Inhibitory effects of telmisartan on culture and proliferation of and Kv1.3 potassium channel expression in peripheral blood CD4+ T lymphocytes from Xinjiang Kazakh patients with hypertension

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Abstract

Introduction: Activation of T lymphocytes, for which potassium channels are essential, is involved in the development of hypertension. In this study, we explored the inhibitory effects of telmisartan on the culture and proliferation of and Kv1.3 potassium channel expression in peripheral blood CD4+ T lymphocytes derived from Xinjiang Kazakh patients with hypertension.

Methods: CD4+ T-cell samples from hypertensive Kazakh patients and healthy Kazakh people were divided into healthy control, case control, telmisartan, and 4-aminopytidine groups. Changes in the expression levels of interleukin (IL)-6 and IL-17 in the blood of the healthy control and case control subjects were detected by enzyme-linked immunosorbent assay. Peripheral blood CD4+ T lymphocytes were first activated and proliferated in vitro and then incubated for 0, 24, and 48 h under various treatment conditions. Thereafter, changes in CD4+ T-lymphocytic proliferation were determined using Cell Counting Kit-8 and microscope photography. Changes in messenger RNA (mRNA) and protein expression of the Kv1.3 potassium channel in CD4+ T lymphocytes were detected using real-time quantitative polymerase chain reaction and Western blots, respectively.

Results: The IL-6 and IL-17 expression levels were significantly higher in the blood of the hypertensive Kazakh patients than in the healthy Kazakh people. Telmisartan inhibited T-lymphocytic proliferation, as well as the mRNA and protein expression of the Kv1.3 potassium channel in CD4+ T lymphocytes, and the inhibitory effects were time-dependent, with the strongest inhibition observed after 48 h and significantly weaker inhibition observed after 24 h of treatment.

Conclusions: Telmisartan may potentially regulate hypertensive inflammatory responses by inhibiting T-lymphocytic proliferation and Kv1.3 potassium channel expression in CD4+ T lymphocytes.

Keywords

Xinjiang Kazakh, essential hypertension, CD4+ T Lymphocytes, Kv1.3 potassium channel, telmisartan

Background

Essential hypertension (EH) is the most common cardio-vascular disease with a multifactorial pathogenesis. Recent studies have confirmed that a large number of EH cases are closely related to the development of inflammation. Activation of inflammatory cells was shown to be involved in the development of hypertension,[1,2] and it has been proposed that hypertension is a chronic, low-grade

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inflammatory disease.[3,4] Animal studies have shown that activation of massive inflammatory cells was obvious in a spontaneously hypertensive rat (SHR) model, and the activation was more significant in adult SHRs than in young SHRs.[5] In fact, massive inflammatory mediators are upregulated in hypertensive patients, and their levels can be used to predict the development of hypertension. Inflammatory reactions in hypertensive patients are characterized by the expression and activation of cytokines.[6,7]

T lymphocytes play an important role in the etiology and development of hypertension and vascular remodeling,[8] and they are important for immune regulation. It is well known that T lymphocytes can be divided into two subsets, CD4+ and CD8+. CD4+ T lymphocytes account for 35–50% of the total peripheral blood lymphocytes and regulate the biological activity of the immune system by secreting cytokines and expressing cell surface molecules. CD4+ T lymphocytes secrete interferon gamma (INF-γ), which can stimulate the expression of major histocompatibility complex (MHC) in endothelial cells (ECs) and smooth muscle cells. This activates the antigen-presenting function of ECs and initiates immune responses. INF-γ also improves T lymphocyte migration through the EC monolayer.

The activation function of T lymphocytes depends on ion channels located on their cell surfaces. Studies have shown that there are mainly three kinds of membrane ion channels on T lymphocytes, a voltage-gated potassium channel (Kv1.3), calcium-activated potassium channel (KCa3.1), and calcium release-activated calcium (CRAC) channel. In 1984, DeCoursey et al.[9] reported the presence of Kvl.3 potassium channels on T cells. The Kv1.3 voltage-gated potassium channel is essential for the activation of T lymphocytes and is involved in some physiological processes, such as T-lymphocytic differentiation and proliferation.[10,11] Kv1.3 on CD4+ T lymphocytes plays a key role in lymphocyte activation in patients with acute coronary syndrome.[12] Opening of the Kv1.3 potassium channel is essential for the membrane depolarization and maintenance of the resting potential. It has been reported that Kv1.3 is involved in the development of various diseases, such as acute coronary syndrome, multiple sclerosis, asthma, tumors, etc.[13–20] The forkhead transcriptional factor FoxP3, expressed in T lymphocytes, is critical for the production of angiotensin II, which is essential for the progression of EH.[8] Some studies have shown that besides lowering blood pressure, angiotensin receptor blockers (ARBs), which are widely used to treat hypertension, also have anti-inflammatory effects.[21–24]

Kazakh individuals are the second largest minority in Xinjiang, and they have a high incidence of hypertension, with EH prevalence rates of 40.5–52.39%.[25] However, rates of disease awareness, cure, and control appear to be lower among Kazakhs than in other ethnic groups. Therefore, studying the pathogenesis of hypertension in Kazakh individuals has social significance. However, few studies have assessed the effects of telmisartan, one of the ARBs, on the proliferation of peripheral blood CD4+ T lymphocytes, as well as messenger RNA (mRNA) and protein expression of the Kv1.3 potassium channel in CD4+ T lymphocytes derived from Xinjiang Kazakh patients with EH.

In this study, we used enzyme-linked immunosorbent assay (ELISA) to detect changes in the expression levels of interleukin (IL)-6 and IL-17 in the blood of hypertensive Kazakh patients. We used magnetic-activated cell sorting (MACS) to isolate CD4+ T lymphocytes from peripheral blood of Xinjiang Kazakh patients with EH and healthy Kazakh people. Then, Cell Counting Kit-8 (CCK-8) and microscope photography were used to analyze the culture and proliferation of the CD4+ T lymphocytes. Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) and Western blotting were used to determine the mRNA and protein expression levels of the Kv1.3 potassium channel in the CD4+ T lymphocytes. Our results may provide the theoretical mechanisms and experimental evidence for the anti-inflammatory effect of telmisartan.

Methods

Subjects

We selected Kazakh hypertensive patients and healthy Kazakh people as the subjects for our experiments. Thirty Kazakh hypertensive patients (mean age: 50.4±3.5 years) and 10 healthy Kazakh people (mean age: 48.8±4.5 years) attending the cardiology clinic of the First Affiliated Hospital of Xinjiang Medical University were enrolled in the study between January–August 2015. The healthy subjects were all assigned to a healthy control group, while the hypertensive subjects were randomised into the following three groups: (a) case controls; (b) telmisartan; and (c) 4-aminopytidine (4-AP). There were 10 individuals (five males and five females) in each group, and they were not undergoing antihypertensive therapy.

The diagnostic criteria of hypertension, used as the inclusion criteria, were as stated in the 2010 Chinese guidelines for the management of hypertension.[26] Briefly, systolic blood pressure of ≥140 mm Hg (1 mm Hg = 0.133 kPa) and/or diastolic blood pressure of ≥90 mm Hg, without treatment with antihypertensive medication, were considered the diagnostic criteria of hypertension.

Subjects with the following types of diseases were excluded from our experiments: (a) secondary hypertension; (b) cerebrovascular disease; (c) coronary, rheumatic, or congenital heart disease; (d) acute or chronic infection; (e) autoimmune disease; (f) important organ failure; (g) diabetes; and (h) carotid atherosclerosis.

The study was conducted in accordance with the World Medical Association Declaration of Helsinki. All procedures were approved by the Ethics Committee of the First

Affiliated Hospital of Xinjiang Medical University (approval number 20141011-2), and informed consent was signed by each subject before the trial.

Reagents

IL-6 and IL-17 ELISA kits were from eBioscience (San Diego, California, USA). Human lymphocyte isolation medium, recombinant human IL-2 (rIL-2), phytohemagglutinin (PHA), and 4-AP were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). The MACS kit was from Miltenyi Biotec (Germany), and telmisartan was from Boehringer–Ingelheim (Germany). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from HyClone (New Zealand). Fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD3 and FITC-labelled mouse anti-human CD4 were from Becton, Dickinson and Company (USA). TRIzol was from Life Technologies (USA). The RT kit, Radio-Immunoprecipitation Assay (RIPA) lysis buffer, protease inhibitor, bicinchoninic acid (BCA) protein assay kit, and enhanced chemiluminescence (ECL) Western blotting kit were from Thermo Fisher (USA). The real-time fluorescent qPCR kit was from Qiagen (Germany). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) reagents were purchased from Bio-Rad (USA). Anti-human monoclonal antibodies against the Kv1.3 potassium channel and β-actin and secondary antibodies were obtained from Abcam (UK). The 5-bromo-4-chloro-3'-indolyphosphate/ nitro-blue tetrazolium chromogenic reagent was from Invitrogen (USA).

CD4+ T lymphocyte isolation

Peripheral venous blood (10 ml) was collected from each subject into a heparinized vacutainer tube. Peripheral blood mononuclear cells were isolated using human lymphocyte isolation medium and density gradient centrifugation. Using the Pan T cell isolation kit (Miltenyi, Germany), human T lymphocytes were isolated by depletion of non-target T cells (negative selection). Non-target T cells were magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies and the Pan T cell microbead cocktail. They were retained on a MACS separation column in the magnetic field of a MACS separator (Miltenyi, Germany), while unlabeled T lymphocytes passed through the column. The purity of the enriched T lymphocytes was evaluated by flow cytometry (Beckman Coulter, USA). The cells were fluorescently stained with FITC–CD3 and analyzed using a MACSQuant analyzer (Miltenyi, Germany). Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence. The results showed that >95% of the cells were T lymphocytes. T lymphocytes were incubated with CD4 monoclonal antibodies. The

suspensions were then incubated with magnetic beads coated with goat anti-mouse Immunoglobulin G (IgG), which bind to antibody-coated cells. Subsequent exposure to a strong magnetic field removed unwanted cells, leaving the desired cell population. The cell-coated magnetic beads were resuspended in complete medium. Anti-human FITC-CD4 was added, and the T lymphocytes were analyzed using flow cytometry. The results showed that >95% of the cells were CD4+ T lymphocytes, which were used for subsequent experiments.

Cell culture

CD4+ T lymphocytes were seeded into 24-well plates containing RPMI 1640 medium with 10% FBS, rIL-2, and PHA and cultured at 37°C, 5% CO_2 in an incubator (Thermo, USA) for 48 h to promote T lymphocyte activation. The cultured CD4+ T lymphocytes were treated as follows: telmisartan (final concentration, 100 µmol/l), 4-AP (final concentration, 3 mmol/l), or an equal volume of a dimethyl sulphoxide (DMSO) vehicle was added to the culture media of cells from the telmisartan, 4-AP, and control groups, respectively. The CD4+ T lymphocytes were then cultured for 0, 24, and 48 h, and the suspensions were harvested. Changes in the T-lymphocytic growth status, shape, and quantity were observed and recorded by microscope photography using an inverted fluorescent microscope (Leica, Germany). Then, the samples were divided into three portions, for CCK-8 analysis and extraction of RNA and protein, respectively.

ELISA assay

Peripheral blood (5 ml) from the healthy Kazakh people in the healthy control group and from the hypertensive Kazakh patients in the case control group was incubated at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant serum was collected and stored at −80°C. Then, levels of IL-6 and IL-17 were analyzed in the serum using the ELISA kits according to the manufacturer's instructions. Both test samples and standard samples were tested in duplicate. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad). Then, standard curves were established, and levels of IL-6 and IL-17 were calculated from the standard curves.

CCK-8 analysis of T-lymphocytic proliferation

CD4+ T lymphocytes, precultured with PHA and rIL-2 for 48 h, were seeded into 96-well plates in 100 µl of medium (less than 2000 cells per well). The cells were treated with telmisartan, 4-AP, or DMSO as described previously (eight replicate wells per treatment) for 0, 24, and 48 h. After the incubation, 10 µl of the CCK-8 reagent was added to the wells. The culture plate was incubated at 37 °C, 5% CO_2 in

Table 1. Primer sequences and amplicon sizes.

Gene	Sequences (5′–3′)	GenBank accession number	Product length (base pairs)
Kv1.3	F: CCAGCACCTCTCCTCTTCAG	NM_002232.3	80
	R: TCACCATATACTCCGACTTACTCA		
ACTB	F: TGGCACCCAGCACAATGAA	NM_001101.3	186
	R: CTAAGTCATAGTCCGCCTAGAAGCA		

an incubator for 3 h, and optical density (OD) values were measured at 450 nm using a microplate spectrophotometer. Then, cellular activities of the different groups were calculated as follows:

$$\text{Cellular activity } (\%) =$$
$$\left(\begin{array}{l} \text{OD value of interventional group} - \\ \text{OD value of control group} \end{array} \right) /$$
$$\left(\begin{array}{l} \text{OD value of interventional group at 0 h} - \\ \text{OD value of control group at 0 h} \end{array} \right) \times 100$$

RNA extraction and qRT–PCR analysis

Total RNA was extracted from CD4$^+$ T lymphocytes using TRIzol, and the A_{260}/A_{280} ratios of the resulting RNA samples were approximately 1.8–2.0. RT reactions were performed using 1 μg of RNA at 42°C for 60 min, followed by incubation at 70°C for 5 min. The total volume of qPCR reactions was 20 μl, including 10 μl of SYBR Green PCR master mix (2×), 2 μl of cDNA template, 0.5 μl of each forward (F) and reverse (R) primer, and 7 μl of H$_2$O. The sequences of the genes encoding the Kv1.3 potassium channel and β-actin (*ACTB* gene) were obtained from GenBank. The primers were synthesized by Sangon Biotech (Shanghai). *ACTB* was used as an internal reference control gene (Table 1). PCR and agarose gel electrophoresis were performed with the cDNA templates, and standard curves were established using the PCR products. The qPCR program was as follows: 95°C for 5 min, followed by 39 cycles of 95°C for 10 s and 60°C for 30 s. Amplification efficiencies were approximately 90–100%. After qPCR, the amplicons were separated by 2% agarose gel electrophoresis. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method as follows:

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ reference gene}$$

$$\Delta\Delta Ct = \Delta Ct \text{ experimental group} - \Delta Ct \text{ control group}$$

The rate of inhibition by telmisartan was calculated as follows:

$$\text{Inhibitory rate } (\%) =$$
$$(\text{mRNA expression in control group}$$
$$- \text{mRNA expression in intervention group})$$
$$/ \text{mRNA expression in control group} \times 100.$$

Western blots

Total protein was extracted from CD4$^+$ T lymphocytes using RIPA lysis buffer supplemented with protease inhibitors. Protein concentrations were measured by the BCA protein assay. Protein samples (20 μl=30 μg) were mixed with 5 μl of loading buffer, then denatured in a water bath at 95°C for 5 min, and separated by SDS–PAGE. Then, proteins were transferred to polyvinylidene fluoride membranes, and the membranes were blocked with 5% skim milk for 1 h. The primary antibodies were diluted as follows: anti-Kv1.3 potassium channel, 1:200; anti-β-actin, 1:5000. The blots were incubated with the primary antibodies overnight at 4°C with shaking and then washed with Tris-buffered saline containing Tween 20 (TBS-T), followed by incubation with secondary antibodies (both 1:4000) at room temperature for 1 h and washing with TBS-T. Visualization of protein bands was performed by the addition of 5 ml of the ECL reagent, followed by the analysis using a Quality Image Analysis System (Bio-Rad). The telmisartan inhibitory rate was calculated as follows:

$$\text{Inhibitory rate } (\%) =$$
$$(\text{protein expression in control group} -$$
$$\text{protein expression in interventional group}) /$$
$$\text{protein expression in control group} \times 100$$

Statistical methods

Data were statistically analyzed using the SPSS 17.0 software and are expressed as the mean±standard deviation (SD) or percentage, as appropriate. Multiple data sets were compared by repeated-measures one-way analysis of variance or a chi-square test. A least significant difference *t*-test was used for comparison between two groups. A *p*-value of <0.05 was considered statistically significant.

Results

Clinical and demographic characteristics of subjects

There were no significant differences among the subjects in the healthy control, case control, telmisartan, and 4-AP groups in regard to the age, smoking history, drinking history, body mass index, fasting blood glucose, C-reactive

Table 2. Comparison of baseline data among the healthy control, case control, telmisartan, and 4-aminopytidine (4-AP) groups (*n*=40).

Parameter	Group				
	Healthy control	Case control	Telmisartan	4-AP	*p*-Value
Number	10	10	10	10	>0.05
(males/females)	(5/5)	(5/5)	(5/5)	(5/5)	
Age (years)	49.8 ± 4.5	48.3 ± 2.3	50.2 ± 3.7	50.7 ± 3.3	>0.05
Smoking (%)	49	52	50	51	>0.05
Drinking (%)	50	49	51	51	>0.05
BMI (kg/m^2)	24.8 ± 2.5	26.1 ± 1.5	25.9 ± 1.4	26.2 ± 2.3	>0.05
FBG (mmol/l)	4.5 ± 0.6	4.6 ± 0.3	4.6 ± 0.7	4.6 ± 0.5	>0.05
CRP (mmol/l)	7.5 ± 0.7	7.7 ± 0.8	7.7 ± 0.5	7.6 ± 1.2	>0.05
TG (mmol/l)	1.6 ± 0.2	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	>0.05
HDL-C (mmol/l)	1.3 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	>0.05
LDL-C (mmol/l)	3.5 ± 0.9	3.6 ± 0.8	3.6 ± 1.0	3.8 ± 0.9	>0.05

BMI: body mass index; CRP: C-reactive protein; FBG: fasting blood glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride.

protein (CRP), triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels (*p*>0.05; Table 2). The family history of hypertension was defined as one or more family members (parent or sibling) of the subject having hypertension.[27] Smoking was defined as a current or previous smoking history.[27] Alcohol consumption was defined as drinking at least once per week for more than half a year.[27]

Levels of IL-6 and IL-17 in peripheral blood

The results showed that the expression of the inflammatory cytokines IL-6 and IL-17 was significantly higher in the peripheral blood of the Kazakh hypertensive patients than in that of the healthy Kazakh people (IL-6: 61.0±4.1 vs 43.0±5.3; IL-17: 72.4±1.9 vs 50.8±2.6, respectively, *p*=0.002) (Figure 1).

CD4$^+$ T-lymphocytic proliferation

The cellular activities of the control groups significantly increased, while those of the telmisartan and 4-AP groups significantly reduced over time (*p*<0.05; Table 3).

Morphological observations of CD4$^+$ T-lymphocytic proliferation with and without drug treatment

CD4$^+$ T-lymphocytic proliferation in the healthy and case control groups stimulated with PHA+rIL-2

There were obvious changes in the growth status, shape, and quantity of CD4$^+$ T lymphocytes, and these changes were similar in the healthy control and case control groups. As the culture time progressed, the cellular volume became larger, and most of the cells were round or could be irregular in

shape, such as multi-tentacled. Cytoplasm vacuolization was observed, and the cells aggregated to form a massive T-lymphocytic mass. A large cellular mass formed by hundreds of cells was visible under macrography, which indicated that the cells were in vigorous growth. The aggregated cells could easily be dispersed to single ones. There also was a significant increase in cell numbers.

CD4$^+$ T-lymphocytic proliferation in the telmisartan group

Before the telmisartan treatment, the CD4$^+$ T lymphocytes grew in good condition, and there were no significant differences with the cells in the control groups under micrography. After 24 h of telmisartan treatment, there were some changes in the growth status, shape, and quantity of CD4$^+$ T lymphocytes. After 48 h, the differences became more obvious as telmisartan started showing an inhibitory effect on T-lymphocytic proliferation. The cellular activity became weaker, the cell numbers were significantly reduced, and there was scattered cell debris, which was apoptotic, on the bottom of the culture dish.

CD4$^+$ T-lymphocytic proliferation in the 4-AP group

At 0 h, the CD4$^+$ T lymphocytes had good cellular activity, and there were large numbers of cells, with large round cells densely populated. At 24 h of 4-AP treatment, the cell numbers were slightly reduced, but there was still some aggregated T-lymphocytic mass, which was sensitive to PHA. The cellular volume became smaller, and there was some cell debris deposited on the bottom of the culture dish. At 48 h, the cell numbers were significantly reduced, and cell debris, which was apoptotic, could be observed on the bottom of the culture dish. There were scattered single cells and some small cell mass of aggregated cells with a low cellular
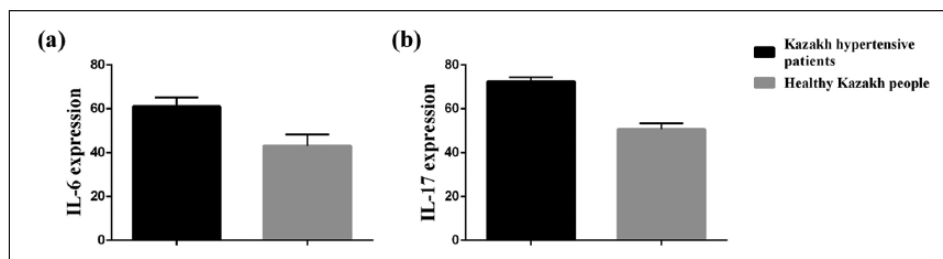
Figure 1. Levels of expression of interleukin (IL)-6 (a) and IL-17 (b) detected by enzyme-linked immunosorbent assay (ELISA) in hypertensive patients and healthy persons.

Table 3. CD4^{+} T-lymphocytic cellular activities detected using Cell Counting Kit-8.

Group	0 h	24 h	48 h
Healthy control	28.13 ± 6.23	36.15 ± 3.19[a]	48.14 ± 5.21[a]
Case control	106.45 ± 10.28	121.63 ± 5.37[a]	135.03 ± 10.74[a]
Telmisartan	102.73 ± 2.96	68.33 ± 5.39[a]	22.27 ± 3.58[a]
4-AP	105.17 ± 5.19	47.32 ± 6.38[a]	11.21 ± 2.01[a]

[a]$p < 0.01$ compared with the cellular activity at 0 h in the same group.

activity. The volume of most cells became smaller, and the CD4^{+} T lymphocytes were dark and had poor cellular activity.

Effect of telmisartan and 4-AP on mRNA expression of Kv1.3 potassium channel in CD4^{+} T lymphocytes

Expression of Kv1.3 potassium channel mRNA in the peripheral blood CD4^{+} T lymphocytes was examined in the four experimental groups. Amplicons of the correct size were observed after agarose gel electrophoresis. The results demonstrated that the mRNA levels of the Kv1.3 potassium channel were lower in the telmisartan and 4-AP groups after 24 h and 48 h of treatment than those in the healthy control and case control groups ($p < 0.05$). There were no statistically significant differences in the expression of Kv1.3 potassium channel mRNA at different time points in the healthy control and case control groups ($p > 0.05$). In addition, the mRNA expression levels of the Kv1.3 potassium channel were significantly reduced in the CD4^{+} T lymphocytes at 24 h and 48 h compared with the levels at 0 h in the telmisartan and 4-AP groups ($p < 0.05$) (Figure 2). Telmisartan inhibited the mRNA expression of the Kv1.3 potassium channel in CD4^{+} T lymphocytes at 24 h and 48 h by 47.2% and 78.5%, respectively.

Effects of telmisartan and 4-AP on Kv1.3 potassium channel protein expression in CD4^{+} T lymphocytes

Expression of the Kv1.3 potassium channel protein was examined in the peripheral blood CD4^{+} T lymphocytes from the four experimental groups. The results
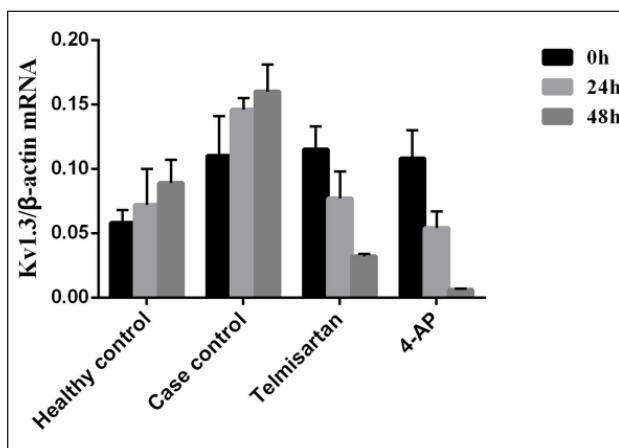

Figure 2. messenger RNA (mRNA) expression of the Kv1.3 potassium channel relative to *ACTB* in samples extracted from activated peripheral blood CD4^{+} T lymphocytes from Xinjiang Kazakh essential hypertension (EH(patients, determined by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Cells were treated with dimethyl sulphoxide (DMSO) (healthy control and case control), telmisartan (100 µmol/l), and 4-aminopytidine (4-AP) (3 mmol/l); $n = 10$ in each treatment group.

demonstrated that the protein levels of the Kv1.3 potassium channel were lower in the telmisartan and 4-AP groups after 24 h and 48 h of treatment than those in the healthy control and case control groups ($p < 0.05$). There were no statistically significant differences in the expression of the Kv1.3 potassium channel protein at different time points in the healthy control and case control groups ($p > 0.05$). In addition, the protein expression levels of the Kv1.3 potassium channel were significantly reduced in the CD4^{+} T lymphocytes at 24 h and 48 h compared with the levels at 0
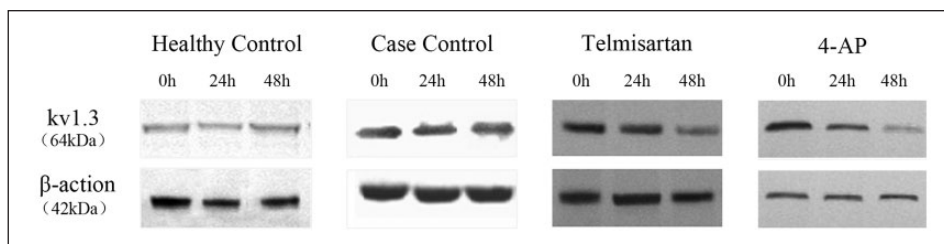
Figure 3. Representative Western blots of protein samples extracted from activated peripheral blood CD4+ T lymphocytes from Xinjiang Kazakh essential hypertension (EH) patients. Blots were probed with antibodies against the Kv1.3 potassium channel and β-actin: healthy control group; case control group; telmisartan group (100 μmol/l); 4-aminopytidine (4-AP) group (3 mmol/l).
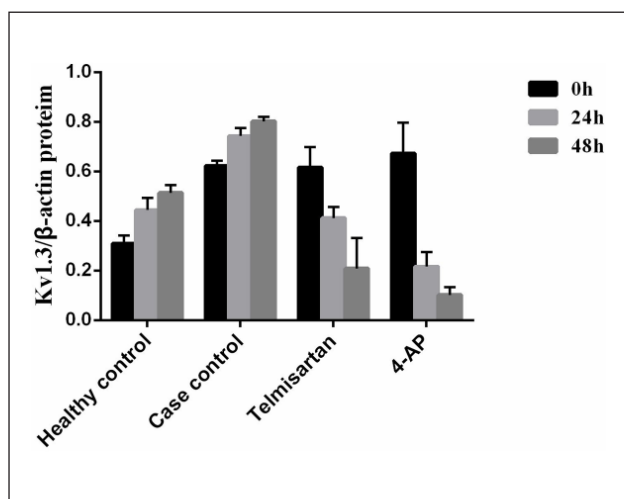

Figure 4. Protein expression of the Kv1.3 potassium channel relative to β-actin in samples extracted from activated peripheral blood CD4+ T lymphocytes from Xinjiang Kazakh essential hypertension (EH) patients, determined by Western blotting. Cells were treated with dimethyl sulphoxide (DMSO) (control), telmisartan (100 μmol/l), and 4-aminopytidine (4-AP) (3 mmol/l); n=10 in each treatment group.

h in the telmisartan and 4-AP groups (Figures 3 and 4). Telmisartan inhibited the protein expression of the Kv1.3 potassium channel in the CD4+ T lymphocytes at 24 h and 48 h by 49.3% and 67.9%, respectively.

Discussion

EH is a chronic, low-grade inflammatory disease with abnormal activation of inflammatory cytokines and inflammatory signaling pathways.[3,4,28] Expression of inflammatory factors causes vascular inflammation and promotes development of hypertension, which further changes the structure and function of blood vessels and causes damage to the heart, kidney, and other target organs. Harrison and Gongora[29] have proposed that the release of various lymphocyte cytokines causes vasoconstriction and sodium and water retention, which further increases the blood pressure to promote the development of EH. Elevated IL-1β and CRP levels are also associated with hypertension. Thus, it has been confirmed recently that

tumor necrosis factor alpha increased the activity of reduced coenzyme II oxidase in polymorphonuclear leukocytes, which led to oxidative stress, an inflammatory response, and elevated blood pressure.[30] Under various conditions, activated T lymphocytes will produce multiple inflammatory cytokines, including IL-6 and IL-17. Consistent with most studies, here we found that the levels of IL-6 and IL-17 in peripheral blood were higher in Kazakh hypertensive patients than in related healthy persons, which indicated that there was an activation of inflammatory cytokines in the hypertensive patients.

The T lymphocyte function depends on the electrical activity of ion channels in their cell membrane.[31] Studies using patch clamps have suggested that Kv1.3 potassium channels are the key effectors of the sustained activation of CD4+T lymphocytes.[32] The main effects of Kv1.3 potassium channels are to maintain the membrane potential and intracellular calcium signaling, which regulates the cell proliferation and cytokine production. Kv1.3 potassium channels are likely targets for the immune or bi-directional prevention of EH and are associated with many clinical diseases. Thus, Beeton and others[33,34] studied peripheral blood of multiple sclerosis patients and postmortem brain lesions and found that the antigen-specific CD4+ T cells were mainly CCR7–CD45RA effector memory T cells, which exhibited increased expression of Kv1.3 channels. Guo et al.[35] detected increased levels of Kv1.3 potassium channel mRNA in rat lymphocytes, suggesting that the elevated expression of Kv1.3 on T lymphocytes plays an important role in atherosclerosis in rats. Xu et al.[14] found that CD4+/CD28– T lymphocytes were potential immunomodulatory targets in patients with acute coronary syndrome and suggested that selective blocking of CD4+/CD28– T lymphocytes could be used to treat the disease. Therefore, this study focused on Kv1.3 channels on CD4+ T lymphocytes derived from Kazakh hypertensive patients.

As a Kvl.3 potassium channel inhibitor, 4-AP can inhibit the activation and proliferation of T lymphocytes and regulate the cytokine production and immune responses by inhibiting the Kvl.3 potassium channel. Some studies have shown that ARBs can effectively inhibit the Kvl.3 potassium channel, regulate the immunity through anti-inflammation and antioxidation, and play an important role as anti-atherosclerosis drugs. By observing

blocking of Kvl.5/Kvl.3 potassium channels in *Xenopus* oocytes, Li et al.[36] found that telmisartan blocked the opening state of the Kv1.3 potassium channel, which is one of the mechanisms that can regulate the immune system and anti-atherosclerotic effects.

Data from our preliminary study[37] have indicated that the mRNA expression levels of the Kv1.3 potassium channel are significantly increased in lymphocytes of SHRs, along with an elevated potassium current density, demonstrating an increase in functional potassium channels in SHR lymphocytes. Moreover, we have also previously reported that the mRNA and protein expression levels of the Kv1.3 potassium channel are significantly elevated in lymphocytes from hypertensive Kazakh patients from Xinjiang[38] and that the Kv1.3 potassium current densities in peripheral blood T lymphocytes of this population were also higher.[39] These studies support the concept that potassium channels of T lymphocytes are involved in the pathogenesis and progression of hypertension through their role in T lymphocyte activation. We have also performed studies demonstrating that telmisartan can block Kv1.3 potassium channels in lymphocytes of SHRs in a concentration-dependent manner.[40] In addition, telmisartan effectively inhibited potassium current densities in peripheral blood T lymphocytes of Kazakh patients with EH in a time-dependent manner.[41] Hence, ARBs, including telmisartan, have the potential to elicit potent anti-inflammatory effects by blocking Kv1.3 potassium channels on T lymphocytes, although the specific underlying mechanisms remain unclear.

In the present study, there were no significant differences between the healthy control and case control groups, which indicated that the findings reflect a general phenomenon characteristic of CD4$^+$ T lymphocytes. Electron microscopy revealed that after the incubation with 4-AP at an effective concentration for 0, 24, and 48 h, the numbers of CD4$^+$ T lymphocytes were gradually reduced, their cellular activity gradually lowered, the shape condensed and gradually shrank, and many apoptotic cells could be seen. qRT–PCR and western blotting revealed that after the incubation with 4-AP at an effective concentration for 0, 24, and 48 h, the mRNA and protein expression levels of the Kv1.3 potassium channel in CD4$^+$ T lymphocytes were significantly reduced. The results showed that 4-AP had an obvious inhibitory effect on T-lymphocytic proliferation, as well as on mRNA and protein expression of the Kv1.3 potassium channel in CD4$^+$ T lymphocytes, which indirectly indicated that the Kvl.3 potassium channel is the key factor of T lymphocyte activation. The 4-AP group was used as a positive control group, and the telmisartan group showed similar changes, although the changes in the T-lymphocytic proliferation and mRNA and protein expression of the Kv1.3 potassium channel in CD4$^+$ T lymphocytes were slower and weaker with telmisartan relative to those in the 4-AP group. This indicates that the anti-inflammatory function and inhibitory effects of telmisartan

on the T lymphocyte Kv1.3 potassium channel are unlikely to affect the normal immune function in hypertensive patients. The CCK-8 analysis, qRT–PCR, and Western blotting showed that the cellular activities and mRNA and protein expression of the Kv1.3 potassium channel in the telmisartan and 4-AP groups were significantly lower at 24 and 48 h than at 0 h, with a slow, time-dependent, descending tendency. Compared with the data for the same time points in the healthy and case control groups, the cellular activities and mRNA and protein expression of the Kv1.3 potassium channel were significantly reduced in the telmisartan group. This indicates that the drug can effectively inhibit the proliferation of CD4$^+$ T lymphocytes, as well as the mRNA and protein expression of the Kv1.3 potassium channel. Furthermore, the inhibitory effects of telmisartan on the T-lymphocytic proliferation and mRNA and protein expression of the Kv1.3 potassium channel were time-dependent over a 48-hour period.

Nataraj et al.[42] demonstrated that the angiotensin II type 1 receptor (AT1R) is strongly expressed on T lymphocytes. AT1R can increase the intracellular Ca^{2+} concentration by activating T lymphocyte potassium channels, which triggers the Ca^{2+}-mediated activation of the calcineurin/ nuclear factor (CaN/NFAT) signalling pathway, leading to T lymphocyte activation and proliferation. We conclude that increased expression of potassium channels on CD4$^+$ T lymphocytes enhances potassium ion efflux, and the increased gradient of the electric potential on both sides of the cytomembrane generates hyperpolarization to promote calcium ion influx. This causes a transient increase in the calcium concentration, with subsequent CaN-mediated dephosphorylation of NFAT and activation of the CaN/ NFAT signaling pathway. The activated CaN/NFAT signalling pathway mediates T lymphocyte proliferation and the release of inflammatory cytokines to promote development of hypertension. Telmisartan can inhibit T lymphocyte potassium channels, which results in decreased influx of extracellular Ca^{2+} into the cytoplasm and inhibition of the CaN/NFAT signalling pathway. Consequently, the activation and proliferation of CD4$^+$ T lymphocytes are strongly suppressed, which promotes an anti-inflammatory environment.

Taken together, here we found that telmisartan could inhibit the culture and proliferation of CD4$^+$ T lymphocytes from Xinjiang Kazakh patients with hypertension, as well as the mRNA and protein expression of the Kv1.3 potassium channel, to exert anti-inflammatory effects. This suggests that hypertension is an inflammatory disease and provides experimental support for the anti-inflammatory activity of telmisartan based on cellular and ion channel biology. However, certain limitations exist in our study. In particular, our experiments were performed in vitro, and the number of subjects was insufficient. Therefore, we plan to complete a clinical follow-up or in vivo study to provide evidence for the antihypertensive effects of telmisartan.

Declaration of conflicting interest

Funding

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