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Original research article

Exenatide (a GLP-1 agonist) expresses anti-inflammatory properties in cultured human monocytes/macrophages in a protein kinase A and B/Akt manner

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ABSTRACT

Background: Incretin-based therapies in the treatment of type 2 diabetes mellitus are associated with significant improvements in glycemic control, which are accompanied by a beneficial impact on atherosclerosis. Macrophages are essential in the development of atherosclerotic plaques and may develop features that accelerate atherosclerosis (classically activated macrophages) or protect arterial walls against it (alternatively activated macrophages). Therefore, we explored whether beneficial actions of exenatide are connected with the influence on the macrophages' phenotype and synthesis of inflammatory and anti-inflammatory cytokines.

Methods: Monocytes/macrophages were harvested from 10 healthy subjects. Cells were cultured in the presence of exenatide, exendin 9-39 (GLP-1 antagonist), LPS, IL-4, PKI (PKA inhibitor) and triciribine (PKB/Akt inhibitor). We measured the effects of the above-mentioned compounds on markers of macrophages' phenotype (inducible nitrous oxide (iNOS), arginase 1 (arg1) and mannose receptors) and concentration of nitrite, IL-1β, TNF-α and IL-10.

Results: Exenatide significantly increased the level of IL-10 and decreased both TNF-α and IL-1β in LPS-treated monocytes/macrophages. Furthermore exenatide increased the expression of arg1–a marker of classical activation and reduced the LPS-induced expression of iNOS–a marker of classical activation. According to experiments with protein kinases inhibitors we found that proinflammatory markers were protein kinase A dependent, whereas the activation of alternative activation was similarly reliant on protein kinase A and B/Akt.

Conclusions: We showed that exenatide skewed the macrophages phenotype toward anti-inflammatory phenotype and this effect is predominantly attributable to protein kinase A and to a less extent to B/Akt activation.

Introduction

Incretin-based therapies are becoming increasingly important in the treatment of type 2 diabetes mellitus. Very promising hypoglycemic results are obtained during treatment with GLP-1 agonists (e.g. exenatide, liraglutide), however, clinical effects seem to stretch beyond the glucose-lowering properties of these drugs. Currently, ongoing long-term studies should provide us with the answer whether the on-top therapy with exenatide will reduce the rate of cardiovascular events (e.g. EXCEL study). The background for improved cardiovascular outcomes may stem from improvements in atherosclerosis during incretin-based therapies that were showed both in *in vitro* and *in vivo* studies [1,2]. These results seem to be connected with the reduction of local inflammation, which takes place in peripheral fat deposits and arterial wall [3]. The above-mentioned phenomena are connected with macrophages activity, which contributes greatly to the development of atherosclerotic plaques. Depending on features of these cells, they are divided into several subpopulations. M1 macrophages (classically activated) are connected with an inflammatory state that deals with invading microbiota but on the other hand it is responsible for a low-level inflammation in arterial walls leading

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to atherosclerosis [4]. In *in vitro* studies these cells are formed using LPS or IFN gamma stimulation and are characterized by high level of reactive oxygen species (ROS) and NO synthesis that is accompanied by an increase in inducible nitrous oxide synthase (iNOS) and elevated expression of pro-inflammatory cytokines, such as IL-1β and TNF-α [5]. On the other side of macrophages' phenotypic continuum there are M2 (alternatively activated) macrophages that are thought to have anti-inflammatory and anti-atherogenic properties. Physiologically, in living organisms, these cells are shaped during the parasitic infestation, but *in vitro* IL-4 is used instead. M2 macrophages are characterized by the high level of arginase 1 (arg1) expression that transforms arginine into proline and polyamines necessary for the tissue repair and plaque stabilization. Additionally, the increased activity of this enzyme leads to a depletion of arginine – a substrate for NO synthesis by iNOS. M2 cells also express higher level of mannose receptors (MR) that are necessary for the phagocytosis and membrane transfer [6].

According to the above-mentioned considerations we conceived a study on the *in vitro* influence of a GLP-1 agonist (exenatide) on human monocytes/macrophages. Experiments included the assessment of the impact of exenatide on the synthesis of pro-inflammatory (i.e. IL-1β and TNF-α) and anti-inflammatory (i.e. IL-10) cytokines. Afterwards, we evaluated the synthesis of NO and changes in the phenotype of macrophages subjected to GLP-1 agonist using commonly accepted markers (iNOS, arg1 and MR). Finally, we performed experiments with antagonist of GLP-1 receptor (exendin 9-39) and inhibitors of the intracellular signaling (protein kinase A inhibitor–PKI (14-22) and protein kinase B/Akt inhibitor–triciribine) to pinpoint the main pathway of cell signaling responsible for observed results.

Materials and methods

Cell culture

Peripheral blood mononuclear cells were separated by the histopaque density gradient centrifugation from the human group including 10 healthy nonsmoking volunteers aged 18–40 years, five women and five men not taking any drugs, using previously described methods [7]. Then, monocytes were isolated from peripheral mononuclear blood cells by negative immunomagnetic separation using Pan-T and Pan-B Dynabeads (Dynal, Oslo, Norway). This procedure enabled us to isolate inactive monocytes without artificial and uncontrolled stimulation. The isolated cells were labeled with a monoclonal antibody (Daco, Glostrup, Denmark) against the monocyte-specific positive antigen CD14+. The procedure gave 92% of CD14+ positive cells in the isolated fraction. Monocytes were suspended in RPMI 1640 medium supplemented with 10% FCS (Fetal Calf Serum; low endotoxin), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mg/ml fungizone (Gibco, Grand Island, NY, USA). Afterwards, cells were counted and checked for viability using an automated cell counter TC-20 (Bio-Rad, Hercules, CA, USA). As a result, a constant number of living cells (10^6 monocytes per well) was placed in a plastic 24-well plate (Becton-Dickinson, Franklin Lakes, NJ, USA) and left intact for 2 h to allow them to adhere to the bottom. Then, the medium was changed and cultures were incubated for 72 h (with additional single medium exchange after first 24 h). Incubations were performed in triplicate at 37 °C in a humidified atmosphere containing 5% CO_2 in the air. The conversion of monocytes into macrophages was confirmed using antibodies against EMR1 (Sigma-Aldrich, Poznań, Poland). The procedure gave 80% of EMR1 positive cells. After a 72 h incubation only with culture medium, the supernatant was carefully removed, discarded and replaced with culture medium supplemented with selected reagents (all supplied by Sigma-Aldrich, Poznań, Poland) dissolved in culture medium or culture medium containing DMSO: inducer of pro-inflammatory phenotype (M1) - LPS (0.5 μg/ml), inducer of anti-inflammatory phenotype (M2)–IL-4 (50 ng/ml), GLP-1 agonist - exenatide (10 nM) and GLP-1 antagonist - exendin 9-39 (50 nM), PKA inhibitor–PKI (14–22) (10 μM), PKB inhibitor–triciribine (20 μM) and in combinations [i.e. exenatide + exendin 9-39, LPS + exenatide, LPS + exenatide + exendin 9-39, exenatide + triciribine, exenatide + PKI (14-22) and exenatide + triciribine + PKI (14-22)]. In control cells only culture medium without experimental compounds was used. The final incubation of cultures with the above-mentioned reagents lasted for 24 h. In experiments with GLP-1 antagonist and protein kinase inhibitor cells were pretreated for 1 h with exendin 9-39, triciribine, PKI (14-22) or both - triciribine and PKI (14-22) prior to the addition of exenatide and/or LPS. Reagents' concentrations and optimal culture duration (96 h) were set according to our preliminary studies on cell viability and literature data (data not shown) [1,8–11]. All reagents used in the above experiments were checked for endotoxin levels using Chromogenic Endotoxin Quantitation Kits according to the manufacturer's recommendation (Thermo Scientific Inc., Waltham, MA, USA).

Viability tests

The viability of cells was estimated in two separate tests at the end of the treatment period. The first was based on 0.4% trypan blue exclusion test according to manufacturer's guidelines. Briefly, aliquots (10 μl) of cultured cells suspended in the medium were mixed with 10 μl of 0.4% trypan blue. After the incubation, cells were loaded on a slide and the viability was assessed in the automated cell counter TC-20 (Bio-Rad, Hercules, CA, USA). The second method relied on tetrazolium salt (MTT) conversion. MTT (final concentration 2.5 mg/ml) was added to the medium 3 h before the scheduled end of the experiment, and then cultures were incubated at 37 °C, 5% CO_2/95% air in proper conditions. At the end of the experiment, after being washed twice with PBS, monocytes were lysed in 100 μl of dimethyl sulfoxide, which enabled the release of the blue reaction product — formazan (room temperature, 10 min in the dark). The lysate (200 μl) was transferred to a 96-well plate (Falcon 353072, Becton-Dickinson, Franklin Lakes, NJ, USA). Absorbance at the wavelength of 570 nm was read using a microplate reader (Dynex Technologies, VA, USA). The results were expressed as a percentage of the control (100%).

Nitrite concentration

NO synthesis was determined by assaying the culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using colorimetric assay kits (Cayman Chemicals, Ann Arbor, MI, USA) according to manufacturer's recommendation. The nitrite concentrations were determined basing on a standard curve of sodium nitrite. Fresh culture media served as the blank in all experiments. The optical density was measured at 540 nm using a microplate reader. Experiments were done in triplicate. The detection limit of the assay was 2.5 μM. The intra-assay CV was <5%.

Cytokine assays

IL-1β, TNF-α and IL-10 levels were assayed using ELISA kits according to the manufacturer's recommendations (Diaclone, Besançon, France). The optical density of each well was measured at 450 nm using a microplate reader (Dynex Technologies, VA, USA). Experiments were done in triplicate. The sensitivity of the assay was determined to be 6.5 pg/ml for IL-1β, <10 pg/ml for