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THE EFFECT OF IBUPROFEN ON bFGF, VEGF SECRETION AND CELL PROLIFERATION IN THE PRESENCE OF LPS IN HMEC-1 CELLS

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Abstract: Ibuprofen belongs to the group of non-selective cyclooxygenase (COX) inhibitors, also known as traditional non-steroidal anti-inflammatory drugs (NSAIDs). Bacterial lipopolysaccharide, an inflammatory mimicking agent, is responsible for the production of prostaglandins and growth factors (VEGF and bFGF), and as inflammation and angiogenesis are closely associated with osteoarthritis, these factors play a functional role in the cardiovascular system. Therefore, the main aim of our study was to examine the effect of ibuprofen on cell viability and proliferation of HMEC-1 cells and VEGF and bFGF secretion under the inflammatory conditions. The effect of NSAID and LPS on bFGF and VEGF was analyzed by ELISA. Cell viability was measured by the MTT method and the proliferation by the [^{3}H]-thymidine test. LPS at 100 μg/mL stimulated the secretion of VEGF and bFGF by HMEC-1 cells. Ibuprofen at concentrations of 0.1 and 1 mM intensified the secretion of LPS-induced VEGF in a statistically significant manner ($p < 0.05$). Both concentrations of ibuprofen inhibited LPS-stimulated bFGF secretion ($p < 0.05$) in HMEC-1 in a concentration-dependent manner. The non-selective COX inhibitor decreased proliferation and cell viability induced by LPS in a concentration-dependent manner. The observed effects of ibuprofen on endothelial cells may further explain its effects as well as other NSAIDs on the cardiovascular system function in cardiovascular diseases.

Keywords: bFGF, ibuprofen, LPS, VEGF, endothelial cells

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely-used drugs worldwide. They are used for their analgesic, antipyretic and anti-inflammatory effects, and their action is mediated by the inhibition of cyclooxygenase (COX) and prostaglandin production. Prostaglandins regulate vascular tone, blood coagulation as well as salt and water homeostasis in the mammalian kidney. Cyclooxygenase exists as two isoforms: COX-1, which is constitutive, and COX-2, which is induced by proinflammatory cytokines and endotoxin at inflammatory sites (1). Generally, NSAIDs are divided into two groups: one which comprises traditional, non-selective inhibitors of COX, such as ibuprofen, and another made up of selective COX-2 inhibitors (coxibes)(1, 2).

The endothelium forms a dynamic barrier between the vascular space and the tissues, and produces a variety of regulatory mediators such as nitric oxide, prostanoids, endothelins, angiotensin II, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), adhesion molecules, cytokines and growth factors. NSAIDs are known to have an influence on endothelial function, and may even induce endothelial dysfunction characterized by reduced vasodilation and increased endothelium-dependent contraction (3-8). Some data also indicate that endothelial dysfunction may be involved in the initiation of vascular inflammation and in the development of vascular remodelling. It is also an early determinant in the progression of atherosclerosis, and it is independently associated with increased risk for cardiovascular adverse events. The risk of cardiovascular complications associated with NSAIDs is currently broadly described (9). Although the mechanism behind the adverse cardiovascular effects of NSAIDs appears to be clear, the differences between the drugs themselves demand further analysis and a

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better understanding of the nature of their relationship with the endothelium.

Endothelial cells may also produce growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are responsible for generation of vasoactive such mediators as the prostaglandins PGI_2, PGE_2. Growth factors regulate proliferation of cells, inflammatory processes and neovascularization under physiological and pathological conditions (10, 11). It has been shown that exogenous bFGF increases angiogenesis and myocardial perfusion, promotes regeneration after myocardial infarction and thereby improves cardiac function (12, 13). From this point of view, it seems important to investigate the effect of ibuprofen, a widely used over-the-counter drug, on the secretion of endothelial growth factors under physiological conditions, and during inflammation. Therefore, the main aim of this study was to examine the effect of a non-selective COX inhibitor, ibuprofen, on cell viability, proliferation of HMEC-1 cells and VEGF and bFGF secretion under inflammatory conditions.

MATERIALS AND METHODS

Chemicals

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

Cell culture

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC, catalog number ATCC-CRL-10636 (depositor Centers for Disease Control, Dr. Edwin W. Ades, Atlanta, USA). For experimentation, the cells between passages 10-31 were used. HMEC-1 cells were cultured in 25 cm^3 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% air and 5% CO_2 at 37°C. Cells were harvested every third day in a trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA). HMEC-1 cells were cultured according to the method described in the literature (14, 15) and the authors own modification.

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).

MTT conversion

HMEC-1 cell viability was measured using the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion method. Cells were seeded (50,000 cells/well) into 96-well plates. The treated cells were incubated for 24 h with 100 µg/mL LPS, 10 or 100 µM ibuprofen, LPS and ibuprofen 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, the cells were exposed to 100 µL dimethyl sulfoxide, 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.

Assay of proliferative response of HMEC-1 cells

Cells were seeded in 96-well plates at a density of 50,000 cells per well in 100 µL of the culture medium and cultured for 1 day. On the day of the experiment, after rinsing the cells twice with PBS, fresh serum-free culture medium was added and the cells were incubated in the presence of drugs for 24 h at 37°C. Cell proliferation was determined by adding [^{3}H]-thymidine (0.5 µCi) 18 h before the end of incubation. The cultures were harvested with an automatic cell harvester (Scatron, Lier, Norway), and [^{3}H]-thymidine was estimated using a liquid scintillation counter MicroBetaTriLux (Perkin Elmer) (16).

Data analysis

All data are presented as the 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-Wilk test. If the data were 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 ibuprofen on VEGF secretion under hypoxia and inflammatory conditions in HMEC-1 cells

It was found that 0.1 mM ibuprofen significantly increased VEGF level by 28%, and 1 mM by 113% (Fig. 1) in comparison with the control ($p < 0.05$). Ibuprofen at both concentrations also augmented secretion of VEGF in the presence of 100 µg/mL LPS and 200 µM $CoCl_2$. Non-selective COX inhibitor at 0.1 mM and 1 mM increased the secretion of VEGF by 5 and 7%, respectively, in comparison with $CoCl_2$ (200 µM). Application of ibuprofen (0.1 and 1 mM) with LPS (100 µg/mL) increased the level of VEGF by 8 and 71%, respectively. The observed effects were statistically significant ($p < 0.05$).

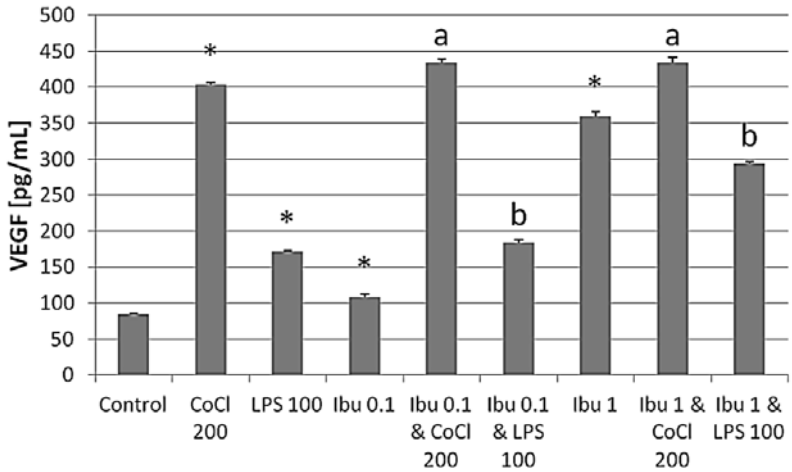

Figure 1. Effects of ibuprofen (0.1 and 1 mM) on VEGF levels in HMEC-1 cells in the presence of $CoCl_2$ (200 µM) or LPS (100 µg/mL). Bars represent the means (±SEM of 3-5 experiments). *$p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* $CoCl_2$ (200 µM); b – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: CoCl 200 - $CoCl_2$ 200 µM; LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

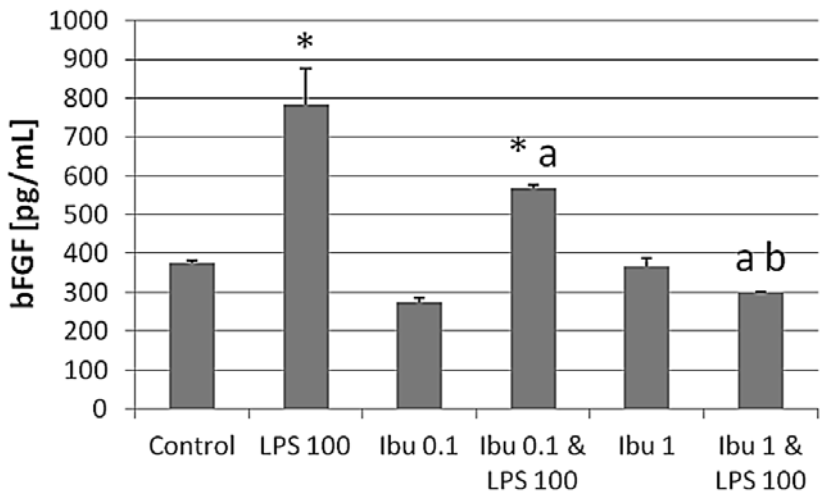

Figure 2. Effects of ibuprofen (0.1 and 1 mM) on bFGF levels in HMEC-1 cells in the presence of LPS (100 µg/mL). Bars represent the means (±SEM of 3-5 experiments). *$p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL); b – $p < 0.05$ *vs.* LPS (100 µg/mL) & ibuprofen (0.1 mM). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

The effect of ibuprofen on bFGF secretion under inflammatory conditions in HMEC-1 cells

Ibuprofen at concentrations of 0.1 and 1 mM had no effect on bFGF secretion (Fig. 2). The addition of 0.1 and 1 mM ibuprofen decreased the secretion of LPS-induced bFGF by 28 and 62%, respectively. The observed effects were statistically significant ($p < 0.05$). Simultaneously, 1 mM ibuprofen decreased the concentration of LPS-stimulated bFGF in cell culture media to a greater degree (by 48%) than 0.1 mM ibuprofen. This effect was also statistically significant ($p < 0.05$). Although 1 mM ibuprofen inhibited the secretion of bFGF in the presence of LPS in tested cells in comparison to control, this effect was not statistically significant.

The effect of ibuprofen on cell viability and proliferation in HMEC-1 cells

The next set of experiments analyzed cell viability based on MTT (Fig. 3), and proliferation by [^{3}H]-thymidine test (Fig. 4). LPS at a concentration of 100 µg/mL increased HMEC-1 cell proliferation by 8%, but only increased cell viability by 32%. Only LPS was found to have a statistically significant influ-

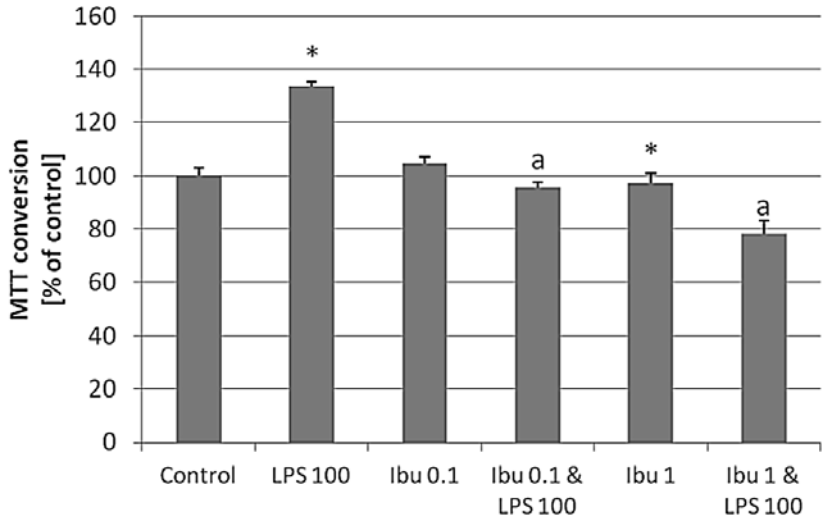

Figure 3. Effects of ibuprofen (0.1 and 1.0 mM) on cell viability of cultured HMEC-1 cells. The results are presented as a percentage in relation to the control value. Bars represent the means (±SEM of 4-15 experiments). * $p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

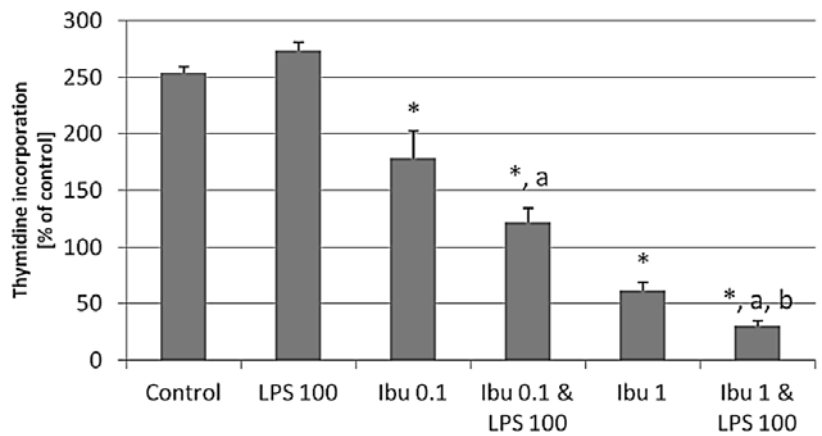

Figure 4. Effects of ibuprofen (0.1 and 1.0 mM) on thymidine incorporation in HMEC-1 cells. Bars represent the means (±SEM of 3-5 experiments). * $p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

ence on cell viability ($p < 0.05$). Ibuprofen did not affect cell viability at 0.1 mM, but decreased it in a statistically significant manner when used at 1 mM. Both concentrations (0.1 and 1 mM) of ibuprofen inhibited proliferation of cells by 76 and 30%, respectively ($p < 0.05$). Moreover, 0.1 and 1 mM ibuprofen inhibited LPS-induced proliferation by 52 and 89% and cell viability by 35 and 51%, respectively. These results were statistically significant ($p < 0.05$).

DISCUSSION

Our previous studies have shown that hypoxia (evoked by 3% O_2) increase the concentration of VEGF in human microvascular endothelial cells (HMEC-1), therefore in this study $CoCl_2$ is used inducing chemical hypoxia (15, 17-20) for comparison with LPS. In this study, bacterial lipopolysaccharide (LPS) is derived from *Salmonella enteritidis* cell walls and it causes the release of inflammatory cytokines. LPS is a less potent stimulator of VEGF secretion than hypoxia and, in contrast to hypoxia, stimulates the secretion of bFGF (17, 18, 20). The aim of this work was to determine the effect of ibuprofen, a non-selective COX inhibitor that inhibits the formation of prostanoids, on the secretion of VEGF under hypoxia and inflammatory conditions, as well as bFGF generation under inflammatory conditions.

Ibuprofen was found to induce production of VEGF and intensify the secretion of LPS-induced VEGF in a statistically significant manner ($p < 0.05$) (Fig. 1). The formation of VEGF is regulated by hypoxia and HIF (hypoxia induced factor) complex (19, 21, 22). NSAIDs have been observed to inhibit hypoxia-induced angiogenesis *via* increasing expression of the VHL tumor suppressor, reducing accumulation of HIF-1α, and consequently decreasing the secretion of VEGF in rat gastric microvascular endothelial cells, although non-selective COX inhibitor was not found to weaken the effect of LPS and $CoCl_2$ on VEGF secretion (21). These observations can be explained according to Palayoor et al. (23), who report that, when 2 mM ibuprofen is used in prostate cancer cells under hypoxic and normoxic conditions, a significantly higher concentration of ibuprofen is needed to inhibit the synthesis of HIF-1α and HIF-regulated gene products (VEGF) than prostaglandins (23). In the present study, ibuprofen used at the maximum concentration of 1 mM did not in fact inhibit VEGF secretion, but even potentiated it.

In a previous study, hypoxia was not seen to have an effect on bFGF level in HMEC-1 cells, although bFGF secretion was stimulated by LPS (20). Ibuprofen, used at both concentrations, inhibited the secretion of bFGF in the presence of LPS in tested cells to a statistically significant degree (Fig. 2.). Ibuprofen decreased level of LPS-induced bFGF in cell culture media to a greater degree when used at 1 mM than 0.1 mM ($p < 0.05$). Akarasereenont et al. demonstrated that LPS, the inflammatory mediator, is responsible for stimulation of COX and prostaglandin production in bovine aortic endothelial cells (BAEC) (24). Thus, the application of COX inhibitor reduces bFGF levels in HMEC-1 cells, which may indicate on participation of inflammation in the bFGF synthesis. The effect of ibuprofen on the secretion of bFGF may be reflected in the cardiovascular risk of NSAIDs, since bFGF (pro-angiogenic factor) is responsible for regeneration after myocardial infarction (11, 12).

According to the MTT and thymidine tests, the incubation of endothelial cells with 100 µg/mL LPS significantly stimulated cell survival (Fig. 3.) and proliferation (Fig. 4) ($p < 0.05$). Ibuprofen used at concentrations of 0.1 and 1 mM inhibited cell viability and the proliferative effect of 100 µg/mL LPS in comparison with the control and endothelial cells incubated with LPS. The cells exposed to LPS were found to release cytokines which activate COX-2. The products, prostaglandins, are responsible for endothelial cell proliferation (25, 26), COX inhibitors such as ibuprofen prevent their synthesis and hence, cell proliferation. Some studies have shown that sulindac and celecoxib inhibit the survival of endothelial cells and even induce their apoptosis (27, 28). Low concentrations of aspirin, which is used as an anti-aggregation drug, protect BAEC from apoptosis, while at relatively higher concentrations, when used as an anti-inflammatory, they induce apoptosis in endothelial cells (26). Taken together, the results imply that the inhibition of endothelial cell viability by high doses of NSAIDs lead to impaired endothelium function, which may have further consequences in terms of cardiovascular risk.

CONCLUSIONS

The obtained findings demonstrate that ibuprofen at concentrations of 0.1 and 1 mM both stimulates VEGF secretion and it increases bFGF reduction in concentration-dependent manner under inflammatory conditions. Ibuprofen decreases proliferation and cell viability induced by LPS in a concentration-dependent manner. The observed effects of ibuprofen on endothelial cells may further explain

its effects as well as other NSAIDs on the cardiovascular system function in cardiovascular diseases.

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