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Original article

# CB1 cannabinoid receptor antagonist attenuates left ventricular hypertrophy and Akt-mediated cardiac fibrosis in experimental uremia

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ABSTRACT

Cannabinoid receptor type 1 (CB1R) plays an important role in the development of myocardial hypertrophy and fibrosis—2 pathological features of uremic cardiomyopathy. However, it remains unknown whether CB1R is involved in the pathogenesis of uremic cardiomyopathy. Here, we aimed to elucidate the role of CB1R in the development of uremic cardiomyopathy via modulation of Akt signalling. The heart size and myocardial fibrosis were evaluated by echocardiography and immunohistochemical staining, respectively, in 5/6 nephrectomy chronic kidney disease (CKD) mice treated with a CB1R antagonist. CB1R and fibrosis marker expression levels were determined by immunoblotting in H9c2 cells exposed to the uremic toxin indoxyl sulfate (IS), with an organic anion transporter 1 inhibitor or a CB1R antagonist or agonist. Akt phosphorylation was also assessed to examine the signaling pathways downstream of CB1R activation induced by IS in H9c2 cells. CKD mice exhibited marked left ventricular hypertrophy and myocardial fibrosis, which were reversed by treatment with the CB1R antagonist. CB1R, collagen I, transforming growth factor (TGF)-β, and α-smooth muscle actin (SMA) expression showed time- and dose-dependent upregulation in H9c2 cells treated with IS. The inhibition of CB1R by either CB1R antagonist or small interfering RNA-mediated knockdown attenuated the expression of collagen I, TGF-β, and α-SMA in IS-treated H9c2 cells, while Akt phosphorylation was enhanced by CB1R agonist and abrogated by CB1R antagonist in these cells. In summary, we conclude that CB1R blockade attenuates LVH and Akt-mediated cardiac fibrosis in a CKD mouse model. Uremic toxin IS stimulates the expression of CB1R and fibrotic markers and CB1R inhibition exerts anti-fibrotic effects via modulation of Akt signaling in H9c2 myofibroblasts. Therefore, the development of drugs targeting CB1R may have therapeutic potential in the treatment of uremic cardiomyopathy.



## 1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in patients with chronic kidney disease (CKD) [1]. CVD mortality rates in end-stage renal failure patients are 10- to >100-fold higher than in the age-matched general population [2]. In addition to vascular calcification, the high rate of CVD death reported in CKD patients is mainly attributed to uremic cardiomyopathy [3,4]. Experimental and clinical investigations have highlighted unique aspects of this disorder, which are characterized by left ventricular hypertrophy (LVH), reduced capillary density, fibrosis, and ventricular remodeling [5]. In general, cardiac remodeling involves molecular, cellular, and interstitial changes that manifest clinically as alterations in cardiac size, shape, and function, for instance in response to cardiac injury or increased cardiac load [6,

*Abbreviations:* α-SMA, α-smooth muscle actin; ANP, atrial natriuretic peptide; β-MHC, myosin heavy chain beta; BNP, brain natriuretic peptide; BW, body weight; CB1R, cannabinoid receptor type 1; CKD, chronic kidney disease; CMR, cardiac magnetic resonance; CVD, cardiovascular disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HW, heart weight; IL, interleukin; IS, indoxyl sulfate; LVH, left ventricular hypertrophy; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PI3K, phosphoinositide-3-kinase protein kinase; PARP, poly ADP-ribose polymerase; PNx, partial nephrectomy; PtdIns, phosphatidylinositol; siRNA, short interfering RNA; TGF, transforming growth factor.

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7]. Compared to non-CKD patients with similar blood pressure levels, CKD patients have a greater left ventricular mass, suggesting the existence of specific CKD-associated factors that contribute to cardiac remodeling [8,9]. However, the molecular mechanisms underlying the development of uremic cardiomyopathy remain largely unknown.

Phosphoinositide-3-kinase protein kinase (PI3K) is a lipid kinase that phosphorylates the 3′-OH of phosphatidylinositol rings, for instance those of phosphatidylinositol (PtdIns) 4-phosphate and PtdIns (4,5)-bisphosphate (P2) to produce PtdIns (3,4)P2 and PtdIns (3,4,5)-trisphosphate, respectively. PtdIns (3,4)P2 and PtdIns (3,4,5)-trisphosphate can then bind to Akt (also known as protein kinase B) via pleckstrin homology domains, leading to its phosphorylation and consequent activation. The PI3K–Akt signaling pathway is activated by a variety of cellular stimuli or toxic insults and participates in the regulation of essential cellular functions including transcription, protein synthesis, proliferation, growth, and survival [10–12]. PI3K also plays an important role in controlling organ sizes in *Drosophila* and the heart size in mice [13,14]. Moreover, mice with cardiac-specific Akt overexpression showed increased heart and cardiomyocyte sizes [15–17]. In contrast, the overexpression of kinase-deficient Akt partially reversed the growth-promoting effect of constitutively active PI3K, while active Akt promoted heart growth to the same extent in mice expressing dominant-negative and wild-type PI3K [18]. These data suggest that PI3K/Akt signaling plays a major role in promoting cardiac hypertrophy, and disturbance of this pathway is associated with cardiac fibrosis and apoptosis, impaired angiogenesis, defective glucose transporter 4 translocation, and downregulation of sarcoplasmic calcium ATPase expression, all of which are features of uremic cardiomyopathy [19].

Endocannabinoids are bioactive lipids consisting of amides, esters, and ethers of long chain polyunsaturated fatty acids. The most important endogenous cannabinoids are anandamide and 2-arachidonoylglycerol. CB1R and CB2R are human endocannabinoid receptors that belong to the family of G protein-coupled receptors, and have distinct tissue distribution and signaling mechanisms. In addition to prominent effects on the central nervous system, CB1R has important roles in cardiovascular disorders [20–24]. CB1R activation by endocannabinoids or synthetic ligands leads to defects such as cardiovascular collapse from various forms of shock [20] and heart failure [25–27]. Moreover, pharmacological inhibition or genetic deletion of CB1R reduces cardiac dysfunction, oxidative stress, inflammation, and fibrosis in diabetic mice and improves cardiac function and remodeling in rats after myocardial infarction induced by left coronary artery ligation [28]. CB1R inhibits adenylyl cyclase by coupling to Gi/o proteins, regulates ion channels, and activates mitogen-activated protein kinase (MAPK). Recent studies have demonstrated that some CB1Rs use Gi/o proteins as molecular scaffolds to recruit PI3K, thereby activating Akt signaling, a pathway that is potentially involved in the pathogenesis of uremic cardiomyopathy [17,29]. We therefore hypothesized that CB1R plays a role in the development of uremic cardiomyopathy via modulation of PI3K/Akt signaling.

To test this hypothesis, the expression of CB1R and uremic cardiomyopathy markers in the heart was assessed by western blotting in a murine 5/6 nephrectomy CKD model, with or without CB1R antagonist treatment. Indoxyl sulfate (IS), a uremic toxin, induces uremic cardiomyopathy in vivo and in vitro. The pathogenic role of CB1R was investigated by treating H9c2 cells with IS with or without CB1R inhibition, which was achieved using a pharmacological antagonist or by small interfering RNA (siRNA)-mediated knockdown of the *CB1R* gene. To investigate the mechanism of uremic cardiomyopathy induction by CB1R, the activation of PI3K/Akt signaling was assessed in IS-treated H9c2 cells. The findings provide insight into the molecular basis of uremic cardiomyopathy as well as potential targets for drugs designed to ameliorate the accompanying LVH and fibrosis.

## 2. Methods

### 2.1. Animal experiments

Uremic cardiomyopathy was induced in vivo via partial nephrectomy (PNx) as described previously [30,31]. Animal experiments were approved by the local animal care committee of the National Defense Medical Center (IACUC-12-024). Animals (n = 24) were housed in a light- and temperature-controlled room with free access to deionized drinking water and standard chow consisting of 0.28% (wt/wt) NaCl, 1.00% (wt/wt) Ca, and 0.22% (wt/wt) Mg (LabDiet, Richmond, IN, USA). After acclimatization, mice were randomly assigned to 3 groups (n = 8/group): sham-operated mice (serving as controls), PNx-treated with a vehicle control, and PNx-treated mice administrated SR141716A (PNx + SR) by intraperitoneal injection (i.p.; 50 mg/kg/week). PNx was performed on 3-month-old male C57BL/6 mice by selective cauterization of the entire upper and lower poles of the left kidney via a Bovie high-temperature fine-tip cautery (Aaron Medical, St. Petersburg, FL, USA), leaving an intact 2-mm segment around the hilum (PNx, stage I). This procedure was followed by the removal of the right kidney after 2 weeks (PNx, stage II). In the CKD + SR group, CBR1 antagonist was administered for 7 times (50 mg/kg/week; i.p.) commencing one week post PNx. The animals were sacrificed 8 weeks after sham operation or PNx by CO2 inhalation. Blood was collected and the hearts were dissected and processed for further analyses. The timeframe of the in vivo experimental protocols was shown in Fig. 1.

### 2.2. Echocardiography

Prior to sacrifice, echocardiography was performed on lightly anesthetized animals using a Philips iE33 ultrasound imaging system (Philips Medical Systems, Best, the Netherlands) equipped with a 7–15-MHz linear array transducer. After 2-dimensional left ventricular (LV) short-axis images were obtained, M-mode traces were acquired for measurements of diastolic and systolic LV wall thickness and chamber dimensions, echocardiography-derived LV masses, fractional shortening, and heart rates. Measurements were averaged from 5 consecutive cardiac cycles and were performed by a cardiologist and an experienced technician who were blinded to the animal groups.

### 2.3. Blood biochemistry and body weight (BW) measurements

The final body weight (BW) was recorded before sacrifice and hematocrit (Hct) was taken measured by the microhematocrit method with micro capillary reader. The serum creatinine (sCr) and blood urea nitrogen (BUN) concentrations were detected by the Jaffe method (Beckman Coulter Synchron LX System; Beckman Coulter Inc., Brea, Calif., USA). The serum inorganic phosphate (Pi) levels were measured by colorimetric methods as previously described [32] on the AU5800 automated chemistry analyzer (Olympus, Tokyo, Japan).

### 2.4. Histological analysis

Paraffin-embedded LV myocardial sections were stained with hematoxylin and eosin for histological examination. To visualize fibrotic deposition, the sections were stained with 0.1% Picrosirius Red (Sirius Red F3BA in saturated picric acid [wt/v]) for 1 h at room temperature, rinsed twice with 0.01 N HCl for 1 min, and then immersed in distilled water. After dehydration in 70% ethanol for 30 s, the sections were mounted with cover slips and observed under a bright-field microscope. At least 3 whole sections from each animal were imaged using a Leica DM2500 fluorescence microscope at 15× magnification (Leica Microsystems GmbH, Wetzlar, Germany). Quantification was performed using an established protocol (http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html). The amount of fibrotic