

Fibrates and Endothelium

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Abstract: Fibrates are a group of drugs which have long been used in the treatment of dyslipidemia patients. Lipid homeostasis disturbances are cardiovascular risk factors of high incidence among the population. In recent years the mechanism of fibrate action has been considerably investigated. Knowledge of the mechanisms of atherosclerotic plaque initiation and progression has increased, which enables the development of new drugs that would affect inflammatory pathways more effectively.

Key words: fibrates, endothelium, atherosclerosis, PPAR

Fibric acid derivatives known as fibrates are the class of drugs which has been used in monotherapy for quite a long time to treat patients with disturbances in lipid homeostasis, in particular associated with elevated levels of triglycerides, low level of high density lipoprotein (HDL) and normal or slightly increased concentration of low density lipoprotein (LDL) in plasma. These components are known as atherogenic dyslipidemia and are characteristic for patients with metabolic syndrome, which also includes hypertension, insulin resistance, glucose homeostasis disorders and visceral adiposity, and leads to a significant increase of the risk of atherosclerosis. Due to actual recommendations it is also possible to add fibrates to statins in treatment of people with severe dyslipidemia who present a big risk of coronary heart disease.

In addition to their beneficial effects in the normalization of lipid homeostasis disorders (mainly through lowering of plasma triglycerides concentration and moderate increase of HDL), fibrates may also influence inflammatory pathways by inhibiting endothelial and hepatic production of proinflammatory cytokines, modulate factors promoting thrombosis, improve insulin sensitivity and have a positive impact on carbohydrate metabolism. Due to their pleiotropic functions, fibrates may prevent initiation or progression of atherosclerotic plaque and also stabilize those that exist, and consequently decrease the risk of myocardial infarction. It has been reported that this class of drugs also reduces the risk of ischemic stroke [1].

Study data suggest that the pleiotropic effects of fibrates depend largely on their influence on peroxisome proliferator-activated receptors (PPARs). There are 3 main subtypes of PPARs: α , β/δ and γ . PPAR α are present in the liver, kidneys, skeletal muscles, and in the cardiovascular system: in endothelial cells, smooth muscle cells, as well as on macrophages located in the vascular wall [2]. They play an important role in the regulation of the process of fatty acids β -oxydation, production of proteins involved in vascular inflammation, creation and stabilization of atherosclerotic lesions, and modulation of factors which promote thrombosis [3].

PPAR γ are mainly present in adipose tissue, but are also present in monocytes, macrophages and foam cells, lymphocytes, β -cells in the pancreatic islets, endothelium, and smooth muscle cells in blood vessels. They take part in the process of adipocytes proliferation and differentiation, intake and storage of fatty acids, the insulin-regulated process of transporting glucose into skeletal muscle cells and adipose tissue and improve insulin sensitivity, but they also exert anti-inflammatory and anti-atherogenic effects [4].

PPAR β/δ are so far the least known subtype of PPARs. They exist in skeletal muscles and heart tissue, probably take part in the activation of fatty acids catabolism, and play a role in the adaptation to environmental changes, such as intensive physical exercise, by controlling the number of oxidative myofibers [5, 6]. Additionally, they have also been found in blood vessels [7]. Natural agonists of this subtype of PPARs are fatty acids derivatives such as hydroxyoctadecadienoid acids and prostaglandins derivatives such as 15-deoxy Δ -prostaglandin I₂ [7]. Treatment with agonists of this subtype of receptors in mice with diet induced obesity leads to overweight reduction and decrease in insulin resistance [8].

Natural agonists of PPAR α and γ are polyunsaturated fatty acids and eicosanoids contained in quite a large amount in fish oil [9]. Fibric acid derivatives include fenofibrate, bezafibrate, ciprofibrate, clofibrate and gemfibrozil, but only fenofibrate is currently widely used in the treatment of people with dyslipidemia. Bezafibrate binds to a comparable degree with all 3 subtypes of PPARs, whereas clofibrate and fenofibrate are dual agonists of PPAR α and γ with a 10-fold higher affinity to α receptors [2, 3]. Drugs belonging to thiazolidinediones, such as rosiglitazone or pioglitazone, are receptor γ agonists.

PPARs belong to the nuclear receptors family of transcription factors. After binding with ligand they heterodimerize, with retinoid receptor X. PPAR being constructed out of an COOH-terminal domain, which decides the ligand specificity, a domain for a cofactor, a domain for the binding with the element in response to PPAR (PPRE), which is localised in the promoter region of target gene, and an NH₂-terminal domain which determines PPARs activity. When inactive, PPAR is bound to the corepressor protein. Due to the action of PPAR agonists the receptor dissociates from corepressor and, in the presence of coactivators—for example, steroid receptor coactivator-1 or PPAR binding protein—it heterodimerizes with retinoid receptor X [2]. Next, such a formatted dimer binds with response elements to PPAR (PPRE) located in the promoter of the target gene and

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Received: 20 November 2007; accepted: 17 December 2007

takes part in regulation of transcription. Data suggest that PPRE consists of direct repetition of 6 nucleotides: AGGTCA, which is half-spaced by 1-2 additional nucleotides (DR 1 or DR 2) [7]. In this way, mainly processes involved in lipid and carbohydrate homeostasis are regulated [2].

It is possible that the regulation of inflammatory processes acts through interactions between PPARs and other transcription factors. This process does not require the binding of the receptor with a ligand [2, 7] and acts through inhibition of the nuclear factor $\kappa\beta$ (NF- $\kappa\beta$) and activator protein-1 (AP-1), and also through the induction of apoptosis [3, 10, 11]. It has also been reported that PPARs activity may be regulated through phosphorylation and interactions with cofactors such as zinc [11, 12]. NF- $\kappa\beta$ and AP-1 are transcription factors controlling the production of the majority of adhesion molecules and cytokines, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and interleukin-6 (IL-6) by the endothelium [3, 10, 11].

The expression of VCAM-1 and ICAM-1 on the surface of endothelial cells is known as the inflammatory activation element. These molecules, both selectin P and E, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), take part in the recruitment of leukocytes from the blood flow. The site of adhesion of leucocytes to the endothelium is defined by particles such as chemokine RANTES and platelet factor-4 (PF-4). This process is also regulated by lipid mediators- platelet activating factor (PAF) and cysteinyl leucotrienes (cysLT) [13]. Migration of monocytes through the vessel wall is an important element improving the initiation of atherosclerotic plaque. Transcription of genes for chemoattractant factors which attract monocytes to the vessel wall depends on NF- $\kappa\beta$ activation [3]. It has been reported that an elevated level of free fatty acids, insulin resistance, and obesity elements, which are characteristic for metabolic syndrome, lead to inflammatory activation through NF- $\kappa\beta$ pathways [3, 14].

It is possible that PPARs need the presence of zinc to inhibit pro-inflammatory cytokines expression. It has been reported that after removing zinc from porcine pulmonary artery endothelial cells by a chelating substance, and following 2-hour pretreatment with fenofibrate, ciprofibrate, troglitazone or thiazolidinedion, all these drugs lost their anti-inflammatory properties, determined by measurement of binding with NF- $\kappa\beta$ and AP-1 capacity and the level of transcription of inflammatory genes such as VCAM-1 and IL-6 mRNA after activation by tumor necrosis factor α (TNF α). After adding zinc again, all the drugs significantly decreased TNF α induced expression of pro-inflammatory transcription factors- NF- $\kappa\beta$ and AP-1, and also inhibited VCAM-1 and IL-6 gene expression [11].

Applying lipopolysaccharide (LPS) to mice without PPAR α led to enhanced inflammatory response, which did not decrease after treatment with fibrates [15].

PPAR α activation reduces the production of endothelin-1 [2], which is a strong vaso-constricting factor. Increased release of endothelin-1 may be induced by epinephrine, thrombine, vasopressin, angiotensine II, cytokines, growth factors, reactive species of oxygen, hypoxia and leptin. Hemodynamic shear stress and nitric oxide (NO) reduce ET-1 production. ET-1 stimulates type A and B endothelin receptors. Type A receptors are situated in the arteries and veins of the systemic and pulmonary circulation systems, and their activation leads to vasoconstriction. Type B receptors are located mainly on

smooth muscle cells in pulmonary vessels and veins – where their stimulation also leads to vasoconstriction – and on epithelial cells. Activation of type B endothelin receptors results in an increase of NO and prostacycline production and vasodilatation. The final result of endothelin-1 activity is therefore the consequence of its impact on both types of receptors on smooth muscle cells and type A of receptors located on the endothelium, but predominantly endothelin is considered a vasoconstricting substance. It has additionally been reported that endothelin may cause smooth muscle cells proliferation [16, 17].

A potential neuroprotective action of chronic fibrate treatment has been investigated. Data suggest that long-term treatment with PPAR α agonist increases the activity of antyoxidative enzymes such as copper/zinc dismutase, glutathione reductase, glutathione peroxidase and glutathione S-transferase. It has been proved that treatment with fenofibrate inhibits enhanced expression of VCAM-1 and ICAM-1 in the animal brain submitted to 1-hour ischemia. On the other hand, fenofibrate influence on the expression of nitric oxide syntase (NOS) in the ischemic brain of mice treated with this drug has not been proved. According to these data it is considered possible that PPAR α agonists may decrease ischemia-induced brain injury. It has been proved that this action does not depend on their influence on lipid homeostasis [1]. In contrast to these reports, some data suggest that PPAR on endothelium cells may be activated by oxidized phospholipids, which resulted in the increase of the MCP-1 and IL-8 expression level. This may suggest that PPAR can enhance inflammatory processes in certain conditions [18].

Vascular tone status depends on the balance between the vasoconstricting influence of the endothelin and vasodilating action of NO released from endothelial cells. NO is produced by endothelial nitric oxide syntase (eNOS) from L-arginine and molecular oxygen with the contribution of 2 important cofactors: tetrahydrobiopterin (BH₄) and nicotinamide adenine dinucleotide phosphate (NADPH) [19]. Hemodynamic shear stress enhances eNOS activity; however, LDL, angiotensine II and pro-inflammatory cytokines decrease its activity through shortening of the mRNA half-life period [10]. It has also been proved that there are binding AP-1, NF- $\kappa\beta$ and IL-6 sites in the promoter region of eNOS gene [20]. However, the presence of binding PPAR region has not been found in this location [21]. The eNOS activity is also regulated through post-translational modifications, probably by acylation and phosphorylation. Inactive eNOS is located in plasmalemmal caveolae and connected with caveoline which inhibits its activity. After the action of the activating agent through the G-protein receptor, the elevated intracellular calcium level causes binding of eNOS with calmoduline, dissociation from caveoline, and activation of eNOS. When the action of the activating factor ends, eNOS binds again with caveoline and becomes inactive [10].

Reduced production of NO by endothelial cells is characteristic of its dysfunction. Oxidative stress, dyslipidemia or diabetes mellitus may negatively interfere with NO synthesis by limited availability of BH₄ (due to its oxidation) and disturbances in balance between NADH and NADPH. Generated free peroxide radicals (O₂⁻) react rapidly with NO, which affects the synthesis of peroxyntirite (OONO⁻) and leads to further increasing of oxidative stress, reduced availability of NO and enhancement of endothelium dysfunction.

Antyoxidative enzymes prevent this, for example, superoxide dismutase converts O_2^- to hydrogen peroxide (H_2O_2) which is further metabolized into H_2O by catalase and glutathione peroxidase [19]. In conditions of intensive oxidative stress, the compensational mechanisms of free radicals neutralisation are insufficient, and the decrease in NO availability causes domination of vascular-constricting factors. Fibrates may also activate the receptor G protein and act through it by activating the transcripton of the NO gene factor. Moreover, due to reduction of endothelin-1 production they cause enhancement of eNOS activity through negative feedback [22, 23].

Fibrates may also influence endogenous inhibitors of the NOS level, such as asymmetric dimethylarginine (ADMA). It has been proved that pretreatment with fenofibrate of cultured endothelial cells limited the increase of ADMA, lactate dehydrogenase and TNF α after the incubation of human umbilical vein endothelial cells (HUVECs) with oxidative LDL. This protective effect of fibrate treatment disappeared after the adding of PPAR α antagonist [24].

Endothelium derived NO is responsible for postischemic flow-mediated vasodilatation. It has been proved that fenofibrate treatment of subjects with type 2 diabetes mellitus significantly improved brachial artery flow-mediated dilatation, but did not affect nitrogliceryn-mediated dilatation [25]. Data suggest that subjects with hypertriglyceridemia who received fenofibrate treatment significantly improved blood flow in the forearm in response to verapamil, nitroprusside or acetylcholine [26]. Bovine aortic endothelial cells (BAECs) treated for 48 hours with fenofibrate showed enlarged expression of eNOS mRNA and protein. This action was antagonized by PPAR α antagonist. However, the effect of fenofibrate on eNOS activity after 1 hour of pretreatment was not revealed. In accordance with this, it has been concluded that PPAR α agonists influence eNOS expression mainly through the stabilization of eNOS mRNA and have no effect on post-translational modification [27]. The beneficial effects of PPAR α on eNOS may be additionally enhanced by recoupling with an adequate amount of antioxidants. It has been reported that oral supplementation with coenzyme Q_{10} increased the endothelial function, normalizing the effect of fenofibrate in the treatment of people with type 2 diabetes mellitus, which is probably due to a scavenger oxidant species which lead to oxidative stress reduction [19].

It is also possible that fenofibrate can inhibit endothelial cells proliferation induced by angiogenesis factors, endothelial cells migration and angiogenesis *in vivo*, and in large concentrations may increase apoptosis. The inhibition of endothelial cells migration probably acts through the disorganization of the actin cytoskeleton. After that, fenofibrate significantly decreased the fibroblast growth factor induced by Akt activation and cyclooxygenase-2 gene expression [28].

Data suggest that fenofibrate inhibits the apoptosis of retina endothelial cells, which is not inhibited by PPAR α antagonist. These properties may be connected with the activation of AMP-activated protein kinase and vascular endothelial growth factor mRNA expression, and is independent of PPAR α activation. This suggests that fibrates may be used in future in the prevention of diabetic retinopathy [29].

PPAR α agonists decrease metalloproteinase 9 (MMP-9) expression [30]. Metalloproteinases are proteolytic enzymes secreted by inflammatory cells such as monocytes and their activity is improved by oxidative stress conditions and inhibited by metalloproteinase tissue inhibitors. They are

produced as inactive zymogenes and their activation depends on the processing of precursors [31]. Endothelial cells *in vitro* secrete MMP-1, MMP-2, MMP-3 and MMP-9 after stimulation by proinflammatory mediators such as IL-1 and TNF- α . It has been reported that C-reactive protein increases MMP-2 and 9 activity, possibly through CD-40, because HUVECs incubation with anti-CD antibodies prevented a CRP-induced increase of MMP-2 and 9 expression [6]. When activated, MMP-9 degrades collagen, elastin, fibronectin and integrines [32]. The domination of extracellular matrix degradation over its production can lead to atherosclerotic plaque destabilisation through the destruction of the fibrous cap and thrombosis activation. Metalloproteinase activation plays also an important role in unfavourable reconstruction of heart tissue in chronic heart failure. Fenofibrates reduce gene transcription, secretion and enzymatic activity of MMP-9 in macrophages [33]. This may be partially caused by the increase of NO synthesis by endothelial cells after PPAR α agonists activation [34].

It has been proved that HUVECs treatment with fenofibrate leads to a decrease of CD40 and CD40 ligand (CD40L) expression and limits the increase of MMP-2 and MMP-9 activity caused by C-reactive protein (CRP). This action is dose-dependent [6]. CD40 receptor is an integral membrane protein with a mass of 50 kD, which belongs to TNF receptor family. This receptor expression is present on antigen-presenting cells, endothelial cells, fibroblasts, and smooth muscle cells in blood vessels. CD 40 ligand is a membrane-bound protein with a mass of 39 kD and is also the member of TNF family. It is present on T-cells, mastocytes and activated platelets [6]. The interaction between CD40-ligand and its receptor located on endothelial cells activates the release of IL-8 and MCP-1 from endothelium. These molecules are important factors determining the migration of neutrophils and monocytes through the blood vessel walls. This interaction also increases the expression of VCAM-1, ICAM-1 and selectine E on endothelial cells surface, which causes further activation of inflammatory response and tissue factor expression leading to thrombosis activation [35].

Healthy endothelium secretes factors that prevent platelet activation, namely, prostacycline (PGI_2), NO and CD39. Inflammatory-activated endothelium is a site for platelet adhesion, not only because of decreased eNOS activity and NO and PGI_2 concentration, but also due to selectine P and the von Willenbrand factor (vWF) expression. The interaction between selectine P and vWF expressed on the surface of endothelial cells, and platelet glycoprotein (GP) Ib and P-selectin glycoprotein ligand-1 (PSGL-1) expressed on platelets, leads to platelets rolling along the surface of the blood vessel wall, followed by constant platelet adhesion to the endothelium, depending on GPIIb/IIIa [36]. Activated platelets release many cytokines, such as IL-1 β , CD40L, β -thromboglobuline, and the following growth factors: platelet-derived growth factor (PDGF), transforming growth factor- β , fibroblasts growth factor, as well as chemokines: regulated on activation normal T expressed and activated (RANTES), platelet factor-4 (PF-4), macrophage inflammatory protein (MIP)-1 α and pro-coagulative factors (plasminogen activator's inhibitor-1, PAI-1) [36]. Chemokine RANTES with the participation of platelet factor-4 determines monocytes adhesion site on the endothelium, and the monocytes then adhere to the endothelium with the participation of platelet's selectine P. Inflammatory-activated monocytes release the

cytokines IL-8, TNF- α , chemokines MCP-1, MIP-1 α and tissue factor into blood [35, 36].

Data suggest that fibrates significantly decrease PAI-1 level in cultured human cells [6] and decrease the fibrinogen level, probably due to fibrinogen gene repression through PPAR α [37, 38, 39]. Fenofibrate treatment leads to an increased plasma level of homocysteine. Hiperhomocysteinemia may be caused by deficiency of Vitamin B₆, Vitamin B₁₂ and folic acid, and genetic reasons such as enzymatic deficiency of cystathione β -synthase or methylene-tetrahydrofolate reductase [40]. Hiperhomocysteinemia may also be the result of fenofibrate treatment. Data suggest that an elevated level of homocysteine may enhance the inflammatory activation of the endothelium and oxidative stress. It has been reported that it is possible to prevent fenofibrate treatment induced hiperhomocysteinemia through oral supplementation with folic acid. Data suggest that fenofibrate in monotherapy induces a significant decrease of LDL, and a 39,5% increase in the homocysteine plasma level; however, it has no influence on oxidized LDL, vWF and thrombomoduline. During combined therapy with fenofibrate and a 10 mg dose of folic acid, the level of homocysteine remained unchanged; nevertheless, the levels of vWF and thrombomoduline decreased [40].

PPAR α agonists may influence hepatic inflammation response through the regulation of CRP, fibrinogen, serum amyloid A (SAA) and α 2 macroglobulin expression in the liver. It has been proved that fibrate treatment decreases the level of these proteins, probably through the action on PPAR α . TNF α , IL-1, and IL-6 gene transcription is regulated by many transcriptional factors, such as CCAAT-Enhancer Binding Protein (C/EBP) Signal Transducer and Activator of Transcription Protein (STAT), NF-K β complex and activator protein-1 (AP-1) complex, including c-fos and c-jun. Cofactors which remodel chromatin and bridge the DNA-bound transcription factors to the transcription machinery, have an influence on transcriptional activity. Due to their action on PPAR α , fibrates may decrease the availability of cofactors, e.g. by binding with GRIP1/TIF2, a cofactor for C/EBP- β , and in this way limit IL-6 gene transcription [6].

PPAR α may also limit the activity of the CRP gene promoter. They decrease the expression level of p50 element of NF-K β and C/EBP- β [6]. The regulation of transcriptional factors activity may also act through their phosphorylation. It has been reported that fenofibrate decreases the level of phosphorylated c-jun [41].

PPAR α can also weaken the inflammatory response mechanisms by binding with p65 element of NF-K β , c-jun, which converts them into inactive complexes. These inactive complexes have a negative influence on the process of transcription regulated by NF-K β and AP-1. The activity of NF-K β is also regulated by IK β , which sequesters NF-K β in the cytoplasm. The expression of IK β is enhanced by PPAR α , which limits the translocation of p50 and p65 proteins, which are included in NF-K β [6].

Data suggest that PPAR α agonists improve insulin-sensitivity and decrease body mass in rodent models. PPAR α and γ agonists increase the density of adiponectin receptors such as Adipo R2 [42]. These properties can be used in the treatment of people with metabolic syndrome. It has been proved that the adiponectin level has an independent influence on flow-mediated dilatation. The increase of adiponectin level leads to the improvement of insulin-sensitivity and prevents arteriosclerosis [3]. Fibrates are used in the monotherapy

of people with hypertriglyceridemia, low HDL and normal or slightly increased levels of LDL. Many of these patients also present other components of metabolic syndrome, such as visceral adiposity, insulin resistance, hyperinsulinemia, glucose homeostasis disorders, hypertension, increased level of inflammatory cytokines, and enhanced prothrombotic activity. Fibrates are also used with statins in combined treatment of patients with dyslipidemia and a large risk of coronary heart disease. It has been proved that during the careful monitoring of patients, the combination of fenofibrate and statin is a safe method of treatment, only to a small degree increases the risk of adverse effects, such as rhabdomyolysis or liver dysfunction. As a result, the combined treatment is an alternative for patients in whom statin in monotherapy does not affect the other components of atherogenic dyslipidemia, despite LDL reduction. Many reports demonstrated that fibrates have an anti-inflammatory action apart from lipid homeostasis disorders normalization. Their effect on coagulation, however, is less certain and is still being investigated. It is known that fibrates owe a considerable part of their properties to their action on nuclear receptors PPAR α , which are present not only in the liver, kidneys and skeletal muscles, but also in the cardiovascular system, endothelium, smooth muscle cells, and vessel wall macrophages. Fibrates reduce the production of VCAM-1, ICAM-1, IL-6 by the endothelium, probably increase the activity of antyoxidative enzymes, indirectly increase NO availability, and improve artery flow-mediated dilatation. Data suggest that these drugs may also inhibit endothelial cell proliferation and migration induced by angiogenesis factors and, in large concentrations, increase apoptosis [28]. It has been reported that they also inhibit the apoptosis of retina endothelial cells [29]. It is also possible that they have neuroprotective properties [1].

Fibrates may also improve insulin-sensitivity, which is also characteristic of PPAR γ agonists. Due to the prediction that in the 21st century there will be an increase in the incidence of metabolic syndrome and its significant influence on the risk of coronary heart disease, studies on dual PPAR α and γ agonist are in progress. One such substance is TAK-559, which decreased the proliferation and migration of smooth muscle cells, the recruitment of macrophages, and causes a significant reduction of intimal thickening in hypercholesterolemic rabbits [43, 44].

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