

The effect of diclofenac on proliferation and production of growth factors by endothelial cells (HMEC-1) under hypoxia and inflammatory conditions

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Diclofenac belongs to non-steroidal anti-inflammatory drugs (NSAIDs) and non-selective COX inhibitors. The aim of this study was to examine the effect of diclofenac on endothelial cell proliferation under the influence of hypoxia or inflammatory conditions. Another goal was to check whether diclofenac modulates the secretion of angiogenic factors such as VEGF and bFGF in human microvascular endothelial cells (HMEC-1) in the presence of CoCl_2 or lipopolysaccharide (LPS), which could influence the endothelial cells in an autocrine manner or other cells in a paracrine manner. HMEC-1 cells were treated with 0.1 and 0.3 mmol L^{-1} diclofenac in the presence of 100 $\mu\text{g mL}^{-1}$ LPS or 200 $\mu\text{mol L}^{-1}$ CoCl_2 . Diclofenac decreased cell viability under hypoxia and inflammatory conditions. The stimulation of bFGF secretion by LPS in microvascular endothelial cells (HMEC-1 cell) was attenuated by diclofenac. Diclofenac increased the secretion of VEGF induced by LPS and hypoxia.

Keywords: LPS, VEGF, bFGF, diclofenac, hypoxia, endothelial cells

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Endothelium, which is a dynamic organ, forms a structural barrier between the vascular space and the tissues. Endothelial cells have contact with numerous smooth muscle cells. Many factors such as LDL (low-density lipoproteins), free radicals, infectious microorganisms, shear stress, hypertension, toxins after smoking and/or others may lead to endothelial dysfunction, which is characterized by decreased nitric oxide synthesis. Disturbance in endothelial function is implicated in several diseases, including atherosclerosis, hypertension, inflammatory diseases and cancer metastasis (1–4). Destruction of endothelium by the above-mentioned agents can lead to the release of cytokines and growth factors such as basic fibroblast growth factor (bFGF), which has mitogenic effects on smooth muscle cells (5). The inflammatory process is also responsible for endothelial dysfunction and generation of some growth factors and plays an important role in the progression of atherosclerotic plaque formation. Hypoxia stimulates vascular endothelial growth factor (VEGF) secretion, which is responsible for the formation of new blood

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vessels and solid tumor progression and metastasis. However, neovascularization need not always be associated with pathological processes. An example of positive impact may be effects on the cardiovascular system in the case of ischemic heart disease (1–5).

Diclofenac, a non-selective COX inhibitor, is often used in inflammatory diseases such as rheumatoid arthritis. However, there is no explicit information about the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the secretion of angiogenic growth factors (VEGF, bFGF) by normal endothelial cells. Many studies have reported antiangiogenic effects of cyclooxygenase COX-2 inhibitors on cancer cells (6). Among traditional NSAIDs, diclofenac is the strongest inhibitor of COX-2. This behavior is responsible for significant cardiovascular toxicity resulting from the use of diclofenac. Cardiovascular toxicity is connected with two major mechanisms. Firstly, the main mechanism is the inhibition of prostaglandin generation in the endothelium, which is associated with increased risk of atherothrombotic vascular events. The second mechanism is also mediated by prostaglandin inhibition, which can induce sodium and water retention. This effect leads to an increase in blood pressure (7). Diclofenac, more often than other traditional NSAIDs, leads to myocardial infarction and has the highest risk of cardiovascular morbidity and mortality, including fatal or nonfatal stroke and coronary death (7). Meta-analysis published in the *Lancet* in 2013 showed that the cardiovascular risk of diclofenac is similar to rofecoxib (selective COX-2 inhibitor) effect (8).

In turn, in ischemic heart disease, collateral vessel formation plays an important role in protecting the myocardium from ischemia. Recent studies show that f.e. bFGF can be responsible for myocardial regeneration and thereby improvement of cardiac function (9, 10). Thus, it seems important to investigate the effect of diclofenac on growth factors (VEGF, bFGF) and to identify additional factors relevant to the cardiovascular effects of NSAIDs.

Therefore, in this study it was decided to examine the effect of the non-selective COX inhibitor – diclofenac on endothelial cell survival under the influence of hypoxia or inflammatory conditions. The other aim was to check whether diclofenac modulates the secretion of angiogenic factors such as VEGF and bFGF in human microvascular endothelial cells (HMEC-1) in the presence of CoCl₂ or bacterial lipopolysaccharide (LPS), which could influence endothelial cells in an autocrine manner or other cells in a paracrine manner.

EXPERIMENTAL

Chemicals

The following substances were used: MCDB 131 medium, fetal bovine serum, penicillin-streptomycin solution (5,000 units mL⁻¹ penicillin and 5,000 µg mL⁻¹ streptomycin sulphate in normal saline), phosphate buffered saline (PBS, pH 7.4) and trypsin-EDTA (0.25 % trypsin, 1 mmol L⁻¹ Na₄-EDTA). They were purchased from Invitrogen (USA). Cobalt chloride, diclofenac, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), human epidermal growth factor (EGF), lipopolysaccharides from *Salmonella enteritidis* (LPS) and hydrocortisone were purchased from Sigma Chemical Co. (USA).

Cell culture

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC (cells deposited by the Centers for Disease Control, Atlanta, USA). The cells were used betwe-

en passages 10–31 and cultured in 25-mL flasks in medium composed of MCDB 131 supplemented with 10 % fetal bovine serum, 10 ng mL⁻¹ epidermal growth factor, 1 µg mL⁻¹ hydrocortisone and penicillin-streptomycin solution, in a humidified atmosphere of 95 % and 5 % CO₂ at 37 °C. Cells were harvested every third day in a trypsin-EDTA solution (0.25 % trypsin, 1 mmol L⁻¹ EDTA).

MTT conversion

HMEC-1 cell proliferation was measured using the MTT conversion method. Cells were seeded (50 000 cells per well) into 96-well plates. The treated cells were incubated for 24 h. After incubation, 50 µL of MTT (1 mg mL⁻¹) 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 release of the blue reaction product – formazan. Absorbance at 570 nm was read on a microplate reader and results were expressed as the percentage of 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, UK).

Data analysis

All data are expressed as the mean ± SEM values. For statistical evaluation of the results, an analysis of variance (ANOVA) was performed, followed by a *post-hoc* Student-Newman-Keuls test.

RESULTS AND DISCUSSION

Endothelial cells produce prostaglandins from arachidonic acid in physiological processes responsible for vasodilatation and anti-aggregation and in pathophysiological processes such as inflammation, arthritis and cancer (11, 12). Diclofenac, as a non-selective COX inhibitor, inhibits cyclooxygenase-1 and 2; it therefore blocks the formation of prostaglandins (PGs).

Effect of diclofenac on cell viability in the presence of cobalt chloride or bacterial LPS in HMEC-1

In these studies, diclofenac, used at a concentration of 0.01–1.0 mmol L⁻¹, inhibited HMEC-1 cell viability (Fig. 1a). The range of concentrations inducing inhibition of cell survival up to 50 % (IC₅₀) was estimated from the concentration-response curve obtained for endothelial cells. IC₅₀ for diclofenac was 0.1 mmol L⁻¹. A statistically significant decrease of cell viability was observed at diclofenac concentrations of 0.1, 0.3 and 1.0 mmol L⁻¹. These findings are consistent with our previous results (unpublished) and those of other authors who found that sulindac (13), aspirin (14) (non-selective COX inhibitors) and celecoxibe (selective COX-2 inhibitor) (15) are related to antiproliferative and proapoptotic activities. Flis *et al.* (13) concluded that antiproliferative effect of sulindac sulfide is due to COX-dependent and COX-independent mechanisms.

In a subsequent set of experiments, we studied the influence of diclofenac at a concentration of 0.01–0.3 mmol L⁻¹ on the human microvascular cell viability under hypoxia (CoCl₂, 200 μmol L⁻¹) and inflammatory (LPS, 100 μg mL⁻¹) conditions (Fig. 1b). LPS deriving from the *Salmonella enteritidis* cell wall induces inflammation in endothelial cells as well as COX-2 and prostaglandins (PGE₂), which are responsible for proliferation of endothelial cells (14, 16). As shown in Fig. 1b, LPS at 100 μg mL⁻¹ stimulated proliferation of HMEC-1 cells by 33 % (*p* < 0.05). Diclofenac, as a non-selective COX inhibitor, used at concentrations from 0.01 to 0.3 mmol L⁻¹ inhibited the proliferative effect of LPS at 100 μg mL⁻¹ by 28, 44 and 46 %, respectively, in comparison with the control and by 53, 64 and 64.5 %, respectively, in comparison with LPS.

Previous reports indicated that LPS was responsible for the induction of cyclooxygenase-2 in bovine aortic endothelial cells (BAEC) and intensified cell proliferation (17).

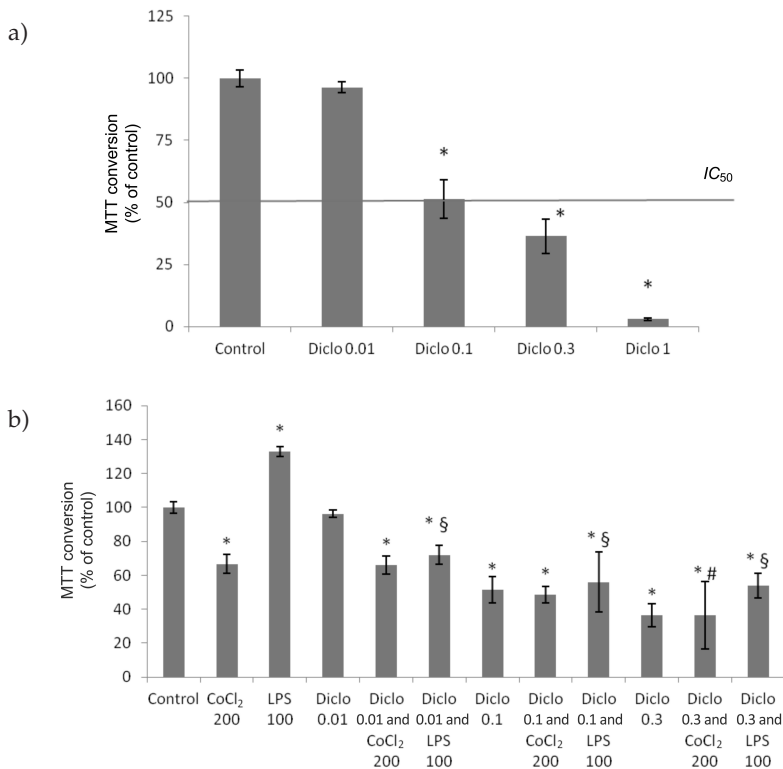


Fig. 1. Effects of diclofenac on viability of cultured HMEC-1 cells: a) diclofenac (0.01–1.0 mmol L⁻¹); b) diclofenac (0.01–0.3 mmol L⁻¹) in the presence of CoCl₂ (200 μmol L⁻¹) or LPS (100 μg mL⁻¹). Bars represent means ± SEM of 4–15 experiments. **p* < 0.05 vs. control; #*p* < 0.05 vs. CoCl₂ (200 μmol L⁻¹); §*p* < 0.05 vs. LPS (100 μg mL⁻¹). Abbreviations: CoCl₂ 200 – CoCl₂ 200 μmol L⁻¹; LPS 100 – LPS 100 μg mL⁻¹; Diclo 0.01 – diclofenac 0.01 mmol L⁻¹; Diclo 100 – diclofenac 0.1 mmol L⁻¹; Diclo 0.3 – diclofenac 0.3 mmol L⁻¹.

Thus, non-selective COX inhibitors (diclofenac), inhibiting prostaglandin synthesis, possess antiproliferative activity (13–15). However, it is emphasized in the literature that COX inhibitors may also enhance apoptosis regardless of the effect on prostaglandin (13, 15). In the present study, diclofenac (non-selective COX-2 inhibitor) reduced the proliferative effect of LPS in dose-dependent manner and in a statistically significant manner.

In contrast, cobalt chloride (CoCl_2), a hypoxia mimicking agent, decreased endothelial cell survival in a statistically significant manner (Fig. 1b). Diclofenac, at concentrations of 0.01 and 0.1 mmol L^{-1} , did not change cell viability weakened by CoCl_2 while increased inhibition of cell viability of HMEC-1 cells at 0.3 mmol L^{-1} was observed. The strongest effect of diclofenac in the presence of LPS and CoCl_2 was observed at a concentration of 0.3 mmol L^{-1} . NSAIDs can augment inhibition of the survival cell effect of hypoxia and decrease the proliferative effect of inflammation.

Effect of diclofenac on bFGF secretion evoked by bacterial LPS in HMEC-1

Antiproliferative and antiangiogenic effect of diclofenac may be associated with the decrease of bFGF after treating HMEC-1 with diclofenac under inflammatory conditions (Fig. 2). LPS induced secretion of bFGF in HMEC-1 cells by 107 % in a statistically significant manner. Diclofenac at concentrations of 0.1 and 0.3 mmol L^{-1} did not influence the secretion of bFGF in HMEC-1 cells. In the presence of LPS, diclofenac inhibited formation of bFGF stronger at higher concentration (0.3 mmol L^{-1}) than at concentration of 0.1 mmol L^{-1} . This observation was statistically significant ($p < 0.05$). Other reports showed that under inflammatory conditions endothelium may secrete bFGF, which may stimulate proliferation of smooth muscle cells (5, 12). This study showed that diclofenac may inhibit secretion of bFGF by endothelial cells and the proliferative effect of bFGF on smooth muscle cells. Effect of diclofenac on bFGF under inflammation conditions is directly proportional to the concentration.

Effect of diclofenac on VEGF secretion evoked by cobalt chloride or bacterial LPS in HMEC-1

According to the previous studies of Namiecinska *et al.* (18) and Loboda *et al.* (19, 20), expression of VEGF in human microvascular endothelial cells (HMEC-1 cells), can be induced by both hypoxia (1 and 3 % O_2) and cobalt chloride, a hypoxia mimicking agent (21) and LPS (22). Similarly, in the present work, the level of VEGF after 24-hour incubation with CoCl_2 at 200 $\mu\text{mol L}^{-1}$ was 4.7-fold ($p < 0.05$) higher than that of the control (Fig. 3). At a concentration of 100 $\mu\text{g ml}^{-1}$, LPS stimulated the generation of VEGF by 101 % in comparison with the control ($p < 0.05$). Increase of VEGF level evoked by hypoxia was 2.35-fold higher than under the influence of inflammation in HMEC-1.

Incubation of HMEC-1 cells with diclofenac, at 0.1 and 0.3 mmol L^{-1} , increased the level of VEGF by 152 and 166 %, respectively, in comparison with the control. In the subsequent set of experiments, the influence of diclofenac (0.1 and 0.3 mmol L^{-1}) on the VEGF secretion under hypoxia and inflammatory conditions was studied. 24-hour incubation with diclofenac (0.1 and 0.3 mmol L^{-1}) under hypoxia conditions further intensified the production of VEGF by 10.5 and 16 %, respectively ($p < 0.05$). Diclofenac (0.1 and 0.3 mmol L^{-1}) when applied with LPS potentiated the secretion of VEGF by 125 and

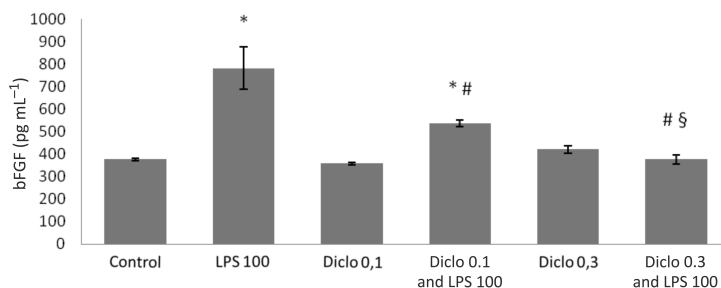


Fig. 2. Effects of diclofenac (0.1 and 0.3 mmol L⁻¹) on bFGF levels in HMEC-1 cells in the presence of LPS (100 µg mL⁻¹). Bars represent means ± SEM of 3–5 experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. LPS (100 µg mL⁻¹); § $p < 0.05$ vs. LPS (100 µg mL⁻¹) and diclofenac (0.1 mmol L⁻¹). Abbreviations: LPS 100 – LPS 100 µg mL⁻¹; Diclo 0.1 – diclofenac 0.1 mmol L⁻¹; Diclo 0.3 – diclofenac 0.3 mmol L⁻¹.

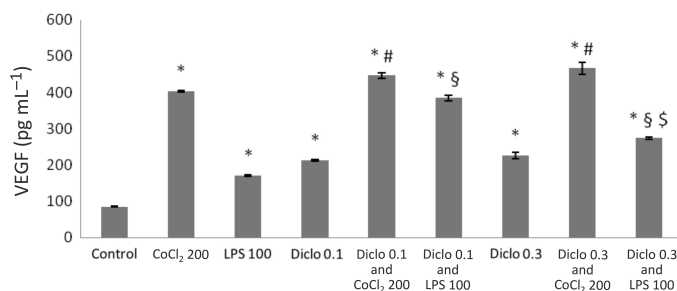


Fig. 3. Effects of diclofenac (0.1 and 0.3 mmol L⁻¹) on VEGF levels in HMEC-1 cells in the presence of CoCl₂ (200 µmol L⁻¹) or LPS (100 µg mL⁻¹). Bars represent mean ± SEM of 3–5 experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. CoCl₂ (200 µmol L⁻¹); § $p < 0.05$ vs. LPS (100 µg mL⁻¹) and diclofenac (0.1 mmol L⁻¹). Abbreviations: CoCl₂ 200 – CoCl₂ 200 µmol L⁻¹; LPS 100 – LPS 100 µg mL⁻¹; Diclo 0.1 – diclofenac 0.1 mmol L⁻¹; Diclo 0.3 – diclofenac 0.3 mmol L⁻¹.

60 %, respectively ($p < 0.05$), in comparison with LPS alone. In the presence of LPS, diclofenac at a concentration of 0.3 mmol L⁻¹ stimulated secretion of VEGF to a lower extent than 0.1 mmol L⁻¹ diclofenac (by 29 %). This observation was statistically significant. In summary, diclofenac stimulated secretion of VEGF in a manner opposite to the dose, the higher dose of diclofenac (0.3 mmol L⁻¹) induced VEGF secretion less than the lower dose (0.1 mmol L⁻¹) under inflammatory conditions.

CONCLUSIONS

The obtained findings demonstrate that LPS could induce HMEC-1 proliferation through a COX-signaling mechanism. The classical NSAID – diclofenac could inhibit this step of HMEC-1 proliferation. This non-selective COX inhibitor potentiated the secretion of VEGF under hypoxia and inflammatory conditions. Diclofenac inhibits LPS-induced

secretion of bFGF in a dose-dependence manner. The observed inhibition of bFGF secretion by diclofenac under inflammatory conditions can play an important role in cardiac function deterioration in coronary diseases. Further studies are required to determine the mechanism of VEGF and bFGF generation under inflammatory conditions and in the presence of diclofenac.

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Acronyms: BAEC – bovine aortic endothelial cells, bFGF – basic fibroblast growth factor, COX – cyclooxygenase, EGF – epidermal growth factor, FGF – fibroblast growth factor, HMEC-1 – human microvascular endothelial cells-1, IC_{50} – half maximal inhibitory concentrations, LPS – bacterial lipopolysaccharide, NSAID – nonsteroidal antiinflammatory drug, PG – prostaglandin, VEGF – vascular endothelial growth factor.

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