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Basic Science

The Arrhythmogenic Effect of Protein-Bound Uremic Toxin *p*-Cresylsulfate: An *In Vitro* Study

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Background: *p*-Cresylsulfate (PCS) is a protein-bound uremic toxin that accumulates in patients with chronic kidney disease. Previous studies have indicated that serum total PCS levels are significantly increased in the presence of abnormal corrected QT (QTc) intervals, and that they are associated with QTc prolongation. However, the QTc prolongation effect of PCS remains unclear. The current study aimed to investigate the arrhythmogenic effect of PCS using *in vitro* experiments and computer simulation.

Methods: The arrhythmogenic effect of PCS was evaluated by incubating H9c2 rat ventricular cardiomyocytes *in vitro* with increasing concentrations of PCS. Electrophysiological studies and mathematical computer simulations were performed.

Results: *In vitro*, the delayed rectifier potassium current (I_K) was significantly decreased in a dose-dependent manner after treatment with PCS. The modulation of PCS on I_K was through regulation of the phosphorylation of the major potassium ion channel protein Kv2.1. In computer simulations, the decrease in I_K induced by PCS prolonged the action potential duration (APD) and sped up the re-entrant wave, which is known to be a trigger mechanism for lethal ventricular arrhythmias.

Conclusions: PCS significantly downregulated the phosphorylation of the I_K channel protein Kv2.1 and I_K current activity, which increased the cardiomyocyte APD. This was observed both *in vitro* and in the computer O'Hara-Rudy dynamic human ventricular model. These findings suggest that PCS may play a key role in the development of cardiac arrhythmias.

Key Words: Action potential duration • Cardiac arrhythmia • Computer O'Hara-Rudy dynamic model • Delayed rectifier potassium current • *p*-Cresylsulfate

INTRODUCTION

The importance of cardiovascular disease (CVD) and cardiac arrhythmia in chronic kidney disease (CKD) is well known. Previous studies have demonstrated that CVD is the principal cause of death in this population.[1] Uremic toxicity, which is caused by various uremic toxins, has been identified to be a potential cause of the increased CVD and mortality observed in CKD patients. Dialysis therapy, which is generally used for patients with end-stage renal disease, cannot totally remove all uremic toxins. According to previous studies, the toxins that cannot be removed are mostly protein-bound or with a molecular weight > 500 Da.[2]

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As renal function deteriorates, levels of uremic retention toxins and proinflammatory cytokines increase.[3] In recent decades, the levels of uremic retention solutes has been shown to be associated with the elevated production of inflammatory mediators and reactive oxygen species in CKD patients.[4] A recent study showed that C-reactive protein and other proinflammatory cytokines such as interleukin-6 and platelet-activating factor are associated with arrhythmias via the modulation of ion channel function.[5,6]

p-Cresol (4-methylphenol, molecular weight 108.1 Da) is a small molecule derived from ingested phenylalanine and plant phenols. In humans, it exists predominantly as the conjugate *p*-cresylsulfate (PCS), which is a protein-bound rich substance. A recent *post hoc* analysis showed that increased free form *p*-cresol levels in dialysis patients were associated with cardiovascular events, suggesting it may be a novel cardiovascular risk factor.[7] *p*-Cresol and PCS have been reported to be important contributors to the inhibition of phagocyte reactive species production and endothelial dysfunction by enhancing baseline leukocyte activity.[8,9] PCS binds strongly to proteins, and therefore has poor clearance by conventional hemodialysis. PCS has also been shown to have a pro-inflammatory effect and to produce free radicals, as evaluated by increased oxidative burst activity of leucocytes at baseline; therefore, PCS may contribute to the propensity to vascular damage observed in CKD patients.[9] We previously showed that increased serum PCS levels may be associated with a higher risk of impaired left ventricular systolic function,[10] which is in turn associated with corrected QT (QTc) prolongation,[11] major adverse cardiac events,[12] and prognosis of coronary atherosclerosis.[13] These reports on the biological toxicity of PCS support its role as a potential cardiovascular risk factor. However, to the best of our knowledge, the arrhythmogenic effect of PCS remains unclear.

Delayed rectifier potassium current (I_K) is one of the major current determinants of cardiomyocyte action potential duration (APD).[14] Current abnormalities are always found in myocardial infarction and heart failure, and they can induce cardiac arrhythmias.[15,16] The present study aimed to evaluate the acute effect of PCS on the I_K of H9c2 rat ventricular cardiomyocytes, as well as its electrophysiological effect using *in vitro* experiments and computer simulation.

MATERIALS AND METHODS

Cell culture

H9c2 rat ventricular cardiomyocytes (BCRC 60096, Bioresource Collection and Research Center, Taiwan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL) in an atmosphere of 95% air and 5% CO_2 at 37 °C. Stocks of myoblasts were propagated in culture flasks for successive passage.

PCS treatment

PCS was purchased from the Shanghai Chemical Co., Ltd. The treatment of H9c2 cells with various concentrations of PCS (10 μM, 100 μM and 500 μM) was performed as in previous studies.[17,18] The cells were treated with PCS for 48 hours before electrophysiological recording and further experiments.

Western blot analysis

The protein expressions of Kv2.1 and phosphorylated Kv2.1 in H9c2 cells was analyzed by Western blotting as previously described.[19,20] In brief, total protein content was extracted using a Bio-Rad Protein assay (Bio-Rad Lab. Inc., Canada) and then separated using 10% denaturing-acrylamide gel. The proteins were transferred to immobilon PVDF membranes (Millipore Corp., USA) and incubated with rabbit polyclonal antibodies against Kv2.1 (Millipore Corp., USA) or phosphorylated-Ser805 Kv2.1 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 hour at room temperature. The membranes were subsequently incubated with a secondary antibody (anti-rabbit, Millipore Corp., USA) and conjugated with horseradish peroxidase. Antigen-antibody complexes were detected by enhanced chemiluminescence (Millipore Corp., USA) and densitometric analysis was conducted using LabWorks 4.5 ImageAcquisition and Analysis software (Ultra-Violet Products Ltd., UK).

Patch-clamp cell electrophysiological studies

To detect the I_K of H9c2 cardiomyocytes, whole-cell potassium outward currents were recorded using an Axopatch 700A amplifier (Axon Instruments, Union City,

CA) in the whole-cell patch-clamp configuration. Full details of these methods have been described in previous studies.[21,22] In brief, H9c2 cells were placed in a recording dish and perfused with a bath solution containing 60 mM NaCl, 80 mM Na-gluconate, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES and 10 mM glucose (pH 7.4, NaOH). Patch pipettes were constructed from soft borosilicate glass capillaries that were double pulled, coated with sticky wax close to the tip and fire polished. The resistances were 3-4 MΩ when filled with the internal solution, which contained 0.5 mM $MgCl_2$, 30 mM KCl, 110 mM K-gluconate, 10 mM EGTA, 5 mM HEPES, 5 mM Na_2ATP and 1 mM GTP-tris (pH 7.2, KOH). The recording electrode was gently lowered onto an H9c2 cell. Negative pressure was briefly applied to rupture the membrane, and a gigaohm seal was obtained. The cells were subsequently voltage clamped. Step-pulse protocols and data acquisition were performed using pCLAMP software (Axon Instruments). For whole-cell current recording, series resistance and capacitance were routinely compensated for by adjusting the internal circuitry of the patch-clamp amplifier. Membrane capacitance was calculated from the peak amplitudes and time constant decay of capacity transients elicited by 10 mV and hyperpolarizing voltage pulses from a holding potential of -50 mV. All electrical recordings were performed at room temperature.

Mathematical computer model for cardiomyocyte action potential and re-entry activity

To evaluate the effect of PCS-induced I_K modulation on human cardiomyocyte APD, the latest mathematical model of the O'Hara-Rudy dynamic human ventricular model (ORd model) was used for a computer simulation experiment.[3] Ventricular cardiomyocyte action potential was mathematically constructed to include ionic currents, ionic pumps and exchangers, and processes regulating the intracellular concentration changes of Na^+, K^+ and Ca^{2+}. Additionally, the model incorporated Ca^{2+}/calmodulin-dependent protein kinase II with a modulated rate dependence for Ca^{2+} cycling. The numerical forward Euler method with integration time-step size (0.001 ms) and the Rush and Larsen method were used.

The Markov model of I_K was derived from previously published K channel models.[24] The I_K data from H9c2 cells with and without PCS treatment was digitized and formulated into a new Markov model computer equation and inserted into the ORd model to evaluate the effect of PCS on human cardiomyocyte action potential.

To obtain stable re-entry, the Na+ current formulation was replaced by the Na+ current formulation from the TP06 model.[25] Each model was embedded in a 2D mono domain tissue model[26] with isotropic diffusion, a diffusion coefficient of 1.171 cm^2s^{-1} (TP06 model), 0.2975 cm^2s^{-1} (ORd human ventricular model), and a specific capacitance of 1 μFcm^{-2} to characterize restitution properties and examine the re-entry period. No-flux boundary conditions were imposed at each edge by setting the gradient of membrane voltage to zero at the boundary condition. Measurements were made from a thin strip of tissue with a dimension of 200 × 200 grid points (50 × 50 mm) for studies of reentry. APD and conduction velocity restitution were measured using an S1S2 protocol where S1 = 1000 ms, and re-entry was initiated by imposing an Archimedian spiral on the tissue as an initial condition.

Statistical analysis

Continuous data are reported as the mean value ± standard deviation. Variables among groups were compared using the Kruskal-Wallis test, and if the p value was < 0.05, follow-up comparisons of the different groups were performed using the Mann-Whitney test. A p value < 0.05 was considered to indicate a statistically significant difference. All analyses were performed by a senior biostatistician using SPSS statistical software (version 17.0, SPSS Institute Inc., Chicago, Illinois).

RESULTS

The I_K in cardiac H9c2 cells did not significantly change following PCS treatment for 8-24 hours over night. However, it was significantly decreased after 48 hours of treatment. The average association between I_K and the membrane potential, as calculated from the measured peak current amplitudes, showed that I_K was significantly decreased at membrane potentials from 0 mV to 50 mV in a dose-dependent manner (Figures 1A and 1B).

The potassium ion channel protein Kv2.1 is the major subunit protein that generates I_K in H9c2 cells.[27] In

the present study, Western blot analysis revealed a significant increase in Kv2.1 protein expression and a significant decrease in phosphorylated Kv2.1 protein expression following treatment with PCS (Figure 2A). Although there was a significant increase in the Kv2.1 level following PCS treatment, there was no significant difference between the groups in higher PCS (500 μM) concentration treatment (Figure 2B). In contrast, the expression of phosphorylated Kv2.1 was significantly decreased in the PCS-treated groups compared with the

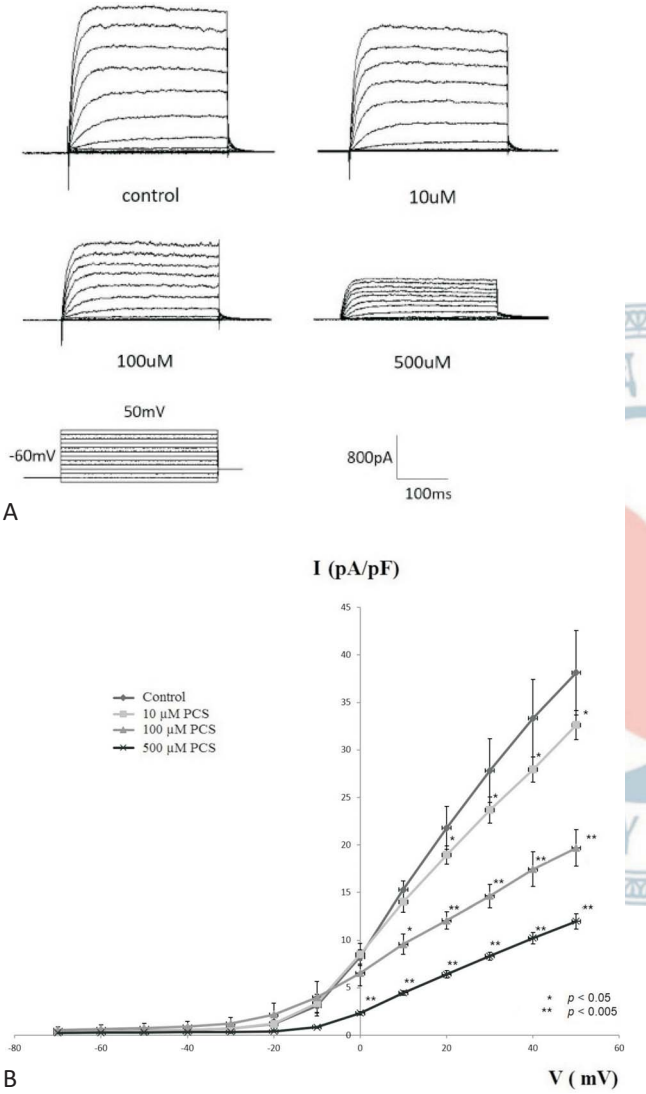

Figure 1. *Representative current traces for delayed rectifier potassium outward currents (I_k) in H9c2 cells treated with increasing concentrations of p-cresylsulfate (PCS) concentration treatment. (A) I_k was elicited by 300 ms depolarizing step pulses from -70 to 50 mV at a holding potential of -60 Mv. The average association between I_k (pA/pF) and the membrane potential in the control, 10 μM PCS, 100 μM PCS and 500 μM PCS groups (n = 6). (B) When comparing the PCS treated groups with the control group, I_k was significantly decreased at membrane potentials from 0 mV to 50 mV in a dose-dependent manner. * p < 0.05 vs. the untreated control group. ** p < 0.005 vs. the untreated control group.*

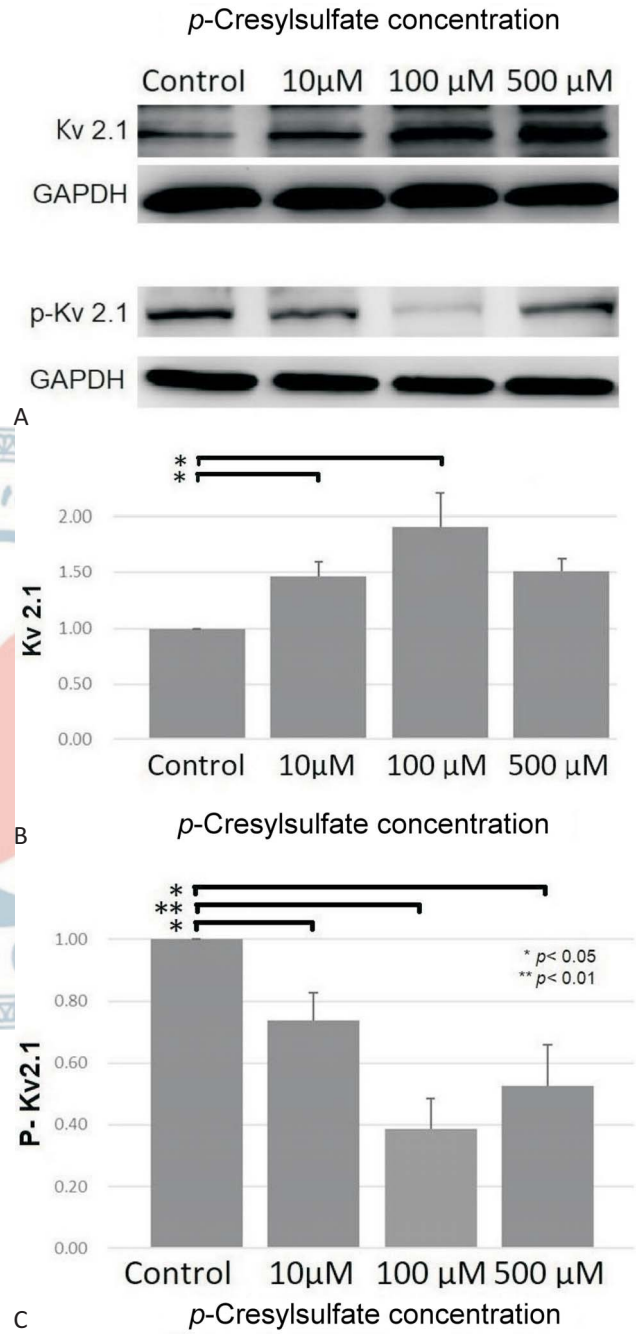

Figure 2. *(A) Protein expression of the delayed rectifier potassium current channel protein Kv2.1 and phosphorylated Kv2.1 (p-Kv2.1) in H9c2 cells was analyzed by western blot. (B) Protein expression of Kv2.1 significantly increased whereas. (C) Protein expression of p-Kv2.1 significantly decreased following treatment. * p < 0.05 vs. the untreated control group. ** p < 0.01 vs. the untreated control group.*

controls, although the expression began to reverse in the highest PCS (500 μM) concentration group (Figure 2C). Furthermore, when the ratio of phosphorylated Kv2.1 to total Kv2.1 protein was compared between groups, it was significantly decreased in the PCS-treated H9c2 groups compared with the untreated control group (Figure 3). In addition, the ratio was also significantly decreased in the 100 μM PCS group compared with the 10 μM PCS group (Figure 3).

The ventricular cardiomyocyte action potential was constructed using the Ord model. According to the computer calculations and simulation results, the decrease in I_K caused by the increase in PCS concentration gradually prolonged the constructed APD (Figure 4). The spiral re-entry behavior in the 2D thin tissue strip computer simulation revealed that the re-entrant wave in PCS-affected tissue was faster and could be maintained for longer (Figure 5).

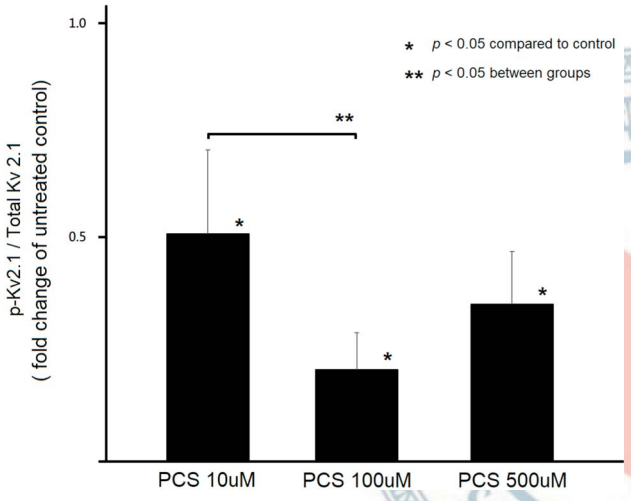

Figure 3. *The level of phosphorylated Kv2.1 (p-Kv2.1) expressed by the ratio of p-Kv2.1 to total Kv2.1 protein. * The ratio significantly decreased in the p-cresylsulfate (PCS)-treated H9c2 groups compared with the untreated control group (p < 0.05). ** The ratio also significantly decreased in the 100 μM PCS group compared with the 10 μM PCS group (p < 0.05).*

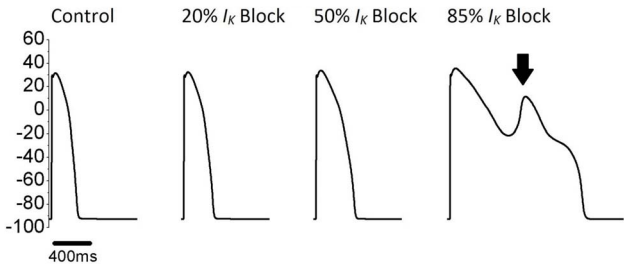

Figure 4. *Ventricular cardiomyocyte action potential (AP) as constructed by the O'Hara-Rudy dynamic human ventricular model. The suppression of I_K mimics the effect of p-cresylsulfate toxicity to ventricular cardiomyocyte AP. The AP duration was gradually increased and early afterdepolarization (arrow) was noted in the higher suppression of I_K.*

DISCUSSION

The present study demonstrated that I_K was significantly decreased in a dose-dependent manner in H9c2 rat ventricular cardiomyocytes after treatment with PCS. Furthermore, the expression of Kv2.1 significantly increased, whereas the expression of phosphorylated Kv2.1 significantly decreased following PCS treatment. The spiral re-entry behavior was aggravated after PCS treatment in the 2D thin tissue strip computer simulation. To the best of our knowledge, this is the first study to observe the PCS arrhythmogenic effect and its down-regulatory effect on I_K channel protein phosphorylation and I_K current activity, which may in turn increase the APD.

Cardiac arrhythmias are often observed in patients with chronic renal diseases. Many causative mechanisms for this have been suggested, including electrolyte imbalance, autonomic nerve dysfunction and rapid

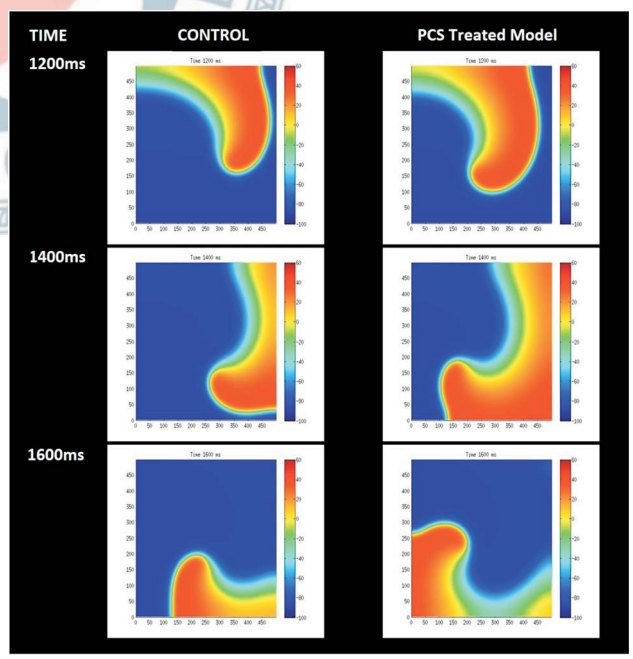

Figure 5. *The spiral re-entry behavior was aggravated after p-cresylsulfate treatment in the 2D thin tissue strip computer simulation.*

changes in electrolyte plasma concentrations during hemodialysis and cardiac hypertrophy.[28] We previously showed that total serum PCS levels were significantly higher in the presence of abnormal QTc intervals, and that they were associated with the QTc prolongation.[14] Cardiorenal syndrome, which states that in the heart and kidneys, acute or chronic dysfunction of one organ may induce acute or chronic dysfunction in the other, is caused by multiple factors, including non-dialyzable uremic toxins such as PCS.[29]

PCS is a protein-bound uremic toxin that is hard to remove from the body. It has a pro-inflammatory effect and produces free radicals (as evaluated by the increased oxidative burst activity of leucocytes at baseline), and therefore it may contribute to the propensity to vascular damage observed in CKD patients.[11] Several studies in recent decades have shown that PCS is associated with coronary artery disease,[16,30] peripheral artery disease, vascular access failure,[31] vascular calcification[32] and general cardiovascular disease.[9,33] It is therefore reasonable to propose that PCS may act as a pro-inflammatory cytokine and play a role in chronic inflammation, thereby contributing to the pathogenesis of atherosclerosis and QTc interval prolongation.[34,35]

I_K is one of the core current determinants of the cardiomyocyte APD, and an increase in APD has been shown to prolong the electrocardiographic QT interval.[36] The Kv2.1 protein is the major subunit protein of the ion channel which generates the I_K in H9c2 cells,[37] and previous studies have shown that Kv2.1 channel activity is regulated by phosphorylation.[38] In the present study, PCS was shown to decrease the protein expression of phosphorylated Kv2.1 in H9c2 cells, downregulate the Kv2.1 channel activity and decrease the I_K, which suggests that PCS may play a role in the development of QTc prolongation. This finding provides evidence that uremic toxins are associated with arrhythmogenesis, and should be considered in addition to traditional risk factors. We previously demonstrated that indoxyl sulfate (IS) could down-regulate I_K channel protein phosphorylation and I_K current activity, which in turn increased the cardiomyocyte APD and QTc interval *in vitro* and in the computer ORd model. These findings suggest that IS may play a role in the development of arrhythmogenesis in CKD patients.[39]

Interestingly, when cells were treated with the highest PCS concentration (500 μM), the protein expressions of Kv2.1 and phosphorylated Kv2.1 protein reversed, while suppression of the potassium current remained the same. The underlying mechanism for this is unknown, however, it might be due to complex homeostatic regulatory mechanisms including a feedback pathway, compensatory pathway, cognate pathway and protein transcriptional regulation.[40,41] In addition, Park et al. showed that the Kv2.1 potassium channel is regulated by variable phosphorylation,[42] in which protein kinase C (PKC) is usually involved.[43,44] Previous studies have demonstrated that PKC was associated with inflammation, oxidative stress and many other pathological states.[45,46] PCS has also been shown to have a pro-inflammatory effect and to produce free radicals, and therefore PCS may contribute to the propensity to vascular damage in CKD patients.[11] In addition, the induction of nephrotoxicity by PCS and IS may be mediated by organic anion transporters (OATs) such as OAT1 and OAT3, which are localized in the basolateral membrane of renal proximal tubular cells.[47,48] Their activity and function is modulated via phosphorylation, which is mediated by the PKC pathway. Therefore, it is possible that PCS could downregulate phosphorylated Kv2.1 via the PKC signaling pathway, however further studies are needed to verify this.

Study limitations

Some limitations the current study need to be considered. PCS affected the I_K in vitro at very low concentrations, even below the normal population serum PCS level.[49] However, it is not easy to demonstrate its electrocardiographic effect, and how it is converted and degraded within the body is unknown. Furthermore, the potassium concentration in the added PCS potassium salt (0.1 uM to 0.3 mM) was relatively low compared with the potassium concentration in the DMEM culture medium (5.3 mM) used in the experiments, which could mean that the additional potassium effect on the action potentials in the experiments was eliminated. As cardiac electrical activity is a complex system, it should be considered that another modulation system exists *in vivo* that competes with the PCS biological effect, such as the equilibrium state of PCS protein binding capacity.[50] In addition, in the patch clamp protocol used in the present study, it was not easy to exclude contamination of

the calcium current. However, the protocol has been used and published in many previous studies, and the authors believe that the main current detected in the current study was the I_K.[21,22] Finally, it remains unclear whether PCS affects the expression of other cardiac ion channels, ion currents and cardiomyocyte electrophysiology. Further investigations are warranted to explore this issue.

CONCLUSIONS

The current study demonstrated that the arrhythmogenic effect of PCS was via the inhibition of I_K. As renal disease progresses and interacts with cardiorenal syndrome, the effect and role of PCS on arrhythmogenesis among CKD patients may be enhanced, and this may then contribute to the advancement of renal function impairment. As a result, the arrhythmogenic effect of PCS should be taken seriously.

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CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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