European Review for Medical and Pharmacological Sciences 2014; 18: 646-656

Importance of large conductance calcium-activated potassium channels (BKCa) in interleukin-1β-induced adhesion of monocytes to endothelial cells

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Abstract. – OBJECTIVE: The present study investigated the role of the large conductance calcium-activated potassium channels (BKCa) in interleukin-1β (IL-1β) induced inflammation.

METHODS: Human umbilical vein endothelial cells (HUVECs) were isolated and cultured. Endothelial cell membrane potential measurements were accomplished using the fluorescent dye DiBAC4(3). The role of BKCa was assessed using iberiotoxin, a highly selective BKCa inhibitor. Changes in the calcium intracellular calcium were investigated using Fura-2-AM imaging. Fluorescent dyes DCF-AM and DAF-AM were further used in order to measure the formation of reactive oxygen species (ROS) and nitric oxide (NO) synthesis, respectively. Endothelial cell adhesion tests were conducted with BCECF-AM adhesion assay and tritium thymidine uptake using human monocytic cells (U937). Expression of cellular adhesion molecules (ICAM-1, VCAM-1) was determined by flow cytometer.

RESULTS: Interleukin-1β induced a BKCa dependent hyperpolarization of HUVECs. This was followed by an increase in the intracellular calcium concentration. Furthermore, IL-1β significantly increased the synthesis of NO and ROS. The increase of intracellular calcium, radicals and NO resulted in a BKCa dependent adhesion of monocytes to HUVECs. Endothelial cells treated with IL-1β expressed both ICAM-1 and VCAM-1 in significantly higher amounts as when compared to controls. It was further shown that the cellular adhesion molecules ICAM-1 and VCAM-1 were responsible for the BKCa-dependent increase in cellular adhesion. Additionally, inhibition of the NADPH oxidase with DPI led to a significant downregulation of IL-1β-induced expression of ICAM and VCAM, as well as inhibition of eNOS by L-NMMA, and intracellular calcium by BAPTA.

CONCLUSIONS: Activation of the endothelial BKCa plays an important role in the IL-1β-induced monocyte adhesion to endothelial cells.

Key Words:
IL-1 β, HUVECs, BKCa, ICAM, VCAM, Monocyte adhesion, Intracellular calcium, ROS, Atherosclerosis.

Abbreviations

BCECF-AM = 2´,7´-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein, acetoxymethyl ester; ICAM-1 = inter-cellular adhesion molecule 1; VCAM-1 = vascular cell adhesion protein 1; DPI = diphenylene iodonium; eNOS = endothelial nitric oxide synthase; L-NMMA = L-NG-monomethylarginine; BAPTA = 1,2-bis(o-aminophenoxyl)ethane-N,N,N´,N´-tetraacetic acid; RPMI = Roswell Park Memorial Institute; FCS = fetal calf serum; DIBAC4(3) = bis-(1,3 dibutylbarbituric acid)-trimethine oxonol; HBSS = Hanks' balanced salt solution; DCF = 2´,7´-dichlorofluorescin diacetate; Ibtx = Iberiotoxin; DAF = diaminofluorescin; Fura-2-AM = Fura-2-acetoxynethyl ester; PBS = phosphate buffered saline.

Introduction

Arteriosclerosis is a chronic, progressive, inflammatory disease of the arteries in which connective tissue, deposits of cholesterol, fatty acids and calcium build up on the inner layer of large and medium sized arteries[1]. It is the underlying cause of approximately 50% of deaths in westernized countries[2]. The rapid uptake of oxidized LDL-C (low-Density Lipoprotein-Cholesterol) by macrophages in the subendothelial space is considered to be the major underlying[3]. Furthermore, atherosclerotic processes involve many highly interrelated processes including dyslipidemia, platelet aggregation, endothelial dysfunc-

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tion, inflammation, oxidative stress, and activation of vascular smooth muscle cells[4,5]. Endothelial cells play an important role in atherosclerotic changes[6-9]. Endothelial dysfunction leads to increased expression of endothelial cell adhesion molecules such as selectins, VCAM-1, and ICAM-1[10]. These molecules trigger an inflammatory response in the subendothelial matrix by secreting pro-inflammatory substances including cytokines, interleukins, and tumor necrosis factor-alpha (TNF)[11,12]. Through these signals, endothelial cells are stimulated to overexpress adhesion molecules and LDL receptors. These processes eventually become a vicious cycle in which more and more white blood cells and oxidized LDL-C accumulate in the subendothelial space. The monocytes uptake the oxLDL by endocytosis via scavenger receptors and become foam cells[13,14]. These cells are responsible for triggering inflammation that leads to the proliferation of vascular smooth muscle cells[15].

The synthesis and release of endothelial mediators and, thus, the control of endothelial function essentially depends on the intracellular calcium concentration and hence the membrane potential. The membrane potential of endothelial cells is determined extensively by potassium (K^+) and chloride (Cl^-) channels. Hyperpolarization of the cell membrane is achieved by an increased opening probability of the calcium-activated potassium channels. Large conductivity and the simultaneously existing high selectivity for K^+ ions of large conductivity calcium-activated potassium channels (BKCa) cause strong potassium efflux from the cell, resulting in hyperpolarization of the cell membrane[7,16]. On the other side, through the depolarization of the cell membrane, which is caused by a reduced potassium channel opening, an increased calcium influx and, thus, a vasoconstriction occurs. The BKCa is on one side a voltage-dependent channel, and on the other side depends on the intracellular Ca^{2+} concentration[17]. Thereby, the ion channel makes an impact on the intracellular calcium homeostasis and strengthens even in its activity.

Interleukins are members of cytokine family that are mainly responsible for white blood cell communication. IL-1β is one of the major interleukin, found in very low concentrations under physiological conditions; however, markedly expressed in pathological conditions[7]. It is synthesized primarily by monocytes, but also by fibroblasts, osteoclasts, lymphocytes and NK cells[18,19]. Two types of receptors have been identified for IL-1β: Interleukin receptor type 1 (IL-1r-1,) found mainly on T lymphocytes and mesenchymal cells; Interleukin receptor type 2 (IL-1r-2) found on B cells, macrophages, and granulocytes[20-22]. A systemic injection of small amounts of IL-1β leads in humans and animals to fever, hypotension, tachycardia, lactic acidosis or activation of the hypothalamic-adrenal axis[7]. It was further shown to play a role in various pathological processes such as in systemic inflammatory response syndrome (SIRS), rheumatoid arthritis, Alzheimer's disease,and acute myeloid leukemia (AML)[23,25]. IL-1r is found on various tissues and cells involved in the development of atherosclerosis, such as vascular smooth muscle cells and macrophages. Macrophages are an extremely potent source of IL-1β, especially after the activation of foam cells[26,27]. Moreover, shear stress-induced expression of IL-1β has been shown in endothelial cells and vascular smooth muscle[16,28]. IL-1β also stimulates the proliferation of vascular smooth muscle cells and enhance the effects of growth factors, in turn cause an increased secretion of IL-1β and IL-1r[8,29,30]. Furthermore; IL-1β leads to increased permeability of the endothelium for neutrophils and increased expression of adhesion molecules such as I-CAM 1, and V-CAM 1, which are essential for the adhesion of monocytes[8,10]

Accordingly, the aim of the present study was to investigate the impact of the large conductance calcium-activated potassium channels in interleukin-1β induced inflammation.

Materials and Methods

Isolation, Cultivation and Identification of Human Umbilical Vein Endothelial Cells (HUVECs)

Isolation and cultivation of HUVECs were performed according to methods described by Jaffe et al[31]. Endothelial cells were isolated from human umbilical cord veins by collagenase digestion procedure[32]. After the endothelial cells had been centrifuged, they were seeded in 5 ml of endothelial basal medium (EBM) and the following substances were added to the primary cell medium: 0.4% endothelial cell growth supplement/heparin (ECGS/H); epidermal growth factor, 0.1 ng/ml; hydrocortisone 1 µg/ml; basic fibroblast growth factor 1 ng/ml; 1% penicillin/streptomycin and 20% fetal calf serum (FCS, PAA, Linz, Austria). The cultures

were later incubated at 37°C with 5% CO_2 concentration. The EBM was changed every 2-3 days. Cell identification was accomplished using a light microscope and immunofluorescence staining using antibodies against von Willebrand factor (VWF) (Dakopatts, Hamburg, Germany).

Cultivation of U-937 Monocytes

Human monocytic cells (U937) were used in the endothelial cell adhesion tests. Primary culture mediums for U937 were prepared in 75 cm^2 flasks with the addition of 15 ml RPMI, 10% FCS and 1% penicillin/streptomycin solution. The medium was changed every 2-3 days until a confluent cell layer was formed. After the confluent layer formed, cells were centrifuged and divided in 4 flasks, followed by addition of 15 ml of RPMI.

Measurement of the Membrane Potential

Endothelial membrane potential measurements were accomplished using the fluorescent dye DiBAC4(3) Molecular Probes, Leiden, Netherlands). HUVECs were seeded at 24-well plates and incubated with 0.5 µmol/L DiBAC4(3) for 15 minutes at 37°C in the dark. The first group was treated only with IL-1β (10 ng/ml). Cells in the second group were treated with the selective BKCa-blocker, iberiotoxin (100 nmol/l) and IL-1β (10 ng/ml). In the third group, cells were incubated only with iberiotoxin (100 nmol/l). Finally, 1 ml of HBSS serum (PAA, Linz, Austria) was added to the unstimulated control group. Changes in membrane potential were measured using Genios microplate-plate reader (Tecan, Vienna, Austria). After 2 minutes of lead-time, fluorescence was excited at 475 nm, and emission was read at 535 nm. Membrane potentials were measured for over a period of 12 minutes at intervals of 1 minute.

Measurement of Reactive Oxygen Species (ROS)

ROS were measured after a 30-minute incubation period with the fluorescent dye DCF (Calbiochem, La Jolla, CA, USA). The investigations were done after 15 minutes of initial stimulation. The following groups of endothelial cells were studied: Control (1 ml HBSS); IL-1β (10 ng/ml); IbTX (100 nmol/l); IL-1β (10 ng/ml) + IbTX (100 nmol/l); DPI (Sigma, Deisenhofen, Germany), an inhibitor of the mitochondrial NADPH-ubiquinone-oxidoreductase (5 µmol/l); IL-1β (10 ng/ml) + DPI (5 µmol/l); L-NMMA (Sigma, Deisenhofen, Germany), a non-specific inhibitor of all NO-synthesis (300 µmol/l); IL-1β (10 ng/ml) + L-NMMA (300 µmol/l). Following a lead-time of 2 minutes, measurement was conducted over a period of 60 minutes at intervals of respectively 5 minutes using Genios plate reader. The fluorescent dye was excited at 475 nm, and emission was read at 535 nm.

Measurement of the NO Synthesis

The endothelial cells were incubated with fluorescent dye DAF for 60 minutes in order to determine nitric oxide synthesis. Study groups were designed similar to the membrane potential measurements: Control (1 ml HBSS); IL-1β (10 ng/ml); IL-1β (10 ng/ml) + IbTX (100 nmol/l); IbTX (100 nmol/l). After cells were distributed on 24-Well Plates, NO synthesis was measured in the Genios plate reader stimulating the fluorescence at 490 nm wavelength and measuring the emission at 535 nm, at intervals of 5 minutes, over a period of 60 minutes.

Fura-2 Imaging for the Measurement of the Intracellular Calcium

Changes in the calcium release of vascular smooth muscle cells was investigated using Fura-2-AM (Molecular Probes, Leiden, The Netherlands) imaging. HUVECs were seeded on gelatin precoated-glass slides and later cultivated for 3 days in RPMI culture medium at 37°C, until a confluent cell monolayer formed. 50 µg Fura-2 was dissolved in 160 µl DMSO (Dimethyl sulfoxide) (Sigma, Deisenhofen, Germany) and respectively 10 µl of the solution was mixed with 1 ml of cell culture medium. After the glass slides were incubated for 60 minutes, solutions were removed from the slides. 1.33 µmol/L $CaCl_2$ and 1 ml Hanks Balanced Salt Solution (PAA Laboratories, Pasching, Austria) was used as cell medium in the chamber. The following groups were studied: Control (1 ml HBSS); IL-1β (10 ng/ml); IL-1β (10 ng/ml) + IbTX (100 nmol/l); IbTX (100 nmol/l); DPI (5 µmol/l); IL-1β (10 ng/ml) + DPI (5 µmol/l); L-NMMA (300 µmol/l); IL-1β (10 ng/ml) + L-NMMA (300 µmol/l).

The measurements were performed under a fluorescent microscope. Fura-2 was excited at 340 nm and 380 nm of light. The emitted light was measured at around 510 nm. After a total test time of 35 minutes, the measurement was terminated and the photo-bleach technique was performed in order to determine the intensity of the

background[33]. Data analysis was conducted with TILL Photonics imaging system (TILL Photonics, Martinsried, Germany).

Adhesion Test Using Tritium Thymidine Uptake

The adhesion behavior of U-937 cells on HUVECs were first measured using the tritium thymidine uptake test, which is required in the DNA-synthesis. Tritium [^{3}H] is the heaviest hydrogen isotope and decays under emission of weak beta radiation. The electrons emitted by tritium were quantitatively measured indirectly via a beta counter. HUVECs were centrifuged and resuspended in 80 ml RPMI and later incubated with [^{3}H]-thymidine (2 µl/10 ml RPMI) for 18 hours. The cells were finally seeded in 24-Well plates covered with endothelial basal medium. After confluent growth, the cells were divided into the following groups, and incubated with the respective stimulants: Control (1 ml HBSS); IL-1β (10 ng/ml); iberiotoxin (100 nmol/l); IL-1β (10 ng/ml) + iberiotoxin (100 nmol/l).

The radioactively labeled U-937 monocytes were centrifuged and resuspended with RPMI (2.5 ml RPMI per 24-Well plate). 100 µl of the suspension was added to the HUVECs. After 6 hours, the solution and non-adherent monocytes were removed by washing twice with 250 µl of PBS. The remainder was lysed with 250 µl of NaOH [0.1 M] and later transferred into scintigraphy vials and the light emission was subsequently measured in the beta-counter (Canberra Packard, Dreieich, Germany).

BCECF Adhesion Test

A second adhesion test was conducted with BCECF (Molecular Probes, Eugene, OR, USA) using same monocytic cell lines (U-937). HUVECs were initially seeded on 24-Well Plates (0.25 million cells/well) and stimulated for 4 hours in the incubator. The following groups were studied: Control (1 ml of HBSS); IL-1β (10 ng/ml); IL-1β (10 ng/ml) + IBTX (100 nmol/l); IBTX (100 nmol/l); DPI (5 mol/l); IL-1β (10 ng/ml) + DPI (5 mol/l); L-NMMA (300 micromol); L-NMMA (300 micromol) + IL-1β (10 ng/ml); BAPTA (10 micromol/L) + IL-1β; BAPTA (10 micromol/L). All measurement groups were prepared three times. Endothelial cells were loaded with I-CAM and V-CAM in the first two studies, respectively. The third study consisted of unstimulated HUVECs. Then, the fluorescent dye BCECF (50 µl) was mixed with 20 µl DMSO, which served as a carrier substance for the cellular uptake, frozen for 15 minutes. 15 ml of RPMI was added.

After confluent growth, U-937 monocytes were centrifuged, resuspended and diluted in 5 ml RPMI, 5% FCS and 1% PS, until 6.5 million of U-937 cells were reached in 5 ml RPMI. For each one of the 8 stimulation groups, 5 ml of the U-937 solution and respectively 1.5 ml of the solution from RPMI and BCECF was transferred and incubated for 30 minutes. After incubation and following centrifugation, the supernatant was aspirated, rinsed with 5 ml PBS, and then treated with 6.5 ml RPMS. 250 µl of the U-937 solution added to each well and incubation of the cell suspension for 1 hour in order to remove non-adhered U-937 cells. The cells were then lysed by adding 500 µl/well NaOH [0.1 M] and loaded with BCECF fluorescent. The fluorescence measurement was performed in the Genios plate reader at 340 nm of wavelength.

Fluorescence Measurement of the Adhesion Molecules ICAM, and V-CAM by Flow Cytometry

HUVECs were firstly seeded in 0.2% gelatin-coated 25 cm² plastic petri dishes. After confluent growth of cells w achieved, the nutrient medium was aspirated and HUVECs were incubated for 4 hours at 37°C in the incubator. The following groups were studied: Control (1 ml of HBSS); IL-1β (10 ng/ml); IL-1β (10 ng/ml) + a selective BKCa blocker, IBTX (100 nmol/l); IBTX (100 nmol/l); An inhibitor of mitochondrial NADPH-ubiquinone oxidoreductase, Diphenyleneidonium chlorides "DPI", (5 mol/l); IL-1β (10 ng/ml) + DPI (5 mol/l); L-NMMA (300 micromol/l); L-NMMA (300 micromol/l) + IL-1β (10 ng/ml); (10 micromol/L) + IL-1β; BAPTA (10 micromol/L). All measurement groups were prepared three times. Cells were loaded with I-CAM and V-CAM in the first two studies, respectively. Furthermore, the third study consisted of the unstimulated HUVECs.

Cells were washed with HBSS, dissolved in trypsin-EDTA solution, centrifuged, and later resuspended within the endothelial cell medium. The endothelial cell concentration was assessed using the Neubauer counting chamber. A portion of the cell suspension (100 l/well) was transferred into the endothelial cell medium, which was prepared for the seeding. The sowing was then examined under the light microscope for its homogeneity. The incubation was terminated by aspiration of cells and rinsing twice with HBSS, dissolving in 4 ml of

trypsin-EDTA solution. The detachment of cells was halted by adding 1 ml of FCS, and cells were later transferred to 10 ml tubes. Thereafter, endothelial cells were centrifuged, the supernatant discarded and cells were resuspended in each case with 1 ml of RPMI. The number of cells in each solution was counted using the Neubauer chamber and then diluted with RPMI in order to achieve a density of 2 million cells per ml.

100 UL of each solution was transferred in 96-well round-bottom plate and centrifuged. The supernatant was discarded. Non-specific bindings were eliminated with 20 µl immunoglobulin and 5 mu/l antibody was added into the respective wells. The 96-well plate was then placed on ice for 30 minutes. Subsequently the individual wells were washed three times with HBSS buffer solution. The same steps followed by using fluorescent mouse antibody, which binds to I-CAM and V-CAM. The groups were finally transferred into the calibrated flow cytometer.

Statistical Analysis

The data were tested statistically using analysis of variance (ANOVA) and post-hoc Tukey's test and presented as mean ± SD in independent experiments. A value of $p < 0.05$ was considered statistically significant. The statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Impact of IL-1b on the Membrane Potential and the Role of BKCa in IL-1b-induced Potential Changes

IL-1β led to a significant reduction in DiBAC4(3) fluorescence of 74% compared to the control group [IL-1β vs. Control, $p < 0.05$]. Specifically, IL-1β induced hyperpolarization of the human umbilical vein endothelial cells. In the following experiment, a selective inhibitor of BKCa, IbTX significantly reduced the hyperpolarizing effect of IL-1β on membrane potential [IL-1β + IbTX vs. IL-1β, $p < 0.05$] (Figure 1). It was reduced by treating cells both with IL-1 β and IbTX to 91% of the control group. These results indicated that IL-1β-induced hyperpolarization of HUVECs depends on the presence of BKCa. Furthermore, no significant difference was shown in the membrane potential for cells treated only with IbTX compared to control group [IbTX vs. Control, $p > 0.05$].

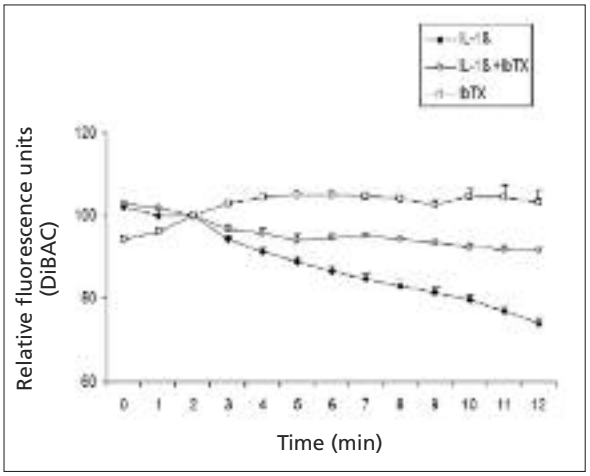

Figure 1. Effect of BKCa on the IL-1ß-induced hyperpolarization of HUVEC.

Effect of IL-1b on Intracellular Calcium and Impact of BKCa and NADPH Oxidase in IL-1b-Induced Calcium Changes

Effects of interleukin-1β on intracellular calcium were analyzed using Fura-2AM fluorescent dye. Stimulation of HUVECs with IL-1β caused a significant increase in the intracellular calcium by 128% compared to the control group after 35 minutes of the initial stimulation [IL-1β vs. Control, $p < 0.05$]. In the next step, co-incubation of IbTX with IL-1β significantly reduced the IL-1 β-induced increase in intracellular calcium from 128% to 107% [IbTX + IL-1β vs. IL-1β, $p < 0.05$]. It was further shown that treating cells with the NADPH oxidase inhibitor, DPI and IL-1β led to a significant reduction in the intracellular calcium compared with sole stimulation of IL-1β by 94% [DPI + IL-1β vs. IL-1β, $p < 0.05$]. The sole stimulation with IBTX or DPI did not cause any significant differences compared to control groups [IbTX vs. Control; DPI vs. Control, $p > 0.05$]. The results suggest that the IL-1β-induced increase in intracellular calcium is dependent on the BKCa and the NADPH oxidase. Results are shown in the Figure 2.

Impact of IL-1b and BKCa on Cellular NO Synthesis

After 60 minutes of co-incubation, a significant enhancement in DAF fluorescence intensity was observed with IL-1β stimulation compared to control group [IL-1β vs. Control, $p < 0.05$]. The endothelial NO synthesis was increased to 151% of the control. Thereafter, the IL-1β-induced increase of intracellular NO was signifi-

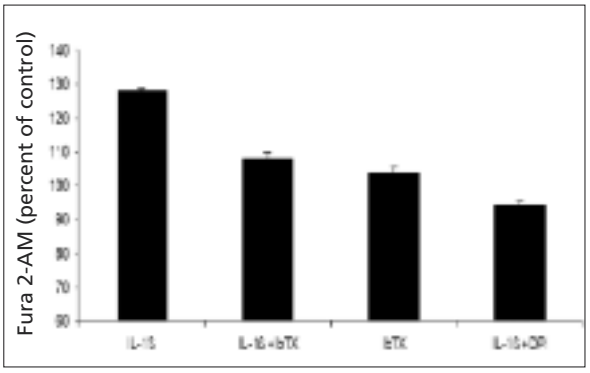

Figure 2. Figure shows the results of intracellular calcium changes.

cantly reduced by addition of IbTX, indicating that BKCa have a significant effect on intracellular NO synthesis [IbTX + IL-1β vs. IL-1β, $p < 0.05$]. The sole administration of IbTX showed no significant changes compared to control [IbTX vs. Control, $p > 0.05$].

Role of IL-1b in the Intracellular ROS Synthesis and Effects of BKCa, Intracellular Calcium, and NADPH Oxidase on the IL-1b-Induced ROS Synthesis

Stimulation of the endothelial cells with IL-1β gave rise to significant augmentation in DCF fluorescence intensity of 141% compared to controls,, which indicated IL-1β as an activator of ROS synthesis [IL-1β vs. Control, $p < 0.05$]. IbTX significantly reduced the IL-1β-induced ROS synthesis to 105% of controls [IbTX + IL-1β vs. IL-1β, $p < 0.05$]. Inhibition of NADPH oxidase by DPI and chelation of intracellular calcium by BAPTA caused a significant reduction in IL-1β-induced ROS synthesis to respectively 111% and 70% compared to IL-1β-stimulated controls, respectively [IL-1β + DPI vs. IL-1β; IL-1β + BAPTA vs. I L-1β, $p < 0.05$]. The measurements were performed 60 minutes after stimulation. The individual administrations of IBTX, DPI and BAPTA caused no significant changes in intracellular ROS synthesis compared to control [DPI vs. Control; L-NMMA vs. Control; BAPTA vs. Control, $p > 0.05$].

Effect of IL-1b on the Monocyte Adhesion and Role of BKCa in IL-1b Induced Adhesion

The first U937 monocyte adhesion studies were conducted using the tritium thymidine uptake test after an incubation period of 4 hours. It was demonstrated that treating HUVECs with IL-1β leads to a significant increment in monocyte adhesion of 476% compared to the control group [IL-1β vs. Control, $p < 0.05$] (Figure 3a). In the following experiment, monocyte adhesion was significantly suppressed from 476% to 204% of control by administration of IbTX [IL, -1β vs. IL-1β + IbTX $p < 0.05$]. This result represents that the signal transduction of IL-1β-induced adhesion is affected by BKCa. Finally, no significant change was shown with sole administration of IBTX compared to control [IbTX vs. Control, $p > 0.05$].

The second measurement was carried out using BCECF-AM fluorescent. HUVECs stimulated with IL-1β showed a significantly increased adhesion of 120% compared to those cells in control group [IL-1β vs. Control, $p < 0.05$]. It was further shown that the IL-1β-induced adhesion of U-937 monocytes to HUVECs was significantly reduced to 111% of the unstimulated control by addition of IbTX [IL-1β vs. IL-1β + IbTX, $p < 0.05$] (Figure 3b). In other words, large conductance calcium-activated potassium channels played a significant role in IL-1β-

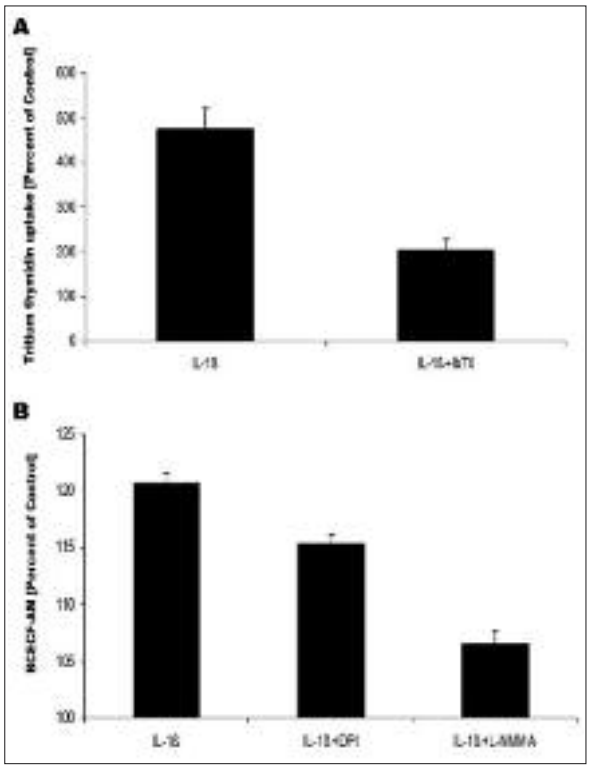

Figure 3. Figure demonstrates the effect of BKCa on IL-1β-induced adhesion using tritium thymidine uptake ***(A)*** and BCECF-AM assay ***(B)***.

induced monocyte adhesion. Incubation of endothelial cells only with IbTX did not lead any significance [IbTX vs. Control, $p > 0.05$]. Results are shown in the Figure 3.

Impact of the NADPH Oxidase and the Endothelial NO-synthase in IL-1b-Induced Adhesion of Monocytes

It is shown that NADPH oxidase inhibitor DPI significantly reduced the IL-1β-induced adhesion of monocytes to 115% of the control [DPI vs. IL-1β vs. IL-1β, $p < 0.05$]. Similarly, treating cells with eNOS inhibitor L-NMMA also led to a significant reduction in the adhesion to 106% of the control [L-NMMA vs. L-NMMA + IL-1β, $p < 0.05$]. A single administration of DPI or L-NMMA did not significantly differ IL-1-induced adhesion compared to control [DPI vs. Control; L-NMMA vs. Control, $p > 0.05$].

Expression of the Intercellular Adhesion Molecules ICAM-1 and VCAM-1

Expression of intercellular adhesion molecules (ICAM, VCAM) under stimulation with IL-1β was detected by use of a flow cytometer. HUVECs treated only with IL-1β expressed both ICAM-1 and VCAM-1 significantly more than unstimulated cells in control group [IL-1β vs. Control, $p < 0.05$]. This upregulation of both adhesion molecules was significantly decreased by the addition of IbTX [IL-1β vs. IL-1β + IbTX, $p < 0.05$]. Additionally, inhibition of NADPH oxidase with DPI caused significant inhibition of IL-1β-induced expression of ICAM-1 and VCAM-1, as well as the inhibition of eNOS by L-NMMA, and calcium by BAPTA [IL-1β vs. IL-1β + DPI; IL-1β vs. IL-1β + L-NMMA; IL-1β vs. IL-1β + BAPTA, $p < 0.05$]. The sole administration of IBX, DPI, L-NMMA and BAPTA did not significantly change in ICAM or VCAM expression in HUVECs compared to control group [DPI vs. Control; L-NMMA vs. Control; BAPTA vs. Control, $p > 0.05$]. Results are shown in Figure 4.

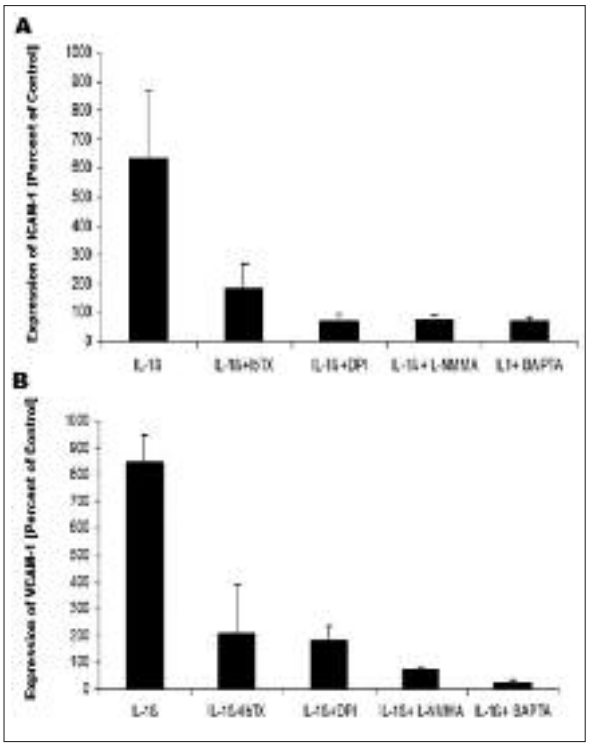

Figure 4. Measurements of IL-1ß-induced ICAM-1 ***(A)*** and VCAM-1 ***(B)*** expression by HUVEC; Figure also demonstrating the influence of BKCa, the NADPH oxidase, eNOS, and the intracellular calcium on the expression of intercellular adhesion molecules.

Discussion

Studies have shown IL-1β to cause hyperpolarization in various cell types. Salter et al[34] showed an IL-1β-induced hyperpolarization in human bone cells through the activation of the small conductance calcium-activated potassium channels. Similarly, a different study[35] reported an IL-1β-induced hyperpolarization caused by activation of voltage-dependent potassium channels in hypothalamic neurons of mice. We investigated the effects of IL-1β on large conductance calcium-activated potassium channels, which have an essential role in the regulation of membrane potential and the concentration of intracellular calcium. Analogous to the literature, HUVECs stimulated with IL-1β were significantly hyperpolarized. Kuhlmann et al. documented an increase in the opening probability of BKCa by ox-LDL and lysophosphatidylcholine, two of which are involved in the development of atherosclerosis[36]. In the present study, iberiotoxin significantly reduced the effect of IL-1β on the membrane potential. These results indicate that IL-1β-induced hyperpolarization of HUVECs depends on the presence of BKCa. However, the regulation of channel activation by IL-1β is unclear. Possible mechanisms involve direct activation by IL-1β or indirect activation via an increase in intracellular calcium and activation of the channel via calcium bowls. Additionally, it is also known that the opening probability of BKCa increases with changes in the membrane potential[37].

Furthermore, IL-1β stimulation led to a significant increase in intracellular calcium to 128% of controls. This increase was significantly blocked by inhibition of BKCa with IbTX. Activation of BKCa via IL-1β is likely involved in the increase of intracellular calcium through extracellular calcium influx. Erdogan et al[38] showed BKCa-dependent increase in intracellular calcium with a calcium peak ensuing approximately 1 minute using other stimulants, such as with apigenin. Several other studies have also shown IL-1β-induced increase in the intracellular calcium in various cell types including renal mesangial cells, astrocytes, and sea bream cells, except in mammalian cells[39-41]. In our work, the rise in the intracellular calcium was abolished by inhibiting the NADPH oxidase. Qinghua et al[42] demonstrated oscillations in intracellular calcium by histamine-induced activation of NADPH oxidase in human aortic endothelial cells. These oscillations were attributed to an increased sensitivity of the endoplasmic reticulum to inositol triphosphate (IP3) caused by ROS. In contrast, Meier et al[43] showed in their experiments on endothelial cells of pig that no relationship exists between ROS synthesis and the BKCa-induced Ca^{2+} increase.

ROS are a family of oxygen-containing molecules and their accumulation in aerobic cells contributes significantly to the development of endothelial dysfunction and, thus, atherosclerotic processes[12,13]. Most important endothelial sources include mitochondrial respiration, cyclooxygenases and the NADH/NADPH oxidases[13]. Several studies have shown an increase in the activity of NADPH oxidase caused by increased stress or by application of thrombin[44,45]. The present study shows a significant increase in intracellular ROS under stimulation with IL-1β. Reduction in the IL-1β-induced ROS synthesis by DPI indicated that NADPH oxidase is a significant cause of 1β-induced ROS formation. Similarly, Dongli et al[37] reported NADPH-dependent increase in ROS synthesis by IL-1β in retinal pigment cells. In our further tests, the increase in ROS was inhibited by chelation of intracellular calcium with BAPTA. Schaefer et al[46] reported a dependence of the cyanide-induced ROS synthesis on intracellular calcium in HUVECs, which was further increased by activation of BKCa. This was interpreted as evidence for the involvement of mitochondrial ROS synthesis[46]. IL-1β led to a significant activation of endothelial NO synthesis to approximately 150% of controls. Rosary et al[47] also found similar results in HUVECs that the inflammatory cytokines increased the activity of nitric oxide syntese activity in cultured human endothelial cells. They further showed that, analogous to the present study, the inhibition of BKCa, which indeed has a decisive role in the regulation of intracellular calcium, leads to a reduction in the IL-1β-induced NO-rise. However, other publications show quite heterogeneous outcomes. Suzschek er al[48] demonstrated in rats with aortic endothelial cells that pro-inflammatory cytokines such as TNF-alpha led to near maximal levels of nitrite formation, which was independent of calcium-concentration. Cooke et al[49] showed an activation of BKCa by H_2O_2. Interestingly, Brake Meier et al[43] showed that the BKCa is activated by NO, but dose-dependently inactivated by ROS. Since we only measured endothelial NO synthesis not the ROS production, the proportion of eNOS in ROS production could not be identified.

Tritium-thymidine uptake tests and BCECF-adhesion assay confirmed that IL-1β has a significant effect on monocyte-endothelial cell adhesion. Michael et al[50] also found increased adhesion of U-937 on HUVECs by about 5-fold after IL-1β stimulation for 4 hours using indium ^{111}In-oxine adhesion test. Kukreti et al[51] showed a similar rise of IL-1β-induced monocyte-endothelial cell adhesion at a concentration of 0.1 ng/ml after 4 hours of incubation. In our both tests, a significant reduction in the adhesion occurred by inhibiting BKCa via IbTX. Furthermore, in BCECF-adhesion assay study, the IL-1β-induced adhesion was suppressed by blocking NADPH oxidase and eNOS. Lynch et al[52] demonstrated an 2.5 fold increase in ICAM-1 dominant adhesion rate of polymorphonuclear neutrophils to HUVECs by triggering them with reactive oxygen species. eNOS had a more significant impact on adhesion compared with NADPH oxidase. This was likely due to partially uncoupled eNOS and shortening of the half-life of NO by the radicals produced from the NADPH oxidase[53].

The importance of adhesion molecules for the development of atherosclerosis was described by Davies et al[54] in which VCAM-1 was demonstrated in nearly 100% of the affected vessels and in about 30% of the plaques found in human biopsies. In this study, measurements with flow cytometer showed an increased IL-1β-induced expression of ICAM-1 and VCAM-1 on HUVEC by about 6 and 8 times compared to control, respectively. Dustin et al[55] found a similar significant increase in the expression of adhesion mole-

cules after stimulation for 4 hours with IL-1β (10 U/ml) on dermal fibroblasts[55]. It was additionally shown in this study that the increased expression of IL-1β was dependent on the intracellular calcium and the free radical formation by NADPH oxidase and eNOS. All three inhibitors (DPI, L-NMMA, BAPTA) used in the study led to a significant reduction of ICAM-1 expression. Barnett et al[56] showed a calcium-dependent I-CAM expression in HUVECs after stimulation with bacterial lipopolysaccharide. Sharma et al[57] reported a reduction of the ICAM-1 expression in thyrocytes by inhibition of NADPH oxidase by DPI. Radisavljevic et al[58] showed in brain microvascular endothelial cells that the vascular endothelial growth factor (VEGF) up-regulates the expression of the ICAM-1 through a pathway that includes phosphatidylinositol 3 OH-kinase (PI3K), AKT, and NO. Marui et al found a significant reduction in IL-1β-induced VCAM rise with the antioxidant PDTC. Also, Erdogan et al[59] showed in their experiments with HUVECs that lysophosphatidylcholine, a pro-inflammatory cytokine, inhibited the increased expression of VCAM-1 by inhibiting NADPH oxidase.

Conclusions

To our knowledge, this is the first study to demonstrate the impact of BKCa in the IL-1β-induced adhesion of monocytes to endothelial cells; thus, represents a new fundamental knowledge. Regulation of intracellular calcium by BKCa have a significant impact on inflammatory processes including adhesion of monocytes to endothelial cells. However, the mechanism of IL-1β-induced BKCa activation remains unclear.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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