Characterization of $[^{125}\text{I}]$GLP-1(9-36), a novel radiolabeled analog of the major metabolite of glucagon-like peptide 1 to a receptor distinct from GLP1-R and function of the peptide in murine aorta

Rhoda E. Kuca, Janet J. Maguirea, Keith Siewa, Sheena Patela, David R. Derksenb, V. Margaret Jacksonb, Kevin M. O'Shaughnesseya, Anthony P. Davenporta,*

a Clinical Pharmacology Unit, Box 110, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK
b Pfizer, Cardiovascular Medicine, Cambridge, MA, USA

A B S T R A C T

Aims: Glucagon-like peptide 1 (GLP-1) is an insulin secretagogue, released in response to meal ingestion and efficiently lowers blood glucose in Type 2 diabetic patients. GLP-1(7-36) is rapidly metabolized by dipeptidyl peptidase IV to the major metabolite GLP-1(9-36)-amide, often thought to be inactive. Inhibitors of this enzyme are widely used to treat diabetes. Our aim was to characterize the binding of GLP-1(9-36) to native mouse tissues and to cells expressing GLP1-R as well as to measure functional responses in the mouse aorta compared with GLP-1(7-36).

Main methods: The affinity of $[^{125}\text{I}]$GLP-1(7-36) and $[^{125}\text{I}]$GLP-1(9-36) was measured in mouse tissues by saturation binding and autoradiography used to determine receptor distribution. The affinity of both peptides was compared in binding to recombinant GLP-1 receptors using cAMP and scintillation proximity assays. Vasoactivity was determined in mouse aortae in vitro.

Key findings: In cells expressing GLP-1 receptors, GLP-1(7-36) bound with the expected high affinities (0.1 nM) and an EC$_{50}$ of 0.07 nM in cAMP assays but GLP-1(9-36) bound with 70,000 and 100,000 fold lower affinities respectively. In contrast, in mouse brain, both labeled peptides bound with a single high affinity, with Hill slopes close to unity, although receptor density was an order of magnitude lower for $[^{125}\text{I}]$GLP-1(9-36). In functional experiments both peptides had similar potencies, GLP-1(7-36), pD$_{2}$ = 7.40 ± 0.24 and GLP-1(9-36), pD$_{2}$ = 7.57 ± 0.64.

Significance: These results suggest that GLP-1(9-36) binds and has functional activity in the vasculature but these actions may be via a pathway that is distinct from the classical GLP-1 receptor and insulin secretagogue actions.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/3.0/).

Introduction

Glucagon-like peptide 1 (GLP-1) is derived from the transcrip- tion product of the proglucagon gene, synthesized in the intestine, released in response to meal ingestion and efficiently lowers blood glucose in Type 2 diabetic patients. The GLP-1R (glucagon-like peptide-1 receptor) controls the physiological response to GLP-1 and is currently a major target for the development of drugs owing to the broad range of potential beneficial effects in Type 2 diabetes (Koole et al., 2013).

GLP-1(7-36) is the biologically active form and has a very short half-life. It is rapidly metabolized by dipeptidyl peptidase IV (DPP-4) to the major metabolite GLP-1(9-36)-amide, (Knudsen and Pridal, 1996) which comprises about 60% of circulating GLP peptides and thought to be the major route of inactivation. Inhibitors of DPP-4 enzyme are widely used to treat diabetes.

GLP-1(9-36) is often thought to be inactive. However, in obese mice acute administration of GLP-1(9-36) potently inhibits hepatic glucose production with a similar effect in lean mice in the presence of GLP-1 antagonist, exendin (9-39) (Elahi et al., 2008). This anti-diabetogenic action of GLP-1(9-36) may have a therapeutic benefit. In addition, vascular actions of GLP-1(7-36) and GLP-1(9-36) have been reported in rodent arteries with comparable potency. These actions include changes in coronary flow in isolated mouse hearts in both wild-type and Gpl1r$^{-/-}$ mice deficient in GLP1-R (Ban et al., 2008), vasorelaxation in rat femoral artery (Nystrom et al., 2005) and in rat aorta (Green et al., 2013).
These studies provide the rationale for using rodent vasculature to explore further similarities (or differences) in the potency of the two peptides. Based on previous studies above, we hypothesized that GLP-1(9-36) would have similar potency to GLP-1(7-36).

It is unclear to what extent GLP-1(9-36) mediates functional activity via the GLP1-R or by other previously uncharacterized receptors. Our aim was to characterize the binding of the novel ligand [125I]GLP-1(9-36) in tissues from mouse and to determine the pharmacodynamic parameters (equilibrium dissociation constant, Kd and receptor density, Bmax) compared with [125I]GLP-1(7-36). Secondly, cell lines artificially expressing GLP1-R were used in scintillation proximity and second messenger cAMP assays to compare the binding of unlabeled GLP1(7-36) and GLP-1(9-36). Our final aim was to compare functional responses of both peptides in mouse aorta.

Materials and methods

[125I]GLP-1(7-36) and [125I]GLP-1(9-36) were both custom synthesized by Anawa Trading SA, Switzerland, using the Chloramine T method, typical specific activity ~ 2175 Ci/mmol (for scintillation proximity assays) [125I]GLP-1(7-36) was from Perkin Elmer, scintillation proximity assay beads were from Life Technologies and cAMP assays were from CISBIO. All peptides were synthesized by Pfizer Cambridge, MA, U.S.A.

Mouse tissue samples

The procedures used in this study were approved by the local animal ethical committee and were performed under the UK Home Office Project Licence authority; the study conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult mice, C57/BL6J (either sex, 20–35 g); were killed with CO2 and the brain and aorta were removed prior to binding and functional experiments.

Radioligand binding

Saturation binding assays were carried out adapting methods previously described (Maguire et al., 2012), to determine the affinity (Kd) and binding density (Bmax) of two custom synthesized ligands [125I]GLP-1(7-36) or [125I]GLP-1(9-36).

Coronal and sagittal sections of the brain containing the hypothalamus and thalamus, regions which have the highest density of mRNA encoding GLP-1 receptor were selected for this study (Allen Brain Atlas, 2008; Regard et al., 2008).

Briefly, 10 μm cryostat-cut sections (Bregma ~ −0.7 to −1.0) of adult mouse brain were pre-incubated at room temperature for 20 min in binding buffer (25 mM Hepes, 2 mM MgCl2, 0.05% Tween-20, 0.2% BSA, 0.01% Bacitracin, pH 7.4) prior to incubation for 90 min in buffer containing increasing concentrations of either [125I]GLP-1(7-36) or [125I]GLP-1(9-36) (4 μM–4 nM) at room temperature. Nonspecific binding was determined in adjacent sections using 10 μM of the relevant unlabeled GLP1(7-36) and GLP-1(9-36) (human, rat, mouse) peptides.

Sections were washed (4 × 30 s in 50 mM Tris–HCl at 4 °C) to break equilibrium and counted in a gamma counter. Autoradiography was carried out as above with a fixed concentration (0.2 nM) of each radioligand in a set of adjacent sections to determine total binding and with the addition of either relevant unlabeled peptide GLP-1(7-36) or GLP-1(9-36) at 10 μM for non-specific binding. Slides were rinsed in de-ionized water dried in a cold-air stream and apposed to a Kodak MR-1 film for 3 days prior to hand processing.

Table 1

| Characterization of peptides in native tissue and recombinant GLP-1 receptors. |
|-----------------|--------|-----------------|---------|
| Saturation binding assay (mouse brain, n = 4) | Kd (nM) | Bmax (fmol/mg) | nH |
| [125I]GLP-1(7-36) | 1.29 ± 0.26 | 570 ± 14.5 | 0.96 ± 0.06 |
| [125I]GLP-1(9-36) | 0.214 ± 0.08 | 2.69 ± 0.74 | 1.06 ± 0.05 |
| SPA binding assay (cell line) | Ki (nM) | % inhibition of specific binding |
| GLP-1(7-36) (n = 19) | 0.102 nM (0.0769–0.134) | 100% (99.8–101) |
| GLP-1(9-36) (n = 6) | 156 nM (101–243) | 97.1% (92.8–101) |
| cAMP assay (cell line) | EC50 (nM) | Emax |
| GLP-1(7-36) (n = 26) | 0.072 nM (0.054–0.0992) | 1040 (101–108) |
| GLP-1(9-36) (n = 6) | 7270 nM (935–56600) | 8.93% |

Scintillation proximity assays in cells expressing GLP-1R

To measure the ability of both GLP-1(7-36) and (9-36) to bind to the GLP-1R, and therefore have the potential to mimic GLP-1 activity, radioligand displacement assays were performed in membranes prepared from CHO cells expressing the hGLP-1R. Unlabeled Exendin-4 at a final concentration of 1 μM was used to determine non-specific binding. Affinity was expressed as a Kd value, defined as the concentration of compound required to decrease [125I]GLP-1(7-36) binding by 50% for a specific membrane batch at a given concentration of radioligand. The Kd for [125I]GLP-1(7-36) was determined by carrying out saturation binding, with data analysis by non-linear regression, fit to a one-site hyperbola (Graph Pad Prism). IC50 determinations were made from competition curves, analyzed with a propriety curve fitting program (SIGHTS) and a 4-parameter logistic dose response equation. Affinity constants were calculated from IC50 values, using the Cheng-Prusoff equation and results were given as mean and confidence interval.

cAMP assays in cells expressing GLP-1R

GLP-1 agonist activity was determined with a cell-based functional assay utilizing an HTRF (Homogeneous Time-Resolved Fluorescence) cAMP detection kit (cAMP dynamic 2 Assay Kit). A competitive immuno-assay that measures cAMP levels in the human GLP-1 CHO-K1 cell line expressing wild type GLP-1R receptors. Fluorescence was read with an Envision 2104 multi-label plate reader using an excitation of 330 nm and emissions of 615 and 665 nm. Raw data were converted to nM cAMP by interpolation from a cAMP standard curve, and Emax and EC50 determinations were made from an agonist-response curve analyzed with a curve fitting program using a 4-parameter logistic dose response equation using Graphpad Prism.

In vitro pharmacology — wire myography in mouse aorta

In functional experiments, thoracic mouse aortae (endothelium intact) were mounted in wire myographs and the effect of increasing concentrations of each peptide was measured. Rings of aorta (~2 mm in length) were dissected and mounted in wire myographs for measurement of isometric tension, as previously described (Maguire et al., 2009, 2011, 2014). The segments were bathed in oxygenated Krebs’ solution at 37 °C and were set to 90% of the internal diameter at 100 mm Hg. Maximal force (mN mm−1) was measured three times with a potassium-rich Krebs’ solution (100 mM) at 15-min intervals before

Table 1

| Characterization of peptides in native tissue and recombinant GLP-1 receptors. |
|-----------------|--------|-----------------|---------|
| Saturation binding assay (mouse brain, n = 4) | Kd (nM) | Bmax (fmol/mg) | nH |
| [125I]GLP-1(7-36) | 1.29 ± 0.26 | 570 ± 14.5 | 0.96 ± 0.06 |
| [125I]GLP-1(9-36) | 0.214 ± 0.08 | 2.69 ± 0.74 | 1.06 ± 0.05 |
| SPA binding assay (cell line) | Ki (nM) | % inhibition of specific binding |
| GLP-1(7-36) (n = 19) | 0.102 nM (0.0769–0.134) | 100% (99.8–101) |
| GLP-1(9-36) (n = 6) | 156 nM (101–243) | 97.1% (92.8–101) |
| cAMP assay (cell line) | EC50 (nM) | Emax |
| GLP-1(7-36) (n = 26) | 0.072 nM (0.054–0.0992) | 1040 (101–108) |
| GLP-1(9-36) (n = 6) | 7270 nM (935–56600) | 8.93% |
constructing cumulative concentration–response curves to GLP peptides (0.1 nM–300 nM). Concentration response curves were expressed as a percentage of the mean of the response to 100 mM KCl. They were analyzed using a 4-parameter logistic equation using Graphpad Prism to obtain values of molar concentration producing 50% of the maximum response (EC50) and maximum response was expressed as a percentage of the mean response to 100 mM KCl (EMAX). Potency values were normalized by logarithmic transformation to pD2 values (−log10 EC50). Selected aortae were also tested for vasodilatation by pre-constriction with 10 μM phenylephrine 1 and concentration response curves were tested using the two GLP peptides. Experiments were terminated by 10 μM SNAP to confirm that all vessels tested were capable of vasodilatation. The purpose was to show that the lack of vasodilator response to GLP agonists was not a consequence of a lack of ability to relax.

Results

Radioligand binding

Saturation binding studies in adjacent coronal sections of mouse brain containing the hypothalamus and thalamus which have the highest density of mRNA encoding GLP-1R (Regard et al., 2008) showed both labeled peptides bound with a single high affinity, with Hill slopes close to unity (Table 1). However, the density of receptors was an order of magnitude lower for [125I]GLP-1(9-36) in this native tissue. Autoradiography using sagittal (Fig. 1A and B) and coronal sections (Fig. 1C and D) demonstrated specific binding of [125I]GLP-1(7-36) to the cerebellum (molecular layer), hippocampus, thalamus and hypothalamus. In agreement with the lower density measured in the saturation binding assays, [125I]GLP-1(9-36) binding was mainly confined in coronal sections to the hippocampus (h).

Fig. 1. Autoradiograph showing localization of specific binding for both [125I]GLP-1(7-36) and [125I]GLP-1(9-36) ligands in mouse sagittal and coronal brain sections. A and B. Sagittal sections showing specific binding of [125I]GLP-1(7-36) in molecular layer of the cerebellum (c), hippocampus (h), thalamus (t) and hypothalamus (ht). C and D. Coronal section showing specific binding in (C only) hippocampus (h), thalamus (t) and hypothalamus (ht). E and F. Coronal section showing [125I]GLP-1(9-36) specific binding in hippocampus (h). Nonspecific binding (NSB) was determined in adjacent sections using an excess (10 μM) of the relevant unlabeled GLP-1(7-36) and GLP-1(9-36) (human, rat, mouse) peptides. (Scale bar = 5 mm).
Table 2
In vitro pharmacology of mouse aorta. Both GLP-1(7-36) and GLP-1(9-36) caused vasoconstriction with similar potencies. There was no evidence for endothelium dependent vasodilatation following pre-constriction with phenylephrine (10 μM, n = 3–6).

| Vasoinhibitory assay (mouse aorta) | pD2 (nM) | EMAX (% KCl maximum) |
|------------------------------------|-----------|----------------------|
| GLP-1(7-36) (n = 6)                | 7.40 ± 0.24 | 34 ± 8%              |
| GLP-1(9-36) (n = 3)                | 7.57 ± 0.64 | 25 ± 7%              |

Scintillation proximity assays

Scintillation proximity assay using GLP-1R artificially expressed in cell lines demonstrated a marked difference between the two GLP-1 peptides. GLP-1(7-36) bound with the expected affinity (0.1 nM) whereas GLP-1(9-36) bound with a 70,000 fold lower affinity than in the radiolabeled binding assay (Table 2).

cAMP assays

Similarly, in cAMP assays using GLP-1R artificially expressed cell lines, GLP-1(7-36) gave an expected EC50 of 0.07 nM whereas GLP-1(9-36) produced a 100,000 fold lower EC50 in the cAMP assay, with a maximum response of <10% (Table 2).

In vitro pharmacology — wire myography

In functional myography experiments in mouse aortae both peptides caused vasoconstriction with similar potencies (Fig. 2, Table 1). There was no evidence for endothelium dependent vasodilatation following pre-constriction with phenylephrine (10 μM, n = 3).

Discussion

This study has shown that both [125I]GLP-1(7-36) and [125I]GLP-1(9-36) bound with a single high affinity, with Hill slopes close to unity, although the density of [125I]GLP-1(9-36) binding was an order of magnitude lower.

Intriguingly, we found in scintillation proximity and cAMP assays using GLP1-R receptors artificially expressed in cell lines that, while GLP-1(7-36) bound with the expected affinities, GLP-1(9-36) bound with a 70,000 fold lower affinity in the SPA binding assay, and a 100,000 fold lower EC50 in cAMP, with a maximum response of <10%. These results are concordant with studies in cells expressing rat (Montrose-Rafizadeh et al., 1997) and human GLP-1R (Knudsen and Pridal, 1996), where these receptors were also reported to have low affinity for GLP-1(9-36). In addition, the cardioprotective actions of GLP-1(9-36) were preserved in cardiomyocytes isolated from GLP-1R knockout mice (Ban et al., 2010).

In a detailed study of forty-two mouse tissues measuring mRNA encoding the GLP-1R by qRT-PCR, Regard et al. (2008) found that expression of the receptor was very limited, with the highest levels in the pancreas and islets of Langerhans, as expected from the known actions of the peptide, with lower levels in the lung, stomach and brain. We choose to study mouse aorta, as messenger RNA (Regard et al., 2008) was not detected in this tissue and this strongly suggested that the actions of both peptides were unlikely to be via the GLP-1R. In agreement, in functional myography experiments in mouse aortae both peptides caused vasoconstriction with similar potencies.

Previous studies have shown that the action of GLP-1(7-36) in the rodent vasculature is complex with both constrictor responses as shown here for mouse aorta, as well as endothelium dependent and independent vasodilator responses. In chronically instrumented rats in vivo, infusion of GLP-1(7-36) had regional hemodynamic effects comprising tachycardia, a rise in blood pressure, renal and mesenteric vasoconstriction but hindquarters vasodilatation (Gardiner et al., 2010). In isolated rat arteries in vitro, where metabolism would be expected to be minimized, GLP-1(7-36) causes both endothelium dependent and independent relaxation (Richter et al., 1993; Golpon et al., 2001; Ozyazgan et al., 2005; Nystrom et al., 2005; Green et al., 2008; Nathanson et al., 2009). Importantly, where studies compared both peptides, for example in rat aorta and femoral artery, GLP-1(9-36) displays similar potency to GLP-1(7-36) (Green et al., 2008; Nathanson et al., 2009) consistent with similar potency of the two peptides in mouse aorta in this study albeit that the functional response that we observed was vasoconstriction.

Taken together, these results show that GLP-1(9-36) can distinguish between GLP-1R and a second, as yet unidentified receptor that may mediate at least part of the vascular response to the two peptides. The mechanism of action is not known but since the peptides produced vasoconstriction, it may involve a number of possible actions such as modulating intracellular calcium, but downstream signaling has not been investigated. Interestingly, Shah et al. (2011) showed that the endothelium-dependent vasorelaxation found in response to the DPP-4 blocker, alogliptin was independent of GLP-1 pathways.

In regions of mouse brain known to express GLP-1R, [125I]GLP-1(9-36) bound with similar affinity but labeled a smaller population of receptors than [125I]GLP-1(7-36). Taken together, these results suggest interaction with a receptor distinct from GLP-1R.

There are four other members of the glucagon family of receptors (glucagon-like peptide 2, growth hormone releasing hormone, gastric inhibitory polypeptide and glucagon) but these all have a restricted distribution (Regard et al., 2008) and are not thought to be activated at physiological concentrations by GLP-1 peptides. Both peptides were present in a library of 10,000 ligands that were screened against eighty-two remaining G-protein coupled receptors that have been predicted to exist in the human genome and designated as ‘orphan’ as the endogenous ligand is not yet know. However, but no hits were identified (Southern et al., 2013; Davenport et al., 2013).

Conclusion

The results suggest that GLP-1(9-36) has functional activity in the vasculature at concentrations similar to those circulating levels in the plasma. It is possible that the increased sensitivity to GLP-1(9-36) in native tissue than in cells artificially expressing GLP-1R is the result of interaction with other factors such as accessory proteins or formation of heterodimers with other receptors not present in artificial cell lines. A more intriguing possibility is that the vascular actions may be via a pathway that is distinct from the classical GLP-1 receptor and insulin secretagogue actions.
Conflict of interest

The authors REK, JJM, KS, KOS and APD declare that there are no conflicts of interest. SP, DD and VMJ are employees of Pfizer. The research was supported in part by an investigator-led grant from Pfizer.

Acknowledgments

This study was supported in part by an investigator-led grant from Pfizer. APD and JJM are recipients of a travel grant from the BPS Bain Memorial Award. JJM was supported by the British Heart Foundation (PG/09/050/27734).

References

Allen Brain Atlas version 1. http://mouse.brain-map.org/, 2008.
Ban K, Noyan-Ashraf MH, Hoefler J, Bolz SS, Drucker DJ, Hussein M. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation 2008;117:2340–50.
Ban K, Kim KH, Cho CK, Sauve M, Diamandis EP, Backx PH, et al. Glucagon-like peptide (GLP)-1(9-36)amide-mediated cytoprotection is blocked by exendin(9-39) yet does not require the known GLP-1 receptor. Endocrinology 2010;151:1520–31.
Davenport AP, Alexander SP, Sharman JL, Pawson AJ, Benson HE, Monaghan AE, et al. International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. Pharmacol Rev 2013;65:967–86.
Elahi D, Egan JM, Shannon RP, Meneilly GS, Khatri A, Habener JF, et al. GLP-1 (9-36) amide, cleavage product of GLP-1(7-36)amide, is a glucoregulatory peptide. Obesity (Silver Spring) 2008;16:1501–9.
Gardiner SM, March JE, Kemp PA, Bennett T, Baker DJ. Possible involvement of GLP-1(9-36) in the regional haemodynamic effects of GLP-1(7-36) in conscious rats. Br J Pharmacol 2010;161:92–102.
Golpon HA, Puechner A, Welte T, Wichert PV, Feddersen CO. Vasorelaxant effect of glucagon-like peptide-7(36)amide and amylin on the pulmonary circulation of the rat. Regul Pept 2001;102:81–6.
Green BD, Hand IV, Dougan JE, McDonnell BM, Cassidy RS, Grieve DJ. GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving KATP and cAMP. Arch Biochem Biophys 2008;478:136–42.