A new ATP-sensitive potassium channel opener protects endothelial function in cultured aortic endothelial cells

Hai Wang, Chaoliang Long, Zhibian Duan, Cuige Shi, Guodong Jia, Yingli Zhang

Abstract

Objective: Endothelial dysfunction is an early risk factor for cardiovascular disease and hypertension. Mechanisms that participate in endothelial dysfunction include reduced nitric oxide (NO) generation and increased endothelin-1 (ET-1) generation. Endothelial ATP-sensitive potassium (KATP) channels are responsible for maintaining the resting potential of endothelial cells and modulating the release of vasoactive compounds. We hypothesized that activation of endothelial KATP channels might result in the protection against endothelial dysfunction.

Methods: Using cultured bovine or rat aortic endothelial cells, we examined the effects of a new KATP channels opener, iptakalim, on the secretion of vasoactive substances. We also investigated its effects on the expression of adhesion molecules in metabolically disturbed cultured endothelial cells.

Results: In cultured aortic endothelial cells, iptakalim caused a concentration-dependent inhibition of ET-1 release and synthesis that correlated with reduced levels of mRNA for ET-1 and endothelin-converting enzyme. These effects of iptakalim were significantly inhibited by pretreatment with glibenclamide (a KATP channel blocker) for 1 h. Similarly, iptakalim enhanced the release of NO in a concentration-dependent manner and increased basal levels of free intracellular calcium. Iptakalim at the concentrations of 100 and 1000 μM increased the activities of NO synthase (NOS) significantly. After the activity of NOS was blocked by L-ω-nitro-arginine methyl ester (L-NAME), the inhibition of iptakalim on ET-1 release was abolished. In endothelial cell models of metabolic disturbance induced by low-density lipoprotein, homocysteine, or hyperglycemia, treatment with iptakalim could inhibit the overexpression of monocyte chemoattractant protein-1 (MCP-1), Intercellular adhesive molecule-1 (ICAM-1), and vascular cell adhesive molecule-1 (VCAM-1) mRNA.

Conclusion: Iptakalim is a promising drug that could protect against endothelial dysfunction through activating KATP channels in endothelial cells.

Keywords: ATP-sensitive potassium channel opener; Iptakalim; Endothelial function; Endothelium

This article is referred to in the Editorial by T. Minamino and M. Hori (pages 448–449) in this issue.

1. Introduction

Dysfunction of the endothelium is present in various forms of cardiovascular disease such as hypertension, coronary artery disease, chronic heart failure, and others [1]. Various degrees and forms of endothelial dysfunction exist, including reduced release of NO and prostacyclin and increased generation of ET-1 [2,3]. In hypertension, endothelial dysfunction is a reliable prognostic indicator of cardiovascular events [1]. Therefore, correction of endothelial dysfunction may be a very attractive means to favorably affect the development of atherosclerosis and cardiovascular events in hypertensive patients.

ATP-sensitive potassium channels (KATP channels) are present in endothelial cells in the vascular system [4,5]. Functionally, the endothelial KATP channels are responsible for maintaining the resting potential of endothelial cells and modulating the release of vasoactive compounds [4]. However, it is not clear whether the activation of endothelial KATP channels plays an important role in the process of endothelial dysfunction. Iptakalim, a novel compound of the potassium channel
opener class, is a promising antihypertensive drug undergoing Phase II clinical trials [6]. By opening the K\(\text{ATP}\) channels in vascular smooth muscle cells, iptakalim induces membrane hyperpolarization, relaxing the vessels and reducing blood pressure. We previously reported that iptakalim antagonizes the vascular contraction evoked by ET-1 in isolated rat aorta ring. In vitro, iptakalim reverses the increase in plasma levels of ET-1 in hypertensive rats [7,8]. These data imply that iptakalim affects the endothelin system. Since increased ET-1 release has been implicated in the vascular remodeling and endothelial dysfunction observed in the hypertensive models, opening the endothelial K\(\text{ATP}\) channels might have protective effects on endothelial functions. In the present study, we found that iptakalim inhibited the release and synthesis of ET-1 in cultured aortic endothelial cells, an effect dependent on its action on K\(\text{ATP}\) channels. In addition, iptakalim modulated endothelial function by increasing NO release, NOS activities, and the basal concentration of free intracellular calcium. In various endothelial cell models of metabolic disturbance induced by oxidatively modified low-density lipoprotein (ox-LDL), homocysteine (Hcy), or hyperglycemia (HG), we first confirmed the protective role of iptakalim on endothelium.

2. Materials and methods

2.1. Chemical compounds

Iptakalim was synthesized by the Thadweik Academy of Medicine (Beijing, China). All other chemicals and materials were obtained from local commercial sources.

2.2. Isolation and culture of endothelial cells

Bovine aortic endothelial cells (BAECs) were isolated from a segment of bovine ascending aorta (bovine aorta were obtained from a local slaughterhouse in Beijing). The minced aortic tissue was incubated in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks solution containing 1% collagenase for 15 min at 37 °C in a sterile tube. The collagenase solution was then drained from the tissue, centrifuged at 1000 rpm for 10 min, and the collected cells were transferred to T25 flasks in DMEM medium containing 10% fetal calf serum, 100 ng/ml streptomycin, and 100 IU/ml penicillin.

Rat aortic endothelial cells (RAECs) were isolated from Wistar rat aorta. The aorta was dissected out, sliced open and rinsed with PBS. It was cut into pieces and placed the inner surface down in T25 flasks with 3 ml of M199 medium containing 20% fetal calf serum without growth supplement. On the third day, the aorta pieces were rinsed and the collected cells were cultured in M199 medium containing 10% fetal calf serum, 100 ng/ml streptomycin, and 100 IU/ml penicillin. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

BAECs and RAECs were grown to confluence at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\). A cobblestone morphology and positive immunofluorescence with antibodies against von Willebrand factor and/or Factor VIII-related antigen confirmed the cells as endothelial.

Table 1

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2.3. Measurement of Ca\textsuperscript{2+}

The intracellular calcium concentration, [Ca\textsuperscript{2+}]\textsubscript{i}, was measured in single cells as described previously [9]. Briefly, the cells were loaded with Fluo-2 for 45 min at 37 °C in D-Hanks. Fluo-2 was prepared fresh daily; it was dissolved in dimethylsulfoxide (DMSO) and diluted to 5 μM (final concentration in PBS containing 0.5% DMSO). The cells were then washed three times and Fluo-2 fluorescence was monitored photometrically at an emission wavelength of 510 nm in a single cell mounted on an inverted microscope illuminate alternatively with 340 and 380 nm light using an intracellular imaging system. Photon counting was performed by a photomultiplier tube positioned so that the field of interest could be restricted by thresholding shutters. Data acquisition was accomplished with software that controlled a light chopper to alternate excitation wavelengths during rationing operations. [Ca\textsuperscript{2+}]\textsubscript{i} was calculated in real time from a standard curve established for the same settings using buffers of known Ca\textsuperscript{2+} concentrations.

2.4. Measurement of NO, NOS and ET-1

To assess the effect of iptakalim on NO and ET-1 release, the cultured BAECs were seeded on 12-well culture plates and left to grow to full confluence (2×10\textsuperscript{5} cells/well). The cells were treated for 24 h with iptakalim (0.1–1000 μM final concentration) and the medium was collected for assay. Because of its instability in physiological solutions, most of the NO is rapidly converted to nitrite (NO\textsubscript{2}) and further to nitrate (NO\textsubscript{3}). Therefore, levels of NO\textsubscript{2}/NO\textsubscript{3} in the culture medium were measured with the NO Detection Kit (Eastern Asia Radioimmunity Research Institute, Beijing, China) according to the manufacturer’s instruction. Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured with the Griess reagent. The absorbance was determined at 540 nm with a spectrophotometer. NOS was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions provided by the manufacturer.

Fig. 1. A. Iptakalim inhibits ET-1 synthesis and ET-1/ECE mRNA expression in endothelial cells. B. Glibenclamide (Gli) inhibits the reduction of ET-1 synthesis and the down-regulation of ET-1/ECE induced in endothelial cells by 10 μM iptakalim (Ipt). The cells were preincubated with Gli for 1 h before incubation with iptakalim. Glibenclamide had no effects on ET-1 synthesis and ET-1/ECE mRNA expression at concentrations of 1–100 μM. Vertical bars denote means±SE. n=6. *P<0.05, **P<0.01 vs control; #P<0.05, ##P<0.01 vs iptakalim (10 μM).
To investigate the effects of NOS inhibitor on endothelin release when the cells were treated with iptakalim, the cells were treated for 1 h with L-NAME 100 μM firstly, then treated for 24 h with iptakalim (0.1–1000 μM final concentration) and the medium was collected for assay. ET-1 was measured using a commercial radioimmunoassay kit (Endothelin RIA Kit, Eastern Asia Radioimmunity Research Institute, Beijing, China) according to the instructions provided by the manufacturer.

2.5. Endothelial cell model of metabolic disturbance

To investigate the protective effect of iptakalim on endothelial cells, endothelial cells were cultured in 6-well plates (3 × 10^5 cells/well) and the cells were incubated for 8, 6 or 16 h in the absence (control incubation) or presence of ox-LDL, Hey, or HG at the concentrations of 100 mg/l, 0.1 mM, and 25 mM, respectively. Total RNA was extracted from endothelial cells and levels of mRNA coding for the adhesion molecules such as monocyte chemoattractant protein-1 (MCP-1), intercellular adhesive molecule-1 (ICAM-1) and vascular cell adhesive molecule-1 (VCAM-1) were measured by RT-PCR.

2.6. RT-PCR

Total RNA (5 μg) extracted from endothelial cells was converted to cDNA using reverse transcriptase with random hexamer priming. PCR was performed using primers (Table 1) selected for ET-1, endothelin-converting enzyme (ECE), MCP-1, ICAM-1 and VCAM-1. β2-Microglubulin and β-actin mRNA were amplified as controls from the same RNA preparation (primers are shown in Table 1). PCR was carried out for 35 cycles of 94 °C for 1 min, 55–60 °C for 30 s, and 72 °C for 1 min. PCR products were analyzed on 2% agarose gels and the intensity of the bands was measured by densitometry. Results were expressed as density of ET-1, ECE, MCP-1, ICAM-1, and VCAM-1 against that of β2-microglubulin or β-actin in the same sample.

2.7. Statistical analysis

The results were expressed as mean±SE. Statistical analysis of the data was performed using the Student’s t test or analysis of variance with the SAS Software Program. Statistical significance was accepted at P<0.05.

3. Results

3.1. Effect of iptakalim on ET-1 release and ET-1/ECE expression in endothelial cells

Incubation with iptakalim caused a concentration-dependent inhibition of ET-1 release from endothelial cells. When endothelial cells were stimulated with iptakalim at concentrations of 1–1000 μM, the levels of ET-1 release significantly decreased (P<0.01, Fig. 1A). ET and ECE mRNA levels also decreased significantly after iptakalim treatment at concentrations of 1–1000 μM (P<0.05–0.01, Fig. 1A). A further experiment showed that glibenclamide suppressed the reduction

Fig. 2. A. Summary of steady-state measurement of the [Ca²⁺]i of endothelial cells in response to cumulative doses of iptakalim (added to bath). Under control conditions, iptakalim at concentrations greater than 10 μM significantly increased the [Ca²⁺]i of endothelial cells. B. Effect of iptakalim on the NO release in endothelial cells. The values of NO had been corrected with blank medium. C. Effect of iptakalim on the NOS activities in endothelial cells. Endothelial cells were treated with iptakalim for 24 h. D. Effect of L-NAME on endothelin release when the endothelial cells treated with iptakalim. Endothelial cells were treated with L-NAME 100 μM for 1 h firstly, then treated with iptakalim for 24 h. The values of NO without and with L-NAME were 4.09±0.14 and 0.71±0.11 μmol/l respectively. Vertical bars denote means±SE. n=6. *P<0.05,**P<0.01 vs control.
in ET-1 release and the down-regulation of ET-1/ECE mRNA induced by 10 μM iptakalim, but had no effects on control cells (Fig. 1B).

3.2. Effect of iptakalim on NO release and [Ca2+]i in endothelial cells

Extensive work has shown that the release of NO from the endothelium is evoked by Ca2+. Treatment with iptakalim produced concentration-dependent NO increases in cultured BAECs. On treatment with increasing concentrations of iptakalim (10–1000 μM) for 24 h, NO levels significantly increased to 2.6±0.5, 2.8±0.6, and 3.5±0.9 μM (control release levels: 2.1±0.5 μM, P<0.05–0.01, Fig. 2A). Under the same experimental conditions, the effects of iptakalim on [Ca2+]i were also observed. When endothelial cells were stimulated with iptakalim at concentrations of 10, 100, and 1000 μM, [Ca2+]i increased to 361.6±86.1, 408.5±98.1, and 497.2±132.4 pM, respectively (control concentration: 331.9±81.1 pM) (Fig. 2B).

The effects of iptakalim on NOS activity were also studied. Iptakalim at the concentrations of 100 and 1000 μM increased the NOS activities significantly (P<0.05–0.01, Fig. 2C).

To further assess whether the increased NO levels by iptakalim influenced the endothelin release, we checked the effects of l-NAME, a NOS inhibitor, on endothelin release when the cells treated with iptakalim. It was showed that after the NOS activity was blocked by l-NAME (100 μM), the inhibition of iptakalim on ET-1 release was abolished (Fig. 2D).

3.3. Effect of iptakalim on expression of adhesion molecules in endothelial cell models of metabolic disturbance

Incubation of RAECs with ox-LDL (100 μg/ml) for 8 h significantly increased the expression of MCP-1, ICAM-1, and VCAM-1 mRNA (P<0.05, compared with control incubation). Pretreatment of RAECs with 0.1–100 μM iptakalim for 18 h significantly decreased the mRNA expression of those adhesion molecules (P<0.05), whereas lower concentrations (1–10 nM) had no effect (Fig. 3A).

Incubation of BAECs with Hcy (0.1 mM) for 6 h significantly increased the expression of MCP-1, ICAM-1, and VCAM-1 mRNA induced in RAECs (A) and BAECs (B, C) by ox-LDL (100 μg/ml), Hcy (0.1 mM), and HG (25 mM glucose) for 8, 6 and 16 h in serum-free medium, 1–1000 μM respectively. MCP-1, ICAM-1 and VCAM-1 cDNA fragments were amplified by specific primer pairs. β-actin and β2-microglobulin (β2-MGB) were amplified as controls from the same RNA preparation. Vertical bars denote mean±SE. n=3–6. *P<0.05, **P<0.01 vs control, #P<0.05, ##P<0.01 vs ox-LDL, Hcy, or HG.
and VCAM-1 mRNA (P<0.05, compared with control incubation). Pretreatment of BAECs with iptakalim (0.1–10 μM) for 20 h significantly decreased the mRNA expression of those adhesion molecules (P<0.05); iptakalim at 0.01 μM had no effect (Fig. 3B).

Incubation of BAECs with glucose (25 mM) for 16 h significantly increased the expression of MCP-1, ICAM-1, and VCAM-1 mRNA (P<0.05, compared with control incubation). Pretreatment of BAECs with iptakalim (0.1–10 μM) for 20 h significantly decreased the expression of MCP-1 mRNA, but not of ICAM-1 and VCAM-1 mRNA; lower concentrations of iptakalim (0.01–0.1 μM) had no effect (Fig. 3C).

4. Discussion

We have evaluated iptakalim, a new type of potassium channel opener, for its protective effects on endothelium using aortic endothelial cell cultures. In vivo experiments have shown the ability of iptakalim to induce outward potassium currents in smooth muscle cells and relax small arteries [6]. In animal models, iptakalim has obvious anti-hypertensive effects and could reverse hypertensive cardiovascular remodeling [10]. Our study has shown that iptakalim has obvious protective effects on cultured endothelial cells: it increases \([\text{Ca}^{2+}]_i\), promotes NO release, inhibits ET-1 synthesis, and inhibits ET-1 and ECE mRNA expression. In addition to regulating endothelial cell secretion, iptakalim could inhibit the overexpression of mRNA for adhesion molecules in endothelial cell models of metabolic disturbance. Altogether, these data suggest that iptakalim is a promising drug to control blood pressure and to protect endothelial cells.

ET-1 is a potent vasoconstrictor and mitogen/proliferation factor for vascular smooth muscle. As such, it has been implicated in vascular wall remodeling [11]. Endothelial cells are considered the main source of ET-1. The endoproteinase ECE is thought to play a physiological role in endothelin biosynthesis. We previously showed that iptakalim reduces endothelin-induced arterial contraction and decreases endothelin-induced hypertension in rats [12]. Our previous study also showed that the ET-1 concentration in plasma and renal tissue and the ET-1 and ECE mRNA expression in renal tissue increase in hypertensive conditions [12]. Anti-hypertensive therapy with iptakalim inhibits the pathological increase of ET-1 and ECE in renal tissue [7]. In the present study, iptakalim inhibited ET-1 synthesis and ET-1/ECE mRNA expression. Therefore, the protective profile of iptakalim may not only be due to its ability to reduce endothelin release, but may also relate to its effects on ET-1 and ECE mRNA expression.

The vasorelaxation of smooth muscle cells may be achieved by the release of vasodilator such as NO from endothelial cells in response to various stimuli [13]. The release of NO depends on the intercellular concentration of \([\text{Ca}^{2+}]_i\), which increases with hyperpolarization [4]. Thus, \(K_{\text{ATP}}\) channels may play a key role in generating the electrical activity of endothelial cells and have profound effects on endothelial function. In our study, we showed that iptakalim increases \([\text{Ca}^{2+}]_i\) in endothelial cells, a finding that is consistent with a similar observation in isolated coronary capillaries [9]. In addition, we first report here that the \(K_{\text{ATP}}\) channel opener, iptakalim, could promote NO release from endothelial cells. Iptakalim also could increase NOS activity significantly at high concentrations. The mechanism of increased NO production upon iptakalim treatment may be related to increases in \([\text{Ca}^{2+}]_i\) and NOS activity.

The interaction between NO and ET-1 has been well documented. ET-1 acts on specific receptors on the endothelium to increase the release of NO, while NO depresses the production and/or release of ET-1 from endothelial cells [14]. Our data showed that the NOS activity was blocked by l-NAME, a NOS inhibitor, the inhibition of iptakalim on ET-1 release is abolished. It was suggested that the inhibitory effects of iptakalim on ET-1 release may be related to increases in NO levels.

Metabolic disturbance in endothelial cells is regarded as a risk marker for cardiovascular disease, especially in patients with hypertension [15]. Its mechanism involves in endothelial dysfunction [16], which causes overexpression of adhesion molecules. We have used endothelial cell models of metabolic disturbance induced by ox-LDL, Hey, or HG to investigate whether iptakalim could significantly inhibit the overexpression of MCP-1, ICAM-1, and VCAM-1 mRNA. Our data show that iptakalim has an obvious protective effect on endothelial cells.

In summary, this study first discovered the protective effect of a \(K_{\text{ATP}}\) channel opener, iptakalim, on endothelium. As shown before, iptakalim not only reduces the blood pressure indefinitely, but also reverses hypertensive cardiovascular remodeling [10]. Therefore, iptakalim can be effective in the treatment of hypertension by reversing cardiovascular remodeling and protecting endothelial cells and should be considered as a promising antihypertensive drug to be developed for clinical application. Accordingly, as preclinical studies have been completed, this drug is now undergoing clinical trials.

Acknowledgments

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References