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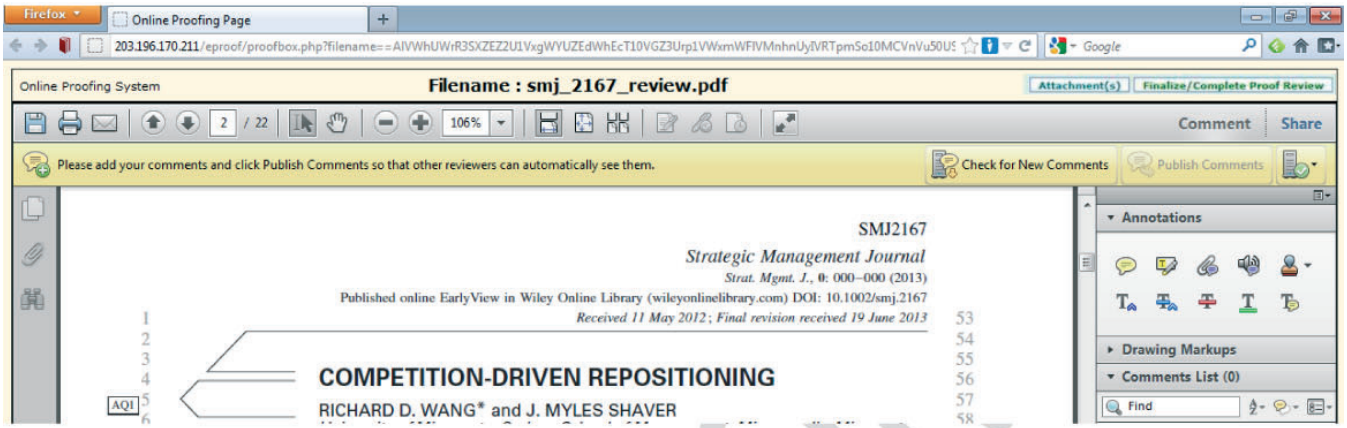

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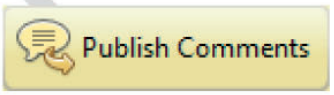

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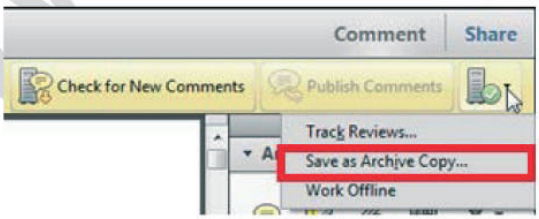

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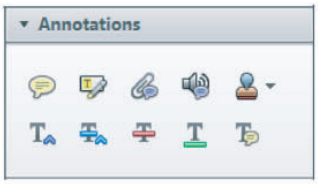

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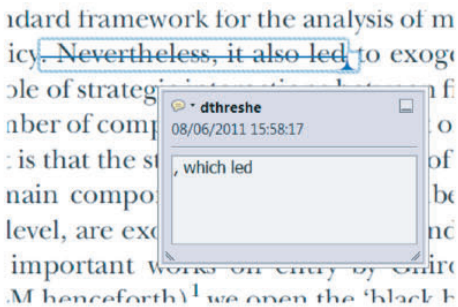

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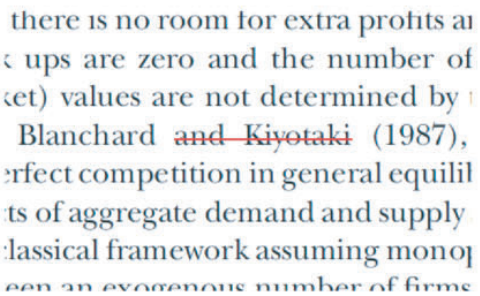

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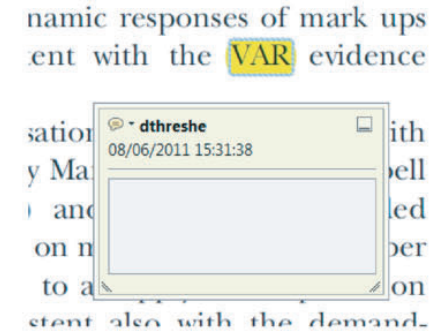

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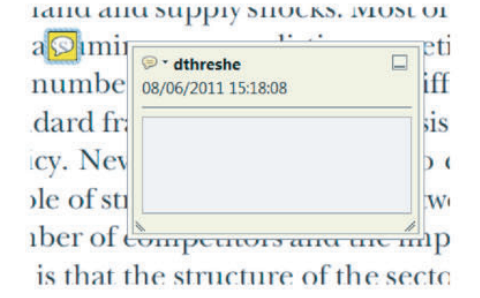

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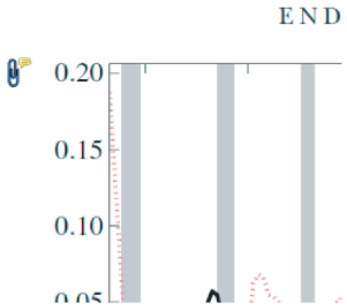

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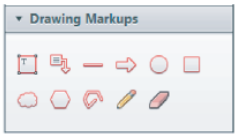

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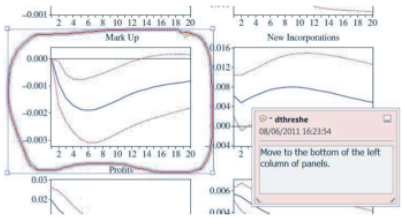

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BRIEF REPORT

Robust GLP-1 secretion by basic L-amino acids does not require the GPRC6A receptor

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The G protein-coupled receptor GPRC6A (GPCR, Class C, group 6, subtype A) has been proposed to be a sensor for basic L-amino acids that are hypothesized to translate ingestive behaviour to endocrine information. However, the contribution of the GPRC6A receptor to L-amino acid-induced glucagon-like peptide 1 (GLP-1) secretion is unclear. Therefore, to discover whether the GPRC6A receptor is indispensible for amino acid-induced secretion of GLP-1, we treated, with oral gavage, GPRC6A knock-out (KO) and wild-type (WT) littermate mice with GPRC6A ligands (L-arginine and L-ornithine) and assessed GLP-1 levels in circulation. We found that oral administration of both L-arginine and L-ornithine significantly increased total plasma GLP-1 levels to a similar level in GPRC6A KO and WT mice 15 minutes after gavage (both amino acids) and accumulated up to 60 minutes after gavage (L-arginine). Conversely, GLP-1 secretion at the 30- and 60-minute time points in the KO mice were attenuated and did not reach statistical significance. In summary, these data confirm that L-arginine is a potent GLP-1 secretagogue and show that the main effect occurs independently of GPRC6A. In addition, this is the first study to show that also L-ornithine powerfully elicits GLP-1 release in vivo.

KEYWORDS

GLP-1 release, GPRC6A, L-arginine, L-ornithine, mouse pharmacology

1 | INTRODUCTION

Pharmacological targeting of the glucagon-like peptide 1 receptor (GLP-1R) is a widely employed strategy to treat obesity and type 2 diabetes.[1] Further, mounting evidence supports the notion that the metabolic benefits of bariatric surgery are coupled with amplification in meal-induced GLP-1 secretion.[2] Therefore, nutritional strategies aiming to boost endogenous GLP-1 release are being explored currently as safe therapeutic alternatives to surgical and pharmacological interventions.[3] It was discovered recently that the basic amino acid L-arginine is a potent GLP-1 secretagogue and the benefits of L-arginine in aiding glycaemic control depend on functional GLP-1R signalling.[4]

A decade ago, we cloned an amino acid-sensing G protein-coupled receptor, termed GPRC6A (GPCR, Class C, group 6, subtype A) and subsequently hypothesized that basic amino acids such as L-arginine and L-ornithine may elicit metabolic benefits, including GLP-1 secretion in a GPRC6A-dependent manner.[5–7] In addition, it has been proposed that testosterone and osteocalcin exert metabolic effects via activation of GPRC6A.[7] This idea was explored partially by Oya and colleagues in 2012; they reported that L-ornithine stimulates GLP-1 secretion in vitro in GLUTag cells via stimulation of GPRC6A.[8] However, in the same study Oya et al. could hardly detect GPRC6A in FACS-sorted intestinal endocrine cells, putting into question the extrapolation of results to the in vivo situation.[8] Very recently Murphy et al. revealed that ablation of the GPRC6A gene does not compromise arginine-induced benefits on glucose tolerance.[9] Likewise, L-arginine-mediated PYY secretion from primary mouse colonic epithelium was not compromised by GPRC6A ablation, whereas L-arginine-mediated GLP-1 release was attenuated,[9] leaving the potential physiological role of GPRC6A in basic amino acid-mediated incretin hormone secretion unresolved.

The aim of the present study was to examine whether the GPRC6A receptor is necessary for L-arginine- and L-ornithine-induced GLP-1 secretion in vivo.

[#]Shared co-first authorship.

2 | MATERIALS AND METHODS

GPRC6A KO mice with a disruption of exon VI from the GPRC6A gene were generated as previously described.[10] GPRC6A KO mice with a deleted region completely covering the GPRC6A locus were obtained from the Knockout Mouse Project (KOMP). GPRC6A KO animals and WT littermate controls were produced by GPRC6A heterozygous C57B1/6N breeding. Studies were performed in male chow-fed mice that were housed under controlled temperature and humidity at a 12-hour light/12-hour dark cycle rodent facility. All experimental work was conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate in Denmark.

GLP-1 secretion in response to oral L-arginine (1 g/kg body weight), oral L-ornithine (1 g/kg body weight) or saline (0.9% NaCl w/v) was examined in 16 to18 week-old non-anesthetized WT animals or animals with disrupted exon VI (Figure 1). Mice were fasted overnight and water was removed 1 hour before study initiation. L-arginine, L-ornithine or saline was administrated by oral gavage. Blood samples were obtained from the retrobulbar intraorbital capillary plexus before gavage (time 0) and 15 minutes after gavage and were placed in ice-cold EDTA blood collection tubes containing a final concentration of 0.1 mM diprotin A and 500 KIU/mL aprotinin (both from Sigma-Aldrich). Plasma was separated by centrifugation (8000 min^{-1}) for 5 minutes at 4°C and stored at −80°C until analysis. GLP-1 was measured using a Mesoscale total GLP-1 kit (Cat.nr. K150JVC-1) on a MSD Sector Imager 240A, Model 1250.

GLP-1 secretion in response to oral L-arginine (0.2 and 1 g/kg body weight) was examined in 8 to12-week old non-anesthetized animals with complete deletion of the GPRC6A locus (Figure 2). Mice were fasted 6 hours before study initiation. L-arginine was administrated by oral gavage. Blood samples were obtained from the tail before gavage (time 0) and 15, 30 and 60 minutes after gavage and were placed in ice-cold EDTA blood collection tubes containing a final concentration of 10 μM dipeptidyl peptidase-4 (DPP-IV) inhibitor valine pyrrolidide. Plasma was separated by centrifugation (8000 min^{-1}) for 10 minutes at 4°C and stored at −80°C until analysis. Total GLP-1 was measured as described above.

Grahpad Prism v. 6.0 (GraphPad Software) was used for statistical analysis and graphical presentation. Unpaired 2-tailed Student's t test or 1-way ANOVA was employed to analyse differences in GLP-1 concentration and area under the curve (AUC) as indicated in the figure legends. Data are presented as mean ± SEM. The statistical level of significance is determined at $P < .05$.

3 | RESULTS

Oral administration of 1 g/kg L-arginine significantly increased total plasma GLP-1 levels within 15 minutes in both GPRC6A exon VI KO and WT mice (Figure 1A) (WT mice, $P < .05$, 33.2 ± 5.9 pM at 0 minutes, 72.4 ± 15.1 pM at 15 minutes, n = 9; GPRC6A KO mice, $P < .05$, 24.4 ± 3.0 pM at 0 minutes, 64.3 ± 16.5 pM at 15 minutes, n = 8). In addition, oral administration of 1 g/kg L-ornithine significantly increased total plasma GLP-1 within 15 minutes in both GPRC6A exon VI KO and WT mice (Figure 1B) (WT mice, $P < .001$, 14.8 ± 2.2 pM at 0 minutes, 52.7 ± 9.0 pM at 15 minutes, n = 9; GPRC6A KO mice, $P < .001$, 9.8 ± 0.86 pM at 0 minutes, 44.6 ± 7.3 pM at 15 minutes, n = 9). A corresponding volume of

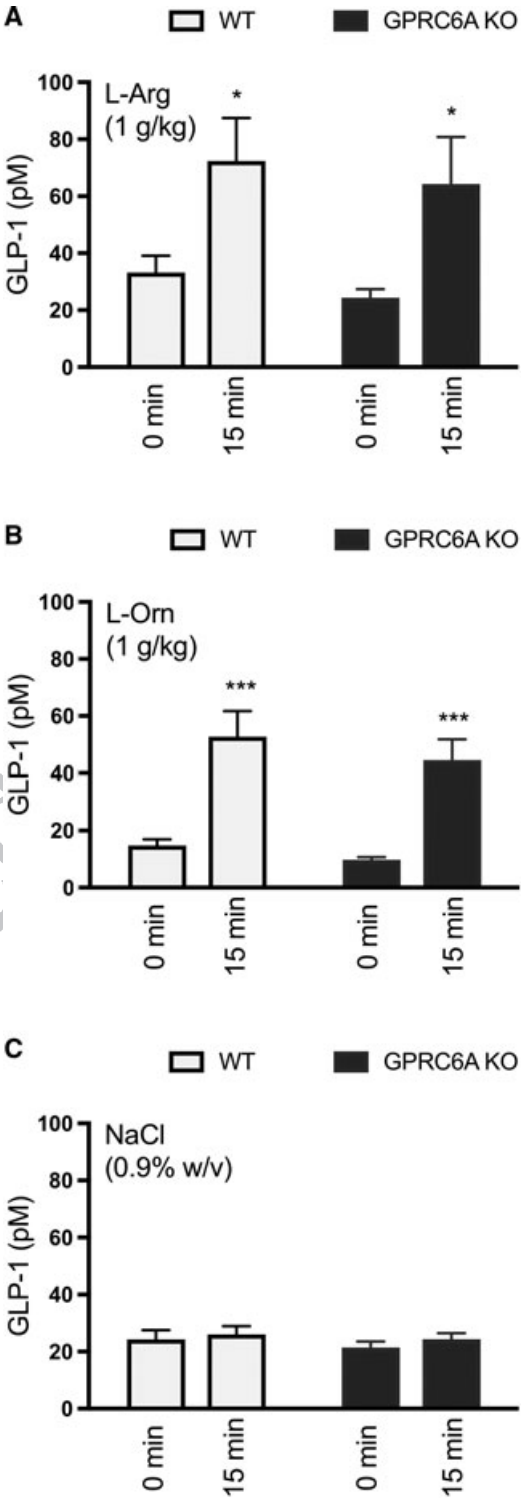

FIGURE 1 L-arginine- and L-ornithine-induced GLP-1 secretion in vivo in a GPRC6A independent manner. GPRC6A exon VI KO mice (dark grey) and WT mice (light grey) received oral gavage of A, L-arginine (L-Arg); B, L-ornithine (L-Orn) or C, saline control. Blood was sampled prior to gavage (0 min) and 15 minutes (15 min) after gavage and assessed for GLP-1 levels. Data presented as mean ± SEM, n = 8 to 9, * $P < .05$, *** $P < .001$, Student's t test.

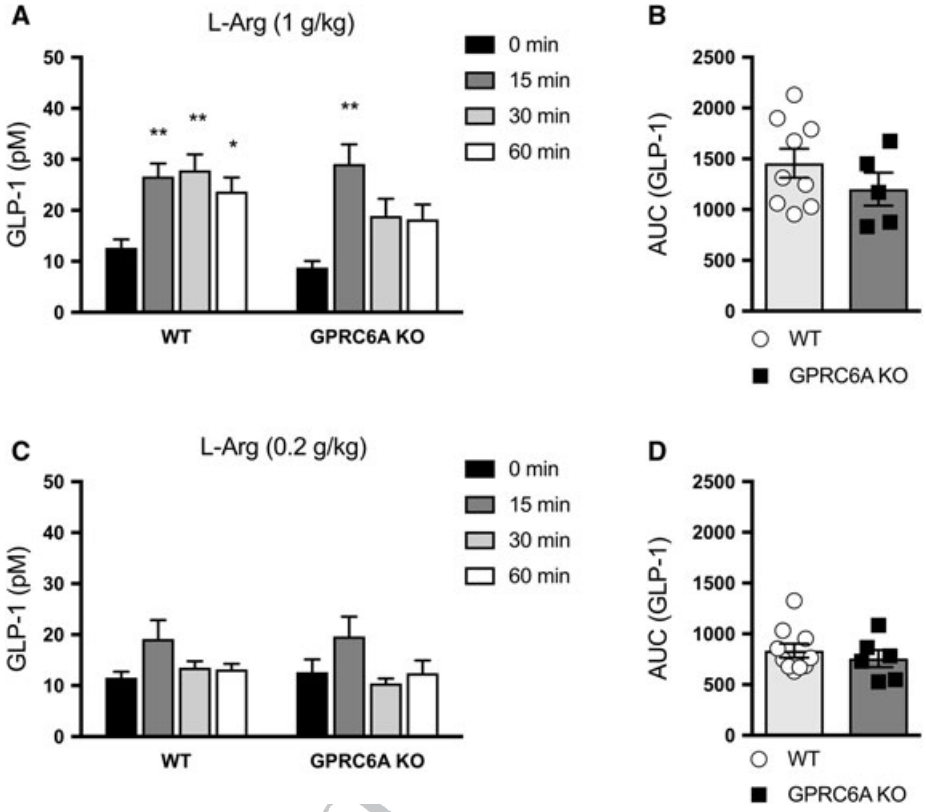

FIGURE 2 Time course of high- and low-dose L-arginine-induced GLP-1 secretion in vivo. GPRC6A full locus KO mice and WT mice received oral gavage of A, high (1 g/kg) or C, low (0.2 g/kg) L-arginine (L-Arg). Blood was sampled prior to gavage (0 min, black) and 15 minutes (15 min, dark grey), 30 minutes (30 min, light grey) and 60 minutes (60 min, white) after gavage and assessed for GLP-1 levels. B and D, show the corresponding areas under the curve (AUC) for 1 and 0.2 g/kg L-arginine, respectively. Data presented as mean ± SEM., n = 5 to10, * $P < .05$, ** $P < .01$, 1-way ANOVA with Bonferroni's multiple comparisons post hoc test (A and C) or Student's t test (B and D).

saline administration by oral gavage did not elicit significant GLP-1 secretion (Figure 1C).

Oral administration of 1 g/kg L-arginine also led to significantly increased total plasma GLP-1 levels after 15 minutes in both GPRC6A full locus KO and WT mice (Figure 2A) (WT mice, $P < .01$, 12.6 ± 1.7 pM at 0 minutes, 26.7 ± 2.5 pM at 15 minutes, n = 9; GPRC6A KO mice, $P < .01$, 8.8 ± 1.3 pM at 0 minutes, 29.1 ± 3.8 pM at 15 minutes, n = 5). In the WT mice, the elevated GLP-1 secretion was retained at the 30-minute and 60-minute time points, whereas an attenuated GLP secretion was observed for the full locus GPRC6A KO mice (WT mice, $P < .01$, 27.9 ± 3.1 pM at 30 minutes; $P < .05$, 23.7 ± 8.2 pM at 60 minutes, n = 9; GPRC6A KO mice, $P > .05$, 18.2 ± 2.9 pM at 30 minutes; $P > .05$, 18.8 ± 3.4 pM at 60 minutes, n = 5).

Oral administration of 0.2 g/kg L-arginine led to only a 1.6-fold increased total GLP-1 level after 15 minutes in both genotypes; however, this did not reach statistical significance (Figure 2C). The concentrations of GLP-1 reverted back to baseline levels at the 30- and 60-minute time points (WT mice, $P > .05$, 11.6 ± 1.2 pM at 0 minutes, 19.2 ± 3.7 pM at 15 minutes, 13.5 ± 1.3 pM at 30 minutes, 13.2 ± 1.1 pM at 60 minutes, n = 10; GPRC6A KO mice, $P > .05$, 12.7 ± 2.5 pM at 0 minutes, 19.6 ± 3.9 pM at 15 minutes, 10.4 ± 1.0 pM at 30 minutes, 12.4 ± 2.5 pM at 60 minutes, n = 6).

There was no statistical difference between AUC of the GPRC6A full locus KO and WT mice in either L-arginine dose (Figure 2B and D).

4 | DISCUSSION

There is a rapidly growing interest in understanding how dietary components can act as signalling molecules to affect human biology beyond their energetic value.[11] L-arginine supplementation has been shown to have widespread metabolic benefits in mice, rats, pigs and humans.[12–15] In addition, research dating back more than 50 years has contributed to positioning L-arginine as a major endocrine regulator, and today we know that arginine exhibits powerful control over the humoral factors governing energy metabolism, including insulin, glucagon, growth hormone and GLP-1.[4,16–18] Despite this knowledge, the molecular underpinnings of the endocrine and metabolic benefits of arginine are incompletely understood.

We have recently shown that L-arginine-mediated insulin release does not require GPRC6A[19] and Murphy et al. have demonstrated that L-arginine reduces food intake in a GPRC6A-independent manner.[9] In agreement with these results, we have observed identical glucose levels after oral glucose gavage in WT and GPRC6A KO mice under normal physiological conditions.[19] But, given the clear metabolic effects of L-arginine, further exploration of the contribution of the GPRC6A receptor, as well as other possible amino acid sensors, in regulating energy homeostasis is an imperative quest. Ultimately, such insights may eventually facilitate the engineering of nutrient-like strategies that could successfully prevent or reverse the metabolic co-epidemics of obesity and type 2 diabetes.

A previous article highlighted the fact that application of L-ornithine to the intestinal GLUTag cell line has GLP-1-releasing effects and further demonstrated that this phenomenon is GPRC6A-dependent. A more recent study relevantly explored whether L-arginine-elicited GLP-1 release also is dependent on the GPRC6A receptor.[9] Like the study by Oya et al. in 2012, the authors used an in vitro system to test this hypothesis and found an attenuated GLP-1 secretion in primary mouse colonic epithelium derived from GPRC6A KO mice as compared to WT cells, which led them to

conclude that GPRC6A "plays at most a minor role in its effects on gut hormone release."[9]

Here we demonstrate that oral delivery of 1 g/kg of the basic amino acids L-arginine and L-ornithine induces a robust increase in GLP-1 plasma levels 15 minutes post administration in WT and 2 different GPRC6A KO mice strains included in this study (Figures 1 and 2). Interestingly, we observe an attenuated, albeit statistically non-significant, GLP-1 release at the 30- and 60-minute time points of the high L-arginine dose in the GPRC6A KO mice (Figure 2A), which corresponds to the finding of Murphy et al.[9] Although this attenuation does not have a major effect on overall GLP-1 release (Figure 2B and D), it indicates that GPRC6A could play a minor role in L-amino acid mediated GLP-1 release, which would be interesting to study in more detail in disease states such as obesity where both GLP-1 signalling[20] and GPRC6A function[21] are altered. A lower dose of 0.2 g/kg L-arginine led to a 1.6-fold increase in GLP-1 release in both WT and GPRC6A KO mice, which returned to baseline levels at the 30- and 60-minute time points (Figure 2). Collectively, our data demonstrate that GPRC6A is not required to elicit robust L-arginine- and L-ornithine-mediated GLP-1 release in vivo in mice. Alternative targets (detailed by Alamshah et al. in 2016) for the L-amino acids could be the calcium-sensing receptor, taste T1R1-T1R3 receptors and membrane depolarization caused by electrogenic transport in the L cells by amino acid transporters such as the sodium-coupled neutral amino acid transporter 2 (SNAT2).[9]

Our findings add to a growing number of studies identifying L-arginine as a GLP-1 secretagogue.[4,9,22] Additionally, to our knowledge, this is the first study to show that L-ornithine is a powerful GLP-1 secretagogue in vivo. A study performed in pigs has previously shown that L-arginine is not catabolized to L-ornithine or other derivatives in the stomach or the duodenum and, moreover, L-arginine has a 60% oral bioavailability,[23] indicating that both L-arginine and L-ornithine are able to elicit the observed GLP-1 release from the intestine directly (Figures 1 and 2).

There are concerns about the clinical safety of L-arginine, given that L-arginine can lead to generation of nitric oxide. A recent review of the use of L-arginine as a dietary supplement in pigs, rats and sheep has not revealed any safety issues related to this,[23] but human doses of 3 × 3 grams per day led to adverse cardiovascular effects in patients with acute myocardial infarction.[23] Identification of the main mechanism(s)-of-action of L-arginine would allow more detailed safety assessments and facilitate the search for alternative ligands with increased potency and safety profile (eg, lack of nitric oxide generation) compared to L-arginine. When more potent and safe ligands have been developed, it would be very interesting to perform head-to-head comparisons of ligands mediating GLP-1 release with or without DPP-IV inhibitors and clinically used GLP-1 analogues. In addition, a wide range of basic, aromatic and aliphatic proteinogenic L-amino acids increase insulin-release in humans.[24] It would therefore be interesting to explore these with respect to GLP-1 release in future studies.

To conclusively determine the potential contribution of the GPRC6A receptor to both L-arginine- and L-ornithine-mediated GLP-1 release in vivo, we tested this in 2 global GPRC6A-deficient mouse models. Contradictory to the study by Oya et al., and in agreement with the conclusion by Alamshah et al. in 2016, we reveal that functional GPRC6A is not required for robust L-arginine- or L-ornithine-induced GLP-1 secretion in vivo. This also correlates well with the very low GPRC6A expression observed in FACS-sorted intestinal endocrine cells.[8] Additional work is now required to delineate the mechanism(s) by which basic L-amino acids lead(s) to robust GLP-1 release both in vitro and in vivo and to carefully gauge if GPRC6A is contributing to the translation of circulating and/or para/autocrine amino acid fluctuations into alterations in metabolism.

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Conflict of interest

The authors have no conflicting interests to declare

Author contributions

C. C., C. V. J. and S. S. performed the experiments and analyzed the data. All authors designed the studies and co-wrote the manuscript.

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