Diclofenac inhibits Kv1.3 and Kir2.1 expressions in human macrophages and affects the membrane potential and foam cell formation

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Abstract: Objective To investigate the effect of diclofenac on the expression of Kv1.3 and Kir2.1 channels in human macrophages and the membrane potential and foaming process of the macrophages. Methods The effect of diclofenac on the expression of Kv1.3 and Kir2.1 channels in cultured human monocyte-derived macrophages was investigated using real-time RT-PCR and Western blotting, and its effect on the membrane potential was analyzed with optical mapping of the membrane potential with voltage-sensitive dyes. The ratio of cholesterol ester (CE) in the macrophages following intake of oxidized low-density lipoprotein (OxLDL) was analyzed by an enzymatic fluorometric method. Results The expression of Kv1.3 and Kir2.1 channels in the macrophages were down-regulated by diclofenac (1.5 μmol/L and 15 μmol/L). Compared with those in the control group, Kv1.3 mRNA expression was reduced by over 80% and 90% (P<0.05), and Kir2.1 mRNA by over 20% and 30% (P<0.05), respectively; both their protein expression was reduced by over 10% and 60% with a dose-dependent effect (P<0.05). Diclofenac at the two doses dose-dependently reduced the surface fluorescence intensity of the macrophage, and the membrane potential was decreased by 28% and 54%, respectively (P<0.05). Incubation of the macrophages with 30 mg/L OxLDL for 60 h caused an obvious enlargement of the cell volume and deposition of numerous lipid granules in cytoplasm, resulting also in a CE/TC ratio over 50% (P<0.05). Diclofenac at 1.5 and 15 μmol/L both significantly decreased the CE/TC ratio to (23.6±4.3.34)% and (13.6±2.916)% (P<0.05), respectively, but this effect did not show a dose-response relationship (P>0.05). Conclusion Diclofenac can significantly down-regulate the expression of Kv1.3 and Kir2.1 channels in human macrophages, lower their membrane potential and inhibit the process of foam cell formation.

Key words: ionic channels; membrane potential; macrophages; diclofenac; cell differentiation

Diclofenac is a new type of non-steroidal anti-inflammatory drug (NSAID) frequently used for treatment of painful autoimmune diseases such as rheumatoid arthritis, and has shown strong anti-inflammatory, anti-rheumatic, analgesic and antipyretic activities. A recent study reveals that in addition to the effects of inhibiting cyclooxygenase and blocking the synthesis of inflammatory mediators, diclofenac may also, through the mechanism of a voltage-dependent potassium channel (VDPC) Kv1.3, weaken the immune response elicited by activated macrophages and T lymphocytes4.

Atherosclerosis (AS) is a special condition of chronic inflammatory fibrous hyperplasia. Macrophages give rise to foam cells following excessive oxidative lipid intake mediated by such scavenger receptors as SR-A and CD36, which serves as a key link in the development of AS plaques35. Inhibition of the differentiation of the macrophages into foam cells is considered an important strategy for anti-AS drug screening and AS prevention16. Studies have shown that Kv1.3 and the inward rectifier potassium channel (Kir) Kir2.1 play a key role in regulating the foaming process of human peripheral blood monocyte-derived macrophages36. These two potassium channels are expected to become new molecular targets in AS prevention and control.

So far no study has been reported to examine the effect of diclofenac on foam cell differentiation of the macrophages and on the immune-mediated injury of the arterial wall in light of the expression and function of Kv1.3 and Kir2.1 channels. In this study, we observed the effect of diclofenac on the expression of Kv1.3 and Kir2.1 channels in human monocyte-derived macrophages.
and examined the changes in the membrane potential and the composition ratio of cholesterol ester (CE) in the macrophages in response to diclofenac treatment.

MATERIALS AND METHODS

Materials

Diclofenac was purchased from Sigma. Low-density lipoprotein (LDL) was provided by Peking Union Medical College, China. Di-4-ANEPPS was the product of Molecular Probes. Rabbit anti-human Kv1.3 and Kir2.1 channel polyclonal antibodies were purchased from Alomone Labs, Inc., and malondialdehyde assay kit was supplied by the Institute of Biomedical Engineering, the Chinese Academy of Medical Sciences. Cholesterol kit was purchased from Cayman Chemical, and the BCA protein quantification kit was from Shenni Pioneer Biotechnology Co., Ltd. RNAiso Plus kits was purchased from Shanghai Flytech Biotechnology Co., Ltd., and RevertAid™ First Strand cDNA Synthesis Kits and Realtime PCR Master Mix were from Fermentas. UnBlot™ gel chemiluminescent detection kit was manufactured by Pierce Company. IQ5.0 real-time quantitative PCR, Protein Electrophoresis Apparatus and protein wet transfer instrument were manufactured by Biorad, and the electrophoresis apparatus by Beijing Liuyi Instrument Factory. The primers were synthesized by Beijing Sunbiotech Co. Ltd.

Preparation of OxLDL

LDL (1.5 g/L) was added in PBS (pH 7.2) containing 10 μmol/L CuSO4, dialyzed for 24 h at 37 °C, and then added in PBS containing 0.2 mmol/L EDTA at 4 °C, followed by a 24-h dialysis to terminate the oxidation. After filtration, the samples were stored at 4 °C. Using malondialdehyde kit, the oxidation level of LDL was identified: the malondialdehyde content was 6.24 μmol/g in non-oxidatively modified LDL, while 16.67 μmol/g in OxLDL.

Cell culture

Using cell density gradient centrifugation, the mononuclear cells were separated from the remaining ingredients of the blood from healthy donors after the platelets were collected. The cells differentiated into macrophages after a 5-day culture.

The obtained macrophages were randomly divided into 4 groups, namely the control group cultured in RPMI 1640 medium (Gibico) containing 10% fetal bovine serum, OxLDL group incubated with 30 mg/L (final concentration) OxLDL, D1.5 group incubated with 30 mg/L OxLDL and 1.5 μmol/L diclofenac, and D15 group incubated with 30 mg/L OxLDL and 15 μmol/L diclofenac. The cells were harvested after incubation for 60 h. In all the experiments, trypan blue dye exclusion test showed a cell viability over 95%.

Oil red O staining

The cell smears were prepared, fixed with 4% paraformaldehyde for 10 min, rinsed with 60% isopropyl alcohol, stained with oil red O dye (Sigma Chemical Company) for 10 min, and then rinsed with 60% isopropyl alcohol and deionized water. Using alum hematoxylin, the solution was stained and the cell nuclei were allowed to condense for 20 s, followed finally by sealing with neutral gum.

Intracellular lipid determination

The cells were collected in which 0.5 ml isopropyl alcohol was added and were disrupted with a ultrasonic disrupter (Shanghai Ultrasonic Instrument Factory) for 3 min with a pulse time of 30 s and a standing time of 30 min at 4 °C. Following centrifugation at 800×g for 5 min, the supernatant was equally divided for analysis of total cholesterol (TC) and free cholesterol (FC) contents using the cholesterol kit. The plate was read using TECAN GENios Pro Fluorescence microplate reader with the excitation wavelength of 565 nm and emission wavelength of 590 nm. The residue sample was dissolved in 0.1 ml/L NaOH (0.3 ml) and the protein content was determined using the BCA Protein Assay Kit. CE was defined as the difference between TC and FC (TC minus FC), expressed in units of mg/g cell protein.

RNA extraction and real-time RT-PCR

According to the manufacturer’s instructions, the total RNA was extracted from the cells using RNAiso Plus kit, and cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit. PCR was carried out using Real-time PCR Master Mix, with a total of 40 thermal cycles of 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The melting curve was analyzed after the amplification, and from 55 °C to 95 °C, the values were read once every 0.5 °C, which should result in a single peak. 3% agarose gel electrophoresis was performed for all the PCR products to confirm the specific bands. The primer sequences and fragments of amplified products were listed in Tab.1. The copied number of the target genes was calculated with 2−△△CT method.

Protein extraction and Western blotting

The cells were lysed with 500 μl ice-cold macrophage lysis buffer (containing 1% Nonidet P-40, 10% glycerol, 50 mmol/L HEPES, 150 mmol/L NaCl, 1 mg/L aprotinin, 1 mg/L leupeptin, 86 mg/L lidoacetamide, and 1 mmol/L PMSF, pH7.5). The supernatant was collected after centrifugation at 12000×g for 10 min at 4 °C. After determination of the protein concentration, the samples were stored at −80 °C until analysis. The samples were boiled for 5 min before electrophoresis at 4 °C with 10% SDS-PAGE (100 V, 1.5 h), and 30 V wet method was used for membrane transfer overnight. After
blocking with TBS containing 5% skim milk at room temperature for 90 min, the membrane was incubated with rabbit anti-human Kv1.3 and Kir2.1 channel polyclonal antibodies overnight at 4 °C, and then with 1:500 secondary antibodies at room temperature for 1 h following TBS-T washing for 15 min×4. Using enhanced chemiluminescence detection method, Kv1.3 and Kir2.1 proteins were detected, and the protein expression levels were expressed as the ratio of integrated optical density of the protein to that of β-actin.

Membrane potential measurement

The membrane potential of the macrophages were measured using voltage-sensitive dye membrane potential mapping. Briefly, the cells were seeded in 6-well plates, and immediately before the measurement, the culture medium was discarded and the cells were washed once with extracellular fluid containing (in mmol/L) 140 NaCl, 4 KCl, 1 MgCl2, 2 CaCl2, 5 D-glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH), followed by a 30-s incubation with 200 μL di-4-ANEPPS (1:1000). After removal of the dye, 1 ml extracellular fluid was added and the recording started. CK40-32P inverted phase contrast microscope (Olympus) was used to continuously shoot the films (exposure time of 20 ms with a 1-s interval), and at the same time, the fluorescence intensity changes were recorded using the Image-pro-plus image capture software. In the treatment groups, the corresponding agent(s) were added 3-7 s after the shooting before the recording started. Using LabVIEW 7.1 analysis software, the fluorescence intensity was converted to Excel data for analysis.

Statistical methods

EXCEL software was used to establish a database, and SPSS 13.0 statistical software was used to perform all the statistical analyses of the data. The data were presented as Mean±SE. Before comparison, the homogeneity of variance was tested and comparisons between the groups were carried out using One-way ANOVA. The significance level of all hypothesis testing was set as α=0.05.

RESULTS

Expression of Kv1.3 mRNA and protein

OxLDL (30 mg/L) down-regulated Kv1.3 mRNA expression in the macrophages by over 30% compared with the control group, but this decrement was not statistically significant (P>0.05); meanwhile, OxLDL slightly up-regulated the protein expression of Kv1.3 (P>0.05). Diclofenac at 1.5 and 15 μmol/L both significantly inhibited Kv1.3 expressions in the macrophages (P<0.05), and compared with those in the control group, the mRNA expression was decreased by over 80% and 90%, respectively (Fig.1A) and the protein expression by over 10% and 60%, respectively (Fig.1B and Fig.2), showing a dosedependent effect.

![Figure 1](image-url)

Fig.1 Effect of diclofenac on Kv1.3 expression in human monocyte-derived macrophages (n=5). A: Expression of Kv1.3 mRNA (*P=0.097 vs control group, **P=0.05 vs control and OxLDL group); B: Expression of Kv1.3 protein (*P=0.148 vs control group, **P=0.05 between groups). D1.5: 1.5 μmol/L diclofenac group; D15: 15 μmol/L diclofenac group.
Expression of Kir2.1 mRNA and protein

Both Kir2.1 mRNA and protein expressions in the macrophages were increased by 30 mg/L OxLDL, and compared with those in the control group, the expressions of mRNA and protein were up-regulated by more than 1.2 folds (Fig.2 and Fig.3), but only the change in the protein expression showed a statistical difference \((P<0.001)\).

Diclofenac at 1.5 and 15 \(\mu\)mol/L both inhibited Kir2.1 expression in the macrophages. Compared with those in the control group, the mRNA expression of Kir2.1 was decreased by more than 20% and 30% (Fig.3A), and the protein expression by more than 10% and 60%, respectively (Fig.2 and Fig.3B), but only the protein expression of Kir2.1 showed a statistically significant change \((P<0.05)\) with an obvious dose-response relationship.

Membrane potential changes of the macrophages

Diclofenac at 1.5 and 15 \(\mu\)mol/L significantly reduced the surface fluorescence intensity of the macrophage in a dose-dependent manner. Compared with that in the control group, the membrane potential of diclofenac-treated cells was decreased by 28% and 54%, respectively \((P<0.05,\) Fig.4).
CE ratio in the macrophages

After incubation with 30 mg/L OxLDL for 60 h, the cell volume of the macrophages increased significantly with deposition of numerous red lipid particles in the cytoplasm (Fig.5). The CE/TC ratio reached more than 50% in the incubated cells, consistent with the cytologic definition of foam cells. Diclofenac at 1.5 and 15 μmol/L significantly reduced the CE ratio in the macrophages with OxLDL intake by \((23.624 \pm 3.34)\%\) and \((13.601 \pm 2.916)\%\), respectively \((P<0.05)\), but the reductions did not show a dose-response relationship \((P>0.05\), Fig.6). 

![Fig.5 Oil red O staining of cultured macrophages (×100).](image)

![Fig.6 Ratio of cellular cholesterol ester in the macrophages (n=5). *P<0.05 vs control group, **P<0.05 vs OxLDL group.](image)

**DISCUSSION**

Kv1.3 plays a key role in the proliferation and activation of T lymphocytes and macrophages. Macrophages are professional antigen-presenting cells actively involved in T cell activation and produce a variety of inflammatory and immunomodulatory substances to modulate the immune response\(^6\). In recent years, Kv1.3 has been identified as a molecular target for selective pharmacological inhibition of the effector memory T cells without affecting the naïve T cells and central memory T cells\(^9\). Patients with autoimmune diseases have an increased number of effector memory T cells characterized by a high expression of Kv1.3, and the antagonist of Kv1.3, in theory, can potentially improve these autoimmune disorders\(^1\).

Diclofenac possesses anti-inflammatory, anti-rheumatic, analgesic and antipyretic activities, mainly through the inhibition of cyclooxygenase and the prevention of arachidonic acid transformation into prostaglandins. A recent study by Villalonga et al\(^1\) found that diclofenac down-regulated Kv1.3 expression in activated macrophages and T lymphocytes, suppressed lipopolysaccharide (LPS)-induced activation and inhibited the synthesis of inducible nitric oxide synthase (iNOS) in Raw 264.7 macrophages; diclofenac also blocked LPS-induced macrophage migration and interleukin-2 production in Jurkat T lymphocytes. These effects could be mimicked by margatoxin, the specific inhibitor of Kv1.3. These findings suggest that diclofenac very likely impairs the immune response through a mechanism involving Kv1.3 to help ameliorate autoimmune diseases.

AS is essentially a condition of chronic inflammatory fibrous hyperplasia characterized by vascular homeostasis destruction, cytokine network dysregulation and cell biological behavior disorders\(^10\). Persistent immunopathological damage within the arterial wall is the most important mechanism responsible for its occurrence and development\(^11\). In this pathophysiological process, the most critical factors are OxLDL, activated T lymphocytes and monocytes-macrophages\(^14\). Excessive OxLDL intake by the macrophages without a feedback inhibition, mediated by such specific receptor as scavenger receptor class A (SR-A) and CD36, ultimately causes the macrophages to differentiate into foam cells\(^15\). T-lymphocytes (2/3 of the CD4\(^+\) cells and 1/3 of the CD8\(^+\) cells) adhering to the endothelial cells are also activated at the same time, thus promoting the antigen presentation by the macrophages and fibroblasts through CD40 ligation and CD40 signaling pathway\(^10,17-18\). This interaction leads to the synthesis and release of a number of proinflammatory cytokines, and results in persistent damage of the arterial wall.

Our preliminary study\(^16\) confirmed that both Kv1.3 and Kir2.1 channels were expressed in human monocyte-derived macrophages and during their differentiation into foam cells. The specific blockers of Kv1.3 and Kir2.1, margatoxin and BaCl\(_2\), respectively, can significantly reduce the CE content in the macrophages to inhibit foam cell formation, suggesting the key roles of Kv1.3 and Kir2.1 channels in regulating the differentiation of the human macrophage-derived foam cells. But so far it remains unclear whether diclofenac affects foam cell formation by regulating the expression and function of Kv1.3 and Kir2.1.

In this study, we investigated the effect of diclofenac on the expressions of Kv1.3 and Kir2.1 channels in human peripheral monocyte-derived macrophages from healthy donors. The results showed that diclofenac inhibited the expression of Kv1.3 and Kir2.1 at both the mRNA and protein levels in the macrophages. At 1.5 and 15 μmol/L, diclofenac caused reductions of Kv1.3 mRNA expression by over 80% and 90% \((P<0.05)\) and the protein expression by over 10% and 60% \((P<0.05)\), resulting also in decreased Kir2.1 mRNA expression by over 20% and 30% \((P<0.05)\) and protein expression by over 10% and 60% \((P<0.05)\), respectively. An obvious dose-dependent effect was noted for diclofenac \((P<0.05)\).
in inhibiting the expression of Kv1.3 and Kir2.1 channels.

The regulation of activation and expression of plasma membrane ion channels is one of the mechanisms for the immune cells to regulate Ca\(^{2+}\) signaling pathway that is essential for their proliferation and differentiation. It has been clarified that voltage-gated K\(^{+}\) channels, inward rectifier K\(^{+}\) channels and Ca\(^{2+}\)-activated K\(^{+}\) channels play major roles in controlling the membrane potential of white blood cells and professional antigen-presenting cells (such as monocytes, macrophages and dendritic cells)\,\(^{[15-20]}\). Using voltage-sensitive dye membrane potential mapping, we found that diclofenac significantly reduced the surface fluorescence intensity of macrophages in a dose-dependent manner. This result shows that diclofenac may also act as a K\(^{+}\) channel blocker and produce a pharmacological effect on the target cells. Thus, diclofenac may, by affecting the Ca\(^{2+}\) influx, lower the membrane potential by down-regulating the expression of Kv1.3 and Kir2.1 channels, and regulate the proliferation, activation, and inflammatory response of the macrophages and the production of immunologically active substances to regulate the immune response.

To further demonstrate the effect of diclofenac on the differentiation of human macrophage-derived foam cells, we observed its effect on the intracellular CE ratio. We found that incubation of the macrophages with 30 mg/L OxLDL for 60 h resulted in an intracellular CE/TC ratio of more than 50\%, indicating that the macrophages had already differentiated into foam cells; diclofenac (15 \(\mu\)mol/L) significantly reduced the CE ratio in the macrophages following OxLDL intake (\(P<0.05\)), thereby inhibiting the formation of foam cells.

Based on the findings in this present study, we conclude that diclofenac, in addition to its classic cyclooxygenase-inhibiting activity, acts also as a K\(^{+}\) channel blocking agent by down-regulating the expression of Kv1.3 and Kir2.1 channels to control the proliferation, activation, and foaming process of macrophages to suppress the immune response and mitigate the damage of vascular wall inflammation in AS, thus producing protective effects against AS. Nevertheless, further study is still needed to clarify the impact of diclofenac on the electrophysiological characteristics of Kv1.3 and Kir2.1 channels and the molecular/signal transduction mechanism involved in its regulation of macrophage proliferation and activation.

References


双氯芬酸对人巨噬细胞钾通道Kv1.3、Kir2.1表达的抑制作用及其对膜电位和泡沫细胞形成的影响

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摘要: 目的 分研究双氯芬酸对人巨噬细胞电压依赖性钾通道Kv1.3、内向整流钾通道Kir2.1表达的影响及意义。方法 以健康人外周血单核细胞源性巨噬细胞为对象, 采用Real-time RT-PCR及Western blot技术研究双氯芬酸对Kv1.3和Kir2.1表达的影响; 电压敏感染料膜电位标测技术分析膜电位的变化, 并用酶荧光化学法检测摄取氧化修饰低密度脂蛋白(OxLDL)的巨噬细胞内胆固醇酯(CE)的构成比率。结果 双氯芬酸(1.5和15 μmol/L)抑制巨噬细胞Kv1.3和Kir2.1的表达。同对照组相比,Kv1.3mRNA下降分别超过80%和90%(P<0.05), Kir2.1mRNA下降分别超过20%和30%(P>0.05); 两种钾通道蛋白水平的下降均分别超过10%和60%, 且存在明显的剂量依赖性(P<0.05)。同时, 双氯芬酸可剂量依赖性减弱巨噬细胞表面的荧光强度, 使膜电位分别下降约28%和54%(P<0.05)。巨噬细胞同30 mg/L OxLDL孵育60 h后, 细胞体积明显增大, 且有许多红色的脂质颗粒沉积于细胞质内, CE/TC的百分比超过50%。1.5和15 μmol/L双氯芬酸分别使摄取OxLDL的巨噬细胞内CE的百分比显著减少到[23.62±3.34]%和[13.60±2.91]% [P<0.05]。结论 双氯芬酸显著下调人巨噬细胞Kv1.3和Kir2.1的表达, 降低细胞膜电位, 并抑制泡沫细胞形成。

关键词: 离子通道; 膜电位; 巨噬细胞; 双氯酚酸; 细胞分化

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