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GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving K_{ATP} and cAMP

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ABSTRACT

Increasing evidence from both clinical and experimental studies indicates that the insulin-releasing hormone, glucagon-like peptide-1 (GLP-1) may exert additional protective/reparative effects on the cardiovascular system. The aim of this study was to examine vasorelaxant effects of GLP-1(7-36)amide, three structurally-related peptides and a non-peptide GLP-1 agonist in rat aorta. Interestingly, all GLP-1 compounds, including the established GLP-1 receptor antagonist, exendin (9-39) caused concentration-dependent relaxation. Mechanistic studies employing hyperpolarising concentrations of potassium or glybenclamide revealed that these relaxant effects are mediated via specific activation of ATP-sensitive potassium channels. Further experiments using a specific membrane-permeable cyclic AMP (cAMP) antagonist, and demonstration of increased cAMP production in response to GLP-1 illustrated the critical importance of this pathway. These data significantly extend previous observations suggesting that GLP-1 may modulate vascular function, and indicate that this effect may be mediated by the GLP-1 receptor. However, further studies are required in order to establish whether GLP-1 related agents may confer additional cardiovascular benefits to diabetic patients.

Glucagon-like peptide-1(7-36)amide (GLP-1) is a gut hormone with potent insulin-releasing and glucose-lowering actions. Recent years have seen the clinical development of a number of GLP-1 analogues and mimetic compounds for the treatment of type 2 diabetes. In 2005, the first anti-diabetic drug to target the GLP-1 receptor, exendin-4 (1-39) (also known as exenatide), received approval by the Food and Drug Administration. Numerous similar compounds, which hold vast therapeutic potential for the improved management of type 2 diabetes, are currently undergoing preclinical and clinical trials [1,2].

Recent decades have witnessed an intensive examination of GLP-1's numerous anti-diabetic and metabolic effects [2]. However, emerging evidence now indicates that this peptide hormone may have a number of additional effects on the cardiovascular system. Recent studies report wide-ranging cardiovascular actions of GLP-1 such as modulation of heart rate, blood pressure and cardiac structure and function [3–8]. Furthermore, GLP-1 has been shown to improve cardiovascular function in experimental heart failure [9,10] and in both diabetic and non-diabetic heart failure patients [11,12]. Chronic GLP-1 treatment has also been found to improve cardiovascular risk factors, such as HDL cholesterol, triglycerides and diastolic blood pressure, in patients with type 2 diabetes [13]. Patients with diabetes are characterised by an increased incidence of cardiovascular disease reported to be up to five times that of the normal non-diabetic population [14]. Given the current epidemic escalation in type 2 diabetes and the associated cardiovascular co-morbidities, it seems pertinent to further investigate potential cardiovascular actions of anti-diabetic drugs, such as GLP-1 which may prove to be beneficial in the treatment of such complications.

Previous studies investigating the *ex vivo* vascular actions of GLP-1 in rat blood vessels have suggested that it causes direct relaxation via an endothelium-independent mechanism [15,16]. The aim of the present study was to significantly extend these preliminary findings to a detailed investigation of the mechanisms by which native GLP-1(7-36)amide, three structurally-related peptides (GLP-1(9-36)amide, exendin-4 (1-39) and exendin (9-39)) and a small molecule GLP-1 receptor agonist, may modulate vascular function.

Experimental

Animals

Male Sprague–Dawley rats (8–12 weeks) were used throughout this study. They were housed under constant climatic conditions with free access to food and water. All experiments were

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performed in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK).

Isolated vessel studies

Rats were killed with a sodium pentobarbitone overdose (200 mg kg^{-1} body weight i.p.) and the thoracic aorta excised, cleared of surrounding connective tissue, and cut into 3 mm rings, taking care to leave the endothelium intact. Rings were suspended between a force transducer and fixed support in organ bath chambers containing 1.5 ml modified Krebs–Henseleit buffer (KHB, composition in mM: 118.5 NaCl, 3.8 KCl, 1.18 KH_2PO_4, 25 $NaHCO_3$, 1.19 $MgSO_4$, 10 glucose and 1.25 $CaCl_2$), bubbled with 95% O_2/5% CO_2 at 37 °C. Vessels were held at a resting tension of 1 g (preliminary experiments found this to be optimal), and allowed to equilibrate for 45 min, before the maximum contractile response to 80 mM KCl was assessed. Following washout and re-equilibration, a cumulative dose response curve to phenylephrine (PE, 1 nM–10 μM) was performed. After washout, rings were then pre-constricted with PE to 70% of their maximal PE-induced contraction before the following experimental protocols were performed.

In order to study the direct effects of GLP-1 compounds on vascular function, cumulative relaxation responses were performed to the peptides GLP-1(7-36)amide, GLP-1(9-36)amide, exendin-4 (1-39) and exendin (9-39) and the non-peptide small molecule GLP-1 receptor agonist 6,7-dichloro-2-methylsulfonyl-3-*N*-*tert*-butylaminoquinoxaline (DMB) (0.1 pM–0.1 μM). Possible mechanisms of action of GLP-1 were further investigated by performing cumulative relaxation responses to GLP-1(7-36)amide in endothelium-denuded vessels and in the presence or absence of the following specific inhibitors of candidate pathways (n = 8–13): (1) N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.3 mM), non-selective nitric oxide (NO) synthase inhibitor; (2) indomethacin (10 μM), cyclooxygenase inhibitor; (3) catalase (1250 U ml^{-1}), hydrogen peroxide scavenger; (4) hyperpolarising potassium (30 mM KCl); (5) glybenclamide (10 μM), ATP-sensitive potassium channel blocker; (6) Rp-8-Br-cAMPS (10 μM), cell-permeable cyclic AMP (cAMP[1]) antagonist. Cumulative relaxation response curves were also performed to the cAMP activator, forskolin (0.1 mM) in the presence and absence of Rp-8-Br-cAMPS, in order to demonstrate the specificity of the inhibitor for cAMP.

Determination of cAMP production

Rats (n = 8–12) were killed and the thoracic aorta removed, cleaned and cut into two sections. Each vessel segment was equilibrated for 30 min in KHB at 37 °C and bubbled with 95% O_2/5% CO_2, before incubation with the non-specific phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 0.1 mM) for a further 10 min. One vessel section was then incubated with GLP-1(7-36)amide or exendin (9-39) (0.1 μM) for 15 min and the other with an equivalent volume of vehicle to act as a control. Vessels were then blotted dry and frozen in liquid nitrogen for further analysis.

For determination of cAMP production, frozen aortae were weighed and homogenised (1500 rpm) using a reusable pellet pestle (Kimble-Kontes, Vineland, New Jersey, USA) fixed into an overhead stirrer (SS10, Stuart Scientific, Surrey, UK). Homogenates were suspended in trichloroacetic acid (5% (w/v); 100 μl/100 mg of tissue), centrifuged (1500*g*, 10 min) and the supernatant was removed. Trichloroacetic acid was extracted from samples using water-saturated diethyl ether before total cAMP production was measured using an enzyme immunoassay kit (Cayman Europe, Tallinn, Estonia).

Real-time RT-PCR

Expression of GLP-1 receptor mRNA was analysed in rat aorta, skeletal muscle (gastrocenimus) and heart by real-time RT-PCR with fluorescent SYBR Green technology on a Prism 7300 HT system (Applied Biosystems, Warrington, UK), using β-actin mRNA for normalisation. Primers (5'–3'): β-actin: forward CGTGAAAAGATGACCCAGATCA, reverse TGGTACGACCAGAGGCATACAG; GLP-1 receptor: forward CATCCACCTGAACCTGTTTGC, reverse GGGCAGCGTCTTTGATGAA. The comparative Ct method was used for relative quantification with validation experiments demonstrating approximately equal efficiencies of primers. All samples were run in triplicate and data expressed in arbitrary units.

Reagents

GLP-1 and exendin peptides (>95% purity) were custom made by EZbiolabs (Indiana, USA). L-phenylephrine hydrochloride (PE), N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), indomethacin, catalase, glybenclamide, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (Poole, UK). Rp-8-Br-cAMPS and 6,7-dichloro-2-methylsulfonyl-3-*N*-*tert*-butylaminoquinoxaline (DMB) were purchased from Merck Chemicals Ltd (Nottingham, UK). All drugs, with the exception of indomethacin, were initially dissolved in deionised water (at 10 mM) and diluted in KHB. Indomethacin was initially dissolved in dimethyl sulphoxide (at 0.1 M) and diluted in KHB. All solutions were freshly prepared on the day of the experiment. Concentrations are expressed as the final concentration of each drug in the organ bath.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). For organ bath studies data was expressed as decrease in tension calculated as a percentage of the initial PE-induced tension, and was plotted against log agonist concentration. Production of cAMP and GLP-1 receptor mRNA were expressed as arbitrary units. Data were analysed by a two-way repeated measures ANOVA, one-way ANOVA with Tukey's post-hoc test or unpaired Student's t test (two-tailed), as appropriate. $P < 0.05$ was considered to be significant.

Results

Effects of GLP-1 and structurally-related peptides on vascular relaxation in isolated rat aorta

Fig. 1 shows cumulative relaxation response curves to (A) GLP-1(7-36)amide and GLP-1(9-36)amide, (B) exendin-4 (1-39) and exendin (9-39) and (C) GLP-1(7-36)amide and DMB. All five compounds elicited significant and concentration-dependent vasorelaxation actions in rat aorta. Maximal relaxations to endogenous GLP-1(7-36)amide and GLP-1(9-36)amide (52.1 ± 7.0 and 45.8 ± 8.2%) appeared to be greater than those to synthetic exendin-4 (1-39) and exendin (9-39) (32.5 ± 7.8 and 33.7 ± 6.6%), although this did not reach statistical significance (P = 0.086, one-way ANOVA). The non-peptide GLP-1 receptor agonist, DMB induced similar maximal relaxations to GLP-1(7-36)amide (55.1_ ± 5.7 vs 60.9 ± 8.9%). These data are summarised in Fig. 1D.

[1] *Abbreviations used:* cAMP, cyclic AMP; DMB, 6,7-Dichloro-2-methylsulfonyl-3-*N*-tert-butylaminoquinoxaline; DPP IV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; IBMX, 3-isobutyl-1-methylxanthine; KHB, Krebs–Henseleit buffer; L-NAME, N_ω-nitro- L-arginine methyl ester hydrochloride; NO, nitric oxide; PE, phenylephrine.