Variants of Glucagon-like Peptide 1 Receptor Modify Intracellular Signaling

Mesgana Dagnachew[1], Richard Printz[2], and Kevin Niswender[2]
[1]Sewanee: The University of the South [2]Vanderbilt University

Introduction

Glucagon-like peptide 1 (GLP1) is a peptide hormone that is mostly produced in the intestinal epithelial endocrine cells and released in response to food intake. The actions of this hormone include stimulating insulin secretion, inhibiting glucagon secretion and gastrointestinal motility, regulating appetite and providing protection from inflammation and other forms of cellular stress. Due to these functions, its receptor, known as the GLP1 receptor (GLP1R), has gained attention as an effective therapeutic target for obesity and type 2 diabetes. GLP1R has a number of naturally occurring genetic variants found in human populations that alter the coding sequence by a single amino acid and that appear to impact health. Low-frequency missense variants of the GLP1R gene, A316T and G168S, are associated with reduced risk of obesity comorbidities, coronary artery disease and type 2 diabetes[1,2].

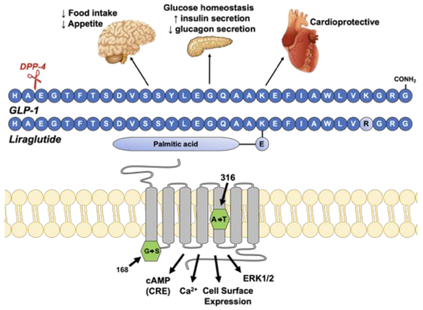


Figure 1. GLP1 function and GLP1R signaling. GLP1R agonists, GLP1 and liraglutide (a dipeptidyl peptidase-4 [DPP-4] resistant analog), have been shown to regulate glucose homeostasis, food intake, appetite, and provide cardioprotection. GLP1R couples to multiple downstream signaling pathways, including cAMP production, Ca^{2+} flux, β-arrestin recruitment leading to changes in cell surface expression, and activation of ERK1/2.

Hypothesis

The amino acid change at position 168 of GLP1R, substituting a Serine for Glycine, and at position 316, substituting a Threonine for an Alanine, may create a protein kinase recognition sequence for phosphorylation.

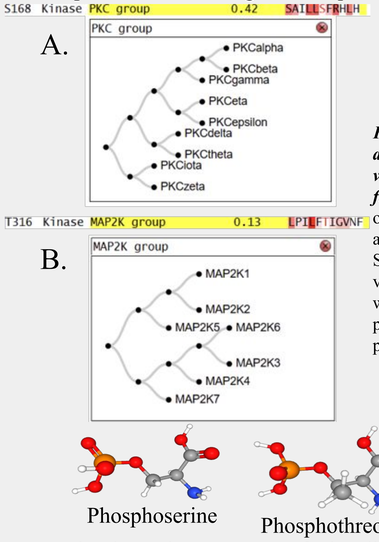


Figure 2. NetPhorest Analysis of the amino acid sequence for each GLP1R variant for possible consensus sequences for phosphorylation. (A) The kinase group of Protein Kinase C (PKC) was identified as the closest match to phosphorylate the Serine site at position 168 of the GLP1R variant. (B) The kinase group of MAP2K was identified as a candidate to phosphorylate the Threonine at the 316 position of the GLP1R variant.

To study the unique pattern of signaling bias that could result from phosphorylation of the Serine in G168S and the Threonine in A316T, we created new forms of GLP1R, G168E and A316E, with a Glutamic Acid (E) substitution for the corresponding Serine and Threonine found in the naturally occuring GLP1R variants. This will introduce a permanent charge at either the 168 or 316 position to mimic phosphorylation at these sites.

Methods

Cells: cDNA encoding the human reference (Ref) GLP1R (GenBank NM_002062.3) was used for site-directed mutagenesis to generate the GLP1R A316T and G168S variants. Stably, as well as transiently, transfected Flp-In T-REx 293 (HEK- 293) cells were used to express recombinant genes that contain a tetracycline-inducible promoter for adjusting the expression levels of the GLP1R Ref, A316T, G168S, A316E or G168E variants.

Gene Expression: The levels of GLP1R mRNA within Ref, A316T and G168S cell lines were quantified by performing quantitative real-time PCR (qRT-PCR) with specific primers for GLP1R and RPL13a, the latter a house-keeping gene used for sample normalization. RNA was isolated using a Direct-zol RNA MiniPrep Kit (Zymo Research). cDNA was synthesized from each RNA sample with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed with the cDNA, gene specific primers and Sso Advanced Universal SYBR Green Supermix (Bio-Rad).

Cell Surface Expression: GLP1R expression on the cell surface was assessed by biotinylation of cell surface proteins, followed by purification and a deglycosylation step (Peptide-N-Glycosidase F) to better visualize differences in GLP1R levels as determined by Western blot analysis.

cAMP Coupling: Cells were transiently transfected with a cAMP response element (CRE)-luciferase reporter gene plasmid to determine the extent of cAMP coupling to CRE-regulated gene expression.

Results

GLP1R Ref and variant genes express equivalent levels of mRNA

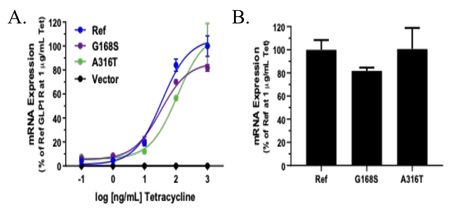


Figure 3. GLP1R mRNA expression in FlpIn-TRex293 cells. (A) FlpIn-TRex293 cells stably transfected with GLP1R Ref, G168S, A316T or empty vector were treated with tetracycline (Tet; 0 – 1 μg/mL) for 18h. (B) GLP1R Ref, G168S and A316T cells were treated with 1 μg/mL Tet (Tet) for 18h. Expression of GLP1R was determined by qRT-PCR and data reflect percentage of mRNA levels (± SEM) relative to GLP1R Ref at 1 μg/mL Tet (n=3 sets of experiments).

GLP1R A316T and G168S have altered cell surface expression in contrast to Ref

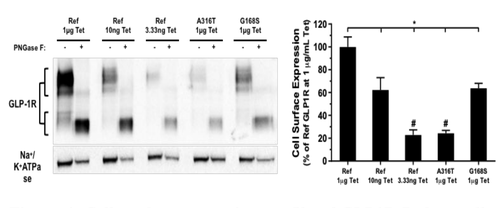


Figure 4. Cell surface expression profile of GLP1R Ref as well as G168S and A316T variants. Cell surface expression of the human GLP1R reference and variants stably transfected into FlpIn-TRex293 cells were determined using cell surface specific biotinylation. GLP1R reference cells were treated with either 3.33 ng/mL, 10 ng/mL or 1 μg/mL tetracycline (Tet) and G168S and A316T cells were treated with 1 μg/mL Tet. Representative immunoblots for GLP1Rs, with graphs quantifying expression of GLP1R and corrected for Na^+/K^+ ATPase. Bars reflect the mean (± S.E.M.) cell surface expression relative to GLP1R Ref at 1 μg/mL Tet (n=4 sets of experiments). *P<0.05 vs Ref 1 μg/mL Tet and #P<0.05 vs all other groups.

Phosphomimetics of A316T and G168S display significant reduction in cell surface expression

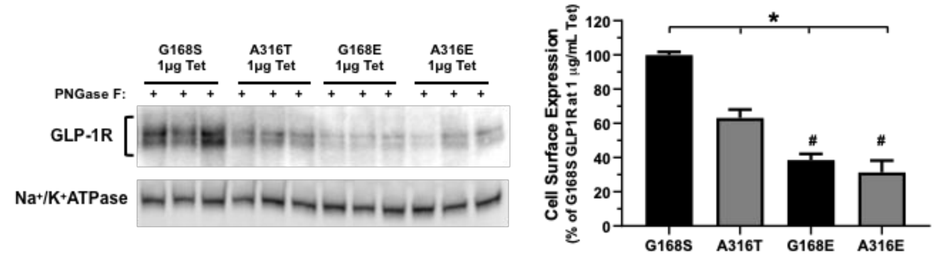
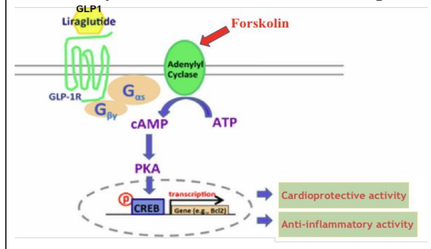


Figure 5. Cell surface expression of GLP1R phosphomimetics in FlpIn-TRex293 cells. Cell surface expression of the GLP1R variants and their phosphomimetics transiently transfected into FlpIn-TRex293 cells were determined using cell surface specific biotinylation. GLP1R Ref, A316T, G168S, A316E and G168E cells were treated with 1μg/mL tetracycline (Tet). Representative immunoblots for GLP1Rs, with graphs quantifying expression of GLP1R and corrected for Na^+/K^+ ATPase. Bars reflect the mean (± S.E.M.) cell surface expression relative to GLP1R Ref at 1 μg/mL Tet (n=3 sets of experiments). *P<0.05 vs Ref 1 μg/mL Tet and #P<0.05 vs all other groups.

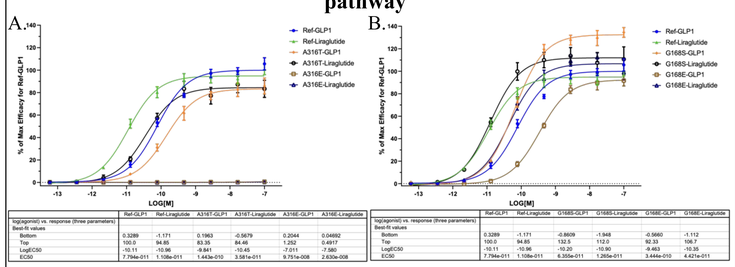
Pathway of CREB Activation in Response to Agonist Binding to GLP1R



Li Y, Bader M, Tamargo I, Rubovitch V, Tweedie D, Pick CG, Greig NH. Liraglutide is neurotrophic and neuroprotective in neuronal cultures and mitigates mild traumatic brain injury in mice. J Neurochem. 2015 Dec;135(6):1203-17. doi: 10.1111/jnc.13169. Epub 2015 Jun 18. PubMed PMID: 25982185; PubMed Central PMCID: PMC4644713.

Figure 6. The adenylate cyclase-cAMP-protein kinase A-CREB/CRE pathway in response to GLP1R activation. Binding of GLP1 or liraglutide to GLP1R activates adenylate cyclase, which induces the conversion of ATP to cAMP. Once activated by cAMP, Protein Kinase A (PKA) can phosphorylate CREB, which can induce transcription of genes involved in cardioprotective and anti-inflammatory activity.

Phosphomimetics reduce signaling of GLP1R through the cAMP/CRE pathway



log(agonist) vs. response (three parameters)	Ref-GLP1	Ref-Liraglutide	A316T-GLP1	A316T-Liraglutide	A316E-GLP1	A316E-Liraglutide
Best-fit values						
Bottom	0.3289	-1.171	0.1963	-0.5679	0.2044	0.04692
Top	100.0	94.85	83.35	84.46	1.252	0.4917
LogEC50	-10.11	-10.96	-9.841	-10.45	-7.011	-7.580
EC50	7.794e-011	1.108e-011	1.443e-010	3.581e-011	9.751e-008	2.630e-008

log(agonist) vs. response (three parameters)	Ref-GLP1	Ref-Liraglutide	G168S-GLP1	G168S-Liraglutide	G168E-GLP1	G168E-Liraglutide
Best-fit values						
Bottom	0.3289	-1.171	-0.8609	-1.948	-0.5660	-1.112
Top	100.0	94.85	132.5	112.0	92.33	106.7
LogEC50	-10.11	-10.96	-10.20	-10.90	-9.463	-10.35
EC50	7.794e-011	1.108e-011	6.355e-011	1.265e-011	3.444e-010	4.421e-011

Figure 7. CRE activity in GLP1R Ref and variant cells. (A) GLP1R A316E has little to no efficacy toward the cAMP/CRE pathway compared to Ref and A316T. (B) GLP1R G168E has a similar efficacy toward the cAMP/CRE pathway compared to Ref and G168S, but requires a higher dose of agonists (and an increased EC50). Data reflect mean (±SEM) relative to the response of 10 μM forskolin (a direct activator of adenylyl cyclase).

Conclusion

- Even though mRNA levels are equivalent, GLP1R A316T cell surface expression is 4-fold lower than Ref at the same Tet exposure (1 μg/mL).
- The phosphomimetics A316E and G168E show a further reduction in cell surface expression levels in comparison to that of A316T and G168S.
- Although A316E and G168E have similar cell surface expression, A316E fails to generate a cAMP/CRE response with agonists, suggesting that the phosphorylation of T316 may inactivate the receptor.
- The phosphomimetic of G168S, G168E, maintains receptor activity but at greater levels of ligand, affecting GLP1 to a greater extent than liraglutide.

Future Directions: We plan to further explore the effects of phosphorylation at the GLP1R variants, A316T and G168S, based on the kinases involved.

References

1. Scott, R.A. et al. A genomic approach to therapeutic target validation identifies a glucose-lowering *GLP1R* variant protective for coronary heart disease. *Sci. Transl. Med.* 8, 341ra76 (2016).
2. Wessel, J. et al. Low-frequency and rare exome chip variants associate with fasting glucose and type 2 diabetes susceptibility. *Nat. Commun.* 6:5897 (2015).

Acknowledgements

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