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Original research article

Effects of incretin agonists on endothelial nitric oxide synthase expression and nitric oxide synthesis in human coronary artery endothelial cells exposed to TNFα and glycated albumin

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ABSTRACT

Background: There have been a number of beneficial effects of incretin agonists on the cardiovascular system. Glycated albumin (GA) and tumor necrosis factor (TNFα) may lead to endothelial dysfunction. Due to reports of cardioprotective effects of incretin agonist, we wanted to determine if GLP-1 and exendin-4 can reverse diminished production of nitric oxide (NO) after treatment with TNFα and GA. The objective of our experiment was to study the interaction between incretin agonists and proinflammatory substances like TNFα and GA on production of NO in HCAEC.

Methods: Human vascular endothelial cells from the coronary artery (HCAEC) were used. The mRNA expression and protein level of endothelial nitric oxide synthase (eNOS) and inducible (iNOS) were quantified. NO production was measured in cells using DAF-FM/DA and flow cytometry.

Results: TNFα (10 ng/mL) decreased eNOS: mRNA by 90% and protein level by 31%. TNFα also decreased NO by 33%. GA (500 μg/mL) neither affected eNOS expression nor the protein level, but inhibited nearly all formation of NO in endothelium. GLP-1 (100 nM) and exendin-4 (1 and 10 nM) decreased the amount of NO compared to control. Incubation of HCAEC with TNFα and incretin agonists did not change or moderately reduce the amount of NO compared to TNFα alone.

Conclusions: TNFα and GA decrease production of NO in HCAEC, presumably by inducing reactive oxygen species or eNOS uncoupling. Incretin agonists in tested concentrations in the presence of L-arginine were not able to reverse this effect and instead led to a further reduction in NO production.

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Introduction

Dysfunction of vascular endothelium is an important contributor to developing atherosclerosis, a frequent complication of type 2 diabetes mellitus. Cardiovascular diseases pose major vascular complications and are the most common cause of death in diabetic patients [1]. Incretin based therapy is more widely used for type 2 diabetes. Incretin agonists like glucagon-like peptide 1 (GLP-1), exendin-4, and liraglutide have been shown to exert beneficial effects beyond controlling hyperglycemia. These substances act through GLP-1 receptors (GLP-1R), affecting glucose-dependent insulin secretion, inhibiting glucagon secretion, delaying gastric emptying, and reducing food intake via earlier apetite satiation [2]. Dipeptydyl-peptidase IV (DPP-IV) is a membrane protein that cleaves N-terminal dipeptides from a range of proteins including GLP-1. There have been a number of beneficial effects of incretin agonists on the cardiovascular system, especially on endothelial function [3]. The infusion of GLP-1 in diabetic patients and healthy subjects has a vasodilatory effect on the brachial artery [4]. Cardioprotective effects of GLP-1 by activating cell survival signal pathways have been demonstrated in various congestive heart failure animal models. Incretin agonists as well as DPP-IV inhibitors decrease blood pressure in patients with type 2 diabetes and hypertension [5]. The GLP-1 receptor is widely expressed in endothelial and smooth muscle cells of blood vessels in the human vasculature and heart [6].

Endothelial nitric oxide synthase (eNOS) synthesizes nitric oxide (NO) which is an important endothelium-derived vasodilator. When the endothelium is undamaged, the vasodilative function remains dominant. Endothelial damage shifts the balance

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toward vasoconstriction and leads to accelerated atherosclerosis. The most important cause of endothelial dysfunction is accelerated NO degradation by reactive oxygen species (ROS) [7]. GLP-1 can elevate NO levels in coronary arteries in mouse models; and its vasodilatory effects can be diminished by an eNOS inhibitor, suggesting that GLP-1 upregulates eNOS [8]. Although eNOS is a constitutively expressed enzyme, it is influenced by certain factors. The inflammatory cytokine tumor necrosis factor (TNFα) plays a pivotal role in the disruption of macrovascular and microvascular circulation both *in vivo* and *in vitro*. In the presence of a pro-inflammatory cytokine like TNFα and IL-6, iNOS can be synthesized in endothelial cells. TNFα can inhibit eNOS gene expression and induce iNOS gene expression in human endothelial cells [9,10]. Endothelial dysfunction associated with TNFα in pathophysiological conditions is linked to excessive production of ROS and thus a decrease in NO bioavailability. Glycated albumin is a product of non-enzymatic glycation of human serum albumin. Glycated albumin reflects mean plasma glucose concentrations over approximately 2–3 weeks. Serum levels of glycated albumin are better correlated with severity of cardiovascular disease and were also found to be a better indicator of glycemic fluctuations than glycated hemoglobin HbA1c [11]. In patients with uncontrolled diabetes, the level of glycated albumin exceeds 16% of the total albumin and could be as high as 8.2 g/l in subjects with stage IV chronic kidney disease [12,13]. A 2006 study found that serum glycated albumin levels were significantly increased in diabetic patients with CAD and exceed 20% of total albumin [14]. In contrast to HbA1c, glycated albumin is in contact with the vascular endothelium. It was revealed that a circulating and abundant modified glycated human serum albumin protein in diabetic patients induced a sustained ROS production in human endothelial cells [15]. Glycated albumin operates through receptors other than Receptor for Advanced Glycation Endproducts (RAGE). The anti-inflammatory activity of native albumin is significantly impaired after *in vitro* glycation [16]. Therefore, GA is also potentially an atherogenic protein and contributes to the development of diabetic complications.

The objective of our experiment was to study the interaction between incretin agonists and proinflammatory substances like TNFα and GA on the production of NO in human coronary artery endothelial cells. Due to reports of the cardioprotective effects of incretin agonists, we wanted to determine if GLP-1 and exendin-4 can reverse diminished production of NO after treatment with TNFα and GA.

Materials and methods

Endothelial cell culture

Human vascular endothelial cells from the coronary artery (HCAEC) were purchased from Lonza and cultured in EBM-2 culture medium supplemented with 10% fetal bovine serum and EGM-2 MV SingleQuots (Lonza) at 37 °C in a 5% CO_2 humidified incubator on fibronectin-coated (Sarstedt) tissue culture flasks. Cells between passages 5–7 were incubated with tumor necrosis factor α (TNFα) (10 ng/ml), human glycated albumin (GA) (500 μg/ml), glucose (20 mM), GLP-1 (10 and 100 nM), exendin-4 (1 and 10 nM) and KR-62436 (6.7 μM) – DPP-IV inhibitor (all from Sigma–Aldrich). HCAECs were stimulated with the test substances via 24 h incubation in culture medium without fetal bovine serum. Cell viability and morphology were assessed for each experimental group. In a preliminary experiment, the cells were incubated with TNFα at a concentration of 2.5 and 10 ng/ml and glycated albumin at concentrations of 100, 500, and 1000 μg/ml. For the remaining experiment, TNFα was used at concentration of 10 ng/ml and glycated albumin at a concentration of 500 μg/ml. Cells treated with GLP-1 were co-incubated with DPP-IV inhibitor KR-62436 at a concentration of 6.7 μM because of significant expression of DPP-IV on the endothelial surface. The independent influence of KR-62436 on eNOS was tested in another experiment and was found not to be significant in our model (data not shown).

eNOS and iNOS mRNA assay

Isolation of RNA and quantitative assays were done as described in previous publications [17]. Briefly, cytoplasmic RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was executed immediately after RNA isolation with a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems). cDNA were mixed with primers, probe, and the TaqMan Gene Expression Master Mix. For the quantitative determination of selected gene expression, ready-to-use Applied Biosystems Gene Assays were used for eNOS Hs01574647_g1 and for iNOS Hs01075529_m1. The expression of selected genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. All assays were performed with LightCycler 480 (Roche) according to the program: 10 min at 95 °C and 45 cycles of 20 s at 95 °C and 1 min at 60 °C. The expression of endothelial NO synthase was determined by means of the comparative $\Delta\Delta CT$ method. Before its use for quantification, a validation experiment was performed. A serial dilution of cDNA was amplified under the same conditions to demonstrate that the efficiency of target (eNOS) and reference (GAPDH) amplifications were approximately equal.

eNOS, iNOS and GLP1-R protein assay

The quantity of endothelial NOS protein was determined via the human eNOS Immunoassay kit in compliance with the manufacturer's instructions (R&D Systems). The amount of eNOS protein was determined by western blot in selected samples, as described below. The results were compared to assays performed via the human eNOS Immunoassay kit (R&D Systems). Linear correlations of both methods were confirmed (R^2 = 0.956) (Fig. 1).

Cell preparations were described previously [17]. Total protein concentration was determined with the bicinchoninic acid method (Sigma–Aldrich). Selected samples containing equal amounts of total protein as well as serial dilutions of samples were separated on 10% polyacrylamide gels and transferred to PVDF Immobilon-FL membranes (Millipore). Membranes were incubated with rabbit antibody against human eNOS (Sigma–Aldrich) or iNOS (Cell Signaling). Subsequently, membranes were incubated with secondary goat anti-rabbit antibody conjugated with infrared fluorescent dye IRDye800 (LI-COR). eNOS protein was visualized with the Odyssey imaging system (LI-COR). Intensities of the bands on the images were quantified by Image Studio software (LI-COR).

To analyze GPL-1 receptor expression, equal amounts of protein lysates were resolved on 10% SDS-PAGE and immunoblotted with anti-GLP-1R antibody (Cell Signaling). Bands were visualized using secondary antibody conjugated to IRDye800. Blots were scanned using an Odyssey infra-red scanner (Fig. 1).

Flow cytometry

NO production was determined by flow cytometry using DAF-FM/DA, which is a cell-permeable fluorescent probe for the detection of NO. A FACSCanto II flow cytometer (Becton Dickinson) was used to quantify fluorescence (excitation wavelength 488 nm; emission wavelength 519 nm). A similar method was previously described by Havenga et al. [18]. Fluorescence in these cells was produced by oxidation of DAF-FM/DA

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