). CHBP, CASP3siRNA, and CHBP + CASP3siRNA treatment significantly decreased the extent of TID in IR kidneys (2.72 ± 0.21 or 2.49 ± 0.30 or 1.43 ± 0.34 vs. 5.85 ± 0.56 , all $P < 0.01$, Fig. 1D). Furthermore, IR mice with cotreatment of CHBP and CASP3siRNA exhibited a lower level of TID in contrast to those treated with CHBP only or CASP3siRNA only (1.43 ± 0.34 vs. 2.72 ± 0.21 or 2.49 ± 0.30 , both $P < 0.05$). In addition, CASP3siRNA-treated IR or IR + CHBP mice demonstrated a significant decrease of TID compared with corresponding NCsiRNA controls (2.49 ± 0.30 vs. 6.22 ± 1.83 ; 1.43 ± 0.34 vs. 3.74 ± 0.58 , both $P < 0.01$).

Alleviated Apoptosis. As antiapoptosis is a shared renoprotective effect used by CHBP and CASP3siRNA, immunolabeling was performed to examine their effectiveness on reducing kidney IR-induced apoptosis. Representative images of apoptotic cells were chosen to present the morphology and localization of apoptotic cells (Fig. 2A). IR significantly raised the number of ISEL+ cells compared with sham controls (0.72 ± 0.05 vs. 0.05 ± 0.01 , $P < 0.01$) but greatly lowered by CHBP, CASP3siRNA, and CHBP + CASP3siRNA (0.72 ± 0.05 vs. 0.25 ± 0.06 or 0.22 ± 0.06 or 0.21 ± 0.05 , all $P < 0.01$, Fig. 2B). Nevertheless, comparable levels of ISEL+ cells were found among these treatments. In contrast to NCsiRNA controls, CASP3siRNA reduced apoptosis in either IR kidneys (0.77 ± 0.22 vs. 0.22 ± 0.06 , $P < 0.01$) or CHBP-modified IR kidneys (0.41 ± 0.09 vs. 0.21 ± 0.05 , $P < 0.01$).

Decreased Active Caspase-3 Staining Positive Cells. Cells labeled with active 17 kDa caspase-3 often having the

TABLE 1
The sequence of primers for qPCR

Gene Symbol	Full Name	Primers (5'-3')
SLC22A7	Solute carrier family 22 member 7	Forward: CTGTCTGCCTGTGTTTATCC Reverse: CTCCCCAAATGCCACAGCT
CFH	Complement factor H	Forward: ACTTTCTCAGATTTTCCTGG Reverse: TGGTTGTTACATGCTTTGGG
ANGPTL2	Angiopoietin-like 2	Forward: GGATGGTTCACAGAGAGAGTAC Reverse: CTCCTTGGAGTTGACACAAATG
GREM1	Gremlin-1	Forward: GCAAGTATCTGAAGVGAGATTG Reverse: CGTCATGGTGGTGAACCTTCTTG
ACTB	β -actin	Forward: GAGACCTTCAACACCCGAGC Reverse: ATGTCACGCACGATTTCCC

morphologic features of apoptosis as condensed nuclei were mainly located in tubular epithelia, tubular lumina, and interstitial areas (Fig. 3A). The number of active caspase-3+ cells was significantly increased by IR (0.66 ± 0.15 vs. 0.01 ± 0.02 , $P < 0.01$) but decreased by CHBP, CASP3siRNA, and CHBP + CASP3siRNA (0.66 ± 0.15 vs. 0.13 ± 0.03 or 0.32 ± 0.15 or 0.17 ± 0.06 , $P < 0.05$ or 0.01 , Fig. 3B). No significant differences between these treatments were observed. Comparing with NCsiRNA, CASP3siRNA significantly reduced the number of 17 kDa caspase-3+ cells in IR kidneys (1.07 ± 0.51 vs. 0.32 ± 0.15 , $P < 0.01$).

Decreased Expression of Active Caspase-3 and HMGB1 Protein. Western blotting was used to determine whether there is a coefficient of CHBP and CASP3siRNA on the expression of 17 kDa active caspase-3. The level of 17 kDa caspase-3 was significantly increased by IR (0.90 ± 0.34 vs. 0.57 ± 0.23 , $P < 0.05$) but reduced by CHBP, CASP3siRNA, and CHBP + CASP3siRNA (0.90 ± 0.34 vs. 0.40 ± 0.12 or 0.52 ± 0.19 or 0.29 ± 0.08 , $P < 0.01$ or 0.05 , Fig. 4, A and B). IR mice with the cotreatment of CHBP and CASP3siRNA showed an even lower expression of 17 kDa caspase-3 compared with IR mice treated with CASP3siRNA (0.29 ± 0.08 vs. 0.52 ± 0.19 , $P < 0.05$). Controlled by

Fig. 2. CHBP and/or CASP3siRNA ameliorated apoptosis in tubulointerstitial areas. (A) The method of ISEL fragmented DNAs was used to detect apoptotic cells in kidney tissues. AEC was used to develop color labeling. Representative photomicrographs of apoptotic cells (indicated by arrows) in cortical areas were shown in each group. Scale bar, 50 μ m. (B) The average number of ISEL+ cells per field are demonstrated for each group ($n = 6$). The sections were blindly reviewed by two researchers independently. Data were shown as means $\pm$ S.D. $n = 6$ animals per group; $*P < 0.05$; $**P < 0.01$. Statistical comparisons were calculated by ANOVA followed by post hoc LSD test.

Fig. 3. CHBP and/or CASP3siRNA decreased the number of active 17 kDa caspase-3-positive cells. (A) Representative photomicrographs of cells positively stained with 17 kDa caspase-3+ were shown in the indicated groups. Detected by immunostaining, 17 kDa caspase-3 was labeled and revealed by AEC. Scale bar, 50 μ m. (B) The average number of 17 kDa caspase-3+ cells per field are demonstrated in each group ($n = 6$). The sections were blindly reviewed by two researchers independently. Data were shown as means $\pm$ S.D. $n = 6$ animals per group; * $P < 0.05$; ** $P < 0.01$. Statistical comparisons were calculated by ANOVA followed by post hoc LSD test.

NCsiRNA, 17 kDa caspase-3 was reduced by CASP3siRNA in IR + CHBP kidneys (0.59 ± 0.23 vs. 0.29 ± 0.08 , $P < 0.05$).

HMGB1 is a proinflammatory factor known to arouse profound innate responses by binding to toll-like receptor 4 on surface of tubular epithelial cells (TECs) and macrophages (Wu et al., 2010; Chen et al., 2017). Western blotting using kidney homogenates demonstrated IR significantly increased HMGB1 expression compared with sham controls (3.21 ± 1.41 vs. 1.51 ± 0.67 , $P < 0.01$, Fig. 4, C and D). However, treatments with CHBP, CASP3siRNA, and CHBP + CASP3siRNA decreased the high level of HMGB1 (1.09 ± 0.63 or 1.57 ± 0.59 or 0.81 ± 0.40 vs. 3.21 ± 1.41 , all $P < 0.01$). Moreover, IR mice with cotreatment of CHBP and CASP3siRNA exhibited an even lower level of renal HMGB1 compared with CASP3siRNA-treated animals (0.81 ± 0.40 vs. 1.57 ± 0.59 , $P < 0.05$). In contrast to NCsiRNA, CASP3siRNA reduced HMGB-1 expression in IR kidneys (4.65 ± 0.86 vs. 1.57 ± 0.59 , $P < 0.01$) as well as in IR + CHBP kidneys (2.63 ± 1.26 vs. 0.81 ± 0.40 , $P < 0.01$).

Identification of Differentially Expressed Genes and Revalidation. To disclose the mechanism of renoprotection induced by CHBP and/or CASP3siRNA, transcriptomic microarray analysis was conducted to identify DEGs affected in the IR kidneys. The three chosen samples from each group could best represent biochemistry and pathologic changes in the group.

Two hundred eighty-one DEGs (153 upregulated, 128 downregulated) were identified in the CHBP-treated IR kidneys versus IR kidneys (Fig. 5A). Four hundred eighteen DEGs (226 upregulated, 192 downregulated) were shown by the additional administration of CASP3siRNA to CHBP-treated IR kidneys versus IR + CHBP kidneys, with 46 genes in common with the comparison of IR + CHBP versus IR groups. In contrast to the NCsiRNA treatment to IR + CHBP kidneys, CASP3siRNA produced 504 DEGs (218 upregulated, 286 downregulated) in IR + CHBP kidneys, of which nine genes were commonly altered with the IR + CHBP kidneys versus IR kidneys and 37 genes in common with the comparison of IR + CHBP + CASP3siRNA versus IR + CHBP. Among the above three comparisons, there were only three genes affected universally. The top five genes upregulated and downregulated in three comparisons are listed (Tables 2–4). Among DEGs, upregulated BCL6 was associated with the negative regulation of apoptosis (Table 2), upregulated secretory leukocyte peptidase inhibitor (SLPI) and SERPINA3M were related to inflammation (Table 3), and upregulated GREM1 and downregulated ANGPTL2 were linked to injury, inflammation, and microvascular homeostasis (Table 4).

To validate the outcome of microarray analysis, four DEGs were selected for qPCR detection: upregulated SLC22A7 by CHBP compared with the IR group (FC = 2.996), associated

Fig. 4. CHBP and/or CASP3siRNA decreased 17 kDa active caspase-3 and HMGB1 expression in IR kidneys. (A) The level of active caspase-3 was measured by Western blotting and typical bands were shown. (B) Semiquantitative analysis showed the expression of 17 kDa caspase-3 corrected by the endogenous control of β -actin in each group ($n = 6$). (C) Representative bands of HMGB1 were shown. (D) The level of HMGB1 protein corrected by β -actin was determined in each group ($n = 6$). The immunoblotting was performed three times independently. Data were shown as means $\pm$ S.D. $n = 6$ animals per group; * $P < 0.05$; ** $P < 0.01$. Statistical comparisons were calculated by ANOVA followed by post hoc LSD test.

with the epithelial function of organic anion transport; upregulated CFH by CASP3siRNA compared with NCsiRNA (FC = 1.949), a negative regulator in the alternative pathway of complement activation; and ANGPTL2 and GREM1 as described above. qPCR results showed that the level of

SLC22A7 was greatly upregulated by CHBP (Fig. 5B); CFH and GREM1 were increased (Fig. 5, C and D), but Angptl2 was decreased by CASP3siRNA compared with NCsiRNA (Fig. 5E). Thus, all results from qPCR were consistent with the output of microarray data.

Fig. 5. Identified DEGs and qPCR validation. (A) Venn diagram illustrating the number of significant DEGs in the three comparisons. The sorting criteria was $FC > 1.414$ and $P < 0.05$. In four groups ($n = 3$), each kidney sample was analyzed separately. (B–E) The expression of SLC22A7, CFH, GREM1, and ANGPTL2 mRNA was detected by qPCR, which was done three times independently. $n = 3$ kidney samples from individual animals in each group; $*P < 0.05$. Statistical comparisons were calculated by two-tailed unpaired Student's t test.

GO Analysis of the DEGs. The identified DEGs were subjected to GO functional enrichment analysis to elucidate biologic processes altered by CHBP and/or CASP3siRNA in the IR kidneys at 48 hours. Top 30 items of biologic process ($P < 0.05$) with enrich factors are presented (Fig. 6). DEGs induced by CHBP were mainly involved in positive regulation of mitotic cell cycle, regulation of protein tyrosine kinase activity, acyl-CoA/glucose/cholesterol metabolic processes, positive regulation of cellular component biogenesis, and organic anion transport (Fig. 6A). For instance, CHBP upregulated signal

transducer and activator of transcription 5B ($FC = 1.478$), a positive regulator of mitotic cell cycle, and SLC22A7, mediating organic anion transport, and also a positive regulator of cellular component biogenesis and glucose metabolic process. Further altered genes by CASP3siRNA treatment in IR + CHBP kidneys versus IR + CHBP were involved in the negative regulation of immune response (Fig. 6B). Compared with the NCsiRNA control, CASP3siRNA further affected biologic processes, including regulation of interleukin-1- β production, positive regulation of cAMP metabolic process, positive regulation of

TABLE 2

IR + CHBP vs. IR, top five upregulated and downregulated genes

The gene in italic was discussed for its potential function in IR-induced AKI. $P < 0.05$.

Accession Number Upregulated	Gene Symbol	Gene Name	Fold Change
AK016105	4930553I04RIK	RIKEN cDNA 4930553I04 gene	8.559
AK046501	SACS	Saccin	5.261
NM_013501	CRYAA	Crystallin, alpha A	4.357
NM_011255	RBP4	Retinol binding protein 4, plasma	4.213
NM_009744	<i>BCL6</i>	<i>B cell leukemia/lymphoma 6</i>	3.622
Downregulated			
NM_001167777	ASXL3	Additional sex combs like 3	−24.005
NM_029070	CLDN26	Claudin 26	−5.985
NM_001167746	DNAH17	Dynein, axonemal, heavy chain 17	−5.922
AK171340	PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	−5.113
NM_007501	NEUROD4	Neurogenic differentiation 4	−5.094

TABLE 3

IR + CHBP + CASP3siRNA vs. IR + CHBP, top five upregulated and downregulated genes

The genes in italic were discussed for their potential function in IR-induced AKI. $P < 0.05$.

Accession Number Upregulated	Gene Symbol	Gene Name	Fold Change
NM_001001450	SSXB2	Synovial sarcoma, X member B, breakpoint 2	14.730
NR_028111	4930523C07RIK	RIKEN cDNA 4930523C07 gene	10.131
NM_011414	<i>SLPI</i>	<i>Secretory leukocyte peptidase inhibitor</i>	8.478
NM_009253	<i>SERPINA3M</i>	<i>Serine (or cysteine) peptidase inhibitor, clade A, member 3M</i>	8.289
NM_175309	UPK3B	Uroplakin 3B	5.483
Downregulated			
AK016105	4930553I04RIK	RIKEN cDNA 4930553I04 gene	-8.735
NM_029747	2410137M14RIK	RIKEN cDNA 2410137M14 gene	-5.134
NM_147025	OLFR380	Olfactory receptor 380	-4.354
NM_134160	MCOLN3	Mucolipin 3	-4.142
NM_001163513	DLG5	Disks large MAGUK scaffold protein 5	-3.700

phosphatidylinositol 3-kinase signaling, and release of cytochrome c from mitochondria (Fig. 6C).

Discussion

The present study demonstrated that a single dose of CHBP or CASP3siRNA markedly ameliorated IR-induced kidney injury in terms of preserving renal function and structure, reducing active caspase-3 and HMGB1 expression. The combination of both further decreased TID, active caspase-3, and HMGB1. In addition, genomic microarray analysis identified DEGs induced by CHBP that were mainly involved in preserving cell division, cellular function, and metabolism. DEGs modified by CASP3siRNA were associated with inhibiting inflammation and maintaining vascular function. Certain genes such as BCL6, SLPI, SERPINA3M, GREM1, and ANGPTL2 might be potential biomarkers in IR-induced AKI.

The present study demonstrated that a single dose of CHBP, with a plasma half-life of 300 minutes (Yang et al., 2014a), administered 15 minutes after reperfusion greatly ameliorated renal IR injury at the early stage of 48 hours. This result was consistent with the evidence that a single dose of CHBP protected the kidney from IR injury at 12 weeks (Yang et al., 2015a). Linear HBSP, with a plasma half-life of about 2 minutes, administered at 1, 6, and 12 hours protected the kidney against IR injury at 24 hours (Brines et al., 2008). Our previous study also showed that daily injection of HBSP protected the kidney from immunosuppressant cyclosporine A-induced damage upon IR injury but did not affect IR injury alone in a 2-week rat model (Wu et al., 2013). It has been also

reported that CHBP protected against aristolochic acid-induced AKI (Zeng et al., 2017). These data imply a variety of potential clinical applications of CHBP or HBSP.

It is the first time verifying that a single dose of CASP3siRNA was comparable to CHBP in renal protection. siRNA is a potent and specific tool that can silence detrimental genes under disease conditions, so siRNA therapy provides perspective in the development of precision medicine (Hawgood et al., 2015). Although there are over 30 siRNA-related clinical trials that have been completed, no siRNA treatment against AKI is available in clinical practice. The result from this study implies that caspase-3 gene may be one of the major affected genes by CHBP in renoprotection; therefore, CASP3siRNA might be an alternative treatment in addition to CHBP for IR-induced renal injury.

The transcriptomic profile, moreover, demonstrated that CHBP-altered genes in biologic processes were mainly linked to cell division, cellular function, and metabolism. For example, signal transducer and activator of transcription 5B upregulated by CHBP was involved in cell proliferation in rodent kidneys (Chen et al., 2007; Fragiadaki et al., 2017), whereas SLC22A7, enriched in organic anion transport, was associated with the extrusion of creatinine from TECs and maintaining SCr level (Shen et al., 2015). BCL6, among the top five DEGs upregulated by CHBP (Table 2), has a broad role on antiapoptosis and cell survival (Baron et al., 2010), promoting the expression of organic anion transporter 1 in TECs and maintaining the secreting function of TECs (Wegner et al., 2014). In addition, metabolic processes were greatly enriched by CHBP, including glucose metabolism, which is beneficial for energy production (Wei et al., 2014). It has also been

TABLE 4

IR + CHBP + CASP3siRNA vs. IR + CHBP + NCsiRNA, top five upregulated and downregulated genes

Genes in italic indicate analysis by qPCR and were discussed as biomarker candidates of IR-induced AKI. $P < 0.05$.

Accession Number Upregulated	Gene Symbol	Gene Name	Fold Change
NM_011824	<i>GREM1</i>	<i>Gremlin 1</i>	15.987
NM_001001450	SSXB2	Synovial sarcoma, X member B, breakpoint 2	14.817
NM_026433	TMEM100	Transmembrane protein 100	9.749
NM_011825	GREM2	Gremlin 2 homolog, cysteine knot superfamily (<i>Xenopus laevis</i>)	9.312
NM_007729	COL11A1	Collagen, type XI, alpha 1	8.978
Downregulated			
AK041888	<i>ANGPTL2</i>	<i>Angiopoietin-like 2</i>	-24.390
AF107847	GNAS	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	-12.195
AK081320	ZFYVE9	Zinc finger, FYVE domain containing 9	-7.576
NM_010258	GATA6	GATA binding protein 6	-6.711
NM_026648	DNAAF1	Dynein, axonemal assembly factor 1	-6.579

A

IR+CHBP (n=3) versus IR (n=3)

B

IR+CHBP+CASP3siRNA (n=3) versus IR+CHBP (n=3)

C

IR+CHBP+CASP3siRNA (n=3) versus IR+CHBP+NCsiRNA (n=3)

Fig. 6. GO analysis of DEGs in IR kidneys. The top 30 significantly enriched GO items of biologic processes were shown from three comparisons, modified by CHBP (A) and then further by CASP3siRNA (B) or NCsiRNA (C). The text on the left indicated the category of GO, and the bar chart indicated the enrich factor in each category. Underlined categories were particularly discussed in this study. $n = 3$ animals in each group.

reported that the proteome profile in IR kidneys at 48 hours changed by CHBP treatment was mainly related to the oxidative stress (Yang et al., 2015c). There may be differentiations between transcriptional and translational changes as well as the mouse strain (BALB/c) and dose of CHBP (8 nmol/kg).

Intriguingly, in contrast to single CHBP or CASP3siRNA treatment, cotreatment with CHBP and CASP3siRNA contributed to further preservation in renal structure, with lower active caspase-3 and HMGB1 in IR kidneys. The negative regulation of immune responses was also revealed by microarray analysis, verifying the effectiveness of further CASP3siRNA against renal IR. SLPI, among the top five DEGs upregulated by CASP3 siRNA (Table 3), was renoprotective in experimental ischemia AKI (Ochi et al., 2017). SLPI inhibits nuclear factor- κ B signaling pathway (Tang et al., 2020) and the maturation of interleukin-1- β (Zakrzewicz et al., 2019), and it was shown to be a biomarker candidate in AKI (Averdunk et al., 2019, 2020). In humans, SERPINA3, a member of the serpin superfamily of protease inhibitors, could limit inflammation by targeting cathepsin family (proinflammatory enzymes) (Horvath et al., 2005; Lannan et al., 2012). SERPINA3 expression was also found in rat kidneys, which can detect renal inflammation and fibrosis after IR injury and also serve as a urinary marker for early detection of AKI to chronic kidney disease transition (Sánchez-Navarro et al., 2019). Because murine SERPINA3M (FC = 8.289, Table 3) is a likely ortholog of human SERPINA3, the two proteins may have similar structural and kinetic characterization. The role of SERPINA3M in renal IR injury is worthy of further exploration.

Similar effects of renoprotection from CASP3siRNA were also revealed by comparing it with NCsiRNA control in CHBP-treated IR kidneys. Microarray data revealed that further CASP3siRNA treatment altered 418 DEGs or 504 DEGs in IR + CHBP kidneys without or with NCsiRNA controls, which was much higher than the 281 DEGs altered by CHBP in IR kidneys, with 46 or 9 genes in common, respectively. It was indicated that CASP3siRNA may have additive effects on renoprotection upon CHBP treatment. GO analysis identified the further altered DEGs by CASP3siRNA that were mainly linked to regulation of renal inflammation and programmed cell death upon CHBP compared with that of NCsiRNA. CFH, a negative regulator of complement alternative pathway that plays crucial roles in IR injury (Goetz et al., 2018), was increased by further CASP3siRNA treatment. Notably, ANGPTL2 was the top downregulated DEG by CASP3siRNA (Table 4). Less Angptl2 could contribute to the reduction of renal inflammation, as ANGPTL2 can activate resident macrophages and induce the secretion of proinflammatory cytokines (Umikawa et al., 2015; Amadatsu et al., 2016). Decreased ANGPTL2 may also ameliorate renal fibrosis in AKI-induced chronic kidney disease by depressing transforming growth factor- β signaling (Morinaga et al., 2016). In addition, GREM1 was the top upregulated DEG by CASP3siRNA (Table 4), which activates vascular endothelial growth factor receptor 2 in endothelial cells to induce angiogenesis (Ravelli et al., 2013; Ji et al., 2016). The effective repair of endothelial cells in IR-injured kidney plays essential roles in maintaining the homeostasis of microvasculature and efficient renal blood flow (Kwon et al., 2010) and subsequently ameliorating tubular damage (Cantaluppi et al., 2012). The GREM1-vascular endothelial growth factor

receptor 2 axis may be a novel therapeutic target for kidney inflammation and fibrosis (Mezzano et al., 2018). In addition, Yang et al., 2018 proposed that caspase-3 deficiency in mice reduced IR injury in kidneys through preserving microvascular density. However, whether the preservation of renal microvasculature in this study links to regulated GREM1 is worthy of further investigation. The above evidence indicates a promising strategy for silencing caspase-3 and administering CHBP at the same time for optimized outcome in improving IR injury in kidneys.

Special attention should be paid to the toxicity of NCsiRNA in the present study, which was evidenced by further elevated SCr, TID, and apoptotic levels in CHBP-modified IR kidneys. These data suggested that the synthetic siRNA duplexes may still modulate immunity and inflammation in IR kidneys, such as releasing cytokines and interferons and activating toll-like receptors on immune and nonimmune cells (Judge et al., 2005; Forsbach et al., 2008; Robbins et al., 2009). It was indicated that NCsiRNA might downregulate the influence of CHBP treatment on IR kidneys, providing an ideal and necessary control for the specific effects of CASP3siRNA in the context of CHBP-treated IR kidneys.

There are also limitations in this study. The additive renoprotective effect of CASP3siRNA upon CHBP should be further studied in the long-term prognosis of IR injury. In addition, DEGs identified by the present microarray analysis should be further validated in more comparisons with the groups, including more samples. Moreover, to select and validate potential biomarkers from identified DEGs, different downstream biologic events at the translational and post-translational level should be further investigated in terms of dynamic expression, regulation, and intervention.

The cotreatment of CHBP and CASP3siRNA exhibited synergistic effects on preserving renal structure and reducing injury markers against 48-hour renal IR in a mouse model. The DEGs induced by CHBP are associated with the preservation of cell division, function, and metabolism, whereas the DEGs caused by CASP3siRNA are linked to improving inflammation and potential microvasculature.

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Authorship Contributions

Participated in research design: B. Yang.
Conducted experiments: Wu, Chen, Zhang, Liu, C. Yang, Wang.
Performed data analysis: Wu.
Wrote or contributed to the writing of the manuscript: Wu, Zhu, Fan, B. Yang.

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