Current Research in Translational Medicine xxx (2016) xxx–xxx

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Original article

Protective effects of bezafibrate against elaidic acid-induced accumulation of lipid droplets in monocytic cells

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ARTICLE INFO

Article history:
Received 1st April 2016
Accepted 5 August 2016
Available online xxx

Keywords:
Elaidic acid
Oleic acid
Palmitic acid
Lipid droplets
Bezafibrate

ABSTRACT

Some factors related to diet, such as trans fatty acids (TFA), are known to be involved in the progression of atherosclerosis in humans. Thus, the aim of our study was (i) to evaluate the effects of three dietary free fatty acids (FFA) (elaidic (EA), oleic (OA) and palmitic acid (PA)) on U937 human monocytes, and (ii) to study the eventual benefits of bezafibrate (BZF), a pan-agonist for PPAR isoforms (α, γ and δ) in U937 cells treated with FFA. Morphologic and functional changes were investigated by microscopic and flow cytometric methods. Cellular lipid content, lipid droplets and FA composition were identified and studied. All analyses were also realized in association with or without BZF. Contrary to OA and PA, EA slightly induced both propidium iodide-positive cells and mitochondrial depolarization. In addition, in contrast to OA and PA, EA induced only a slight increase in superoxide anion production. However, EA and OA promoted cytoplasmic lipid droplets accumulation. Only EA and OA significantly increased CD36 expression. It is noteworthy that BZF had a more or less pronounced protective effect against EA-, OA- and PA-induced side effects: BZF attenuated the impaired cell viability and inflammatory response, decreased superoxide anion production and prevented the accumulation of neutral and polar lipids. The effects were less pronounced with OA and PA than with EA. Altogether, our data revealed a benefit of BZF on the side effects induced especially with EA. It may thus be of interest in preventing the early stages of atherosclerotic plaque formation.

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1. Introduction

The relationship between diet and coronary artery disease has been studied intensively for nearly a century and fatty acid (FA) lipotoxicity has gradually become a new field of interest. Free fatty acids (FFA) may play an important role in promoting monocyte differentiation and foam cell formation. In nature, the predominant geometry displayed by unsaturated fatty acids (UFA) is *cis*. Oleic acid (C18:1 *cis* 9, OA) is one of the most important UFA described [1]. However, FAs can take on another geometry, known as *trans* fatty acids (TFA), due to industrial processing and during the partial hydrogenation ('hardening') of vegetable fat. Elaidic acid (C18:1 *trans* 9, EA), the *trans* 9 isomer of OA, is the predominant industrial TFA [1,2] and is found in a variety of food products, especially baked goods, snacks, fried foods, fast foods, creams and margarines. The consumption of industrial TFA has different effects on cardiovascular risks, including the induction of pro-inflammatory cytokines, the dysfunction of endothelial cells and increased levels of serum pro-atherogenic lipids [3,4]. Different studies suggest an association between an increased risk of coronary artery disease (CAD) and a high dietary intake of TFA [5,6]. Some epidemiological studies suggest that the consumption of TFA, especially EA, might increase the risk of CAD more than do saturated fatty acids (SFA), but the mechanisms of their atherogenicity are still controversial [7,8]. In addition, recent studies reported that the origin of TFA might modulate their deleterious effects. Natural ruminant TFA in dairy products appear to be innocuous or may even have a protective effect against CAD [8,9]. Industrially produced TFA, however, were found to be clearly atherogenic [6].

Atherosclerosis is an inflammatory disease characterized by the accumulation of lipids and their metabolites in the arterial wall. Monocytes/macrophages play a crucial role in the inflammatory

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http://dx.doi.org/10.1016/j.retram.2016.08.001
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Please cite this article in press as: Haj Ahmed S, et al. Protective effects of bezafibrate against elaidic acid-induced accumulation of lipid droplets in monocytic cells. Curr Res Transl Med (2016), http://dx.doi.org/10.1016/j.retram.2016.08.001

process and in the development and progression of atherosclerosis [10]. They are known to be among the first cells present in lesion areas, and they accumulate during plaque formation. They are characterized by rapid differentiation into tissue macrophages in the vascular intima, after exposure to a variety of signals. The infiltration of monocytes into the sub-endothelial space of large arteries and their phagocytosis of local lipids are major steps in atherogenesis. These are followed by differentiation into monocyte-derived foam cells, which are a source of large inflammatory cytokines and free radicals that promote inflammation and atherosclerosis [11]. These steps contribute to a vicious cycle and are known to directly result in the development of myocardial infarction and stroke [12]. The anti-inflammatory action of PPARs, especially PPAR-α, has been highlighted [13]. Fibrates, which are synthetic PPAR-α ligands, are widely used as lipid lowering drugs and they contribute to lipid control and immune regulation [14]. They can suppress the expression of inflammatory mediators induced by cytokines in various types of cells [14,15]. Activators of PPAR-α are capable of reducing cholesterol accumulation through the activation of genes involved in cholesterol homeostasis [16] as well as in ox-LDL uptake [17]. All fibrates are PPAR-α agonists. However, bezafibrate (BFZ) has a unique characteristic profile of action. It can activate all three PPAR subtypes (α, γ and δ) [18]. Therefore, BZF operates as a pan-agonist for all three PPAR isoforms.

The aim of the present study was:

- to compare the biological activities of three dietary FFA, namely the monounsaturated fatty acid, oleic acid, (OA) which is naturally present in food, the TFA, elaidic acid (EA), which is a major component in industrially produced TFA, and the saturated fatty acid, palmitic acid (PA);
- to determine the eventual benefit of bezafibrate (BZF).

These activities were tested on U937 human monocytes.

2. Materials and methods

2.1. Cells and treatments

Human monocytic leukaemia cells (U937) obtained from the American Type Culture Collection (Manassas, VA, USA) were used. U937 cells were grown in RPMI 1640 medium with GlutaMAX I (Gibco, Eragny, France) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) (Invitrogen, Cergy-Pontoise, France) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco). Cells were seeded at 100,000/mL of culture medium, passaged twice a week, and incubated at 37 °C under a 5% CO_2/95% air atmosphere. The BZF, EA, OA and PA (Sigma-Aldrich) were solubilized in ethanol (Sigma-Aldrich). The final maximum concentration of ethanol (vehicle) in the culture medium was 0.1%. At this concentration, ethanol did not affect cell growth characteristics [19]. U937 cell cultures were first exposed to 35 μM of BZF during one night, then to 140 and 210 μM of EA, OA or PA either alone or with BZF for 24 h.

EA concentrations were chosen according to measurements made on the plasma of healthy subjects (EA, less than 100 μM) and of patients with coronary artery disease (EA from 100 to 400 μM). The same concentrations of OA and PA were used to compare their effects with those of EA. BZF was chosen among other PPAR agonists due to its remarkable effect in U937. At a concentration of 35 μM, BZF had no toxic effect on cell growth characteristics.

2.2. Flow cytometric evaluation of cell death and/or increased permeability of cytoplasmic membrane with propidium iodide

Cell viability and/or increased permeability of cytoplasmic membrane was determined with propidium iodide (PI; $\lambda_{Excitation\ max}$: 540 nm, $\lambda_{Emission\ max}$: 625 nm; Sigma). A stock solution of PI was prepared in phosphate-buffered saline (PBS) at a concentration of 100 μg/mL and kept in the dark at room temperature. PI was used at a final concentration of 1 μg/mL on the cell suspension. Red fluorescence of PI was detected through a 625 ± 20 nm band pass filter, and fluorescent signals were measured on a logarithmic scale. For each sample, 10,000 cells were acquired and data were analyzed using Flomax (Partec) or Flow Jo (Tree Star Inc) software.

2.3. Staining conditions with Hoechst 33342

Nuclear morphology was analyzed by fluorescence microscopy after staining with Hoechst 33342 (10 μg/mL). It was prepared extemporaneously in distilled water at 1 mg/mL and was added to the culture medium at a final concentration of 10 μg/mL. After 2 h of incubation at 37 °C, cells were washed twice in PBS and re-suspended at a concentration of 10^6 cells/mL in PBS containing 1% (w/v) paraformaldehyde. Cell deposits of approximately 40,000 cells were applied to glass slides by cytocentrifugation for 5 min at 15000 rpm with a cytospin 2 (Shandon, Cheshire, UK), mounted in buffered glycerin (BioMerieux, Marcy L'Étoile, France), and coverslipped. Viable cells are characterized by round and regular nuclei, apoptotic cells by condensed and/or fragmented nuclei, and necrotic cells by nuclei with irregular sizes and shapes. Cell deposits were observed under ultraviolet light by fluorescence microscopy with an Axioskop upright microscope (Zeiss). For each sample, at least 300 cells were examined with an × 63 oil immersion objective.

2.4. Flow cytometric measurement of transmembrane mitochondrial potential with $DiOC_6(3)$

Variations of the mitochondrial transmembrane potential ($\Delta\Psi_m$) were measured by flow cytometry with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$ (Invitrogen)), which allows the percentage of cells with a low $\Delta\Psi_m$ to be determined. With $DiOC_6(3)$, mitochondrial depolarization was indicated by a decrease in green fluorescence collected through a 520/10 nm band pass filter. $DiOC_6(3)$ was used at 40 nM [20]. This cyanine dye was initially prepared at 1 mM in DMSO (Sigma) and further diluted in distilled water in order to obtain an intermediate concentration of 20 μM. To obtain a final concentration of 40 nM, 2 μL of this intermediate solution were added to cell suspensions adjusted to 1×10^6 cells/mL, and after 15 min of incubation at 37 °C, $DiOC_6(3)$, mitochondrial transmembrane potential related fluorescence was immediately recorded by flow cytometry with a GALAXY flow cytometer (partec). Then, 10,000 cells were acquired for each sample. Data were analyzed with Flomax software (Partec).

2.5. Flow cytometric measurement of the intracellular production of superoxide anions with dihydroethidium

Overproduction of reactive oxygen species (ROS), mainly superoxide anions ($O_2^{\bullet-}$), was detected with dihydroethidium (DHE; Invitrogen/Life Technologies), which is a non-fluorescent compound that can diffuse through cell membranes, and is rapidly oxidized in hydroethidine (HE) under the action of $O_2^{\bullet-}$ [21]. DHE was initially prepared at 10 mM in dimethyl sulfoxide (DMSO) [22] and used at a final concentration of 2 μM on cell samples of 1×10^6 cells/mL of RPMI 1640 medium. Cells were incubated for 15 min at 37 °C before flow cytometric analysis. The fluorescent signals were collected through a 580 ± 20 nm band pass filter on a logarithmic scale of four decades of log on a GALAXY flow cytometer (Partec). For each sample, 10,000 cells were acquired. Data were analyzed with Flomax software (Partec).

2.6. Measurement of tumor necrosis factor-alpha (TNF-α) and monocyte chemotactic protein-1 (MCP-1) secretion by ELISA

To measure tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) secretion, U937 cells were incubated for 24 h with EA, OA and PA in the absence (control) or in the presence of ethanol (vehicle (1 mg/mL)). The EA, OA or PA were used at 140 and 210 μM in association with or without 35 μM of BZF. At the end of the incubation time, the culture medium was collected by centrifugation and stored at −80 °C. Samples were thawed just before ELISA, which was carried out according to the manufacturer's procedure (Bender MedSystems, Vienna, Austria) [23]. Human plasma containing or not TNF-α and MCP-1 was used as the positive and negative control, respectively.

2.7. Staining conditions with Nile Red

Nile Red (NR) is a phenoxazine dye used on living cells to localize and quantify neutral and polar lipids (Sigma). NR stains neutral lipids yellow (570–590 nm) and polar lipids orange/red (590 nm and above), when excited at 488 nm [24]. When excited at 532 nm, NR can identify polar lipids, which are colored orange/red [25]. In the present investigation, NR was prepared at 100 μg/mL in DMSO and used at a final concentration of 0.1 μg/mL as previously described [25]. After 15 min of incubation at 37 °C, flow cytometric analyses of NR stained cells were immediately performed on a Galaxy flow cytometer. For each sample, 10,000 cells were acquired and analyzed with Flowmax software.

2.8. Transmission electron microscopy

For transmission electron microscopy, 10×10^6 cells were fixed for 30 min at room temperature with a mixture of glutaraldehyde (1.5%) and paraformaldehyde (4%) prepared in a 0.1 M of phosphate buffer solution (pH 7.3), post fixed in osmium

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