The Effect of Valdecoxib on the Production of Growth Factors Evoked by Hypoxia and Bacterial Lipopolysaccharide in HMEC-1 Cells*

Wpływ waldekoksybu na wydzielanie czynników wzrostu wywołane hipoksją i bakteryjnym lipopolisacharydem w komórkach HMEC-1

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Abstract

Background. Endothelial cells produce prostaglandins (PGE2 and PGI2) and growth factors (VEGF and bFGF). These substances regulate proliferation of cells, inflammatory processes and neovascularization under physiological and pathological conditions.

Objectives. The aim of this study was to check whether valdecoxib – a selective COX-2 inhibitor – inhibits VEGF and/or bFGF secretion in the presence of LPS or cobalt chloride in normal human microvascular endothelial cells (HMEC-1).

Material and Methods. HMEC-1 cells were treated with valdecoxib at a concentration of 10 and 100 µM in the presence of 100 µg/ml LPS or 200 µM CoCl2. The effect of NSAIDs and LPS on VEGF and bFGF proteins was analyzed by ELISA kit (R&D Systems). Cell viability was measured using the 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) method.

Results. Valdecoxib inhibited LPS-induced proliferation of endothelial cells and bFGF secretion in a dose-dependent manner. Valdecoxib stimulated VEGF formation via HMEC-1 under inflammatory conditions.

Conclusion. Ultimately, the anti-angiogenic effect of the selective COX-2 inhibitor was a result of inhibition of HMEC-1 cell proliferation and bFGF generation under inflammatory conditions (Adv Clin Exp Med 2013, 22, 6, 795–800).

Key words: LPS, VEGF, bFGF, valdecoxib, endothelial cells.

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Growth factors such as VEGF and bFGF are responsible for angiogenesis. Vascular endothelial growth factor (VEGF) is one of the factors regulated by hypoxia and the HIF complex [1–3]. Numerous cells such as tumor cells, neutrophils, fibroblasts and some endothelial cells (e.g. HMEC-1 cells) can secrete the most potent pro-angiogenic factors [3]. This growth factor influences the endothelial cells via VEGFR-1 (also known as Flt-1 – fms like tyrosine kinase) and VEGFR-2 (Flk-1 – fetal liver kinase-1 in mouse/KDR – kinase domain region in human) receptors and leads to permeability enhancement, cellular proliferation and migration of cells [4–6]. Apart from VEGF, fibroblast growth factors (FGFs) possess a pro-angiogenic activity by binding to high affinity tyrosine kinase FGFRs on the surface of target cells. FGFs are a large family with 22 distinct members among which two of them were the first to be isolated from the peptide family: FGF1 and FGF2 (also named as acidic FGF; aFGF and basic FGF; bFGF). FGFs are responsible for mitogenesis, differentiation, migration and cell survival of endothelial cells and other cells [7–10].

Hypoxia promotes endothelial cells via VEGF. Bacterial cell wall lipopolysaccharide (LPS, endotoxin) causes VEGF and bFGF secretion and is also responsible for generation of vasoactive mediators such as prostaglandins: PGI$_2$, PGE$_2$ [11]. Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX). This enzyme exists in at least two isoforms: the constitutive isoform, COX-1, and the inducible isoform, COX-2. COX-2 is activated in response to inflammatory mediators such as cytokines, as well as mitogens, growth factors, oncogenes and carcinogens, and also is constitutively expressed in the brain, kidney vascular endothelium, ovaries and uterus [12]. Both enzymes are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs). Valdecoxib and celecoxib and other substances belong to selective COX-2 inhibitors and they block PGI$_2$ formation by endothelial cells. COX inhibitors show anti-angiogenic effect that is the result of endothelial cell survival inhibition and induction of apoptosis [1, 4, 12].

Endothelial cells produce PGs (PGE$_2$ and PGI$_2$) [13] and growth factors (VEGF and bFGF) [14]. These substances regulate proliferation of cells, inflammatory processes and neovascularization under physiological and pathological conditions. Therefore, the aim of this study was to check whether valdecoxib inhibits VEGF and bFGF in the presence of LPS and cobalt chloride in normal human microvascular endothelial cells (HMEC-1).

### Material and Methods

#### Chemicals

The substances used were the following: MCDB 131 medium, fetal bovine serum, penicillin-streptomycin solution (5,000 units/mL penicillin and 5,000 µg/mL streptomycin sulphate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA (0.25% trypsin, 1mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, CA, USA). The cobalt chloride, thiazoyl blue tetrazolium bromide (MTT), EGF human, lipopolysaccharides from *Salmonella enteritidis* (LPS) and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, USA). Valdecoxib was purchased from Cayman Chemical Company (Michigan, USA).

#### Cell Culture

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC with catalog number ATCC-CRL-10636 (depositor Centers for Disease Control, Dr. Edwin W. Ades, Atlanta). The cells were used between passages 10–31 and cultured in 25 cm$^2$ flasks in MCDB 131 medium supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone and penicillin-streptomycin solution, in a humidified atmosphere of 95% O$_2$ and 5% CO$_2$ at 37°C. Cells were harvested every 3rd day in a trypsin-EDTA (0.25% trypsin, 1 mM EDTA) solution. HMEC-1 cells were cultured basing on the method described in the literature [15, 16] and the author’s own modification.

#### MTT Conversion

HMEC-1 cells proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) conversion method. Cells were seeded (50,000 cells/well) into 96-well plates. The cells were incubated for 24 h with LPS 100 µg/mL, CoCl$_2$ 200 µM, valdecoxib 10 or 100 µM, LPS and valdecoxib or CoCl2 and valdecoxib or without tested chemicals (control group). All the substances were added at the same time. After incubation, 50 µL MTT (1 mg/mL, Sigma) was added and the plates were incubated at 37°C for 4 h. At the end of the experiment, cells were exposed to 100 µL dimethyl sulphoxide, which enabled the release of the blue reaction product – formazan. The absorbance at 570 nm was read on a microplate reader and results were expressed as a percentage of the absorbance measured in control cells.
ELISA Assays

VEGF and bFGF concentrations in cell culture media were determined by commercially available ELISA kits according to the vendor’s protocols (R&D System, Abingdon, UK).

Data Analysis

All data was presented as means ± SD (standard deviation). Statistical comparisons between the groups were performed using ANOVA, and post-hoc comparisons were performed using the Student-Newman-Keuls test. The normal distribution of parameters was checked by means of the Shapiro-Wilks test. If the data was not normally distributed or the values of the variance (test F) were different, ANOVA with Kruskal-Wallis and Mann-Whitney’s U test were used. All parameters were considered significantly different if p < 0.05. The statistical data analysis was performed using Statgraphics 5.0 plus software.

Results

The Effect of Valdecoxib on VEGF Secretion Evoked by Cobalt Chloride or Bacterial LPS in HMEC-1

Basal HMEC-1 cells released only small amounts of VEGF (25.6 pg/mL) as measured by ELISA, but cobalt chloride (CoCl₂), a hypoxia mimicking agent, at a concentration of 200 µM in 24-h incubation stimulated the secretion of VEGF by 505% (p < 0.05) (Fig. 1). 100 µg/mL LPS, which mimics inflammation, increased the level of VEGF by 106% (p < 0.05) in a statistically significant manner. Valdecoxib – a selective COX-2 inhibitor – at a concentration of 10 µM did not influence the level of VEGF, but at 100 µM stimulated its generation (p < 0.05). Neither of the valdecoxib concentrations changed VEGF secretion under hypoxia conditions. However, valdecoxib at a higher concentration intensified VEGF secretion in the presence of LPS in comparison with LPS by 130% (p < 0.05) in a statistically significant manner and by 108% in comparison with simultaneous application of valdecoxib 10 µM and LPS (p < 0.05).

The Effect of Valdecoxib on bFGF Secretion Evoked by Bacterial LPS in HMEC-1

The control group of HMEC-1 released approximately 435 pg/mL bFGF (Fig. 2). LPS stimulated the release of bFGF by 50% (p < 0.05) in a statistically significant manner. Valdecoxib at the concentration of 10 µM increased the generation of bFGF by 13% (p < 0.05). The simultaneous application of the selective COX-2 inhibitor and LPS induced a higher secretion of bFGF by 8% (p < 0.05) in comparison with LPS. The observed results were statistically significant. In the next set of experiments, the influence of valdecoxib at 100 µM on the generation of bFGF was studied. 100 µM valdecoxib decreased the level of bFGF by 11% (p < 0.05) in comparison with the control. It also inhibited the secretion of bFGF induced by LPS by 13.5% (p < 0.05) in comparison with LPS and by 20% (p < 0.05) in comparison with simultaneous application of valdecoxib 10 µM and LPS.

Fig. 1. Effects of valdecoxib (10 and 100 µM) on VEGF levels in HMEC-1 cells in the presence of CoCl₂ (200 µM) or LPS (100 µg/mL). Bars represent the means (± SEM of 3–5 experiments).

* p < 0.05 vs. control; a – p < 0.05 vs. LPS (100 µg/mL); b – p < 0.05 vs. LPS (100 µg/mL) & valdecoxib (10 µM)

Ryc. 1. Wpływ waldekoksybu (10 i 100 µM) na poziom VEGF w komórkach HMEC-1 w obecności CoCl₂ (200 µM) lub LPS (100 µg/ml). Kolumny przedstawiają średnie (± SEM z 3–5 doświadczeń).

* p < 0.05 w stosunku do grupy kontrolnej; a – p < 0.05 w stosunku do grupy LPS (100 µg/ml) & waldekoksyb (10 µM)
The Effect of Valdecoxib on Cell Viability in the Presence of Cobalt Chloride or Bacterial LPS in HMEC-1

Cobalt chloride at 200 µM decreased HMEC-1 cell viability (Fig. 3) in a statistically significant manner (p < 0.05). In contrast to hypoxia, LPS at 100 µg/mL stimulated proliferation of HMEC-1 cells by 33% (p < 0.05). Valdecoxib at a concentration of 10 and 100 µM did not change cell viability under hypoxia conditions. However, valdecoxib at both of the concentrations inhibited the proliferative effect of LPS by 14% (p < 0.05) and 35% (p < 0.05), respectively. These observed effects were statistically significant. The decrease in cell viability was statistically significant (p < 0.05) after the application of valdecoxib 100 µM with LPS (100 µg/mL) in comparison with valdecoxib 10 µM with LPS (100 µg/mL).

Discussion

The major aim of this work was to check the effect of valdecoxib (a selective COX-2 inhibitor; valdecoxib inhibits the generation of prostanoids) on the secretion of growth factors (VEGF
In this study, LPS-induced inflammation and bFGF, which are the most potent angiogenic factors. In previous studies the expression of VEGF by human microvascular endothelial cells (HMEC-1 cells) under hypoxia conditions evoked by low oxygen partial pressures (1 and 3%) and cobalt chloride was observed [2, 14, 16]. In the present work, the selective COX-2 inhibitor, valdecoxib, did not change the production of VEGF by HMEC-1 cells under chemical hypoxia (Fig. 1). Hypoxia as well as cobalt chloride potently inhibits HIF-1α degradation and activates transcription of VEGF via binding of HIF-1 to HRE (hypoxia response element) on VEGF genes [2]. Palayoor et al. [17] have shown that higher concentrations of NSAIDs, than those needed to inhibit prostaglandin synthesis, can reduce VEGF formation by affecting HIF-1α. In the present study, valdecoxib at 10 and 100 µM not only did not block VEGF generation in the absence or presence of LPS but also, when used at the higher concentration of 100 µM, it stimulated VEGF production in HMEC-1 cells.

In the next set of experiments, the influence of valdecoxib (at 100 µM) on the generation of LPS-stimulated bFGF in HMEC-1 cells was studied (Fig. 2). In the previous study, the lack of a CoCl2 effect on bFGF production in HMEC-1 cells was determined [14], therefore in this study valdecoxib was used only in the presence of LPS. In this study, valdecoxib increased bFGF generation in a statistically significant manner only when used at 10 µM in the presence of LPS (100 µg/mL). In contrast to valdecoxib used at 10 µM, at a higher concentration (100 µM) valdecoxib inhibited the secretion of bFGF in the presence and absence of LPS in tested cells. Akarasereenont et al. demonstrated that LPS, as a potent inflammatory mediator, is responsible for stimulation of COX-2 and prostaglandins production in bovine aortic endothelial cells (BAEC) [18]. Thus, application of the selective COX-2 inhibitor evoked reduction of the bFGF levels in HMEC-1 cells that may indicate the participation of prostaglandins in bFGF synthesis. The inhibitory effect on bFGF of the high concentration of valdecoxib in this experiment may be associated with the anti-angiogenic effect of valdecoxib. bFGF secreted by endothelial cells (HMEC-1 cells) and under inflammatory conditions (induced by LPS) can also affect other, non-endothelial cells. It is also known as a chemotactic and mitogenic factor for smooth muscle cells (SMC) [19]. The bFGF-evoked increase of SMC migration and proliferation underlies the mechanism responsible for atherosclerotic plaque formation [4]. In this study LPS-induced inflammation evoked bFGF and VEGF secretion, which could stimulate SMC. Moreover, the decrease of bFGF in the presence of valdecoxib at the higher concentration (100 µM) could inhibit the migration and proliferation of SMC induced by LPS.

Incubation of endothelial cells with cobalt chloride at 200 µM decreased cell survival in a statistically significant manner (p < 0.05) but valdecoxib did not influence the viability of HMEC-1 cells under chemical hypoxia (Fig. 3). Moreover, 100 µg/mL LPS stimulated proliferation of cells after 24-h incubation, which was attenuated by the selective COX-2 inhibitor. Application of valdecoxib reduced HMEC-1 cell survival in the presence of LPS (100 µg/mL) and the observed effect of the drug was dose-dependent. These results are in close agreement with those of Flis et al. [13] who demonstrated that sulindac sulfide (a non-selective COX-2 inhibitor) and celecoxib (a selective COX-2 inhibitor) inhibited the survival of HMEC-1 cells and induced their apoptosis. A similar effect was shown by Niederburger et al. [20] in human umbilical vein endothelial cells (macrovessel-derived endothelial cells).

In conclusion, the anti-angiogenic effect of the selective COX-2 inhibitor is the result of both the inhibition of HMEC-1 proliferation and bFGF generation under inflammatory conditions. Prostaglandins (products of COX-2) are responsible for proliferation of endothelial cells [21, 22]. LPS can activate COX-2 and PGs formation [18, 23] but NSAIDs inhibit COX-2 and therefore they can reduce endothelial cell survival. The obtained results and previous studies provide evidence that NSAIDs, due to inhibition of COX, can have an anti-angiogenic effect. NSAIDs can also inhibit bFGF formation and proliferation of microvascular endothelial cells (HMEC-1 cells) thus the inhibitory effect of these drugs on migration and proliferation processes can also be observed in other, non-endothelial cells. However, whether the observed valdecoxib action in HMEC-1 can be described by a similar mechanism is still an open question and needs further studies. Angiogenesis and cell migration have been found to occur in the pathogenesis of rheumatoid arthritis [24]. NSAIDs, including valdecoxib, are used in the treatment of rheumatoid arthritis; therefore their anti-angiogenic action can have an additional therapeutic importance [25].

The author concluded that valdecoxib inhibited the LPS-induced proliferation of endothelial cells in a dose-dependent manner. The anti-angiogenic effect of valdecoxib was associated with inhibiting bFGF secretion. Valdecoxib stimulated VEGF formation under inflammatory conditions.

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References


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