Introduction

Endothelial dysfunction, which induces disequilibrium in vascular tension, coagulation, and fibrinolysis, contributes to the pathological progression of cardiovascular diseases. It is a common initial risk factor for cardiovascular diseases, such as hypertension and coronary artery diseases\[1\]. Recent clinical trials have demonstrated that endothelial dysfunction is a predictor of the development of cardiovascular events \[2\]. Targeting endothelial dysfunction is an important therapeutic strategy for cardiovascular diseases. Iptakalim is a promising endothelial protective drug that opens K\(_{ATP}\) channels by preferentially activating the SUR2B/Kir6.1 subtype of K\(_{ATP}\) channels. Iptakalim protects endothelial function by enhancing the endothelial nitric oxide (NO) system\[3\]. It inhibits the endothelin-1 (ET-1) system by opening K\(_{ATP}\) channels, and it prevents the overexpression of adhesive molecules, such as intracellular adhesive molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1)\[3\]. Iptakalim also improves endothelial function by the activation of K\(_{ATP}\) channels, thereby preventing the progression of cardiac hypertrophy to congestive heart failure induced by pressure overload\[4\]. Improved endothelial function plays a critical role in the iptakalim-mediated prevention of the pathogenetic progression in cardiovascular diseases. The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms. However, the mechanism whereby iptakalim activates K\(_{ATP}\) channels to protect against endothelial dysfunction is not completely understood.

ChemR23, a G protein-coupled receptor, when activated by the fish oil derivative, resolving E1 (RvE1), reduced inflammation by inhibiting the transcriptional activation of NF-κB in neutrophils and by selectively counter-regulating leukocytes and platelets, which may correlate with peripheral atherosclerosis\[5\-7\]. Chemerin, the endogenous ligand of ChemR23, is a newly discovered adipokine that regulates adipocyte differentiation and modulates chemotaxis and recruitment of NK cells, dendritic cells, and macrophages\[8\-10\]. Chemerin is strongly associated with markers of inflammation, metabolic syndrome components, and atherosclerosis\[11\], for which endothelial dysfunction is a fundamental etiological factor. The chemerin/
ChemR23 system also plays an important role in endothelial cells.\textsuperscript{[12, 13]} ChemR23 is reported to be a multifunctional receptor, but little is known about the relationship between the chemerin/ChemR23 axis and endothelial dysfunction.\textsuperscript{[16]} We investigated the regulatory mechanisms of the chemerin/ChemR23 axis in dysfunctional endothelial cells induced by homocysteine, uric acid, glucose, and oxidized low-density lipoprotein (ox-LDL) and found that the chemerin/ChemR23 axis restores the impaired NO production and is upregulated by the K\textsubscript{ATP} channel opener, iptakalim, in endothelial cells. Our data suggest that augmentation of the chemerin/ChemR23 axis may be a pivotal mechanism for protecting endothelial function by opening K\textsubscript{ATP} channels.

Materials and methods

Chemicals

Iptakalim was synthesized by Nhwa Thad Pharmaceutical Co Ltd (Xuzhou, China). All other chemicals and materials were obtained from local commercial sources.

Cell culture

Rat aortic endothelial cells (RAECs) were isolated from normal Sprague-Dawley rats as described previously.\textsuperscript{[19]} The aorta was dissected out, sliced open, and rinsed with phosphate-buffered saline. It was cut into pieces and placed in a dish containing 100 mm-culture dish with 6 mL of M199 medium containing 20% fetal bovine serum (FBS) without growth supplements. On the third day, the aorta pieces were rinsed, and the collected cells were cultured in M199 medium containing 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin. The RAECs were grown to confluency at 37 °C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. A cobblestone morphology and positive immunofluorescence with antibodies against von Willebrand factor, factor VII-related antigen, or CD31 confirmed that the cells were endothelial in nature. The culture medium was replaced every other day, and the cells were subcultured until they were approximately 90% to 95% confluent. Cells were seeded in 100-mm culture dishes or 6- or 24-well plates and used after three to five passages.

Experimental treatment

Prior to the experimental procedures, cell growth was arrested by culture in phenol red-free M199 supplemented with 1% FBS and 1% penicillin-streptomycin for 6 h. RAECs were treated with homocysteine and glucose at two different concentrations (0.5 or 1 mmol/L) for various time periods (6, 32, or 40 h) to determine the effect of iptakalim on chemerin secretion and ChemR23 gene/protein expression. RAECs were pretreated with iptakalim (0.1, 1, or 10 μmol/L) for 8 h before treatment with homocysteine, uric acid, glucose or ox-LDL; 1 μmol/L of glibenclamide was applied 1 h before iptakalim. RAECs were treated with 1 μg/mL of ChemR23 antibody for 1 h, iptakalim and recombinant chemerin for 6 or 8 h, and then with 7.5 mg/L of uric acid for 16 h, to determine NO production.

Western blot analysis

Protein expression of ChemR23

After the treatments, RAECs were lysed with a denaturing lysis buffer, and the protein content of the supernatant was determined. The protein extract was then boiled with sample buffer (Tris 100 mmol/L, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS) and 0.2% bromophenol blue) at a protein extract to buffer ratio of 4:1. Protein samples were separated using 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) in a Tris–glycine transfer buffer. The membranes were washed in Tris-buffered saline with Tween 20 (TBS-T, 10 mmol/L Tris, pH 7.5, 0.1 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Tween 20) three times for 5 min, incubated in TBS-T with 5% skimmed milk for 2 h and then incubated overnight at 4 °C with a ChemR23-specific antibody (42 kDa, goat, 1:500, Santa Cruz, CA, USA) and an α-tubulin-specific antibody as a loading control (55 kDa, mouse, 1:500, Santa Cruz) in TBS-T with 5% skimmed milk. The blot was then incubated with a secondary antibody (1:1000, R & D Systems, Abingdon, Oxon, UK) for 2 h. Immunoblots were developed with an ECL Plus substrate (PerkinElmer, LAS GmbH, Rodgau, Germany). The fluorograph film was developed with a Kodak GBX developer after the optimum exposure time and fixed with Kodak GBX (Kodak, Rochester, New York, USA). Protein expression was quantified by scanning the films and analyzing the densitometric value of bands using a Personal Densitometer and Bio-Rad Quantity One software. The relative protein levels of ChemR23 were normalized to α-tubulin for each lane.

Secretion of chemerin

RAECs were cultured in 6-well plates for Western blot analysis of chemerin secretion. The cell culture supernatant and the total cell lysate were collected after treatment. The cell culture supernatant was mixed with an equal volume of sample buffer and subjected to SDS-PAGE before transfer to polyvinylidene fluoride (PVDF) membranes (0.22 μm). Primary antibodies for chemerin (18 kDa, mouse, 1:2000, Alexis Biochemicals, San Diego, CA, USA) in the cell culture supernatant and the control β-actin (43 kDa, mouse, 1:500, Santa Cruz, CA, USA) in the cell lysates were incubated overnight with the membranes in TBS-T with 5% skimmed milk at 4 °C. Membranes were subsequently washed with TBS-T and incubated with a secondary antibody (1:1000, R & D Systems, Abingdon, Oxon, UK) for 2 h at room temperature. The procedures for immunodetection and development of the fluorograph film were the same as mentioned above. Relative levels of chemerin secretion were normalized to β-actin in the corresponding samples of total cell lysates.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from RAECs and reverse transcribed to cDNA using reverse transcriptase with random hexamer priming. PCR was performed using primers selected for
ChemR23 and β-actin. As a control, β-actin mRNA was amplified from the same RNA preparation. The primer sequences for ChemR23 were as follows: sense, 5'-ACCTATGCCGC-TATGGAC-3'; and antisense, 5'-GACGTGAGCAGAAA-GACG-3', 108 bp. The primer sequences for β-actin were as follows: sense, 5'-GTGTTGCTGCTGCTGCTGCTG-3'; and antisense, 5'-GCTGTGGTGGTAAGGTGGCAGTGGCC-3', 197 bp. The PCR was conducted for 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s. The final cycle included an extension at 72 °C for 10 min. The PCR products were analyzed on a 2% agarose gel, and the intensity of the bands was measured with densitometry. Results were expressed as the density of ChemR23 normalized to β-actin in the same sample.

NO measurement
To assess the effects of iptakalim and recombinant chemerin on NO release, the cultured RAECs were seeded on 24-well culture plates and grown to full confluence. Because of the instability of NO in physiological solutions, most of the NO is typically rapidly converted to nitrite (NO$_2^-$), which is further broken down to nitrate (NO$_3^-$). Therefore, the level of the NO$_2^-/NO_3^-$ ratio in the culture medium was measured with a Nitric Oxide Assay Kit (Nanjing Jincheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured using the colorimetric method of the Griess assay as previously described[15].

Statistical analysis
The Student’s $t$-test and one-way ANOVA for paired values were used. Data are expressed as mean ± SD. $P<0.05$ was considered to be statistically significant.

Results
ChemR23 gene expression is downregulated by homocysteine, uric acid, glucose, and ox-LDL as a function of concentration and time
Endothelial dysfunction is independently induced by hyperhomocysteinemia, hyperuricemia, hyperglycemia, and hyperlipemia. ChemR23 protein/gene expression was downregulated in homocysteine-induced dysfunctional endothelial cells, so we investigated whether ChemR23 expression was also downregulated by uric acid, glucose, and ox-LDL. RAEC treatment with homocysteine, uric acid, glucose, and ox-LDL resulted in decreased ChemR23 gene expression levels as a function of concentration and time. Increased homocysteine, uric acid, glucose, and ox-LDL levels represent risk factors for cardiovascular diseases associated with endothelial dysfunction. Gene expression levels of ChemR23 are decreased in dysfunctional endothelium (Figure 2).

Iptakalim opens K$_{ATP}$ channels to reverse the downregulation of ChemR23 gene expression induced by uric acid, glucose, and ox-LDL
Iptakalim upregulated the chemerin/ChemR23 axis in homocysteine-induced dysfunctional endothelial cells, so we hypothesized that iptakalim would have the same effect on the chemerin/ChemR23 axis in dysfunctional endothelial cells induced by hyperuricemia, hyperglycemia, and hyperlipemia. A concentration of 0.1–10 μmol/L of iptakalim restored ChemR23 gene expression in a dose-dependent manner in endothelial cells treated with uric acid, glucose, and ox-LDL, and this restoration was antagonized by glibenclamide (Figure 3). Chemerin secretion and ChemR23 protein expression were correlated with ChemR23 gene expression, so they may also be upregulated by iptakalim via the opening of K$_{ATP}$ channels when endothelial dysfunction is induced by hyperuricemia, hyperglycemia, and hyperlipemia. Thus, the chemerin/ChemR23 axis may be upregulated by iptakalim via the activation of endothelial K$_{ATP}$ channels in dysfunctional endothelial cells.

Iptakalim and recombinant chemerin promote NO production, which is involved in the activation of ChemR23 in dysfunctional endothelial cells
A ChemR23 antibody was used to block the effects of iptakalim and recombinant chemerin on endothelial NO production, which helped clarify the role of the chemerin/ChemR23 axis in iptakalim protection against endothelial dysfunction. NO production was decreased when the RAECs were treated with uric acid. Iptakalim (10 μmol/L) upregulated the NO production impaired by uric acid and the effect of iptakalim was blocked by the ChemR23 antibody (Figure 4A). Recombinant chemerin (10, 100, and 300 ng/L) increased NO production compared to uric acid (Figure 4B). The effects of 10 μmol/L of iptakalim and 100 ng/L of chemerin were blocked in the presence of the ChemR23 antibody (1 μg/mL; Figure 4B). Thus, ChemR23 activation directly participates in enhancing the endothelial NO production and is correlated with the opening
of \( K_{\text{ATP}} \) channels in endothelial cells.

**Discussion**

In this study, we showed that iptakalim may regulate the chemerin/ChemR23 axis via the opening of \( K_{\text{ATP}} \) channels in the protection against endothelial dysfunction. Iptakalim restored the impaired chemerin secretion and ChemR23 gene/protein expression induced by homocysteine, uric acid, glucose, and ox-LDL in RAECs. The effects of iptakalim were mediated via \( K_{\text{ATP}} \) channel opening, which was antagonized by glibenclamide. Furthermore, iptakalim and recombinant chemerin restored the NO production that was decreased by uric acid, and this effect was abrogated by anti-ChemR23 antibodies. Our data suggest that the chemerin/ChemR23 axis is a novel molecular pathway that is involved in iptakalim-mediated protection against endothelial dysfunction and cardiovascular pathology.

ChemR23 can be activated by its endogenous ligand
Chemerin and its exogenous ligand RvE1, which is an endogenous anti-inflammatory mediator derived from eicosapentaenoic acid. ChemR23, when activated by RvE1, induces the phosphorylation of the ribosomal protein S6 of the PI3K-Akt pathway, which may contribute to the resolution of vascular inflammation and ADP-dependent platelet activation in pathological cardiovascular events [16, 17]. The chemerin/ChemR23 axis may become weaker in pathological conditions, which may not only impair endothelial function, but may also accelerate cardiovascular inflammation [18]. We demonstrated that the expression of the chemerin/ChemR23 axis fluctuates with endothelial function. We found that homocysteine, uric acid, glucose, and ox-LDL all decreased ChemR23 mRNA levels as a function of concentration and time. Iptakalim protected endothelial function by opening endothelial KATP channels, and it restored the impaired chemerin secretion and ChemR23 gene/protein expression caused by homocysteine, uric acid, glucose, and ox-LDL in endothelial cells. Glibenclamide antagonized the effects of iptakalim, which indicates that the effects of iptakalim are mediated by KATP channel opening. In aortic endothelial cells, KATP channels consist of a heteromultimeric complex of Kir6.1, Kir6.2, and SUR2B subunits [19]. Glibenclamide and iptakalim bind with different sites on the regulatory subunit, the sulfonylurea receptor [20]. However, some investigators reported that chemerin expression and secretion were enhanced in lupus erythematosus dermal endothelial cells and in interleukin-1β-stimulated adipocytes [13, 21]. This paradox can be reconciled because these cell types differ in their distinctive physiological functions. ChemR23 expression was downregulated by cardiovascular risk factors, which is consistent with other reports, that showed ChemR23 is downregulated by interleukin-2 or interleukin-15 in NK cells and by proinflammatory cytokines and Toll-like receptor ligands in macrophages [9, 22]. The downregulation of ChemR23 expression was correlated with the inhibition of cell migration in response to chemerin in NK cells, which may indicate that the receptor function of ChemR23 is decreased along with its downregulation [9]. Our data suggest that KATP channel opening upregulates the chemerin/ChemR23 axis in dysfunctional endothelial cells, but the mechanism remains unclear.

NO is one of the most important vasodilating substances released by the endothelium, and it inhibits growth and

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**Figure 2.** Downregulation of ChemR23 gene expression as a function of concentration and time by homocysteine (A), uric acid (B), glucose (C), and ox-LDL (D) in RAECs. RAECs were treated for various time periods and various concentrations of homocysteine (Hcy), uric acid, glucose (Glu), and ox-LDL. Data are expressed as mean±SD. n=4. The t-test was used. aP<0.05, bP<0.01 vs the corresponding vehicle control.
inflammation, has anti-aggregant effects on platelets and plays an important role in cardiovascular diseases. The ability to synthesize NO reflects the functional status of endothelial cells. The endothelial NO system can be downregulated by a number of cardiovascular risk factors, such as hyperhomocysteinemia, hyperuricemia, hyperglycemia, and hyperlipemia[3, 15]. We detected effects of the chemerin/ChemR23 axis on NO-related endothelial function. We found that iptakalim and chemerin restored NO production; this was decreased by uric acid in RAECs, and this decrease was abolished by anti-ChemR23 antibodies. This suggests that the activation of ChemR23 is necessary for iptakalim and chemerin improvements in endothelial function. Endothelial NO is mainly produced by eNOS, the enzymatic activity of which is regulated at several levels, including Ca²⁺/calmodulin binding and the interaction of eNOS with associated proteins[23]. ChemR23 activation resulted in intracellular calcium release promoted by recombinant chemerin in ChemR23-expressing CHO-K1 cells[24]. Calcium enhanced the phosphorylation of eNOS and NO release by binding to calmodulin[25]. Although several studies have reported that the chemerin/ChemR23 axis is strongly associated with cardiovascular disease and aspects of metabolic syndrome[11, 26, 27], there was no direct evidence for the role of the chemerin/ChemR23 axis in maintaining endothelial function. The chemerin/ChemR23 axis enhances the NO system, so it is likely that the chemerin/ChemR23 axis contributes to the maintenance of endothelial function.

A previous study demonstrated that iptakalim upregulated the NO system and downregulated the endothelin system and the production of adhesion molecules ICAM-1/VCAM-1/MCP-1 in endothelial cells to protect against the progression of cardiac hypertrophy to congestive heart failure[3, 4]. Endothelial dysfunction is characterized by the reduced bioavailability of NO, an alteration in the production of prostanoids, impairment of endothelium-dependent hyperpolarization, and an increased release of endothelin-1, all of which separately or in

Figure 3. Iptakalim restores ChemR23 gene expression decreased by uric acid (A), high glucose (B), and ox-LDL (C) in RAECs. RAECs were pretreated with/without iptakalim (Ipt) for 8 h and then stimulated with 7.5 mg/L uric acid for 24 h, 25 mmol/L glucose (Glu) for 16 h, and 100 μg/mL ox-LDL for 12 h. Glibenclamide (Glib, 1 μmol/L) was applied 1 h before iptakalim. Data are expressed as mean±SD. n=4. The ANOVA test was used. *P<0.05, **P<0.01 vs the corresponding control; *P<0.05, **P<0.01 vs uric acid, glucose, or ox-LDL stimuli; *P<0.01 vs iptakalim pretreatment, respectively.
association contribute to endothelial dysfunction[1]. Elevated NO contributes to the equilibrium of vasoactive mediators involved in the regulation of endothelial function and vascular homeostasis. We suggest that the chemerin/ChemR23 axis may attenuate the endothelin system and the overexpression of adhesion molecules in dysfunctional endothelial cells because the chemerin/ChemR23 axis enhances the NO system. Upregulation of the chemerin/ChemR23 axis is a well-supported explanation for how iptakalim improves endothelial function. The chemerin/ChemR23 axis is connected with endothelial function by two lines of evidence. The first is that the chemerin/ChemR23 axis is an endogenous pathway which iptakalim upregulates the chemerin/ChemR23 axis to improve endothelial function. A detailed molecular and functional analysis of the role of the chemerin/ChemR23 axis in endothelial function should provide further understanding of the relationship between the chemerin/ChemR23 axis and endothelial function.

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Author contribution
Hai WANG designed the study, and Rui-jun ZHAO performed the research, analyzed data, and wrote the paper.

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