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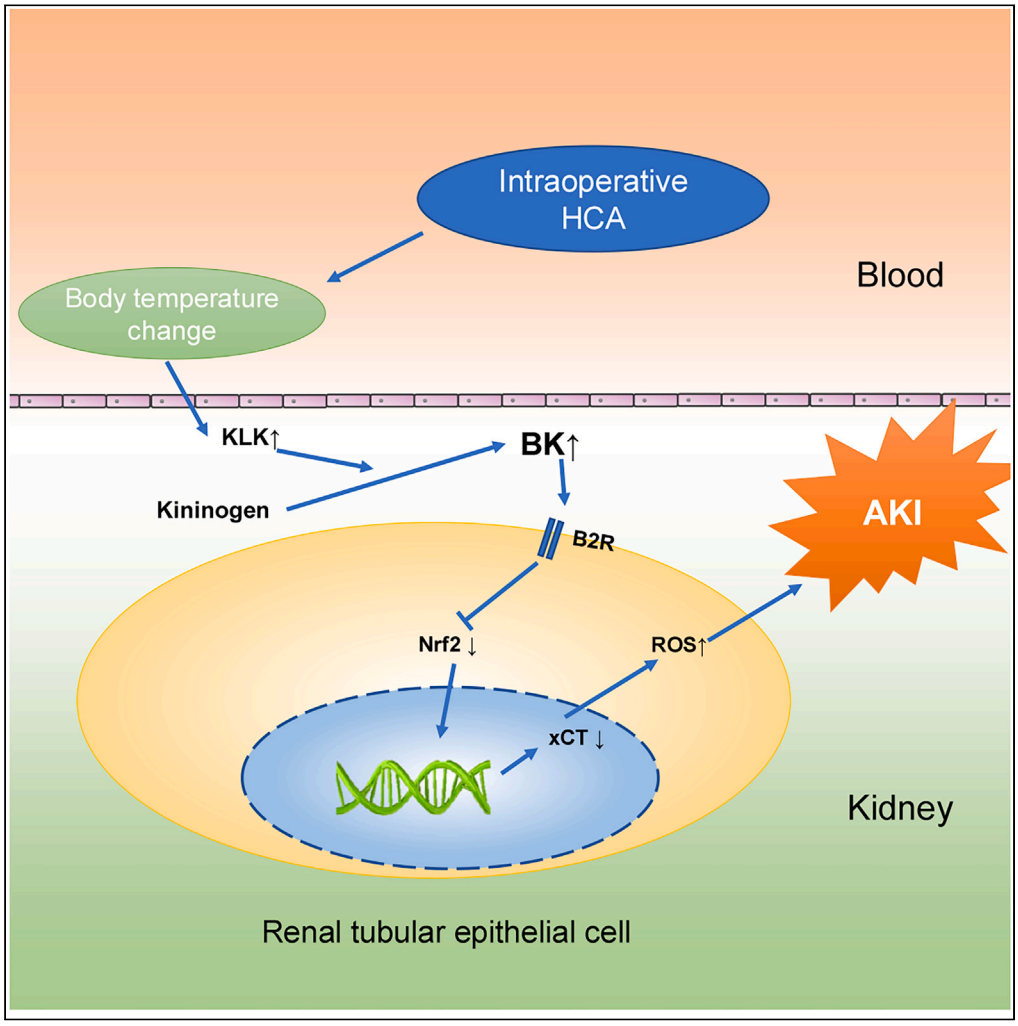

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Highlights

Hypothermia during hypothermic circulatory arrest can lead to postoperative AKI

The kallikrein-kinin system is activated due to temperature changes during HCA

Bradykinin inhibits the Nrf2-xCT pathway and causes renal oxidative stress injury

Icatibant may be used to prevent and treat AKI after surgery with HCA

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Bradykinin induces acute kidney injury after hypothermic circulatory arrest through the repression of the Nrf2-xCT pathway

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SUMMARY

Postoperative acute kidney injury (AKI) is a common complication in patients undergoing deep hypothermic circulatory arrest (HCA); however, its underlying pathogenesis is unclear. In this study, we established a rat cardiopulmonary bypass model and demonstrated that hypothermia during HCA, rather than circulatory arrest, was responsible for the occurrence of AKI. By recruiting 56 patients who underwent surgery with HCA and analyzing the blood samples, we found that post-HCA AKI was associated with an increase in bradykinin. Animal experiments confirmed this and showed that hypothermia during HCA increased bradykinin levels by increasing kallikrein expression. Mechanistically, bradykinin inhibited the Nrf2-xCT pathway through B2R and caused renal oxidative stress damage. Application of Icatibant, a B2R inhibitor, reversed changes in the Nrf2-xCT pathway and oxidative stress damage. Finally, Icatibant reversed hypothermia-induced AKI *in vivo*. This finding reveals the pathogenesis of AKI after HCA and helps to provide therapeutic strategy for patients with post-HCA AKI.

INTRODUCTION

Heart surgery-related acute kidney injury (AKI) is a common complication that can occur after cardiac surgery, and affects up to 30% of patients undergoing these procedures[1–4]; this complication worsens short- and long-term outcomes and increases morbidity and mortality rates.[5–7] Compared with other types of surgery, cardiac and aortic surgeries involving hypothermic circulatory arrest (HCA) are associated with a greater incidence of postoperative AKI,[8] the underlying mechanisms of which are complex and multifactorial. Currently, treatment for postoperative AKI is limited,[9] and there are no proven effective clinical trials or applicable drugs available to prevent or treat AKI.[10] In addition, the complex factors related to AKI are usually associated rather than causal, and there is a need to explore the mechanism of postoperative AKI and identify potential therapeutic targets for renal protection.

The pathophysiology of AKI is complex and multifactorial. Previous studies have identified renal ischemia reperfusion injury, inflammation, oxidative stress, hemolysis, and nephrotoxins as possible causes of AKI.[9] The body's inflammatory response triggered by heart surgery can contribute to kidney damage by releasing cytokines and other inflammatory mediators. In addition, the use of a cardiopulmonary bypass (CPB) procedure can activate complement and coagulation cascades, further contributing to inflammation and renal tubular cell damage. During cardiac surgery, multiple pathological factors induce tubular epithelial cell injury and the production of reactive oxygen species, which can cause oxidative stress injury to renal tubular cells.[11] Severe oxidative stress can lead to apoptosis through protein and lipid peroxidation and DNA damage, ultimately resulting in AKI.[12,13]

Circulatory arrest is an important technique that allows for certain cardiac surgeries. During this process, hypothermia is used to protect organs from ischemic damage.[14] However, a previous large cohort retrospective study from our group suggested that hypothermia during circulatory arrest procedures may be an independent risk factor for AKI.[15] Consistent with our research, independent studies by other research teams have also demonstrated an association between AKI and surgeries involving HCA.[16–19] All these studies raised concerns about the risk of HCA in the occurrence of AKI. However, a deeper understanding of the underlying mechanism, which is necessary to guide clinical decisions effectively, is lacking.

Hypothermia and circulatory arrest are two associated but independent interventions that occur during HCA and may contribute differently to the etiology of AKI. However, currently, there is a lack of a suitable *in vivo* model to study the effects of these two factors on AKI. In this

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study, we successfully established a rat model that successfully mimics the progression of surgery in humans. Using this model, we demonstrated the role of hypothermia, rather than circulatory arrest, in the occurrence of postoperative AKI. Mechanistically, elevated bradykinin, which results from a temperature reduction during HCA, suppressed the Nrf2-xCT pathway and increased oxidative stress, which led to postoperative AKI.

RESULTS

Exacerbated acute kidney injury was induced after hypothermic circulatory arrest

To investigate the underlying mechanism of renal injury associated with HCA, we established a cardiopulmonary bypass (CPB) rat model (Figure S1). First, we determined the changes in renal function 24 h after HCA in rats. The postoperative levels of serum creatinine (Scr) and blood urea nitrogen (BUN) were significantly greater in the CPB group than in the sham-operated group and further increased in the CPB with HCA group (Figures 1A and 1B). Renal HE and PAS staining were performed to validate the biochemical results. The results showed that the structure of the kidneys of the rats in the sham-operated group was normal, as indicated by structurally normal proximal and distal renal tubules with an orderly arrangement of preserved brush borders. In contrast, loss of the tubular brush border and degeneration of renal tubular epithelial cells were observed in the CPB group. Notably, it was further aggravated in the CPB with HCA group (Figures 1C–1E).

We next analyzed changes in renal apoptosis. TUNEL staining of kidney sections showed that mild apoptosis was induced in the CPB group but was significantly aggravated after CPB with HCA (Figures 1F and 1G). Western blot analysis confirmed these findings, as evidenced by a decreased ratio of Bcl-2 to Bax (Figures 1H and 1I). These results suggested that renal injury was aggravated during the HCA process.

Hypothermia during hypothermic circulatory arrest account for the occurrence of acute kidney injury

During HCA, both the circulatory arrest process and changes in body temperature are significant pathological factors that can contribute to renal injury. To further understand the impact of the circulatory arrest procedure on renal injury, we extended the duration of circulatory arrest. Plasma tests revealed that, compared to those in the HCA (circulatory arrest for 10 min) group, increased BUN levels were demonstrated in the HCA20 (circulatory arrest for 20 min) group, while the Scr levels did not significantly change. (Figures S2A and S2B). Consistently, HE and PAS staining showed no significant changes in kidney morphology (Figures S2C–S2E). In addition, no change in apoptosis was observed, as revealed by TUNEL staining (Figure S2F–S2G). In clinical aortic surgery, the duration of circulatory arrest usually does not exceed 20 min. These results suggested that the process of circulatory arrest was not the leading cause of renal injury during HCA within this time frame.

We then analyzed the effect of body temperature changes on kidney injury during HCA. The rat body temperature was lowered to 28°C or 18°C for 10 min during CPB, respectively, and then recovered to 37°C. Renal function analysis revealed that lowering the body temperature to 28°C during CPB (MH group) had no significant impact compared with the effect of CPB procedures without HCA. However, a decrease in body temperature to 18°C during CPB (DH group) caused a significant decrease in renal function, which is similar to that of HCA (Figures 2A and 2B). In addition, HE and PAS staining and TUNEL staining supported the findings of renal function analysis (Figures 2C–2G). These results suggested that hypothermia during HCA was the main cause of kidney injury.

Inflammation during hypothermic circulatory arrest does not play a major role in acute kidney injury

Given that inflammation is an important contributor to AKI via both hemodynamics-dependent and hemodynamics-independent pathways,[9] we first detected inflammatory changes in rat kidneys to explore the mechanism of hypothermia-induced AKI during HCA. We found that HCA induced an increase in renal IL-1β at the mRNA level (Figure S3A). However, it did not change the protein level (Figure S3B), and there was no obvious inflammatory cell infiltration in the kidney (Figure S3C). Moreover, the inflammatory factor IL-1β and renal inflammatory cell infiltration did not significantly change with changes in body temperature or the duration of circulatory arrest (Figures S3C–S3E).

We collected human blood samples for analysis to further explore the changes in renal inflammatory response during HCA. We estimated the incidence of acute renal failure after aortic surgery without an HCA procedure to be 2%, while HCA is estimated to increase the risk of acute renal failure by 9-fold. According to our database of aortic procedures, 20% of the procedures involved HCA. Considering a dropout rate of 10%, we aimed to collect data from at least 267 patients. In collaboration with nine medical centers in China, we obtained data from 56 patients who underwent aortic surgery with HCA from our jointly maintained aortic surgery database (Table S2). As a control group, we collected data from 225 patients who underwent aortic surgery with CPB but without circulatory arrest and without cooling during the same period (Table S3). Consistent with our previous results, the analysis indicated that hypothermia during HCA was associated with postoperative acute renal failure. Circulatory arrest surgery with hypothermia was associated with a higher risk of postoperative acute renal failure compared with non-circulatory arrest surgery without hypothermia (Table 1; Figure S4A).

We conducted a longitudinal sampling of 56 patients who underwent the HCA procedure at four time points: before surgery (T0), after intraoperative protamine neutralization (T1), 4 h after surgery (T2), and 24 h after surgery (T3). We first selected TNF-α, IL-1, IL-2, and IL-6 as representative inflammatory factors. The results suggested that compared with patients without postoperative acute renal failure, inflammatory factor levels did not change significantly in patients with postoperative acute renal failure (Figures S3F–S3I). We obtained similar results in human studies to those in rats, indicating that the pathological changes in the rat model broadly reproduced the findings in the human patients. This suggests that hypothermia does not cause kidney injury through the inflammatory response in the early stages of kidney injury.

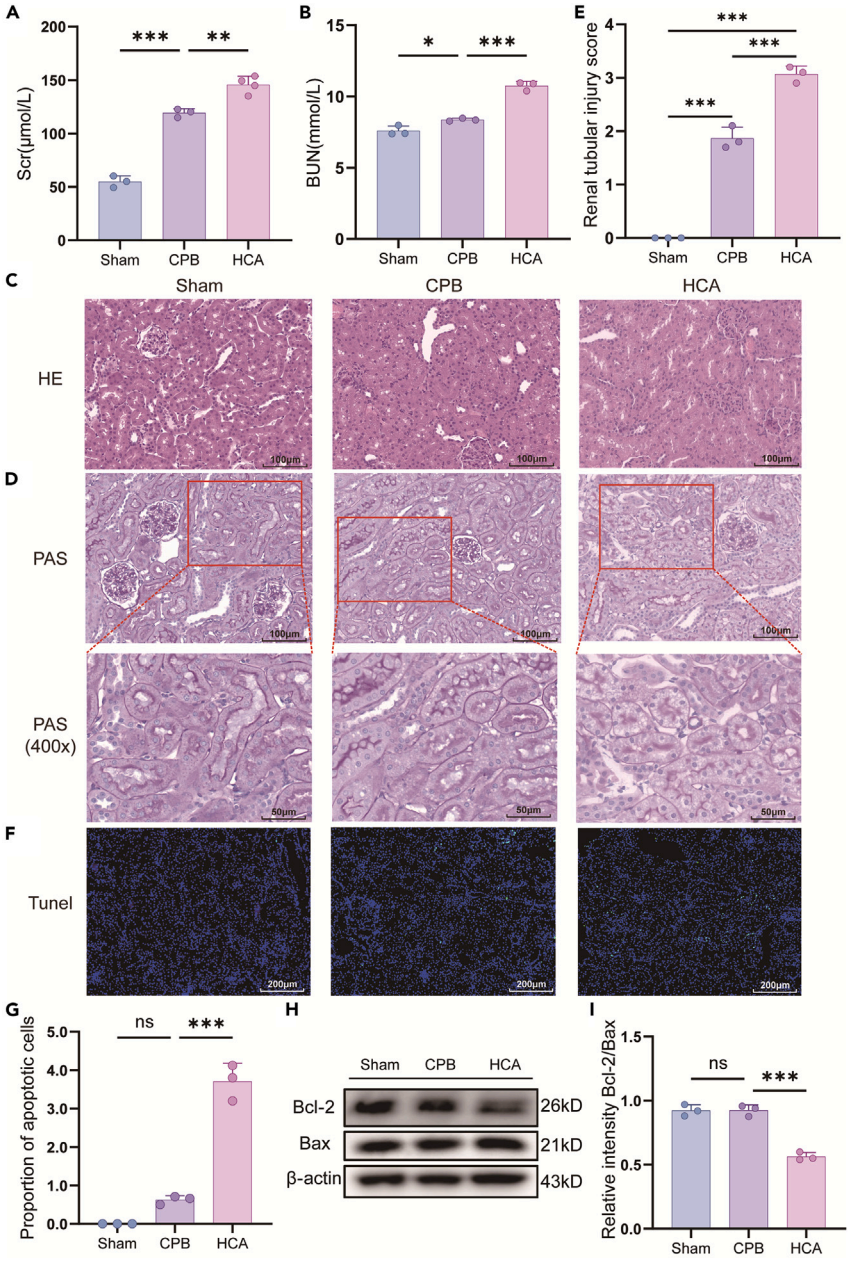

Figure 1. Aggravated AKI occurred after cardiopulmonary bypass with HCA

(A) Scr levels were measured 24 h after surgery in the rats in the Sham, CPB, and HCA groups.

(B) BUN levels were measured 24 h after the operation in the rats in the indicated groups. Representative images of HE (C) and PAS (D) staining of kidney sections harvested 24 h after surgery from the rats in the indicated groups.

(E) Scores of PAS staining according to Paller's renal tubular scoring criteria.

(F) Representative images of TUNEL-stained kidney sections harvested at 24 h after surgery.

(G) Quantification of the proportion of apoptotic cells according to TUNEL staining.

(H) Representative Western blot images of Bcl-2 and Bax expression in the renal tissues of the indicated groups.

(I) Quantitative analysis of the ratio of Bcl-2 to Bax. Scale bar: 100 μm (C, D upper part), 50 μm (D lower part), 200 μm (F). The data in Figures 1A–1C, 1G, and 1I are expressed as the means ± SDs. Statistical significance of Figures 1A–1C, 1G, and 1I was determined by ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. Scr, serum creatinine; BUN, blood urea nitrogen; Sham, sham-operated; CPB, cardiopulmonary bypass; HCA, hypothermic circulatory arrest.

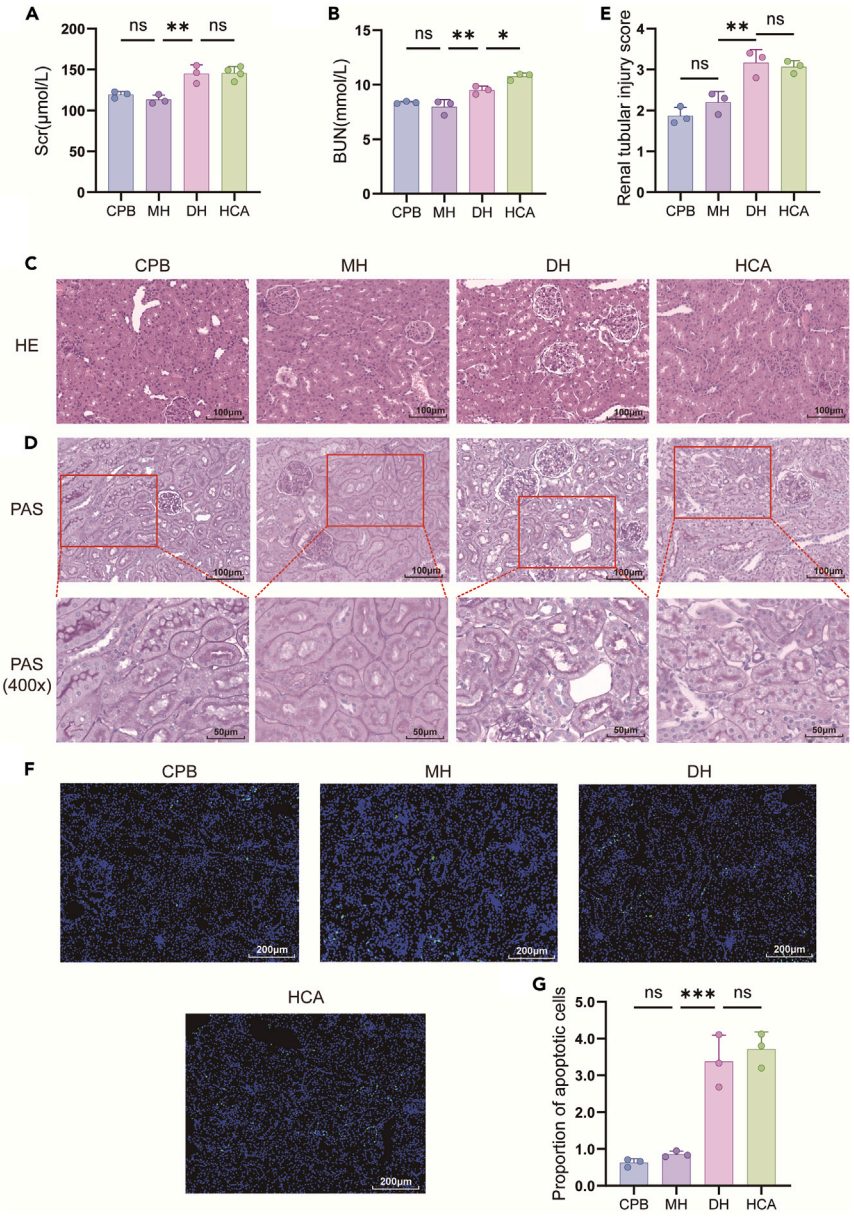

Figure 2. Hypothermia change during HCA accounts for AKI

(A) Scr levels measured 24 h after surgery in the rats in the CBP, MH, DH and HCA groups.

(B) BUN levels were measured 24 h after the operation in the rats in the indicated groups. Representative images of HE (C) and PAS (D) staining of kidney sections harvested 24 h after surgery from the rats in the indicated groups.

(E) Scores of PAS staining according to Paller's renal tubular scoring criteria.

(F) Representative images of TUNEL-stained of kidney sections harvested at 24 h after surgery.

(G) Quantification of the proportion of apoptotic cells according to TUNEL staining. Scale bar: 100 μm (C, D upper part), 50 μm (D lower part), 200 μm (F). The data in Figures 2A, 2B, 2E, and 2G are expressed as the means ± SDs. Statistical significance of Figures 2A, 2B, 2E, and 2G was determined by ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. Scr, serum creatinine; BUN, blood urea nitrogen; Sham, sham-operated; CPB, cardiopulmonary bypass; MH, CPB with moderate hypothermia; DH, CPB with deep hypothermia; HCA, hypothermic circulatory arrest.

Acute kidney injury is associated with elevated bradykinin levels during hypothermic circulatory arrest

We previously found that bradykinin is a risk factor for severe hypoxemia after surgery with HCA,[20] suggesting that bradykinin might act as an endogenous pathogenic factor. We explored the relationship between bradykinin and the HCA process and postoperative AKI. We assessed bradykinin expression in animal models. As shown in Figure 3A, renal bradykinin levels were increased in the CPB group, and further increased after HCA. We further examined the changes in bradykinin and the bradykinin regulatory factor FXII in human plasma. Results showed that

Table 1. Clinical characteristics and risk factor analysis results

Clinical characteristics				Multivariable binary Logistic regression results[a]		
	Without ARF	With ARF	*p* value	OR	95%CI of OR	*p* value
Number of patients (case)	269	12				
Gender, female (case)	67 (24.9%)	6 (50.0%)	0.085	–	–	–
Age (years)	48.3 ± 13.4	55.3 ± 9.5	0.076	–	–	–
Height (cm)	171.2 ± 9.4	167.3 ± 7.0	0.159	–	–	–
Weight (kg)	73.9 ± 15.6	66.8 ± 12.1	0.120	–	–	–
Body mass index (kg/m^2)	25.1 ± 4.4	23.8 ± 3.6	0.303	–	–	–
Hypertension (case)	59 (21.9%)	7 (58.3%)	0.009	–	–	–
Coronary artery disease (case)	3 (1.1%)	0 (0.0%)	1.000	–	–	–
Diabetes (case)	13 (4.8%)	0 (0.0%)	1.000	–	–	–
Left ventricular ejection fraction (%)	61.0 ± 8.0	62.8 ± 8.0	0.457	–	–	–
Absolute value of leukocyte (10^9/L)	9.39 ± 31.72	8.92 ± 3.01	0.959	–	–	–
Platelet (10^9/L)	200.7 ± 65.1	141.7 ± 45.6	0.002	0.982	0.966–0.998	0.032
Hemoglobin (g/L)	140.2 ± 17.8	125.3 ± 10.2	0.005	0.944	0.901–0.989	0.016
Creatinine (μmol/L)	75.8 ± 32.8	74.5 ± 22.5	0.889	–	–	–
AST (U/L)	40.5 ± 178.0	31.8 ± 19.4	0.867	–	–	–
ALT (U/L)	35.5 ± 108.9	24.7 ± 19.5	0.730	–	–	–
Aortic root size (mm)	44.1 ± 11.4	43.6 ± 9.8	0.904	–	–	–
Cardiopulmonary bypass time (min)	142.6 ± 51.9	216.8 ± 63.8	<0.001	–	–	–
Sun's procedure	48 (17.8%)	8 (66.7%)	<0.001	–	–	–
Wheat procedure	12 (4.5%)	0 (0.0%)	1.000	–	–	–
David procedure	20 (7.4%)	0 (0.0%)	1.000	–	–	–
Bentall procedure	133 (49.4%)	9 (75.0%)	0.083	14.804	2.188–100.147	0.006
Surgery with ascending aorta replacement	99 (36.8%)	3 (25.0%)	0.546	–	–	–
Surgery with CABG	13 (4.8%)	1 (8.3%)	0.465	–	–	–
Surgery with HCA (case)	48 (17.8%)	8 (66.7%)	<0.001	8.306	1.828–37.748	0.006
HCA time (min)	28.2 ± 10.3	28.6 ± 10.0	0.920	–	–	–
Rectal temperature when circulatory arrest (°C)	25.6 ± 2.1	23.2 ± 2.2	0.004	–	–	–
Intraoperative blood loss (mL)	1262.5 ± 464.3	1650.0 ± 378.0	0.030	–	–	–
RBC transfusion volume (mL)	319.4 ± 464.8	708.3 ± 512.5	0.005	–	–	–
Plasma transfusion volume (mL)	172.0 ± 278.6	616.7 ± 493.3	0.010	1.003	1.001–1.005	0.009

Abbreviations: ARF, Acute Renal Failure; HCA, Hypothermic Circulatory Arrest; CABG, Coronary Artery Bypass Grafting; OR, Odd Ratio; CI, Confidence Interval.
NOTE. The categorical variables in the table are presented by the number of cases (with percentage) and the continuous variables are expressed by the mean (with standard deviation).
[a]Because rectal temperature was only recorded in patients who underwent surgery with HCA, rectal temperature was not included in the multivariable binary logistic regression.

patients with postoperative acute renal failure had higher plasma bradykinin levels 24 h after surgery (Figures 3B–3D). To further explore the relationship between bradykinin and AKI after HCA, we analyzed the changes in perioperative blood bradykinin levels in 56 patients. The results showed that the plasma bradykinin level increased during surgery (T1) and remained elevated 4 h after surgery (T2) in all patients. However, 24 h after surgery (T3), in patients without postoperative acute renal failure, the blood bradykinin concentration recovered to the preoperative level or decreased slightly. Moreover, in patients with postoperative acute renal failure, it maintained a relatively greater value (Figure 3E). In addition, 24 h after surgery (T3), higher serum bradykinin concentrations were associated with acute renal failure (stage 3 AKI) (Table S2). Linear correlation analysis revealed that the postoperative bradykinin concentration was positively correlated with the postoperative Scr concentration (Figure S4B). These results suggested that the elevated blood bradykinin concentration was closely related to the HCA process and postoperative AKI.

To investigate the mechanism underlying bradykinin elevation, we analyzed the changes in kallikrein expression and found that the expression of kallikrein 1 (KLK-1), the enzyme that catalyzes bradykinin synthesis, was not affected by CPB but was dramatically increased after HCA in

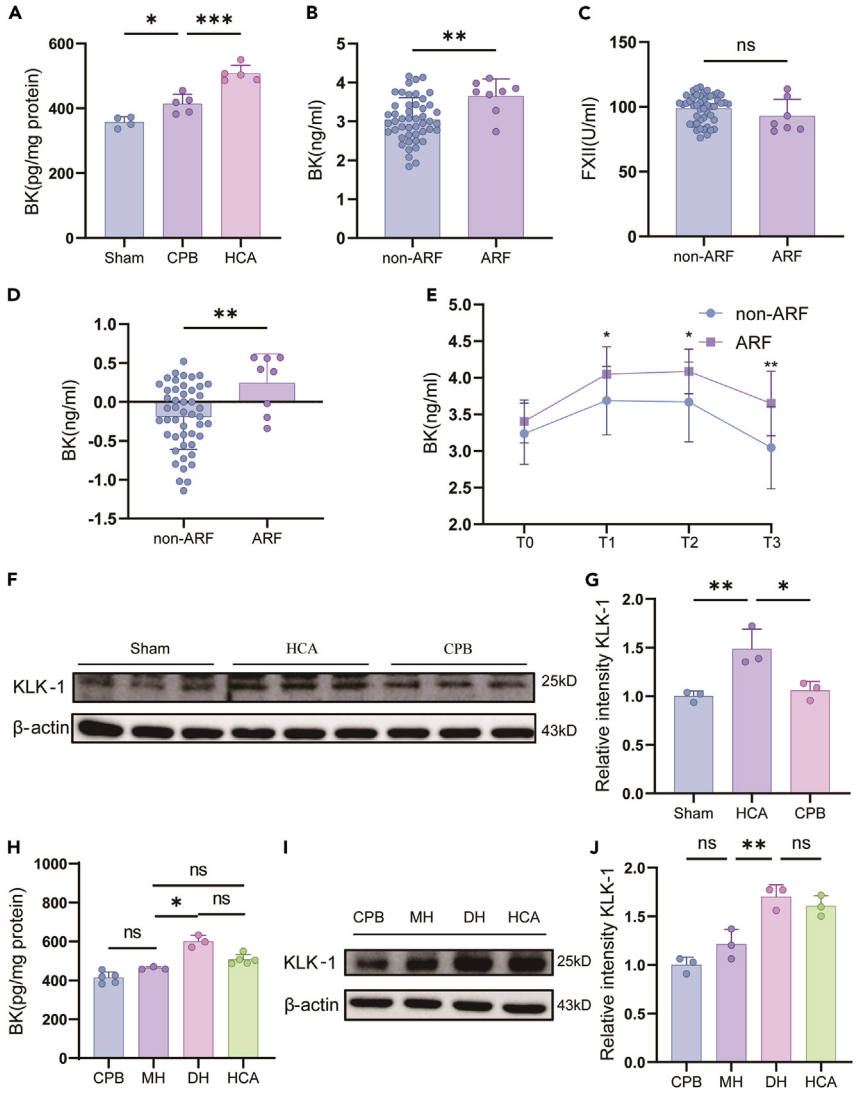

Figure 3. An increase in the serum bradykinin concentration during HCA was associated with AKI

(A) Contents of bradykinin in renal tissues harvested 24 h after surgery from the rats in the Sham, CPB and HCA groups.

(B and C) Serum levels of bradykinin and FXII at 24 h after emergency aortic surgery in patients with and without postoperative ARF.

(D) Differences in the serum bradykinin concentration between 24 h after surgery and before surgery in patients with and without postoperative ARF.

(E) Curve graphs showing the serum bradykinin levels before surgery (T0), after heparin neutralization (T1), at 4 h after surgery (T2), and at 24 h after surgery (T3) in patients with and without postoperative ARF. Representative Western blot image (F) and quantitative analysis (G) of KLK-1 expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups.

(H) Contents of bradykinin in renal tissues harvested at 24 h after the operation of rats in the CPB, MH, DH, and HCA groups. Representative Western blot image (I) and quantitative analysis (J) of KLK-1 expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups. The data in Figures 3A–3E, 3G, 3H, and 3J are expressed as the means ± SDs. Statistical significance of Figures 3A, 3G, 3H, and 3J was determined by ANOVA. Statistical significance of Figures 3B–3E was determined by Student's t test (two-tailed); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. BK, bradykinin; FXII, coagulation factor XII; ARF, acute renal failure; Sham, sham-operated; CPB, cardiopulmonary bypass; HCA, hypothermic circulatory arrest; KLK-1, kallikrein-1, MH, CPB with moderate hypothermia; DH, CPB with deep hypothermia.

the kidney (Figures 3F and 3G). Subsequently, we investigated whether the alterations in temperature during HCA contributed to the upregulation of bradykinin and KLK-1. Our findings, as illustrated in Figures 3H–3J, indicated that, compared to that in the CPB group, the renal expression of bradykinin and KLK-1 in rats subjected to a body temperature of 28°C during CPB (MH group) was unchanged. However, when the body temperature was further reduced to 18°C during CPB (DH group), there was a substantial increase in renal KLK-1 expression and bradykinin concentration, comparable to what was observed in the HCA group. Conversely, when the duration of circulatory arrest was extended, no significant alterations in renal KLK-1 expression or bradykinin concentration were observed (Figures S5A–S5C). In addition,

the results of KLK-1 immunohistochemistry showed that renal KLK-1 was mainly distributed in renal tubular cells, suggesting that the renal bradykinin concentration may be regulated mainly by renal tubular cells (Figure S5D). Further analysis showed that the expression of renal bradykinin receptor 1 (B1R) and bradykinin receptor 2 (B2R) was increased in the CPB group and further increased in the HCA group (Figures S5E and S5F). These findings indicate that hypothermia during HCA is accountable for the increase in KLK-1 and bradykinin concentrations in the kidneys.

Hypothermia during hypothermic circulatory arrest causes increased oxidative stress in the kidney

Previous studies have shown that a low body temperature leads to the production of ROS in the kidney and triggers AKI.[13,21] Therefore, we examined the changes in renal oxidative stress. The results showed that oxidative stress was increased in the CPB group, as evidenced by a decrease in superoxide dismutase (SOD) activity and an increase in malondialdehyde (MDA) content. Strikingly, oxidative stress was further enhanced in the HCA group that underwent CPB with HCA (Figures 4A and 4B). These results confirmed that kidney injury during the HCA process was attributable to enhanced oxidative stress.

Given the observed oxidative stress during hypothermic conditions, we focused on Nrf2, a key regulator of antioxidants, and its downstream targets related to oxidative stress pathways, including xCT and GPX4.[13,22] The results showed that GPX4 expression was unaffected by CPB or HCA (Figures 4C and 4D). Notably, HCA caused a significant decrease in renal Nrf2 and xCT expression (Figures 4E–4G). To further elucidate this change, the expression of renal Nrf2 and xCT with time were further analyzed. The results showed that renal Nrf2 and xCT did not significantly decrease at the end of the HCA process, but significantly decreased 6 h later and lasted till 48 h later (Figure S6A). These results revealed the role of the Nrf2-xCT pathway downregulation in enhanced oxidative stress during HCA and implicated it as a potential target for intervention in mitigating renal oxidative stress.

Subsequently, we analyzed the role of temperature change and circulatory arrest time in Nrf2-xCT signaling pathway and the resulting renal oxidative stress during HCA. The results suggested that hypothermia during HCA was linked to the downregulation of the Nrf2-xCT signaling pathway and oxidative stress in the kidney (Figures 4H–4L and S6B–S6F).

Bradykinin increases oxidative stress by downregulating the Nrf2-xCT pathway

Since the changes in the renal Nrf2-xCT signaling pathway during HCA paralleled the changes in bradykinin, we detected the relationship between bradykinin and the renal Nrf2-xCT signaling pathway in HK-2 cells *in vitro*. HK-2 cells were incubated with different concentrations of bradykinin and H_2O_2. The results showed that the expression of Nrf2 and xCT decreased in response to bradykinin in a dose-dependent manner (Figure 5A). Moreover, oxidative stress in HK-2 cells was aggravated, and intracellular ROS production was significantly increased (Figures 5B–5F). These results suggested that bradykinin induced the suppression of the Nrf2-xCT pathway and aggravated oxidative stress in renal tubular epithelial cells.

To further explore the link between the Nrf2-xCT signaling pathway and bradykinin-induced oxidative stress, we utilized the Nrf2 agonist CDDO to determine whether this pathway could rescue injury. Our findings revealed that treating HK-2 cells with bradykinin resulted in increased cellular ROS production and a decreased SOD concentration. However, the administration of CDDO reversed the changes in ROS and SOD induced by bradykinin (Figure 6). Taken together, these results suggest that decreased Nrf2 expression plays a role in mediating bradykinin-induced oxidative stress in renal tubular epithelial cells.

Bradykinin receptor 2 blockade alleviates the changes in the Nrf2-xCT pathway and oxidative stress *in vitro* and *in vivo*

To investigate the impact of bradykinin receptors on bradykinin-induced renal oxidative stress and changes in the Nrf2-xCT signaling pathway, we used siRNA to silence both B1R and B2R (Figures 7A–7H). The results showed that silencing B1R in HK-2 cells intensified the downregulation of the Nrf2-xCT signaling pathway induced by bradykinin. Conversely, silencing B2R significantly reversed the decrease in Nrf2 and xCT expression. Moreover, it completely restored the alterations in the SOD and MDA levels. Consistently, silencing of B1R further exacerbates bradykinin-induced ROS production, whereas B2R silencing reverses this effect.

Using specific antagonists, we determined the involvement of bradykinin receptors *in vivo*. Similar to what was observed in HK-2 cells, both bradykinin treatment and B1R blockade prior to the HCA procedure aggravated oxidative stress in the kidneys. However, B2R blockade markedly reversed HCA-induced renal oxidative stress injuries (Figures 7I and 7J). Consistently, bradykinin treatment decreased xCT expression. In addition, the expression of Nrf2 and xCT was downregulated by blockade of B1R. However, blockade of B2R reversed the HCA -induced changes in the Nrf2-xCT pathway (Figures 7K–7M). These results suggested that elevated bradykinin production during HCA induced the downregulation of the Nrf2-xCT pathway mainly through B2R, which resulted in increased renal oxidative stress and kidney injuries.

Bradykinin receptor 2 blockade alleviates hypothermic circulatory arrest-induced renal injury *in vivo*

Finally, we determined the role of bradykinin and B2R blockade in renal injury *in vivo*. After treatment with bradykinin or Icatibant, a B2R antagonist, the changes in kidney injury were analyzed. We found that bradykinin aggravated whereas Icatibant alleviated the functional and structural damage induced during the HCA process (Figure 8). These results suggested that bradykinin induced kidney injury through B2R during HCA.

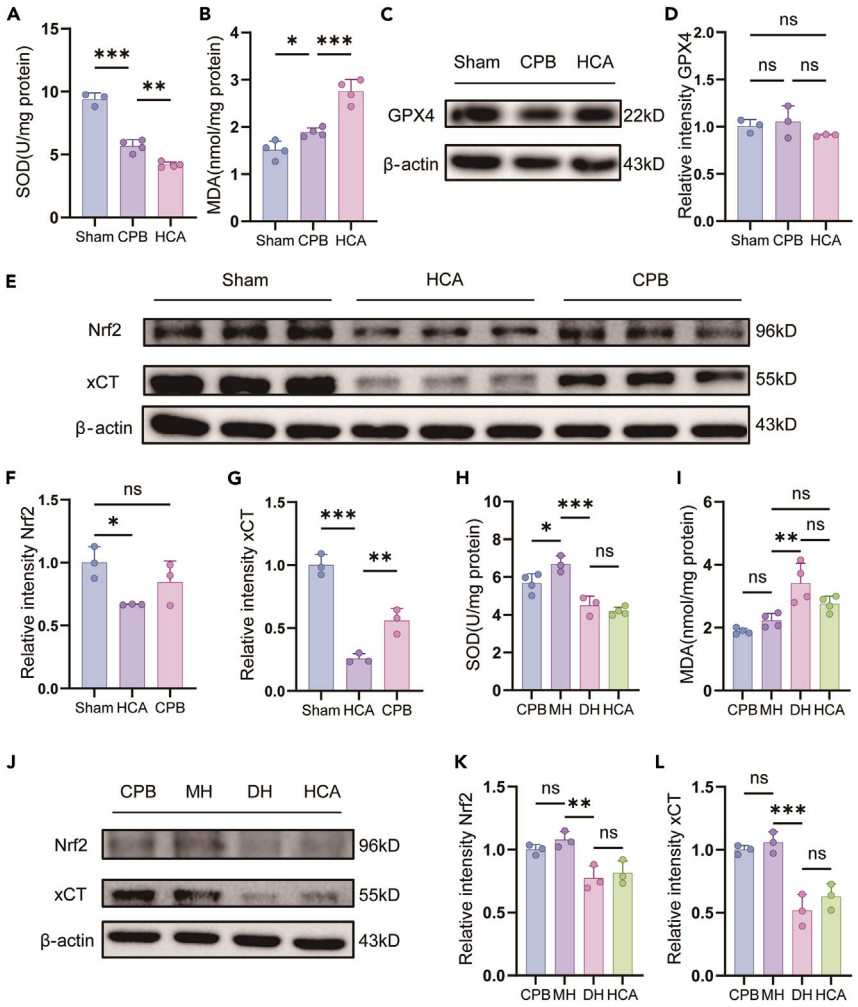

Figure 4. Hypothermia during HCA led to increased oxidative stress via Nrf2-xCT suppression

Contents of SOD (A) and MDA (B) in renal tissues harvested 24 h after surgery from the rats in the Sham, CPB and HCA groups. Representative Western blot image (C) and quantitative analysis (D) of GPX4 expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups. Representative Western blot images (E) and quantitative analysis of Nrf2 (F) and xCT (G) expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups. Contents of SOD (H) and MDA (I) in renal tissues harvested 24 h after surgery from the rats in the CPB, MH, DH, and HCA groups. Representative Western blot images (J) and quantitative analysis of Nrf2 (K) and xCT (L) expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups. The data in Figures 4A, 4B, 4D, 4F–4I, 4K, and 4L are expressed as the means ± SDs. Statistical significance of Figures 4A, 4B, 4D, 4F–4I, 4K, and 4L was determined by ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. Sham, sham-operated; CPB, cardiopulmonary bypass; HCA, hypothermic circulatory arrest; MH, CPB with moderate hypothermia; DH, CPB with deep hypothermia.

DISCUSSION

AKI is a serious complication that is associated with increased postoperative mortality in patients who undergo cardiac and aortic surgeries with HCA. During HCA, there are multiple pathological factors that can lead to renal injury. In this study, we found that hypothermia during HCA can increase bradykinin expression and induce renal injury.

Patients with postoperative AKI are at increased risk of prolonged ICU stays, prolonged ventilator use, and various complications. Additionally, even if renal function recovers completely, these patients have a greater risk of death within 10 years after surgery than patients without AKI.[7] Despite previous studies investigating the pathogenesis of AKI after cardiac and aortic surgeries, the pathophysiological mechanism is not fully understood.[23–25]

In this study, we used a rat model to investigate the mechanism of HCA -related renal injury. Since the HCA procedure is used mainly in cardiovascular surgery, it was not possible to obtain human renal tissues to support our research hypotheses. However, rodent animal models have been widely used in the study of AKI because they can reflect pathological changes similar to those in humans. While mice are commonly used, performing HCA surgery is challenging due to the small size of the mice. Although other large animals, such as pigs, rabbits, and sheep,

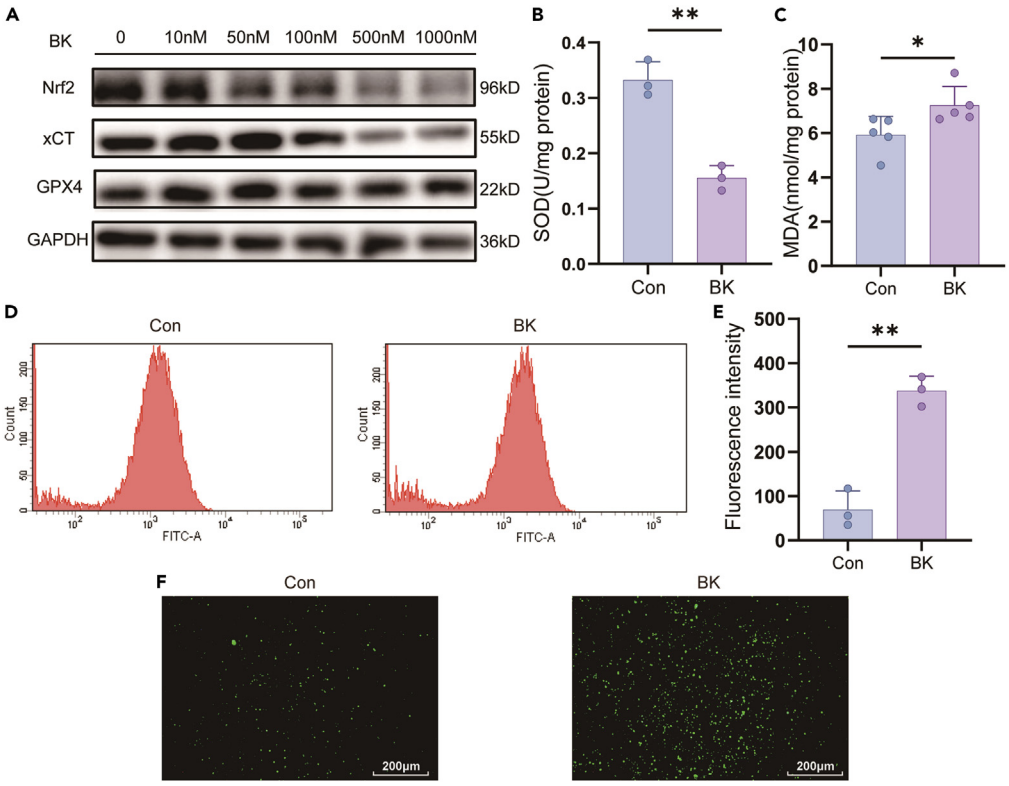

Figure 5. Bradykinin suppressed the Nrf2-xCT signaling pathway and increased oxidative stress in renal tubular epithelial cells

(A) Representative Western blot images of Nrf2, xCT and GPX4 expression in HK-2 cells treated with 100 μM H_2O_2 and different concentrations of bradykinin for 24 h. Contents of SOD (B) and MDA (C) in HK-2 cells treated with 100 μM H_2O_2 alone or 100 μM H_2O_2 plus 100 nM bradykinin. Flow cytometry (D) and statistical analysis (E) of reactive oxygen species (ROS) in HK-2 cells treated with 100 μM H_2O_2 alone or 100 μM H_2O_2 plus 100 nM bradykinin.

(F) Representative microscopy images of H_2DCFDA-stained HK-2 cells treated with 100 μM H_2O_2 alone or 100 μM H_2O_2 plus 100 nM bradykinin to detect ROS. Scale bar: 200 μm (F). The data in Figures 5B, 5C, and 5E are expressed as the means ± SDs. Statistical significance of Figures 5B, 5C, and 5E was determined by Student's t test (two-tailed); *$p < 0.05$, **$p < 0.01$, ns, not significant.

can also undergo CPB and HCA,[26–28] a wealth of molecular biology tools are available for use in rats, and then we chose rats as the animal model for studying the mechanism of HCA -related renal injury.

In this study, we found that compared with the circulatory arrest process, changes in body temperature had a more significant effect on kidney injury. In previous studies, hypothermic kidney transplantation was generally considered the basic protective strategy for donor organs.[29] Moreover, the potential risk of ischemia-reperfusion injury caused by circulatory arrest was more likely to cause kidney injury.[30] However, recent study shows that although low temperature can reduce renal metabolism and reduce oxygen consumption, more ROS are produced in the kidneys.[21] This is consistent with our finding that hypothermia induced further oxidative stress damage in the kidneys, which is more serious than the injury caused by circulatory arrest. A recent study by Vekstein et al. also examined the association between circulatory arrest temperatures and AKI in patients undergoing proximal aortic surgery with HCA.[31] Due to the considerable variability in the surgical approaches, different diagnostic criteria for AKI and others, there seems to be some differences from our results. However, the findings of Vekstein et al. still support that hypothermia at less than 20°C may have damaging effects on the kidneys. In fact, in our preliminary studies and meta-analysis results that combined the results of multiple studies, it was confirmed that excessively low hypothermia (18°C–30°C) is a risk factor for postoperative AKI.[15,32]

Previous studies have shown that AKI may involve factors such as hypoperfusion, inflammation, oxidative stress, and nephrotoxic drugs.[9] Although the process of circulatory arrest can lead to renal hypoperfusion, our present study showed that the duration of circulatory arrest did not affect renal injury. Blood analysis of the patients in this study revealed no difference in the blood inflammatory factor levels between patients with and without postoperative ARF. In addition, no nephrotoxic drugs were used in the animal model of this study.

Oxidative stress is currently recognized as an important pathogenic factor leading to AKI.[9] Studies on the Nrf2 signaling pathway have confirmed that Nrf2 plays a key role in maintaining the ability of cells to resist oxidative stress.[33] Previous studies have shown that the overactivation of Nrf2 can enhance the ability to prevent oxidative stress and reduce oxidative stress injury in renal tubular cells.[34] Our present study revealed that hypothermia-induced renal oxidative stress injury is achieved by inhibiting the Nrf2-xCT signaling pathway. CDDO is

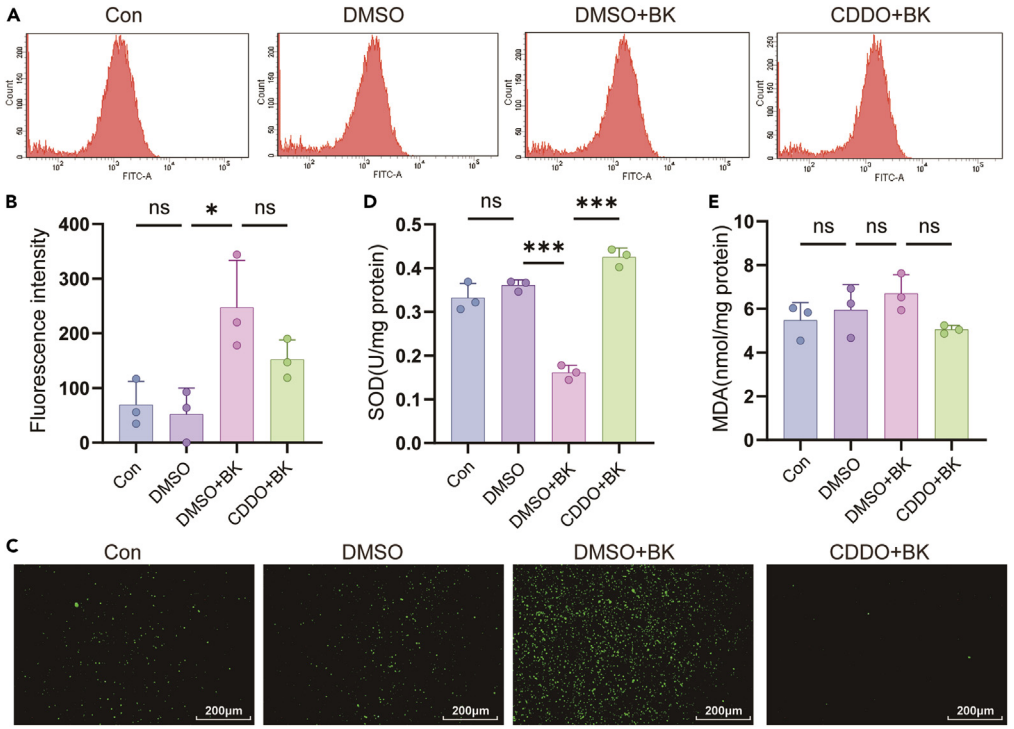

Figure 6. Nrf2 activation with bardoxolone (CDDO) reversed bradykinin -induced oxidative stress in renal tubular epithelial cells

Flow cytometry (A) and statistical analysis (B) of reactive oxygen species (ROS) in HK-2 cells treated with 100 μM H_2O_2 alone or in combination with the indicated stimulus for 24 h.

(C) Representative microscopy images of H_2DCFDA-stained HK-2 cells treated with 100 μM H_2O_2 alone or with the indicated stimulus for 24 h for the detection of ROS. Contents of SOD (D) and MDA (E) in HK-2 cells treated with the indicated stimuli. Scale bar: 200 μm (C). The data in Figures 6B, 6D, and 6E are expressed as the means ± SDs. Statistical significance of Figures 6B, 6D, and 6E was determined by ANOVA; *$p < 0.05$, ***$p < 0.001$, ns, not significant. DMSO, dimethyl sulfoxide; BK, bradykinin; CDDO, bardoxolone.

an agonist of Nrf2 that reduces cellular oxidative stress by activating Nrf2. In addition, CDDO can activate Nrf2 to increase the transcription of a series of downstream anti-oxidative stress proteins, allowing cells to synthesize additional reducing substances to neutralize ROS, thereby protecting cells from oxidative stress injury.[34]

The tissue kallikrein-kinin system (KKS) is an endogenous homeostatic pathway.[35,36] As the main effector molecule of KKS, bradykinin is considered to have cardioprotective effects on cardiac injury.[37,38] However, previous studies have provided contradictory results on the role of bradykinin in the kidney. Several previous studies have suggested that bradykinin is protective against kidney injury. Injection of exogenous kallikrein can inhibit renal fibrosis.[39] In renal ischemia reperfusion injury, knockout of the bradykinin receptor can aggravate renal ischemia reperfusion injury.[40] On the other hand, studies have also shown that bradykinin can aggravate kidney damage. Bradykinin upregulates the expression of IL-6, CCL-2 and TGF-β through the MAPK pathway in a dose- and time-dependent manner, thereby aggravating inflammation and fibrosis.[41] In addition, in renal ischemia reperfusion, the application of kallikrein can aggravate the injury caused by renal ischemia reperfusion.[42] Based on the above findings, we believe that bradykinin may play different roles in the kidney in different diseases. In the present study, we found that bradykinin exacerbates renal oxidative stress and thereby leads to renal injury, which confirms the role of bradykinin in aggravating renal injury. Hypothermia in HCA, rather than the circulatory arrest process, has a greater impact on the kidney. The CPB process creates an environment of high oxidative stress,[43] which increases the susceptibility of the kidney to oxidative stress injury, and this is not the same as renal ischemia reperfusion.

Bradykinin acts via two G protein-coupled receptors, inducible B1 (B1R) and constitutive B2 (B2R). Under physiological conditions, the expression of B1R is quite low, and its expression increases under stress conditions. In the majority of cases, bradykinin signals through B2R, the main receptor for bradykinin in the physiological state.[44] The clinically validated drug icatibant was used in this study. Icatibant is a known bradykinin B2R antagonist, and the only approved drug used in the clinic to treat hereditary angioedema.[45] Our present findings imply that icatibant may play a role in treating AKI after cardiac and aortic surgeries, which will help broaden the clinical application of icatibant.

In conclusion, this study revealed that hypothermia during HCA can increase renal bradykinin levels and that bradykinin suppresses the Nrf2-xCT signaling pathway by activating B2R in renal tubular epithelial cells, which leads to increased oxidative stress and ultimately kidney injury. The bradykinin B2R and Nrf2-xCT signaling pathways may be potential therapeutic targets for treating AKI after HCA.

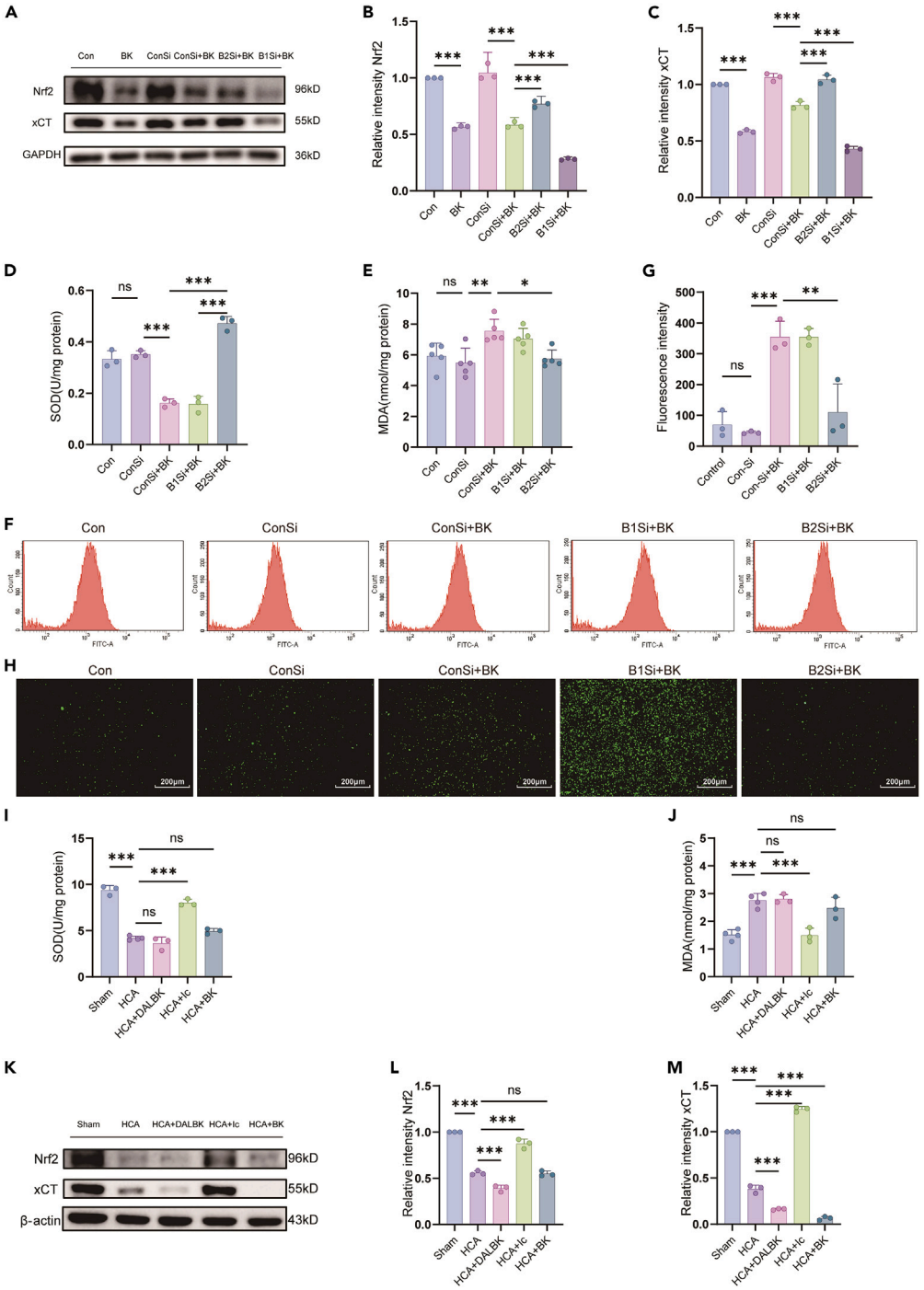

Figure 7. The effect of bradykinin receptor blockade on bradykinin -induced oxidative stress injury and the Nrf2-xCT signaling pathway *in vitro* and *in vivo*

Representative Western blot images (A) and quantitative analysis of Nrf2 (B) and xCT (C) expression in HK-2 cells treated with the indicated siRNA and then 100 μM H_2O_2 alone or 100 μM H_2O_2 plus 100 nM bradykinin for 24 h. Contents of SOD (D) and MDA (E) in HK-2 cells treated with the indicated stimuli. Flow cytometry (F) and statistical analysis (G) of reactive oxygen species (ROS) in HK-2 cells treated with the indicated stimulus.

(H) Representative microscopy images of H_2DCFDA-stained HK-2 cells detected ROS in response to the indicated stimulus. Contents of SOD (I) and MDA (J) in renal tissues harvested 24 h after surgery from the rats in the Sham, HCA, HCA+DALBK, HCA+Ic and HCA+BK groups. Representative Western blot images

Figure 7. *Continued*

(K) and quantitative analysis of Nrf2 (L) and xCT (M) expression in renal tissues harvested 24 h after surgery from the rats in the indicated groups. Scale bar: 200 μm (H). The data in Figures 7B–7E, 7G, 7I, 7G, 7L, and 7M are expressed as the means ± SDs. Statistical significance of Figures 7B–7D, 7E, 7G, 7I, 7G, 7L, and 7M was determined by ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. HCA, hypothermic circulatory arrest; HCA+DALBK, HCA treated with des-Arg9-[Leu8]-BK; HCA+Ic, HCA treated with icatibant; HCA+BK, HCA treated with BK.

Limitations of the study

This study has several limitations. First, the rat model used in this study is not based on corresponding diseases, such as cardiac or aortic diseases, which most patients who undergo HCA surgery suffer from in the clinic. Inducing aortic dissection in rats is also not feasible due to the high mortality rate, and humans have a greater tolerance for aortic dissection. However, despite this limitation, the pathogenesis of AKI revealed in this study can still provide a preventive and therapeutic target for postoperative AKI. Second, because other organ tissues were not collected from the rats in this study, the main source of bradykinin was not explored. Third, this study only measured inflammatory factors 24 h after surgery and found no difference in inflammatory factor levels, and did not explore changes in inflammatory factors at later time points. Fourth, this study did not analyze the reversal of HCA-induced kidney injury by CDDO. In clinical trials, CDDO-Me, a CDDO-derived drug, was also found to increase the incidence of heart failure in patients.[46] This may explain why all the rats died after CDDO treatment in our study. Finally, this study also revealed that B1R has a renal injury effect. The mechanism of B1R-induced kidney injury warrants further investigation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

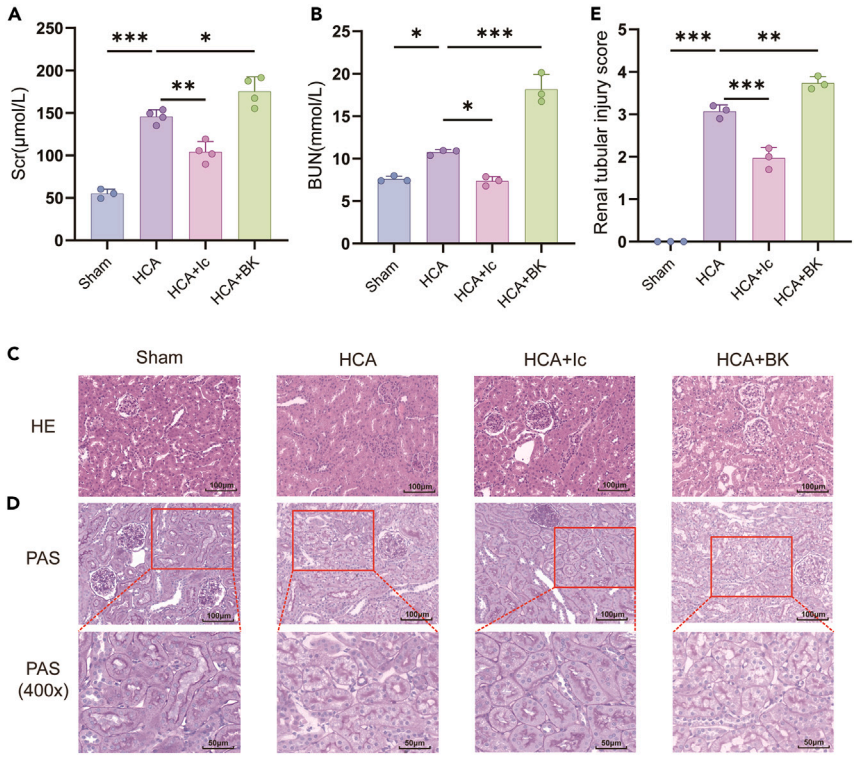

Figure 8. Blockade of bradykinin receptor 2 relieves AKI during HCA *in vivo*

(A) Scr levels were measured 24 h after surgery in the rats in the Sham, HCA, HCA+Ic and HCA+BK groups.

(B) BUN levels were measured 24 h after the operation in the rats in the indicated groups. Representative images of HE (C) and PAS (D) staining of kidney sections harvested 24 h after surgery from the rats in the indicated groups.

(E) Scores of PAS staining according to Paller's renal tubular scoring criteria. Scale bar: 100 μm (C, D upper part), 50 μm (D lower part). The data in Figures 8A, 8B, and 8E are expressed as the means ± SDs. Statistical significance of Figures 1A, 8A, 8B, and 8E was determined by ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant. Scr, serum creatinine; BUN, blood urea nitrogen; Sham, sham-operated; HCA, hypothermic circulatory arrest; HCA+Ic, HCA treated with icatibant; HCA+BK, HCA treated with BK.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110075.

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AUTHOR CONTRIBUTIONS

Research idea and study design: HZ and MG; data acquisition: JL, MLW, MZW, HS, and WW; data analysis/interpretation: JL and MLW; statistical analysis: JL and MLW; supervision or mentorship: HZ, MG, and WW. Each author contributed important intellectual content during article drafting or revision and agreed to be personally accountable for the individual's contributions and to ensure that questions about the accuracy or integrity of any portion of the work, even one in which the author was not directly involved, were appropriately investigated and resolved, including documentation in the literature if appropriate.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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REFERENCES

1. Ko, T., Higashitani, M., Sato, A., Uemura, Y., Norimatsu, T., Mahara, K., Takamisawa, I., Seki, A., Shimizu, J., Tobaru, T., et al. (2015). Impact of Acute Kidney Injury on Early to Long-Term Outcomes in Patients Who Underwent Surgery for Type A Acute Aortic Dissection. Am. J. Cardiol. *116*, 463–468. https://doi.org/10.1016/j.amjcard.2015.04.043.
2. Englberger, L., Suri, R.M., Greason, K.L., Burkhart, H.M., Sundt, T.M., Daly, R.C., and Schaff, H.V. (2011). Deep hypothermic circulatory arrest is not a risk factor for acute kidney injury in thoracic aortic surgery. J. Thorac. Cardiovasc. Surg. *141*, 552–558. https://doi.org/10.1016/j.jtcvs.2010.02.045.
3. Zhao, H., Pan, X., Gong, Z., Zheng, J., Liu, Y., Zhu, J., and Sun, L. (2015). Risk factors for acute kidney injury in overweight patients with acute type A aortic dissection: a retrospective study. J. Thorac. Dis. *7*, 1385–1390. https://doi.org/10.3978/j.issn.2072-1439.2015.07.19.
4. Kim, W.H., Lee, J.H., Kim, E., Kim, G., Kim, H.J., and Lim, H.W. (2016). Can We Really Predict Postoperative Acute Kidney Injury after Aortic Surgery? Diagnostic Accuracy of Risk Scores Using Gray Zone Approach. Thorac. Cardiovasc. Surg. *64*, 281–289. https://doi.org/10.1055/s-0034-1396082.
5. Kuitunen, A., Vento, A., Suojaranta-Ylinen, R., and Pettilä, V. (2006). Acute renal failure after cardiac surgery: evaluation of the RIFLE classification. Ann. Thorac. Surg. *81*, 542–546. https://doi.org/10.1016/j.athoracsur.2005.07.047.
6. D'Onofrio, A., Cruz, D., Bolgan, I., Auriemma, S., Cresce, G.D., Fabbri, A., and Ronco, C. (2010). RIFLE criteria for cardiac surgery-associated acute kidney injury: risk factors and outcomes. Congest. Heart Fail. *16*,

S32–S36. https://doi.org/10.1111/j.1751-7133.2010.00170.x.
7. Hobson, C.E., Yavas, S., Segal, M.S., Schold, J.D., Tribble, C.G., Layon, A.J., and Bihorac, A. (2009). Acute kidney injury is associated with increased long-term mortality after cardiothoracic surgery. Circulation *119*, 2444–2453. https://doi.org/10.1161/CIRCULATIONAHA.108.800011.
8. Ma, X., Li, J., Yun, Y., Zhao, D., Chen, S., Ma, H., Wang, Z., Zhang, H., Zou, C., and Cui, Y. (2020). Risk factors analysis of acute kidney injury following open thoracic aortic surgery in the patients with or without acute aortic syndrome: a retrospective study. J. Cardiothorac. Surg. *15*, 213. https://doi.org/10.1186/s13019-020-01257-1.
9. Wang, Y., and Bellomo, R. (2017). Cardiac surgery-associated acute kidney injury: risk factors, pathophysiology and treatment. Nat. Rev. Nephrol. *13*, 697–711. https://doi.org/10.1038/nrneph.2017.119.
10. Hausenloy, D.J., Candilio, L., Evans, R., Ariti, C., Jenkins, D.P., Kolvekar, S., Knight, R., Kunst, G., Laing, C., Nicholas, J., et al. (2015). Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. N. Engl. J. Med. *373*, 1408–1417. https://doi.org/10.1056/NEJMoa1413534.
11. Haase, M., Bellomo, R., and Haase-Fielitz, A. (2010). Novel biomarkers, oxidative stress, and the role of labile iron toxicity in cardiopulmonary bypass-associated acute kidney injury. J. Am. Coll. Cardiol. *55*, 2024–2033. https://doi.org/10.1016/j.jacc.2009.12.046.
12. Gallogly, M.M., Shelton, M.D., Qanungo, S., Pai, H.V., Starke, D.W., Hoppel, C.L., Lesnefsky, E.J., and Mieyal, J.J. (2010). Glutaredoxin regulates apoptosis in cardiomyocytes via NFkappaB targets Bcl-2 and Bcl-xL: implications for cardiac aging. Antioxid. Redox Signal. *12*, 1339–1353. https://doi.org/10.1089/ars.2009.2791.
13. Habib, E., Linher-Melville, K., Lin, H.X., and Singh, G. (2015). Expression of xCT and activity of system xc(-) are regulated by NRF2 in human breast cancer cells in response to oxidative stress. Redox Biol. *5*, 33–42. https://doi.org/10.1016/j.redox.2015.03.003.
14. Sun, L., Qi, R., Zhu, J., Liu, Y., and Zheng, J. (2011). Total arch replacement combined with stented elephant trunk implantation: a new "standard" therapy for type a dissection involving repair of the aortic arch? Circulation *123*, 971–978. https://doi.org/10.1161/CIRCULATIONAHA.110.015081.
15. Li, J., Gong, M., Joshi, Y., Sun, L., Huang, L., Fan, R., Gu, T., Zhang, Z., Zou, C., Zhang, G., et al. (2021). Machine Learning Prediction Model for Acute Renal Failure After Acute Aortic Syndrome Surgery. Front. Med. *8*, 728521. https://doi.org/10.3389/fmed.2021.728521.
16. Arnaoutakis, G.J., Bihorac, A., Martin, T.D., Hess, P.J., Jr., Klodell, C.T., Ejaz, A.A., Garvan, C., Tribble, C.G., and Beaver, T.M. (2007). RIFLE criteria for acute kidney injury in aortic arch surgery. J. Thorac. Cardiovasc. Surg. *134*, 1554–1561. , discussion 1560-1551. https://doi.org/10.1016/j.jtcvs.2007.08.039.
17. Goldstein, D.J., DeRosa, C.M., Mongero, L.B., Weinberg, A.D., Michler, R.E., Rose, E.A., Oz, M.C., and Smith, C.R. (1995). Safety and efficacy of aprotinin under conditions of deep hypothermia and circulatory arrest. J. Thorac. Cardiovasc. Surg. *110*, 1615–1622. , discussion 1621-1612. https://doi.org/10.1016/S0022-5223(95)70021-8.
18. Mora Mangano, C.T., Neville, M.J., Hsu, P.H., Mignea, I., King, J., and Miller, D.C. (2001). Aprotinin, blood loss, and renal dysfunction in deep hypothermic circulatory arrest. Circulation *104*, I276–I281. https://doi.org/10.1161/hc37t1.094702.
19. Augoustides, J.G., Floyd, T.F., McGarvey, M.L., Ochroch, E.A., Pochettino, A., Fulford, S., Gambone, A.J., Weiner, J., Raman, S., Savino, J.S., et al. (2005). Major clinical outcomes in adults undergoing thoracic aortic surgery requiring deep hypothermic circulatory arrest: quantification of organ-based perioperative outcome and detection of opportunities for perioperative intervention. J. Cardiothorac. Vasc. Anesth. *19*, 446–452. https://doi.org/10.1053/j.jvca.2005.05.004.
20. Guan, X., Li, L., Li, J., Jiang, W., Li, H., Wang, X., Han, L., Liu, Y., Gong, M., and Zhang, H. (2023). High preoperative bradykinin level is a risk factor for severe postoperative hypoxaemia in acute aortic dissection surgery. Exp. Physiol. *108*, 683–691. https://doi.org/10.1113/EP091054.
21. Hendriks, K.D.W., Brüggenwirth, I.M.A., Maassen, H., Gerding, A., Bakker, B., Porte, R.J., Henning, R.H., and Leuvenink, H.G.D. (2019). Renal temperature reduction progressively favors mitochondrial ROS production over respiration in hypothermic kidney preservation. J. Transl. Med. *17*, 265. https://doi.org/10.1186/s12967-019-2013-1.
22. Dodson, M., Castro-Portuguez, R., and Zhang, D.D. (2019). NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. Redox Biol. *23*, 101107. https://doi.org/10.1016/j.redox.2019.101107.
23. Schunk, S.J., Zarbock, A., Meersch, M., Küllmar, M., Kellum, J.A., Schmit, D., Wagner, M., Triem, S., Wagenpfeil, S., Gröne, H.J., et al. (2019). Association between urinary dickkopf-3, acute kidney injury, and subsequent loss of kidney function in patients undergoing cardiac surgery: an observational cohort study. Lancet *394*, 488–496. https://doi.org/10.1016/S0140-6736(19)30769-X.
24. Yang, X., Chen, C., Teng, S., Fu, X., Zha, Y., Liu, H., Wang, L., Tian, J., Zhang, X., Liu, Y., et al. (2017). Urinary Matrix Metalloproteinase-7 Predicts Severe AKI and Poor Outcomes after Cardiac Surgery. J. Am. Soc. Nephrol. *28*, 3373–3382. https://doi.org/10.1681/ASN.2017020142.
25. Zhang, W.R., Garg, A.X., Coca, S.G., Devereaux, P.J., Eikelboom, J., Kavsak, P., McArthur, E., Thiessen-Philbrook, H., Shortt, C., Shlipak, M., et al. (2015). Plasma IL-6 and IL-10 Concentrations Predict AKI and Long-Term Mortality in Adults after Cardiac Surgery. J. Am. Soc. Nephrol. *26*, 3123–3132. https://doi.org/10.1681/ASN.2014080764.
26. DiVincenti, L., Jr., Westcott, R., and Lee, C. (2014). Sheep (Ovis aries) as a model for cardiovascular surgery and management before, during, and after cardiopulmonary bypass. J. Am. Assoc. Lab. Anim. Sci. *53*, 439–448.
27. Qureshi, S.H., Patel, N.N., and Murphy, G.J. (2018). Vascular endothelial cell changes in postcardiac surgery acute kidney injury. Am. J. Physiol. Renal Physiol. *314*, F726–F735. https://doi.org/10.1152/ajprenal.00319.2017.
28. Yu, L., Gu, T., Zhang, G., Cheng, S., Fang, Q., and Mao, N. (2014). The deep hypothermic circulatory arrest causes more kidney malfunctions based on a novel rabbit model. Ann. Saudi Med. *34*, 532–540. https://doi.org/10.5144/0256-4947.2014.532.
29. Territo, A., Piana, A., Fontana, M., Diana, P., Gallioli, A., Gaya, J.M., Huguet, J., Gavrilov, P., Rodríguez-Faba, Ó., Facundo, C., et al. (2021). Step-by-step Development of a Cold Ischemia Device for Open and Robotic-assisted Renal Transplantation. Eur. Urol. *80*, 738–745. https://doi.org/10.1016/j.eururo.2021.05.026.
30. Sutton, T.A., Fisher, C.J., and Molitoris, B.A. (2002). Microvascular endothelial injury and dysfunction during ischemic acute renal failure. Kidney Int. *62*, 1539–1549. https://doi.org/10.1046/j.1523-1755.2002.00631.x.
31. Vekstein, A.M., Yerokun, B.A., Jawitz, O.K., Doberne, J.W., Anand, J., Karhausen, J., Ranney, D.N., Benrashid, E., Wang, H., Keenan, J.E., et al. (2021). Does deeper hypothermia reduce the risk of acute kidney injury after circulatory arrest for aortic arch surgery? Eur. J. Cardio. Thorac. Surg. *60*, 314–321. https://doi.org/10.1093/ejcts/ezab044.
32. Shimamura, J., Yokoyama, Y., Kuno, T., Fujisaki, T., Fukuhara, S., Takayama, H., Ota, T., and Chu, M.W. (2023). Systematic review and network meta-analysis of various nadir temperature strategies for hypothermic circulatory arrest for aortic arch surgery. Asian Cardiovasc. Thorac. Ann. *31*, 102–114. https://doi.org/10.1177/02184923221144959.
33. Stenvinkel, P., Painer, J., Kuro-O, M., Lanaspa, M., Arnold, W., Ruf, T., Shiels, P.G., and Johnson, R.J. (2018). Novel treatment strategies for chronic kidney disease: insights from the animal kingdom. Nat. Rev. Nephrol. *14*, 265–284. https://doi.org/10.1038/nrneph.2017.169.
34. Nezu, M., Souma, T., Yu, L., Suzuki, T., Saigusa, D., Ito, S., Suzuki, N., and Yamamoto, M. (2017). Transcription factor Nrf2 hyperactivation in early-phase renal ischemia-reperfusion injury prevents tubular damage progression. Kidney Int. *91*, 387–401. https://doi.org/10.1016/j.kint.2016.08.023.
35. Bekassy, Z., Lopatko Fagerström, I., Bader, M., and Karpman, D. (2022). Crosstalk between the renin-angiotensin, complement and kallikrein-kinin systems in inflammation. Nat. Rev. Immunol. *22*, 411–428. https://doi.org/10.1038/s41577-021-00634-8.
36. Hariri, M.A., Jaffa, M.A., Saoud, R., Zhao, J., Zhu, R., Jaffa, A.A., El-Achkar, G.A., Moussa, M., Kobeissy, F., Hassan, A., et al. (2020). Vascular Cells Proteome Associated with Bradykinin and Leptin Inflammation and Oxidative Stress Signals. Antioxidants *9*, 1251. https://doi.org/10.3390/antiox9121251.
37. Feng, W., Xu, X., Zhao, G., Zhao, J., Dong, R., Ma, B., Zhang, Y., Long, G., Wang, D.W., and Tu, L. (2016). Increased Age-Related Cardiac Dysfunction in Bradykinin B2 Receptor-Deficient Mice. J. Gerontol. A Biol. Sci. Med. Sci. *71*, 178–187. https://doi.org/10.1093/gerona/glu210.
38. Potier, L., Waeckel, L., Vincent, M.P., Chollet, C., Gobeil, F., Jr., Marre, M., Bruneval, P., Richer, C., Roussel, R., Alhenc-Gelas, F., and Bouby, N. (2013). Selective kinin receptor agonists as cardioprotective agents in myocardial ischemia and diabetes. J. Pharmacol. Exp. Ther. *346*, 23–30. https://doi.org/10.1124/jpet.113.203927.
39. Jin, J.Z., Li, H.Y., Jin, J., Piao, S.G., Shen, X.H., Wu, Y.L., Xu, J.C., Zhang, L.Y., Jiang, Y.J., Zheng, H.L., et al. (2020). Exogenous pancreatic kininogenase protects against renal fibrosis in rat model of unilateral ureteral obstruction. Acta Pharmacol. Sin. *41*,

1597–1608. https://doi.org/10.1038/s41401-020-0393-7.

40. Kakoki, M., McGarrah, R.W., Kim, H.S., and Smithies, O. (2007). Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury. Proc. Natl. Acad. Sci. USA *104*, 7576–7581. https://doi.org/10.1073/pnas.0701617104.
41. Tang, S.C.W., Chan, L.Y.Y., Leung, J.C.K., Cheng, A.S., Chan, K.W., Lan, H.Y., and Lai, K.N. (2010). Bradykinin and high glucose promote renal tubular inflammation. Nephrol. Dial. Transplant. *25*, 698–710. https://doi.org/10.1093/ndt/gfp599.
42. Chiang, W.C., Chien, C.T., Lin, W.W., Lin, S.L., Chen, Y.M., Lai, C.F., Wu, K.D., Chao, J., and Tsai, T.J. (2006). Early activation of bradykinin B2 receptor aggravates reactive oxygen species generation and renal damage in ischemia/reperfusion injury. Free Radic. Biol. Med. *41*, 1304–1314. https://doi.org/10.1016/j.freeradbiomed.2006.07.011.
43. Clermont, G., Vergely, C., Jazayeri, S., Lahet, J.J., Goudeau, J.J., Lecour, S., David, M., Rochette, L., and Girard, C. (2002). Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass. Anesthesiology *96*, 80–87. https://doi.org/10.1097/00000542-200201000-00019.
44. Dutra, R.C. (2017). Kinin receptors: Key regulators of autoimmunity. Autoimmun. Rev. *16*, 192–207. https://doi.org/10.1016/j.autrev.2016.12.011.
45. Cicardi, M., Banerji, A., Bracho, F., Malbrán, A., Rosenkranz, B., Riedl, M., Bork, K., Lumry, W., Aberer, W., Bier, H., et al. (2010). Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema. N. Engl. J. Med. *363*, 532–541. https://doi.org/10.1056/NEJMoa0906393.
46. de Zeeuw, D., Akizawa, T., Audhya, P., Bakris, G.L., Chin, M., Christ-Schmidt, H., Goldsberry, A., Houser, M., Krauth, M., Lambers Heerspink, H.J., et al. (2013). Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. N. Engl. J. Med. *369*, 2492–2503. https://doi.org/10.1056/NEJMoa1306033.
47. Kellum, J.A., and Lameire, N.; KDIGO AKI Guideline Work Group (2013). Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). Crit. Care *17*, 204. https://doi.org/10.1186/cc11454.
48. Levey, A.S., Stevens, L.A., Schmid, C.H., Zhang, Y.L., Castro, A.F., 3rd, Feldman, H.I., Kusek, J.W., Eggers, P., Van Lente, F., Greene, T., et al. (2009). A new equation to estimate glomerular filtration rate. Ann. Intern. Med. *150*, 604–612. https://doi.org/10.7326/0003-4819-150-9-200905050-00006.
49. Liu, M., Li, Y., Gao, S., Yan, S., Zhang, Q., Liu, G., and Ji, B. (2020). A novel target to reduce microglial inflammation and neuronal damage after deep hypothermic circulatory arrest. J. Thorac. Cardiovasc. Surg. *159*, 2431–2444.e7. https://doi.org/10.1016/j.jtcvs.2019.06.115.
50. Paller, M.S., and Neumann, T.V. (1991). Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation. Kidney Int. *40*, 1041–1049. https://doi.org/10.1038/ki.1991.312.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bcl-2	Abcam	Cat#ab196495; RRID:AB_2924862
Bax	Abcam	Cat#ab32503; RRID: AB_725631
β-actin	Cell Signaling Technology	Cat#3700S; RRID: AB_2242334
KLK-1	Thermo Fisher	Cat#PA-79738; RRID:AB_2746853
GPX4	Abcam	Cat#ab125066; RRID:AB_10973901
Nrf2(Rat)	Proteintech Group	Cat#16396-1-AP; RRID:AB_2782956
Nrf2(Human)	Abcam	Cat#ab62352; RRID: AB_944418
xCT	Abcam	Cat#ab175186; RRID: AB_2722749
GAPDH	Cell Signaling Technology	Cat#97166S; RRID: AB_2756824
Bax	Abcam	Cat#ab32503; RRID: AB_725631
β-actin	Cell Signaling Technology	Cat#3700S; RRID: AB_2242334
KLK-1	Thermo Fisher	Cat#PA-79738; RRID:AB_2746853
GPX4	Abcam	Cat#ab125066; RRID:AB_10973901
Nrf2(Rat)	Proteintech Group	Cat#16396-1-AP; RRID:AB_2782956
Nrf2(Human)	Abcam	Cat#ab62352; RRID: AB_944418
xCT	Abcam	Cat#ab175186; RRID: AB_2722749
GAPDH	Cell Signaling Technology	Cat#97166S; RRID: AB_2756824
Biological samples		
Peripheral venous blood of 56 patients after HCA surgery, the average age of males and females was 48.3 years old	Homo sapiens	The Ethics Committee of Beijing Anzhen Hospital approved this study (Approval Number: 2014019) and confirmed that informed consent was obtained from all subjects.
Rat kidney and blood samples, all from male SD rats	Rat	The Institutional Animal Research and Use Committee of Capital Medical University approved the experiments and confirmed that all experiments complied with relevant regulatory standards.
Chemicals, peptides, and recombinant proteins		
DALBK	Sigma Aldrich	Cat#B6769
Icatibant	MedChemExpress	Cat#HY-17446
Bradykinin	MedChemExpress	Cat#HY-P0206
CDDO	MedChemExpress	Cat#HY-14909
Critical commercial assays		
Creatinine test kit	Nanjing Jiancheng Bioengineering Research Institute Co., Ltd.	Cat#C011-2-1
BUN detection kit	Nanjing Jiancheng Bioengineering Research Institute Co., Ltd.	Cat#C013-2-1
Bradykinin ELISA kit	Wuhan Cloud Clone Technology Co., Ltd.	Cat#CEA874Ra
SOD detection kit	Beijing Solarbio Science & Technology Co.,Ltd.	Cat#BC5165

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MDA detection kit	Beijing Solarbio Science & Technology Co.,Ltd.	Cat#BC0025
ROS detection kit	Beyotime Biotechnology	Cat#S0033S
Apoptosis detection kit	Beyotime Biotechnology	Cat#C1088
Experimental models: Cell lines		
Human proximal renal tubular epithelial cell line, HK-2	National Collection of Authenticated Cell Cultures	Cat#SCSP-511
Experimental models: Organisms/strains		
Rat: Wild type	Beijing HFK Bioscience Co., Ltd.	Sprague dawley Rat
Oligonucleotides		
B1R	SinoGenoMax	Seq-Forward:5′- GGTGGCAGCAACGACAGAG -3′ Seq-Reverse:5′- GCAGAGGTCAGTTCCGAAGG -3′
B2R	SinoGenoMax	Seq-Forward:5′- GCCTGCGTCATTGTCTAC -3′ Seq-Reverse:5′- ACTTCTTCATCTCGTTGTTCC -3′
GAPDH	SinoGenoMax	Seq-Forward:5′- TCA TTG ACC TCA ACT ACA -3′ Seq-Reverse:5′- CAAAGTTGTCATGGA TGACC -3′
Software and algorithms		
R 4.0.4	The R Project	https://www.r-project.org/
GraphPad Prism version 9.0.0	GraphPad Software	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: Prof. Hongjia Zhang, M.D.; zhanghongjia722@ccmu.edu.cn.

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Participants

This study recruited 281 adult patients who underwent aortic surgery at 9 medical centers in China between January 1, 2015, and January 1, 2016. Among them, 56 patients (18 females and 38 males) underwent HCA, and 225 patients (55 females and 170 males) did not. All patients were Han nationality. The Ethics Committee of Beijing Anzhen Hospital approved this study (No. 2014019) and confirmed that informed consent was obtained from all subjects. Informed consent was obtained from the 56 patients who underwent HCA or from their family members. No specimens were collected from the 225 patients who did not undergo an HCA procedure; therefore, the need for written informed consent was waived. All patients were diagnosed with Stanford type-A aortic dissection through aortic computed tomography angiography (CTA) by experienced imaging specialists and cardiovascular surgeons. The surgeries were performed by the surgical team of the medical center at the time of the patient's admission. Patients who had renal failure before surgery, incomplete surgical data, or surgery involving the abdominal aorta and below were excluded. The diagnosis of ARF was established according to the Kidney Disease: Improving Global Outcomes guidelines.[47] Postoperative ARF was defined as patients whose serum creatinine (Scr) rises more than 3 times within 7 days after surgery compared with the latest Scr before surgery, or whose absolute value rises by more than 4.0 mg/dL (353.6 μmol/L), or who require RRT treatment. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration formula (CKD-EPI).[48]

All patients underwent surgery requiring sternotomy and CPB using a CPB machine via the axillary or femoral artery and venous cannulation. Patients undergoing HCA procedures were cooled at a rate of 0.5∼1.0°C/min. After lowering to the required temperature, the CPB machine was completely stopped, and a bloodless distal aortic anastomosis was performed with antegrade cerebral perfusion, followed by recovery of body temperature at a rate of 0.3∼0.5°C/min.

Animals

Sprague–Dawley rats were purchased from HFK Bioscience (Beijing, China). Male rats (400-500 g body weight, 15 weeks old) were used in the experiments. The procedures involving the animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Institutional Animal Research and Use Committee of Capital Medical University. Animals were humanely cared for and euthanized by carbon dioxide inhalation. The Institutional Animal Research and Use Committee of Capital Medical University approved the experiments and confirmed that all experiments complied with relevant regulatory standards.

METHOD DETAILS

Rat model of hypothermic circulatory arrest

The rat HCA model was established by modifying a previously described model.[49] Briefly, rats were anesthetized with 5% isoflurane, endotracheally intubated, connected to an anesthesia machine, mechanically ventilated with 3% isoflurane plus pure oxygen, and maintained under anesthesia. Life indices, including the rectal temperature and electrocardiogram results, were monitored. The cardiopulmonary bypass pipeline and membrane were filled with hydroxyethyl starch. The right external jugular vein and tail artery were dissected from the right neck and tail of the rats. A venous cannula prewetted with 250 U/ml heparin was placed into the right external jugular vein, and 500 IU/kg heparin was injected. The venous cannula was inserted into the right atrium. An arterial cannula was then placed in the tail artery. The venous catheter and arterial catheter were connected to the cardiopulmonary bypass pipeline, and a mixture of 5% isoflurane and pure oxygen was passed into the membrane lung. The cardiopulmonary bypass pump was started and maintained at a flow rate of 100-120 ml/kg·min. During that time, the blood was drained by a venous catheter, driven by the cardiopulmonary bypass pump, oxygenated in the membrane lung, temperature adjusted in the temperature exchanger, and then returned to the tail artery through the arterial cannula. Then, the HCA process would start. The body temperature of the rats was lowered to 18°C or 28°C within 50 mins through a temperature exchanger. After the electrocardiogram showed cardiac arrest, the cardiopulmonary bypass pump was turned off and maintained for 10 or 20 minutes. The cardiopulmonary bypass pump was restarted, and the rats' body temperatures were restored to 37°C within 50 minutes. We adjusted the $PaCO_2$ concentration and pH by adjusting the ventilator parameters and administering sodium bicarbonate. As the body temperature recovered, the rats' heartbeats also recovered. Then, the venous and arterial cannulas were removed, and the incisions were sutured. After the rats resumed spontaneous breathing, the tracheal cannulas were removed. The rats were euthanized after 24 hours, and blood and kidneys were collected for experiments. Representative images of the surgery are shown in Figure S1. The preoperative, immediate postoperative, and 24-hour postoperative blood gas results of the main groups are shown in Table S1.

Sham-operated rats underwent arterial and venous cannulation without cardiopulmonary bypass or HCA. The CPB group underwent cardiopulmonary bypass without HCA. In the HCA group, rats underwent cardiopulmonary bypass with HCA for 10 minutes, after which their body temperature was lowered to 18°C. The MH group underwent moderate hypothermic cardiopulmonary bypass, during which their body temperature was lowered to 28°C without circulatory arrest. The DH group underwent deep hypothermic cardiopulmonary bypass, in which their body temperature was reduced to 18°C without circulatory arrest. The HCA-20 group received cardiopulmonary bypass and HCA for a 20-minute duration.

For DALBK treatment, the rats were administered DALBK 30 minutes before anesthesia (1.5 mg/kg, i.p.). For icatibant treatment, the rats were administered icatibant 30 minutes before anesthesia (1.5 mg/kg, i.p.). For bradykinin treatment, the rats received synthesized bradykinin 30 minutes before anesthesia (3.0 mg/kg, i.p.).

Histological examination

Kidneys were fixed with 10% neutral formaldehyde, cut into 4 μm thick sections after paraffin embedding, and then deparaffinized and rehydrated. The specimens were stained with HE and PAS. Paller's renal tubular scoring criteria were applied to assess renal injury.[50] The scores were evaluated according to cell necrosis, brush border loss, cast formation, and tubular dilatation (0, none; 1, ≤25%; 2, 26-50%; 3, 51-75%; and 4, >75%). The scores were evaluated in a blinded manner by a nephrologist. Twenty visual fields were randomly selected for each sample to calculate the kidney injury score.

TUNEL staining

A TUNEL assay was performed with paraffin sections using a kit (apoptosis detection kit, C1088, Beyotime Biotechnology) according to the manufacturer's instructions. Nuclei were stained with DAPI. Images were captured under a fluorescence microscope, and positively labeled cells were counted using ImageJ software to calculate the proportion of positive cells.

Assessment of kidney function

Scr and blood urea nitrogen levels were measured using assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

The Scr levels of the patients were detected in the Laboratory Department of Anzhen Hospital. The definition of acute renal failure was based on the criteria defined by the Kidney Disease Improving Global Outcomes.[47]

Assessment of oxidative stress in the kidney

After the kidney tissues or cells were homogenized, superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured using assay kits (Solarbio Life Science) according to the manufacturer's instructions. The levels of SOD and MDA were normalized to the protein concentration of the homogenate.

ELISA

The plasma bradykinin, TNF-α, IL-1, IL-2, IL-6 and FXII levels were measured using ELISA kits (Cloud-Clone Corp.) according to the manufacturer's instructions.

After homogenizing the rat kidney tissues, bradykinin and IL-1β levels were measured using ELISA kits (Cloud-Clone Corp.) according to the manufacturer's instructions. The levels of bradykinin were normalized to the protein concentration of the homogenate.

Western blotting

Kidney tissues or cells were homogenized. Total protein was extracted using RIPA lysis buffer (Beijing Robi Biotechnology) containing protease and phosphatase inhibitors. Proteins were separated using 10% or 12% SDS–PAGE, transferred to PVDF membranes, and incubated with the corresponding primary and secondary antibodies.

Immune complexes were visualized with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and detected with an AI600 Chemiluminescence Imager (GE Healthcare Life Sciences). The band densities were analyzed by ImageJ software, are expressed relative to those of GAPDH or β-actin and were normalized to the mean density of the controls.

Cell culture

The human proximal tubule epithelial cell line HK-2 (ATCC, CRL-2190) was purchased from the National Collection of Authenticated Cell Cultures. HK-2 cells were cultured in a 1:1 mixture of DMEM containing 1.0 g/L glucose (Life Technologies) and F12 medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Life Technologies).

HK-2 cells were serum starved for 24 hours before the indicated treatments were applied. To simulate the high oxidative stress state of renal tubular epithelial cells during HCA, the HK-2 cells were incubated with 100 μM hydrogen peroxide (H_2O_2) for 24 hours and simultaneously incubated with bradykinin, DMSO or CDDO dissolved in DMSO.

siRNA-mediated knockdown of HK-2

After the HK-2 cells were incubated at 50-70% confluence in 6-well plates for 24 hours, the cells in each well were treated with 100 nM B1R siRNA (Santa Cruz Biotechnology, sc-39878), B2R siRNA (Guangzhou RiboBio) or control siRNA (Santa Cruz Biotechnology, sc-37007). The corresponding siRNAs were mixed with an equal volume of Lipofectamine 3000 Transfection Reagent (Life Technologies) in Opti-MEM (Life Technologies). Cells were harvested 72 hours after transfection.

ROS measurement

For ROS detection, cells were treated with 20 μM DCFDA (Beyotime Biotechnology), and the mean fluorescence intensity was calculated by flow cytometry or observed under a fluorescence microscope. Hoechst 33342 (10 μM; Solarbio Life Science) was used for nuclear staining.

QUANTIFICATION AND STATISTICAL ANALYSIS

Basic statistical analysis of clinical data was performed using R 4.0.4. Continuous variables are expressed as the mean ± standard deviation, and categorical variables are expressed as the frequency (n) and percentage (%). The data were analyzed using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA) and are expressed as the mean ± SD of independent replicates. Risk factors for acute renal failure were assessed using multivariable logistic regression analysis. *P* values less than 0.05 were considered to indicate statistical significance. Statistical significance was determined by Student's t test (two-tailed) for two groups and ANOVA followed by Bonferroni post hoc correction for multiple groups and set at $*P<0.05$, $**P<0.01$, $***P<0.001$.