Published in final edited form as:

Kidney Int. 2019 April ; 95(4): 815–829. doi:10.1016/j.kint.2018.11.021.

Wnt/ β -catenin signaling mediates both heart and kidney injury in type 2 cardiorenal syndrome

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Abstract

In type 2 cardiorenal syndrome, chronic heart failure is thought to cause or promote chronic kidney disease; however, the underlying mechanisms remain poorly understood. We investigated the role of Wnt signaling in heart and kidney injury in a mouse model of cardiac hypertrophy and heart failure induced by transverse aortic constriction (TAC). At 8 weeks after TAC, cardiac hypertrophy, inflammation, and fibrosis were prominent, and echocardiography confirmed impaired cardiac function. The cardiac lesions were accompanied by upregulation of multiple Wnt ligands and activation of β -catenin, as well as activation of the renin-angiotensin system (RAS). Wnt3a induced multiple components of the RAS in primary cardiomyocytes and cardiac fibroblasts *in vitro*. TAC also caused proteinuria and kidney fibrosis, accompanied by klotho depletion and β -catenin activation in the kidney. Pharmacologic blockade of β -catenin with a small molecule inhibitor or the RAS with losartan ameliorated cardiac injury, restored heart function, and mitigated the renal lesions. Serum from TAC mice was sufficient to activate β -catenin and trigger tubular cell injury *in vitro*, indicating a role for circulating factors. Multiple inflammatory cytokines were upregulated in the circulation of TAC mice, and tumor necrosis factor- α was able to inhibit klotho, induce β -catenin activation, and cause tubular cell injury *in vitro*. These studies identify Wnt/ β -catenin signaling as a common pathogenic mediator of heart and kidney injury in type 2 cardiorenal syndrome after TAC. Targeting this pathway could be a promising therapeutic strategy to protect both organs in cardiorenal syndrome.

Graphical Abstract

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DISCLOSURE

All the authors declared no competing interests.

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Supplementary information is available at Kidney International's website.

Keywords

Wnt; β -catenin; TAC; cardiac hypertrophy; fibrosis; chronic kidney disease

INTRODUCTION

Heart failure is a complex and progressive condition characterized by reduced cardiac output to meet the metabolic demands of peripheral organs. It affects at least 26 million people worldwide, and its prevalence is increasing.^{1,2} Currently there are 5.7 million people in the US having heart failure, and it is estimated that by 2030 more than 8 million people will have this condition, accounting for a 46% increase in prevalence.² Heart failure is associated with serious morbidity and high mortality, and its healthcare expenditures rise rapidly. Despite the significant advances in therapies during the last decades, the mortality rate of heart failure remains high, with 17-45% death incident in the first year after diagnosis and remaining death in the coming 5 years.^{3,4} In short, heart failure is a major and growing public health challenge worldwide.

The heart and kidneys are two vital organs that communicate reciprocally each other, and dysfunction in one organ would inevitably affect the health of the other. This close relationship is reflected through cardiorenal syndrome (CRS).⁵ Based on the organ that initiates the insult, as well as the time-frame and nature of disease, CRS is classified into five subtypes. CRS type 2 (CRS2) is specifically defined as chronic abnormalities in cardiac function causing progressive chronic kidney disease (CKD).⁶ As reported, over 50% of patients with cardiac failure are in the condition of renal insufficiency.⁷ Furthermore, even a slight reduction in renal function is associated with high mortality rate in patients with cardiovascular diseases.^{8,9} As heart-kidney interactions are bidirectional, CKD can have deleterious effects on cardiovascular function as well.¹⁰ Clearly, CRS2 patients with functional disorders in both organs will be more vulnerable and have a high rate of mortality.

The mechanism underlying the cardio-renal connection in pathologic conditions remains incompletely understood. Several potential explanations have been postulated, including hemodynamic abnormalities, sympathetic nervous system hyperactivity, renin-angiotensin system (RAS) activation, systemic inflammation and oxidative stress.¹¹⁻¹⁵ However, it remains elusive whether there is a common pathogenic mediator that triggers tissue injury in

both organs. Clearly, this question is significant and important not only for understanding the mechanism of CRS2 but also for designing future treatment strategies.

Wnt/ β -catenin is an evolutionarily conserved signaling pathway involved in organ development, injury repair, tissue remodeling and inflammation.¹⁶⁻¹⁸ Wnt signaling is generally silent in adult tissues, but reactivated after injury. Mounting studies indicate a potential role of Wnt/ β -catenin activation in the pathogenesis of CKD,^{19,20} as well as in other organ disorders.²¹⁻²⁴ These observations prompted us to hypothesize that Wnt/ β -catenin could be a common pathogenic mediator of both organs in CRS2.

In this study, we investigated the expression, activation and potential role of Wnt/ β -catenin in mediating heart and kidney lesions in mouse model of transverse aortic constriction (TAC). We demonstrate that Wnt/ β -catenin is activated in the heart and kidney after TAC, and blockade of this signaling ameliorate tissue lesions in both organs. Our studies suggest Wnt/ β -catenin as a unified pathogenic mediator of heart and kidney disorders, and therefore targeting this signaling might be a novel strategy for the treatment of cardiorenal syndrome.

RESULTS

Heart failure induced by transverse aortic constriction causes kidney injury

To study the effect of heart failure on kidney integrity, we employed mouse model of pressure overload-induced cardiac hypertrophy and heart failure by TAC. As shown in Figure 1a, Western blotting demonstrated a marked increase of hypertrophic marker β -myosin heavy chain (β -MHC), matrix protein fibronectin and proinflammatory tumor necrosis factor- α (TNF- α) in the heart at 8 weeks after surgery. Figure 1, b through d, shows the quantitative data on β -MHC, fibronectin and TNF- α , in sham and TAC groups. Cardiac crosssections also revealed overt hypertrophic change in mice subjected to TAC (Figure 1e). These results indicate that TAC causes substantial heart lesions characterized by cardiac hypertrophy, inflammation and fibrosis.

We found that TAC-triggered heart failure also caused kidney lesions in mice. As shown in Figure 1, f through i, TAC repressed renal expression of podocalyxin, a marker of glomerular podocytes,²⁵ in mice. Meanwhile, renal expression of fibronectin and Snail1 was induced at 8 weeks after TAC (Figure 1, f through i). Masson's trichrome staining revealed substantial interstitial fibrosis in mice at 8 weeks after TAC (Figure 1j). Therefore, these results indicate that chronic cardiac failure causes kidney injury presumably via cardiorenal inter-organ crosstalk.

Wnt/ β -catenin is activated in the heart after TAC

To investigate the mechanism underlying TAC-induced cardiac lesions, we studied the potential role of Wnt/ β -catenin signaling in this process. Using quantitative, real-time RT-PCR (qRT-PCR) approach, we systematically examined the expression of all 19 Wnt ligands in the heart after TAC. As shown in Figure 2a, several Wnt ligands were induced in the heart of mice at 8 weeks after TAC, including Wnt1, Wnt3a, Wnt7a, Wnt8b and Wnt10b. Western blot analysis also confirmed cardiac induction of Wnt1 and Wnt3a proteins in TAC mice (Figure2b). Since β -catenin is the common intracellular mediator of all canonic Wnt

signaling, we then examined the expression of both active and total β -catenin in the heart. As shown in Figure 2, b through f, TAC induced cardiac β -catenin activation, as defined by induction of active, dephosphorylated β -catenin. Not surprisingly, activation of β -catenin led to its stabilization, resulting in an increase in total β -catenin (Figure 2, b and f). Consistently, immunostaining illustrated that Wnt3a and β -catenin were induced in hypertrophic cardiomyocytes of the heart after TAC. Collectively, these findings indicate that Wnt/ β -catenin signaling is activated in the heart of TAC mice.

Blockade of Wnt/ β -catenin prevents TAC-induced heart injury and dysfunction

To determine whether Wnt/ β -catenin activation plays a role in mediating TAC-induced cardiac injury, we next examined the effect of inhibition of this signaling. To this end, ICG-001, a specific small molecule inhibitor of β -catenin-mediated gene expression,^{24,26} was administered at 5 mg/kg body weight to the mice, starting at 4 weeks after TAC (Figure 3a). As shown in Figure 3, b through h, treatment with ICG-001 abolished TAC-induced expression of β -MHC, TNF- α , fibronectin and type I collagen in the heart. Meanwhile, ICG-001 also inhibited cardiac β -catenin activation, compared to TAC alone (Figure 3, b through h). Interestingly, blockade of angiotensin II type 1 receptor (AT1), a downstream target of Wnt/ β -catenin,²⁷ with losartan also inhibited TAC-triggered expression of β -MHC, fibronectin and collagen I and TNF- α in the heart (Figure 3, b through f).

Consistent with Western blot results, morphological assessment and immunostaining of heart sections gave rise to the same results. As shown in Figure 3i, TAC caused noticeable cardiomyocytes hypertrophy and led to expanded cardiomyocyte sizes, and induced β -MHC in cardiomyocytes and fibronectin in cardiac interstitium (Figure 3i), which were inhibited by ICG-001 or losartan.

We further studied cardiac function in different groups of mice by using echocardiography. TAC caused cardiac dysfunction, as reflected by increased values of left ventricular internal dimension at end-systole (LVIDs) and at end-diastole (LVIDd) (Table 1), left ventricular end systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV), as well as decreased left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) (Table 1). ICG-001 or losartan ameliorated these changes (Table 1), indicating a critical role for canonic Wnt/ β -catenin and its downstream RAS in mediating TAC-induced cardiac injury and dysfunction.

Wnt/ β -catenin activates cardiac RAS *in vivo* and *in vitro*

The finding that losartan is effective in ameliorating TAC-mediated cardiac injury prompted us to investigate the possibility that RAS may mediate Wnt/ β -catenin action *in vivo*. To test this hypothesis, we examined the regulation of RAS components in the heart after TAC. As shown in Figure 4, a through d, TAC induced angiotensin converting enzyme (ACE), renin and AT1 expression in the heart, and treatment with ICG-001 or losartan hampered the induction of these RAS components.

To provide direct evidence for a role of Wnt/ β -catenin in cardiac RAS activation, we used *in vitro* models of primary cardiomyocytes and cardiac fibroblasts, as previously reported.²⁸ As shown in Figure 3, e through j, incubation of primary cardiomyocytes with Wnt3a induced the

expression of ACE, renin and AT1. Furthermore, this induction of RAS components was abolished by ICG-001 (Figure 3, e through j). Similarly, Wnt3a also induced ACE, angiotensinogen (AGT) and AT1 in primary cardiac fibroblasts (Figure 3, k through p). These results indicate that Wnt/ β -catenin is an upstream regulator of RAS in the heart after TAC.

Inhibition of Wnt/ β -catenin/RAS also protects kidneys against TAC-induced injury

We next examined whether inhibition of Wnt/ β -catenin/RAS axis by ICG-001 or losartan affects TAC-triggered kidney injury. As shown in Figure 5, a through e, Western blot analysis confirmed that ICG-001 or losartan inhibited TAC-induced kidney injury and fibrotic responses, as illustrated by down-regulation of renal expression of fibronectin, Snail1 and kidney injury molecule-1 (Kim-1). In addition, ICG-001 or losartan preserved renal expression of podocalyxin after TAC (Figure 5, a and c). Furthermore, ICG-001 not only suppressed renal β -catenin activation (Figure 5, a and f), but also reduced the levels of total β -catenin in the kidney after TAC (Figure 5, a and g). TAC also induced renal expression of ACE, renin and AT1, and ICG-001 or losartan abolished their induction in the kidney (Supplementary Figure S1).

Similar results were obtained by immunostaining of kidney sections. As shown in Figure 5h, TAC inhibited glomerular podocalyxin expression and induced β -catenin predominantly in kidney tubules, but ICG-001 or losartan largely restored podocalyxin and inhibited renal β -catenin protein. Consistently, Masson trichrome staining (MTS) revealed that significant renal fibrotic lesions were observed after TAC and ICG-001 or losartan ameliorated these lesions (Figure 5h).

We next assessed urinary albumin levels of the mice, in view of a diminished expression of podocalyxin after TAC. As shown in Figure 5i, TAC caused an albuminuria at 8 weeks, and ICG-001 or losartan abolished TAC-triggered albuminuria. ICG-001 or losartan also showed the tendency of reducing serum creatinine after TAC, although it did not reach statistical significance (Supplementary Figure S2). We further examined the linkage of proteinuria/kidney impairment to heart injury in this model. As presented in Figure 5j, there was a close correlation between albuminuria and serum BNP, a marker for heart injury.^{21,29} Hence, these data suggest that inhibition of Wnt/ β -catenin/RAS not only protects heart from injury but also preserves kidney integrity after TAC.

Circulating factors link heart failure to kidney lesion

We then investigated the potential mechanism that links heart failure to kidney injury after TAC. Of several potential pathways, blood circulation is clearly a perceivable way linking two organs. Therefore, we sought to test whether circulating factors are responsible for mediating heart-kidney interactions. To this end, serum was collected from different groups of mice, and used to treat kidney proximal tubular cells (HKC-8). As shown in Figure 6, serum from TAC-mice at a concentration of 2.5% was able to induce β -catenin activation and Snail1 and Kim-1 expression in HKC-8 cells, compared with serum from control mice. However, serum from TAC mice treated with ICG-001 or losartan exhibited a reduced effect on β -catenin, Snail1 and Kim-1 expression. These data suggest that circulating factors in the

serum from TAC mice are sufficient to cause kidney tubular cell injury by triggering β -catenin activation.

To further confirm the role of β -catenin activation triggered by TAC serum in mediating kidney injury, we knocked down β -catenin expression in HKC-8 cells by small hairpin RNA (shRNA) lentiviral vector. As shown in Figure 6, f through k, knockdown of β -catenin inhibited its activation, and abolished the expression of fibronectin, Kim-1, Snail1 induced by TAC serum. Similarly, knockdown of β -catenin also blunted the induction of multiple RAS components such as AGT, renin and AT1 by TAC serum in HKC-8 cells (Figure 6, f, l, m and n).

Circulating levels of inflammatory cytokines correlate with heart and kidney injury after TAC

To elucidate the potential factors in the circulation responsible for mediating kidney injury, we examined the levels of major pro-inflammatory cytokines, as systemic inflammation is a characteristic feature of this model.^{30,31} As shown in Figure 7, a through c, the levels of several pro-inflammatory cytokines such as TNF- α , monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β) were markedly elevated in the circulation in TAC mice, compared to the controls. However, ICG-001 or losartan treatment decreased the circulating levels of these cytokines (Figure 7), suggesting that inhibition of Wnt/ β -catenin/RAS axis suppresses TAC-initiated systemic inflammation. Similarly, serum BNP was increased in TAC mice, which was reduced after ICG-001 or losartan treatment (Figure 7d). There was a close correlation between circulating TNF- α and BNP (Figure 7e), suggesting a positive linkage of systemic inflammation to heart injury. Similarly, the levels of serum TNF- α and urinary albumin were also tightly linked (Figure 7f), highlighting a positive association between systemic inflammation and kidney injury. Therefore, it is conceivable that systemic inflammation might play an important role in linking heart failure to kidney injury after TAC.

TNF- α triggers β -catenin activation and tubular injury *in vitro*

To provide direct evidence linking systemic inflammation to kidney injury, we studied the effect of TNF- α on tubular epithelial cells. As shown in Figure 8a, immunofluorescence staining revealed that TNF- α induced β -catenin accumulation predominantly in the nuclei of HKC-8 cells, suggesting TNF- α is sufficient to induce β -catenin activation in kidney cells. Furthermore, treatment with ICG-001 or losartan abolished TNF- α -induced β -catenin activation. Similarly, Western blot analysis of total and activated β -catenin gave rise to the same results as immunofluorescence staining (Figure 8b). Meanwhile, TNF- α stimulated both Snail1 and Kim-1 expression in HKC-8 cells, and ICG-001 or losartan abolished the induction of these proteins (Figure 8, b through f). These data indicate that TNF- α , a cytokine that is upregulated in the circulation after TAC, can adequately trigger β -catenin activation and tubular cell injury.

Depletion of renal Klotho further aggravates cardiac lesion after TAC

Klotho is an anti-aging protein mainly produced in the kidney, but it exerts protective effect on both kidney and heart.^{32,33} We also investigated klotho expression in the kidney and in

blood circulation. As shown in Figure 9, a and b, renal expression of klotho was dramatically suppressed in TAC mice, compared with the controls. ICG-001 or losartan treatment restored, at least partially, klotho expression in the kidneys (Figure 9, a and b). We further assessed the levels of circulating klotho in different groups by ELISA. As shown in Figure 9c, circulating Klotho was decreased in TAC mice, but ICG-001 or losartan partially restored serum klotho level. Similar results were obtained by immunostaining for renal klotho protein (Figure 9d).

To investigate the mechanism by which TAC causes klotho depletion, we examined the effect of systemic inflammation on klotho expression in kidney tubular cells *in vitro*. As shown in Supplementary Figure S3, a and b, TNF- α suppressed klotho expression in HKC-8 cells. However, incubation with ICG-001 did not restore klotho expression *in vitro*, suggesting that TNF- α -induced klotho depletion is not mediated by β -catenin. Therefore, the restoration of klotho expression after ICG-001 treatment *in vivo* probably reflects the consequence of an ameliorated nephropathy and systemic inflammation.

We finally examined the effects of klotho deficiency on cardiac hypertrophy and fibrosis by using *in vitro* system. As shown in Figure 9, e through h, Wnt3a induced β -catenin activation and β -MHC and α -actin expression in primary cardiomyocytes, which was abolished by exogenous klotho. Similarly, klotho also inhibited fibronectin and α -SMA expression induced by Wnt3a in primary cardiac fibroblasts (Figure 9, I through m). These results suggest that loss of klotho could enhance Wnt/ β -catenin signaling, further aggravating cardiac injury and dysfunction after TAC (Figure 9n).

DISCUSSION

The results presented in this study demonstrate that TAC, a model of non-ischemic, hypertrophic and pressure overload-induced heart failure, not only causes chronic heart disease as widely reported,³⁴ but also triggers secondary kidney injury, thereby creating a model of CRS2.³⁵ As depicted in Figure 9n, we propose a cascade of events of TAC-mediated CRS, in which TAC triggers Wnt/ β -catenin activation in the heart (Figure 2), leading to cardiac RAS activation (Figure 4) and the release of pro-inflammatory cytokines into the circulation (Figure 7). Circulating cytokines such as TNF- α , in turn, repress klotho expression in the kidney, leading to renal β -catenin and RAS activation, and kidney injury. As kidney is the main source of circulating klotho,³⁶ damage to this organ reduces its ability to produce and secrete klotho,³⁷ leading to klotho deficiency (Figure 9c). Because klotho is cardio-protective,^{32,33} this state of klotho deficiency will then further exacerbate cardiomyocyte hypertrophy and cardiac fibroblast activation induced by Wnts (Figure 9, e through m), creating a vicious cycle of heart-kidney interactions. The present study represents the first to characterize both heart and kidney disease in this model at the same time, and provide a unique paradigm for interrogating the mechanism of CRS2 and evaluating novel strategies for treatment in the preclinical setting.

One interesting finding of this study is that Wnt/ β -catenin is a common pathogenic mediator of both heart and kidney lesions after TAC. Not surprisingly, TAC causes primary heart injury, as manifested by cardiomyocyte hypertrophy, inflammatory cytokine over-

production, cardiac fibrosis (Figure 1) and left ventricular systolic and diastolic functional deterioration (Table 1). Of interest, these changes are associated with cardiac upregulation of multiple Wnt ligands and activation of β -catenin (Figure 2). Blockade of Wnt/ β -catenin by ICG-001 is able to prevent cardiac lesions and dysfunction. These findings are supported by earlier genetic studies in which either activation or interruption of canonic Wnt signaling by manipulating *Dapper-1* or *disheveled* gene regulates cardiac remodeling and hypertrophy.^{21,29} Notably, Wnt/ β -catenin could elicit its action by activating RAS, as Wnt3a directly induces multiple components of RAS in primary cardiomyocytes and fibroblasts (Figure 4). Consistently, blockade of AT1 by losartan is equally effective in ameliorating cardiac injury.

TAC also induces β -catenin activation in the kidneys, which is accompanied by the development of mild to moderate proteinuria and fibrosis in this model (Figure 5). The activation of renal β -catenin is also associated with loss of klotho (Figure 9a), an endogenous antagonist of Wnt signaling,³⁷ and induction of renal RAS components (Figure S1), the downstream targets of Wnt/ β -catenin.²⁷ These findings are in harmony with previous observations that Wnt/ β -catenin plays a crucial role in the onset and progression of CKD.^{38,39} Taken together, our studies uncover Wnt/ β -catenin as a unified pathogenic mediator of provoking both heart and kidney injury in the evolution of CRS2, and therefore this signaling could be a promising target for the treatment of cardiorenal syndrome.

The present study also explores the mechanism underlying the heart-kidney connection after TAC. Although multiple potential pathways could explain for renal dysfunction in the setting of chronic cardiac failure, our results highlight a crucial role of systemic inflammation in mediating heart-kidney crosstalk. This conclusion is supported by several lines of evidence. First, pro-inflammatory cytokines such as TNF- α , MCP-1 and IL-1 β are increased in the circulation of TAC mice, which is closely correlated with the markers of cardiac (BNP) and renal (albuminuria) injury (Figure 7). Second, either serum from TAC mice or TNF- α alone is able to mediate kidney injury by activating β -catenin and its downstream target genes (Figure 6 and 8). Third, alleviation of the TAC-elicited systematic inflammation by ICG-001 or losartan is associated with reduction of kidney lesion. Therefore, it is conceivable that elevated inflammatory cytokines in the condition of chronic cardiac failure may serve as a cardiorenal connector and plays a key role in causing secondary kidney lesions.^{14,40} These findings are also consistent with earlier studies showing that systematic inflammation is connected to tissue injury in different organs.^{30,41,42} Of note, although numerous Wnt ligands are induced in the heart after TAC, it is unlikely that cardiac Wnts directly trigger renal activation of β -catenin, because Wnts do not freely spread over long distances *in vivo*.⁴³ However, we cannot exclude the possibility that other abnormalities such as hypoperfusion, sympathetic activation and RAS activation in the setting of chronic cardiac failure may also contribute to secondary kidney injury in this model of CRS2.

How inflammatory cytokines in the circulation causes kidney injury remains incompletely understood. Our studies suggest that Klotho, an anti-aging protein highly expressed in renal tubules of normal kidneys,³⁷ could be a mediator that links systematic inflammation to renal β -catenin activation in CRS2. Serum from TAC mice or purified TNF- α alone is sufficient to repress renal klotho expression *in vivo* and *in vitro*, which leads to low klotho level in the

circulation after TAC (Figure 9). These results are supported by previous studies demonstrating an intrinsic connection between inflammation and klotho downregulation.^{44,45} As klotho is a Wnt antagonist,³⁷ decreased klotho would result in β -catenin activation (Figure 5), which then leads to the expression of its downstream target genes such as fibronectin, Snail1 and multiple components of RAS.^{38,39} Notably, ICG-001 does not reverse the klotho downregulation by TNF- α *in vitro* (Figure S3), suggesting that the restoration of renal klotho expression *in vivo* is a consequence, but not a cause, of an improved kidney histology after ICG-001 treatment. It is worthwhile to point out that because klotho elicits its protective activities in multiple organs including heart and kidney, klotho depletion by inflammatory cytokines would aggravate tissue injury in both kidney and heart of TAC mice.

The results in the present study may have significant implications in designing future therapeutics for patients with cardiorenal syndrome. Currently, clinicians are often faced with competing therapeutic options when taking care of CRS patients, as pharmacological drugs used in the management of heart failure may sometimes deteriorate kidney function.^{6,46} In this regard, the identification of Wnt/ β -catenin as a common pathogenic mediator of heart and kidney lesions in CRS2 suggests that strategies to block this signaling will benefit both organs. Although anti-TNF- α may also be effective, it is conceivable that blockade of β -catenin with ICG-001 could protect heart and kidney through multiple mechanisms, because it is the upstream master regulator of multiple RAS components and inflammation.⁴⁷

There are some limitations and pitfalls of this study. One issue is the uncertainty of its clinical relevance. Future studies are warranted to investigate human samples of CRS2 patients. Another issue is related to the use of C57/BL6 mice, as this strain is relatively resistant to kidney injury and fibrosis. This may explain for a moderate increase in serum creatinine level in this CRS2 model (Figure S2). Finally, some genetic models including cell type-specific conditional knockout mice are needed for interrogating the patho-mechanism of CRS2. Clearly, more future studies are warranted in these areas.

In summary, we demonstrate herein that Wnt/ β -catenin is a unified pathogenic mediator of both heart and kidney lesions in mouse model of CRS2 induced by TAC. The heart-kidney connection is likely to be mediated by systematic inflammation, by which circulating cytokines repress klotho expression in the kidney, leading to renal β -catenin activation and secondary kidney injury. This study is unprecedented in that it fully characterizes the lesions of both heart and kidney at the same time, and therefore provides a complete landscape of the cardiorenal syndrome. Our studies also suggest that inhibition of β -catenin could be a novel strategy for protecting both heart and kidney in the therapy of patients with cardiorenal syndrome.

METHODS

Animal models

Male C57BL/6 mice were purchased from the Experimental Animal Center of the Southern Medical University. Mice were randomly divided into four groups (n=6): 1) sham controls;

2) TAC model; 3) TAC mice treated with ICG-001; 4) TAC mice treated with losartan. TAC was carried out as described elsewhere.^{21,35,48} At 4 weeks after TAC surgery, mice were administrated with daily intraperitoneal injection of ICG-001 (#847591-62-2; Chembest, Shanghai, China) at dosage of 5 mg/kg body weight, or given oral losartan (Merck sharp & Dohme, Kenilworth, NJ) daily at dosage of 10 mg/kg body weight, for additional 4 weeks (Figure 3a). At 8 weeks after TAC, mice were sacrificed, serum, urine, heart and kidney tissues collected for various analyses. All animal studies were approved by the Animal Ethics Committee at the Nanfang Hospital, Southern Medical University.

Cell culture

Human kidney proximal tubular epithelial cells (HKC-8) were described previously.²⁷ Cells were serum-starved for 12 h, and then incubated with mouse serum obtained from various groups as indicated at final concentration of 2.5%. HKC-8 cells also treated with TNF- α at the dosage of 5 ng/ml (410-MT; R&D Systems, Minneapolis, USA) in the absence or presence of ICG-001 (5 μ M). HKC-8 cells were also infected with lentiviral β -catenin shRNA (sc-29209-v) and control shRNA (sc-108080; Santa Cruz Biotechnology, Santa Cruz, CA) for 48 h, followed by incubation with serum obtained from TAC or sham control mice at final concentration of 2.5% for 24 h.

Isolation and culture of primary cardiomyocytes and cardiac fibroblasts

Primary neonatal rat ventricular cardiomyocytes (NRVCs) were isolated and cultivated according to the protocols previously described.²⁸ The purity of primary cardiomyocyte population was more than 95% based on the morphology and the staining for α -actin. Cardiomyocytes were treated with Wnt3a (5036-WN; R&D Systems, Minneapolis, MN) in the absence or presence of ICG-001 (5 μ M) and Klotho (100 ng/ml) (5334-KL; R&D Systems, Minneapolis, MN). Primary cardiac fibroblasts were also isolated from the hearts of neonatal rats.²⁸ Cardiac fibroblasts within 3 passages were subjected to various treatments as indicated.

Western blot analysis

Western blot analysis for specific protein expression was carried out according to standard procedures, as reported previously.¹⁹ The primary antibodies used were described in the Supplementary Detailed Methods.

qRT-PCR

Total RNA was extracted with TRIzol reagent according to the instruction of the manufacturer (Life Technologies, Grand Island, NY). Quantitative, real-time RT-PCR (qRT-PCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), as described elsewhere.²⁷ The primer pairs for qRT-PCR are described elsewhere.¹⁹

Histology, immunohistochemical and immunofluorescence staining

Tissue sections were stained with hematoxylin-eosin (H.E.) or Masson's trichrome staining (MTS) according to standard protocol. Immunohistochemical and immunofluorescence

staining was carried out by using standard protocol.¹⁹ The primary antibodies were described in the Supplementary Detailed Methods.

Echocardiography

Cardiac function was assessed by Doppler echocardiography (VisualSonics Vevo2100 Imaging system, Toronto, Ontario, Canada) with a 21-MHz transducer (MS400). Mice were mild anesthetized by inhalant 3.0% isoflurane and oxygen at rate of 1 L/min. Images were standardized to short axis view at the LV mid-papillary level. Two-dimensional image for 3 sequential cardiac cycles were recorded.

ELISA

Serum levels of TNF- α (SEA133Mi; USCN life Science, Wuhan, China), MCP-1 (SEA087Mi; USCN life Science, Wuhan, China), IL-1 β (SEA563Mu; USCN life Science, Wuhan, China), Klotho (SEH757Mu; USCN life Science, Wuhan, China) and BNP (SEA541Mu; USCN life Science, Wuhan, China) were determined by using specific enzyme-linked immunosorbent assay (ELISA) kits according to the protocols specified by the manufacturer. Albumin in urine was assessed with mouse albumin ELISA quantitation set (E90-134; Bethyl Laboratories, Montgomery, TX). Albuminuria was presented after normalization with urinary creatinine levels, and expressed as mg per mg urine creatinine.

Statistical analyses

All data examined were expressed as mean $\pm$ SEM. Statistical analysis of the data was carried out using GraphPad Prism software (La Jolla, CA). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Kuels test. $P < 0.05$ was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China Grant 81521003 and 81770715, and National Institute of Health Grant DK064005.

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Figure 1. Transverse aortic constriction (TAC) causes heart disorder and kidney injury in mice. (a) Western blot analyses show protein expression of β -MHC, fibronectin, TNF- α in the heart of mice subjected to TAC for 8 weeks. (b-d) Quantitative data on β -MHC, fibronectin, TNF- α protein abundances in different groups as indicated. Relative levels (fold induction over the controls) of proteins were presented. * $P < 0.05$. (e) Representative micrographs show the histology (H.E staining) of cardiac sections of control and TAC mice. Heart cross-sections show overt cardiac hypertrophy in TAC mice. Scale bar, 1 mm. (f) Western blot analyses show protein expression of podocalyxin, fibronectin, Snail1 in the kidney of mice subjected to TAC for 8 weeks. (g-i) Quantitative data on podocalyxin, fibronectin, Snail1 proteins in different groups as indicated. Relative levels (fold induction over the controls) of proteins were presented. * $P < 0.05$. (j) Representative micrographs show Masson's trichrome staining of kidney sections of control and TAC mice. Yellow arrow indicates collagen deposition. Scale bar, 20 μ m.

Figure 2. Wnt/ β -catenin is activated in the heart of TAC mice.

(a) qRT-PCR shows that a battery of Wnt genes was induced in the heart of mice at 8 weeks after TAC. * $P < 0.05$ vs controls (n=6). (b-f) Western blot analyses confirm the induction of Wnt1, Wnt3a, active β -catenin and total β -catenin protein in the heart of mice at 8 weeks after TAC. Representative Western blots (b) and quantitative data (c-f) were presented. * $P < 0.05$ vs controls (n=4). (g) Representative micrographs show that Wnt3a was induced in cardiomyocytes in mice at 8 weeks after TAC. Black arrow indicates positive staining. (h) Representative micrographs show the immunohistochemical staining for β -catenin in heart. The β -catenin protein was induced and predominantly localized in the cytoplasm of cardiomyocytes in mice after TAC (black arrow), whereas β -catenin in sham control mice was mainly localized in the site of cell-cell junction (empty arrow). Scale bar, 20 μ m.

Figure 3. Blockade of Wnt/β-catenin signaling by ICG-001 prevented cardiac injury in mice after TAC.

(a) Experimental design. Red arrow indicates the timing of TAC surgery, while black arrow show the timing of sacrifice. (b) Representative Western blots show cardiac protein levels of β-MHC, fibronectin, collagen I, TNF-α, active β-catenin, total β-catenin in various groups as indicated. (c-h) Quantitative data on protein levels of β-MHC, fibronectin, collagen I, TNF-α, active β-catenin and total β-catenin are presented in given groups as indicated.

* $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (i) Representative micrographs show histology and β-MHC and fibronectin protein expression in the heart of mice at 8 weeks after TAC. Upper panel, H.E. staining of heart sections in various groups as indicated; Middle panel, immunostaining for cardiac β-MHC; Bottom panel, immunostaining for fibronectin in the heart of mice as indicated. Yellow arrows indicate positive staining. Scale bar, 20 μm.

Figure 4. Wnt/β-catenin activates renin-angiotensin system in the heart after TAC.

(a) Representative Western blots show cardiac expression of ACE, renin, AT1 in various groups as indicated. (b-d) Quantitative data on cardiac expression of ACE (b), renin (c), AT1 (d) proteins are presented in various groups as indicated. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone ($n = 6$). (e) Representative Western blots show that Wnt3a induced the expression of active β-catenin, β-catenin, ACE, renin, AT1 in primary cardiomyocytes. (f-j) Quantitative data show the induction of active β-catenin, β-catenin, ACE, renin, AT1 by Wnt3a in cardiomyocytes. * $P < 0.05$ vs controls; † $P < 0.05$ vs Wnt3a alone ($n = 3$). (k) Representative Western blots show protein expression of active β-catenin, β-catenin, ACE, angiotensinogen, AT1 in primary cardiac fibroblasts after various treatments as indicated. (l-p) Quantitative data show the protein levels of β-catenin, active β-catenin, ACE, angiotensinogen, AT1 in primary cardiac fibroblasts after various treatments as indicated.

* $P < 0.05$ vs controls; † $P < 0.05$ vs Wnt3a alone (n=3). Ctrl, control; TAC, transverse aortic constriction; ICG, ICG-001; Los, losartan.

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Figure 5. Blockade of Wnt/ β -catenin by ICG-001 also abolishes renal β -catenin signaling and ameliorates kidney injury after TAC.

(a) Representative Western blots show protein expression of fibronectin, podocalyxin, Snail1, Kim-1, active β -catenin and total β -catenin in the kidney of mice as indicated. (b-g) Quantitative data on renal expression of fibronectin, podocalyxin, Snail1, Kim-1, active β -catenin and total β -catenin proteins are presented in various groups as indicated. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (h) Representative micrographs show renal collagen deposition and the expression of podocalyxin and β -catenin proteins in various groups as indicated. Upper panel, Masson's trichrome staining of kidney sections; Middle panel, immunohistochemical staining for podocalyxin; Lower panel, immunohistochemical staining for β -catenin. Arrows denote positive staining. Scale bar, 20 μ m. (i) Urinary albumin levels in various groups as indicated. Albuminuria was expressed after normalization with urinary creatinine. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (j) Graph shows a linear correlation between urinary albumin level and BNP in serum. Ctrl, control; TAC, transverse aortic constriction; ICG, ICG-001.

Figure 6. Circulating factors in mouse serum after TAC mediated renal β -catenin activation and kidney injury *in vitro*.

Human kidney proximal tubular epithelial cells (HKC-8) were incubated with mouse serum (2.5%) from different groups as indicated. **(a)** Representative Western blots show the expression of active β -catenin, total β -catenin, Snail1 and Kim-1 in HKC-8 cells after incubation for 24 hours. **(b-e)** Quantitative data on protein levels of active β -catenin, total β -catenin, Snail1 and Kim-1 are presented. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). **(f-n)** Knockdown of β -catenin abolishes kidney injury and renin angiotensin system activation *in vitro*. HKC-8 cells were infected with lentiviral β -catenin shRNA vector (β -cat-shR) or control vector (ctrl-shR), and followed by incubation with mouse serum (2.5%) from different groups as indicated. Representative Western blots **(f)** show the expression of active β -catenin, total β -catenin, fibronectin, Kim-1, Snail1, angiotensinogen, renin and AT1 in HKC-8 cells after incubation for 24 hours. Quantitative data on protein levels of β -catenin **(g)**, active β -catenin **(h)**, fibronectin **(i)**, Snail1 **(j)**, Kim-1 **(k)**, angiotensinogen **(l)**, renin **(m)** and AT1 **(n)** are presented. * $P < 0.05$ vs control (ctrl) serum; † $P < 0.05$ vs ctrl-shR (n=3). Ctrl, β -cat-shR.

control; TAC, transverse aortic constriction; ICG, ICG-001; Lentiviral Ctrl shR, lentiviral control shRNA; Lentiviral β -catenin-shR, lentiviral β -catenin shRNA.

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Figure 7. Circulating levels of pro-inflammatory cytokines after TAC correlate with heart and kidney injury.

(a) ELISA shows the circulating levels of TNF- α in various groups at 8 weeks after TAC. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (b) Circulating MCP-1 level in various groups at 8 weeks after TAC. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (c) Circulating IL-1 β level in various groups at 8 weeks after TAC. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (d) Circulating BNP level in various groups at 8 weeks after TAC. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (e) Circulating level of TNF- α correlates with cardiac injury. Diagram shows a linear correlation between TNF- α (pg/ml) and BNP (pg/ml) in serum. (f) Circulating level of TNF- α correlates with kidney injury. Diagram shows a linear correlation between albuminuria (mg/mg Ucr.) and TNF- α (pg/ml) in serum. Ctrl, control; TAC, transverse aortic constriction; ICG, ICG-001; Los, Losartan.

Figure 8. TNF-α activates β-catenin and triggers injurious response in kidney tubular cells *in vitro*.

(a) Representative micrographs show immunostaining for β-catenin in HKC-8 cells after various treatments as indicated. HKC-8 cells were pretreated with ICG-001 (5 μM) or losartan (10 μM) for 1 hour before incubating with TNF-α for 12 hours. White arrows denote positive nuclear staining. (b) Representative Western blot analyses revealed protein level of β-catenin, active β-catenin, Snail1 and Kim-1 in HKC-8 cells after various treatments for 24 hours as indicated. (c-f) Quantitative data for β-catenin, active β-catenin, Snail1 and KIM1 proteins in various groups are shown. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TNF-α alone (n=6).

Figure 9. Depletion of Klotho aggravates cardiomyocyte hypertrophy and cardiac fibroblast activation induced by Wnt/ β -catenin.

(a, b) Western blot analyses show that TAC caused renal depletion of Klotho in mice, which was abrogated by ICG-001 or losartan. Representative Western blotting for Klotho in the kidney in various groups (a) and quantitative data on Klotho protein levels were presented (b). * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (c) ELISA assay shows serum levels of Klotho protein in mice after various treatments as indicated. * $P < 0.05$ vs sham controls; † $P < 0.05$ vs TAC alone (n=6). (d) Representative micrographs show renal expression of Klotho protein in mice after various treatments as indicated. Black arrows

indicate positive staining. **(e)** Representative Western blots show the protein levels of β -MHC, α -actin, active β -catenin in primary cardiomyocytes after various treatments as indicated. Rat primary cardiomyocytes were incubated with Wnt3a (100 ng/ml) in the absence or presence of Klotho (100 ng/ml) for 24 hours. **(f-h)** Quantitative data on the protein levels of β -MHC (f), α -actin (g), active β -catenin (h) in various groups as indicated. **(i)** Representative Western blot show the protein levels of fibronectin, α -SMA, active β -catenin, β -catenin in rat primary cardiac fibroblasts after various treatments as indicated. Rat primary cardiac fibroblasts were incubated with Wnt3a (100 ng/ml) in the absence or presence of Klotho (100 ng/ml) for 24 hours. **(j-m)** Quantitative data on the protein levels of fibronectin (j), α -SMA (k), active β -catenin (l), β -catenin (m) in various groups as indicated. **(n)** Schematic diagram depicts cardiorenal inter-organ crosstalk after TAC. Heart injury following TAC activates Wnt/ β -catenin and RAS, leads to systemic inflammation with up-regulation of TNF- α , IL-1 β , MCP-1 in the circulation. Pro-inflammatory cytokines in the circulation subsequently suppresses renal Klotho expression, which leads to activation of Wnt/ β -catenin and RAS in the kidney and cause podocyte and tubular injury, and fibroblast activation. As the main source of systemic Klotho, depressed renal production of Klotho in the damaged kidney directly causes Klotho deficiency in the circulation, which further aggravates Wnt-mediated cardiomyocyte hypertrophy and cardiac fibroblast activation after TAC, forming a vicious cycle. Ctrl, control; TAC, transverse aortic constriction; ICG, ICG-001; Los, losartan.

Table 1.

Echocardiographic Measurements in TAC mice.

	Control	TAC+Vehicle	TAC+ICG-001	TAC+Losartan
LVSD (mm)	0.74±0.02	0.96±0.02 [*]	0.98±0.03	0.79±0.02 [†]
LVSs (mm)	1.11±0.05	1.32±0.04 [*]	1.34±0.03	1.15±0.03 [†]
HR (bpm)	577.74±17.02	581.24±27.84	602.19±22.68	580.19±15.24
LVIDs (mm)	2.48±0.14	3.89±0.24 [*]	2.45±0.12 [†]	2.34±0.09 [†]
LVIDd (mm)	3.58±0.18	4.68±0.23 [*]	3.50±0.14 [†]	3.34±0.1 [†]
LVESV (μl)	22.60±3.26	67.29±8.57 [*]	21.65±2.83 [†]	19.28±1.81 [†]
LVEDV (μl)	54.83±6.60	103.20±10.83 [*]	51.57±5.01 [†]	45.7±3.16 [†]
LVSV (μl)	32.23±3.90	35.91±2.64	29.92±2.61	26.42±1.45
LVEF (%)	58.88±2.68	36.00±2.78 [*]	58.34±2.38 [†]	58.16±1.41 [†]
LVFS (%)	30.64±1.84	17.29±1.49 [*]	30.18±1.62 [†]	29.84±0.92 [†]
CO (ml/min)	18.48±2.15	20.71±1.38	17.83±1.28	15.37±1.01
LVPWd (mm)	0.76±0.04	0.89±0.05 [*]	0.87±0.04	0.77±0.02 [†]
LVPWs (mm)	0.99±0.06	1.22±0.02 [*]	1.17±0.06	1.04±0.03 [†]

^{*} $P < 0.05$ vs controls;[†] $P < 0.05$ vs Vehicle (n=6).