Title

Trp-Tyr is a dipeptide structure that potently stimulates GLP-1 secretion in a murine enteroendocrine cell model, identified by comprehensive analysis

Author names and affiliations.

Hayate Taguchi[1], Kana Murai[1], Tohru Hira[1,2]

[1]Graduate School of Agriculture, Hokkaido University

[2]Research Faculty of Agriculture, Hokkaido University

Corresponding author

Tohru Hira

hira@eis.hokudai.ac.jp

Research Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, JAPAN

Highlights

・Structure-activity relationship in peptide-induced GLP-1 secretion is unknown

・Dipeptide library was used to identify potent GLP-1-releasing dipeptides

・Nine of 339 dipeptides had potent GLP-1 releasing activity, with Trp-Tyr (WY) most

・Addition of Phe to N- or C- terminus of WY further enhanced the potency of WY

・WY is a specific dipeptide sequence that potently stimulates GLP-1 secretion

Abstract

Dietary peptides potently stimulate glucagon-like peptide-1 (GLP-1) secretion, however, the underlying molecular mechanisms, such as structure-activity relationships and sensing mechanisms are only partly elucidated. In this study, we used a dipeptide library to identify dipeptides that potently stimulate GLP-1 release and to clarify the underlying structure-activity relationship.

Murine enteroendocrine GLUTag cells were exposed to 339 dipeptides for 60 min, and the concentration of GLP-1 released into the supernatant was measured. Subsequently, selected dipeptides were examined for their reproducibility and dose responsiveness. In addition, we investigated the role of constituent amino acids in the secretion of GLP-1, and whether tripeptides containing the active dipeptide structures maintained their activity.

In a concentration range of 1– 5 mg/mL, twelve dipeptides had reproducible and concentration-dependent GLP-1-releasing activity. Among them, nine dipeptides (FY, KF, NI, PM, QL, QY, WF, WN, WY) were novel, with WY exhibiting the most potent activity. The reverse sequences and most free amino acids did not induce GLP-1 secretion, indicating that GLP-1-producing cells recognize the structure of each peptide to induce GLP-1 secretion. However, no apparent similarities were found between the active peptides. A comparison between the six tripeptides composed of F, W, and Y revealed the further potent tripeptides FWY and WYF, than WY.

In the present study, a comprehensive analysis revealed nine novel dipeptides with high potential to stimulate GLP-1 secretion. Furthermore, the results indicate that 'WY' is a specific dipeptide sequence that potently stimulates GLP-1 secretion.

Key words

glucagon-like peptide-1, dipeptides, tripeptides, dietary peptides

Introduction

The secretion of gut hormones including cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1), is stimulated by meal ingestion. Among nutrients, monosaccharides (glucose and fructose), fatty acids, peptides and amino acids induce GLP-1 secretion [1]. GLP-1 is an incretin hormone that enhances glucose-induced insulin secretion. Owing to the incretin effect, GLP-1 receptor agonists (GLP-1RAs) and dipeptidyl peptidase (DPP)-4 inhibitors are widely used in the treatment of type-2 diabetes. Besides its incretin effect, GLP-1 has multiple physiological functions including beta cell proliferation/protection, satiety induction, and glucagon secretion suppression [2].

Since GLP-1 secretion is stimulated by meal ingestion, researchers have been investigated t gated the positive modulation of GLP-1 secretion through multiple dietary components. Using *in vitro* and *in vivo* models, we have demonstrated that various dietary proteins/peptides can enhance GLP-1 secretion and subsequently attenuate the *in vivo* glycemic response [3,4,5]. However, the active peptides have not been identified in a majority of the studies. Compared to polysaccharides (e.g., starch) and triglycerides, dietary proteins can release large quantities of distinct fragments, i.e., peptides, in the gastrointestinal lumen during the digestion process.

Although peptidomic analysis using LC-MS/MS is likely a valuable strategy to comprehensively decode peptide fragment sequences from protein hydrolysate, the number of peptide fragments identified with GLP-1 releasing activity remains limited [6-10]. This includes both natural and synthetic peptides, with less than 30 identified thus far [10,11], as summarized in a supplementary Table (Table S1). The identified fragments do not appear similar in terms of structures or characteristics, such as molecular size and amino acid composition or sequences. Therefore, these peptides might stimulate GLP-1 secretion through mechanisms that are either specific to the respective peptide or based on an unidentified mutual characteristic. Consequently, the structure-activity relationship between peptides and their ability to induce GLP-1 release remains unclear.

In the present study, we aimed to identify peptide sequences responsible for stimulating GLP-1 secretion, primarily via a dipeptide library containing 336 dipeptides. The library was employed previously to identify ghrelin releasing peptides [12]. GLP-1-releasing activity of dipeptides was evaluated in a murine GLP-1-producing enteroendocrine cell line GLUTag. We further investigated the role of free amino acids that compose active dipeptides, and the role of amino acid sequence in tripeptides that contain active dipeptides identified in the present study.

Materials and methods

Cell culture consumables, such as Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The dipeptide library was purchased from AnaSpec (Fremont, CA, USA). Dipeptides GW (Gly-Trp), KA (Lys-Ala), KF (Lys-Phe), PM (Pro-Met), QG (Gln-Gly), QK (Gln-Lys), QL (Gln-Leu), QY (Gln-Tyr), YK (Tyr-Lys), and tripeptides FWY (Phe-Trp-Tyr), FYW (Phe-Tyr-Trp), WFY (Trp-Phe-Tyr), WYF (Trp-Tyr-Phe), YFW (Tyr-Phe-Trp), YWF (Tyr-Trp-Phe) were synthesized by Greiner Bio-One Co. (Tokyo, Japan). NI (Asn-Ile), WY (Trp-Tyr), WN (Trp-Asn) were purchased from AS ONE (Osaka, Japan); FY (Phe-Tyr) from Combi-Blocks, Inc. (San Diego, CA , USA); VY (Val-Tyr) from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan); WF (Trp-Phe) from Bachem AG (Bubendorf, Switzerland); GL (Gly-Leu) from Peptide Institute Inc. (Ibaraki-shi, Japan). Unless otherwise specified, all other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) or Sigma Aldrich (St. Louis, MO, USA).

Cell culture

GLUTag cells (a gift from D. J. Drucker, University of Tronto, Canada) were grown in DMEM (Cat. No. 12100-038) supplemented with 10% FBS, 50 IU/ml penicillin, and 500 µg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured by trypsinization upon reaching 80%–90% confluence. Cells at passage numbers 20–40 were used for the experiments.

GLP-1 secretion study

GLUTag cells were seeded at a density of 1.0 × 105 cells/well in a 96- or 48-well plate (depending on the experiment) and grown for 2 days till they reached 80–90% confluency [3]. For the dipeptide library-based assay, a 96-well plate was used since the library contained 1 mg of each peptide. Commercially available dipeptides, FP (Phe-Pro), GL (Gly-Leu), and PL (Pro-Leu) were included in the study. In subsequent studies, 48-well plate was used. Cells were washed twice with HEPES buffer to remove the culture medium and then exposed to the test agents dissolved in HEPES buffer for 60 min at 37°C. When hydrophobic peptides were examined, 0.2% DMSO was added to improve their solubility. Neither GLP-1-releasing nor cytotoxic effects were observed under the experimental conditions. The HEPES buffer (pH 7.4) had the following composition: 140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 0.1% BSA. Following incubation, the supernatants were collected and centrifuged at 800 *g* for 5 min at 4°C to remove the remaining

cells. The supernatants were then stored at −30°C for the measurement of GLP-1 using an ELISA kit (GLP-1 ELISA Kit Wako, High Sensitive, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Measurement of cytotoxicity

The cytotoxic effects on GLUTag cells were determined by measuring the release of lactate dehydrogenase (LDH) from the cells into the supernatant after the cells were exposed to the test agents as described earlier [13]. LDH activity was measured using a commercial kit (Cytotoxicity LDH Assay Kit-WST, Dojindo, Mashiki, Kumamoto, Japan), according to the manufacturer's instructions. Cytotoxicity was calculated as the relative release (%) of LDH after exposure to the test agents compared to the total LDH (100%) released upon treatment with the lysis reagent supplied in the kit.

Statistical analysis

The results are expressed as the mean ± SEM. Statistical analyses were performed using JMP Pro version 16.0.0 software (SAS Institute, Inc., Cary, NC, USA). Statistically significant difference was determined using one-way analysis of variance (ANOVA) followed by Tukey–Kramer's test or Dunnett's test, as stated in each figure legend. In all analyses, $p < 0.05$ was considered statistically significant.

Results and discussion

The potential of stimulating GLP-1 secretion of 339 dipeptides (at 5 mg/mL) was examined. As shown in supplementary figures (Fig. S1A-S), only a limited number of dipeptides increased GLP-1 concentrations in the supernatant after exposure to GLUTag cells for 1 hour. In total, 20 dipeptides increased GLP-1 concentrations more than 1.5-fold compared to the control, as summarized in Fig. 1. These results indicate that GLUTag cells do not respond broadly to dipeptides, but are able to distinguish each dipeptide, prpbably based on their structures (sequences).

To validate their GLP-1 releasing activity, these 20 dipeptides were respectively procured as single peptides, not from the library. Their GLP-1 releasing activity was further investigated on a relatively larger experimental scale (48-well plate) than the library-based study (96-well plate). GL and GW were included because these peptides induced GLP-1 secretion in our preliminary study. Among the 22 peptides, 10 peptides at a dose of 5 mg/mL significantly increased GLP-1 concentrations in the supernatant compared to that in the blank control (Fig. 2). Among the 10 positive peptides, WY was observed to demonstrate highest potency to induce GLP-1 secretion. The reason behind the absence of GLP-1 releasing activity in 12 of the 22 peptides in the second study could not be identified. None of these dipeptides demonstrated any cytotoxic effect, as determined by LDH release assay (data not shown).

The dose-response study further confirmed the GLP-1 releasing activity of 12 dipeptides (Fig. 3). Majority of the dipeptides demonstrated the highest potency at a concentration of 5 mg/mL; however, WF demonstrated higher activity at 2 mg/mL than at 5 mg/mL. This could be the reason that WF failed to induce a significant increase of GLP-1 release at 5 mg/mL (Fig. 2B). Again, WY had the highest potency among the dipeptides at a dose of 5 mg/mL. NI induced significant GLP-1 secretion even at a low dose (1 mg/mL), while further increments in GLP-1 concentration were not observed by increasing the dose (2 and 5 mg/mL). The potency is not very high, but NI is a specific peptide having relatively low threshold to induce GLP-1 secretion.

The results of study investigating reproductivity and dose response were summarized in Fig. 4. Although N-terminal Trp (WF, WN, WY) and C-terminal Tyr (FY, QY, WY) seems to be partly associated with the GLP-1-releasing activity, there are no apparent mutual characteristics between the 12 active peptides. GL [14], GW [15], KA [6], but not other nine dipeptide sequences, are included in short peptides previously reported to have GLP-1-releasing activity in different cell models. Thus, these nine dipeptides (FY, KF, NI, PM, QL, QY, WF, WN, WY) are newly identified to induce GLP-1 release, at least in GLUTag cells.

Previously, ghrelin release-modulating dipeptides were identified by using a dipeptide library, in ghrelin-producing MGN3-1 cells [12]. Among 336 peptides, N-terminal Ser-containing dipeptides play a dominant role in stimulating ghrelin secretion. They found Ser-Val (SV) most strongly stimulates, whereas Leu-Ile (LI) suppresses ghrelin secretion. Thus, the peptides identified in the previous study are not similar to the ones identified in the present study, indicating that specific enteroendocrine cells, such as ghrelin-producing and GLP-1-producing cells, equip different peptide-sensing mechanisms.

Combinations of free amino acids (corresponding to 5 mg/mL of active dipeptides, except for K+A and G+W) did not induce GLP-1 secretion (Fig. 5A), suggesting that free amino acids possibly liberated by extra- or intra-cellular digestion from these peptides are not responsible for the GLP-1-releasing activity of dipeptides. Dipeptides having reversed sequences of the active peptides did not induce GLP-1 secretion in the library-based study (e.g., WY vs YW), supporting the notion that the cells sense the dipeptide structure, instead of the individual constituent amino acids.

In additional experiments, the combination of K+A and G+W again induced GLP-1 secretion (Fig. 5B and C). However, single amino acids, Ala and Gly, but not Lys and Trp, also induced GLP-1 secretion equivalent to the respective combinations (K+A and G+W). These results suggest that Ala [16,17] and Gly [18] are solely able to stimulate GLP-1 secretion via mechanisms distinct from those underlying dipeptide-induced GLP-1 secretion. Some amino acids such as Asn, Gln, Met, Phe, Pro and Trp reportedly stimulate GLP-1 secretion in enteroendocrine cell models [17,19]. The failure of these amino acids to stimulate GLP-1 secretion in the present study could be attributed to differences in experimental conditions.

Because WY demonstrated potent activity throughout the present study, we further explored the role of the sequence in the GLP-1 releasing activity. We examined the GLP-1 releasing activity of six tripeptides composed of Trp (W), Tyr (Y) and Phe (F). Phe (F) was selected to combine with Trp (W) and Tyr (Y) because three of 12 active dipeptides contained Phe (FY, KF, WF). At a dose of 5 mM (2.6 mg/mL), FWY and WYF potently (> 6-folds vs control) induced GLP-1 secretion (Fig. 6A). YWF at 5 mM (Fig. 6A) had also an adequately potent effect (4-folds vs control) on stimulating GLP-1 secretion. At 1 mM (0.5 mg/mL), YFW significantly, while FWY slightly increased GLP-1 secretion. We observed no increment in LDH release in the experiment (Fig. 6B). WY at 5 mM (1.85 mg/mL) did not significantly increase the GLP-1 concentration, but the relative increments (1.2-folds) compared to the blank control are within the range expected from the dose (1 and 2 mg/mL)-response study (Fig. 3). These results demonstrate that FWY and WYF have potent GLP-1 releasing activity in vitro, and the core sequence “WY” is critical for stimulating GLP-1 secretion. It is very interesting to investigate whether these peptides exert GLP-1 releasing activity in vivo models. Oral administration of WY derived from beta lactoglobulin and related peptides (GTWY, WM, WV, WL) reportedly prevents cognitive decline via suppressing microglial activation [20,21]. Such an effect may partly attribute to the neuroprotective effect of GLP-1 [1,2] whose secretion is induced by WY peptide.

There are several limitations in the present study. First, some dipeptides and dipeptides containing cysteine were not included due to limited availability. Second, the molecular mechanisms underlying the effect of each or a group of peptides remain unclear. Because a limited number of peptides induced GLP-1 secretion in the present study and oligopeptide transporter 1(Pept1) is barely detectable in GLUTag cells [22], the dipeptide-induced GLP-1 secretion would not be attributed to Pept1. Third, in vivo effects of the active dipeptides on GLP-1 secretion are unknown. It is assumed that orally administered di- and tri-peptides are unable to stimulate GLP-1-producing cells primarily located in the distal small intestine, because such small peptides would be absorbed or digested in the proximal small intestine. However, longer and slowly digestible peptides containing these sequences may stimulate GLP-1 secretion in the intestine. To address these concerns, further studies are necessary in the future.

In conclusion, we discovered novel nine dipeptides (FY, KF, NI, PM, QL, QY, WF, WN, and WY) that have GLP-1-releasing activity by the comprehensive analysis using a dipeptide library. Although no apparent mutual characteristics were identified in these active peptides, the WY peptide demonstrated the highest potency. Furthermore, adding Phe residue on either the N- or C-terminal side of WY largely potentiated the GLP-1-releasing activity. These findings provide novel insight into the peptide-sensing mechanisms in enteroendocrine cells and a translational perspective that dietary proteins/peptides or compounds having these novel peptide structures prevent glucose intolerance and/or overeating through effectively stimulate GLP-1 secretion.

Acknowledgements

We thank The Tojuro Iijima Foundation for Food Science and Technology, Kieikai Research Foundation, and Urakami Foundation for Food and Food Culture Promotion, for financially supporting this research project.

References

[1] T.D. Müller, B. Finan, S.R. Bloom, D. D'Alessio, D.J. Drucker, P.R. Flatt, A. Fritsche, F. Gribble, H.J. Grill, J.F. Habener, J.J. Holst, W. Langhans, J.J. Meier, M.A. Nauck, D. Perez-Tilve, A. Pocai, F. Reimann, D.A. Sandoval, T.W. Schwartz, R.J. Seeley, K. Stemmer, M. Tang-Christensen, S.C. Woods, R.D. DiMarchi, M.H. Tschöp, Glucagon-like peptide 1 (GLP-1), Mol Metab 30 (2019) 72-130. 10.1016/j.molmet.2019.09.010.

[2] F.M. Gribble, F. Reimann, Metabolic Messengers: glucagon-like peptide 1, Nat Metab 3 (2021) 142-148. 10.1038/s42255-020-00327-x.

[3] N. Higuchi, T. Hira, N. Yamada, H. Hara, Oral administration of corn zein hydrolysate stimulates GLP-1 and GIP secretion and improves glucose tolerance in male normal rats and Goto-Kakizaki rats, Endocrinology 154 (2013) 3089-3098. 10.1210/en.2012-2275.

[4] Y. Ishikawa, T. Hira, D. Inoue, Y. Harada, H. Hashimoto, M. Fujii, M. Kadowaki, H. Hara, Rice protein hydrolysates stimulate GLP-1 secretion, reduce GLP-1 degradation, and lower the glycemic response in rats, Food Funct 6 (2015) 2525-2534. 10.1039/c4fo01054j.

[5] T. Hira, M. Sekishita, H. Hara, Blood Sampling From Rat Ileal Mesenteric Vein Revealed a Major Role of Dietary Protein in Meal-Induced GLP-1 Response, Front Endocrinol (Lausanne) 12 (2021) 689685. 10.3389/fendo.2021.689685.

[6] J. Caron, B. Cudennec, D. Domenger, Y. Belguesmia, C. Flahaut, M. Kouach, J. Lesage, J.F. Goossens, P. Dhulster, R. Ravallec, Simulated GI digestion of dietary protein: Release of new bioactive peptides involved in gut hormone secretion, Food Res Int 89 (2016) 382-390. 10.1016/j.foodres.2016.08.033.

[7] Y. Komatsu, Y. Wada, H. Izumi, T. Shimizu, Y. Takeda, T. Hira, H. Hara, Casein materials show different digestion patterns using an in vitro gastrointestinal model and different release of glucagon-like peptide-1 by enteroendocrine GLUTag cells, Food Chem 277 (2019) 423-431. 10.1016/j.foodchem.2018.10.123.

[8] S. Theysgeur, B. Cudennec, B. Deracinois, C. Perrin, I. Guiller, A. Lepoudère, C. Flahaut, R. Ravallec, New Bioactive Peptides Identified from a Tilapia Byproduct Hydrolysate Exerting Effects on DPP-IV Activity and Intestinal Hormones Regulation after Canine Gastrointestinal Simulated Digestion, Molecules 26 (2020). 10.3390/molecules26010136.

[9] M. Santos-Hernández, L. Amigo, I. Recio, Induction of CCK and GLP-1 release in enteroendocrine cells by egg white peptides generated during gastrointestinal digestion, Food Chem 329 (2020) 127188. 10.1016/j.foodchem.2020.127188.

[10] M. Santos-Hernández, S.M. Vivanco-Maroto, B. Miralles, I. Recio, Food peptides as inducers of CCK and GLP-1 secretion and GPCRs involved in enteroendocrine cell signalling, Food Chem 402 (2023) 134225. 10.1016/j.foodchem.2022.134225.
[11] T. Hira, A. Trakooncharoenvit, H. Taguchi, H. Hara, Improvement of Glucose Tolerance by Food Factors Having Glucagon-Like Peptide-1 Releasing Activity, Int J Mol Sci 22 (2021). 10.3390/ijms22126623.
[12] J. Nakato, H. Aoki, Y. Tokuyama, Y. Yamamoto, H. Iwakura, S. Matsumura, K. Inoue, K. Ohinata, Comprehensive analysis of a dipeptide library to identify ghrelin release-modulating peptides, FEBS Lett 593 (2019) 2637-2645. 10.1002/1873-3468.13522.
[13] K. Ochiai, R. Hirooka, M. Sakaino, S. Takeuchi, T. Hira, 2-Arachidonoyl glycerol potently induces cholecystokinin secretion in murine enteroendocrine STC-1 cells via cannabinoid receptor CB1, Lipids 56 (2021) 603-611. 10.1002/lipd.12323.
[14] P. Richards, R. Pais, A.M. Habib, C.A. Brighton, G.S. Yeo, F. Reimann, F.M. Gribble, High fat diet impairs the function of glucagon-like peptide-1 producing L-cells, Peptides 77 (2016) 21-27. 10.1016/j.peptides.2015.06.006.
[15] B. Le Nevé, H. Daniel, Selected tetrapeptides lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716, Regul Pept 167 (2011) 14-20. 10.1016/j.regpep.2010.10.010.
[16] R. Pais, F.M. Gribble, F. Reimann, Signalling pathways involved in the detection of peptones by murine small intestinal enteroendocrine L-cells, Peptides 77 (2016) 9-15. 10.1016/j.peptides.2015.07.019.
[17] F. Reimann, L. Williams, G. da Silva Xavier, G.A. Rutter, F.M. Gribble, Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells, Diabetologia 47 (2004) 1592-1601. 10.1007/s00125-004-1498-0.
[18] A. Gameiro, F. Reimann, A.M. Habib, D. O'Malley, L. Williams, A.K. Simpson, F.M. Gribble, The neurotransmitters glycine and GABA stimulate glucagon-like peptide-1 release from the GLUTag cell line, J Physiol 569 (2005) 761-772. 10.1113/jphysiol.2005.098962.
[19] Y. Osuga, K. Harada, T. Tsuboi, Identification of a regulatory pathway of L-phenylalanine-induced GLP-1 secretion in the enteroendocrine L cells, Biochem Biophys Res Commun 588 (2022) 118-124. 10.1016/j.bbrc.2021.12.043.
[20] Y. Ano, T. Ayabe, T. Kutsukake, R. Ohya, Y. Takaichi, S. Uchida, K. Yamada, K. Uchida, A. Takashima, H. Nakayama, Novel lactopeptides in fermented dairy products improve memory function and cognitive decline, Neurobiol Aging 72 (2018) 23-31. 10.1016/j.neurobiolaging.2018.07.016.
[21] Y. Ano, R. Ohya, Y. Takaichi, T. Washinuma, K. Uchida, A. Takashima, H. Nakayama, β-Lactolin, a Whey-Derived Lacto-Tetrapeptide, Prevents Alzheimer's Disease Pathologies and Cognitive Decline, J Alzheimers Dis 73 (2020) 1331-1342. 10.3233/JAD-190997.
[22] E. Diakogiannaki, R. Pais, G. Tolhurst, H.E. Parker, J. Horscroft, B. Rauscher, T. Zietek, H.

Daniel, F.M. Gribble, F. Reimann, Oligopeptides stimulate glucagon-like peptide-1 secretion in mice through proton-coupled uptake and the calcium-sensing receptor, Diabetologia 56 (2013) 2688-2696. 10.1007/s00125-013-3037-3.

Figure Legends

Fig. 1. GLP-1 secretory response to 339 dipeptides in GLUTag cells

GLUTag cells were exposed to dipeptide solutions (5 mg/mL) for 60 min. Values are expressed as means of GLP-1 levels relative to the blank control (n=3, expect NI). Two of three supernatants collected from NI treated cells had very high optical absorbance (> 3.0) in GLP-1 ELISA. Relative GLP-1 levels higher than 1.5 are highlighted by black background and white letters. Capital letters indicate N- and C-terminal amino acids within the dipeptides, respectively. Gray cells indicate unavailable dipeptides.

Fig. 2. GLP-1 secretory response to selected dipeptides in GLUTag cells

GLUTag cells were exposed to dipeptide solutions (5 mg/mL) for 60 min. Values are means ± SEM expressed as GLP-1 levels relative to the blank control (n=3). Asterisks indicate significant differences compared to the blank control ($P<0.05$, Dunnett's test).

Fig. 3. Dose-responsive effects of dipeptides on GLP-1 secretion

GLUTag cells were exposed to dipeptide solutions (1, 2, 5 mg/mL) for 60 min.

Values are means ± SEM expressed as GLP-1 levels relative to the blank control (n=3). Bars not sharing the same letter indicate significant differences ($P<0.05$, Tukey's HSD test).

Fig. 4. Dipeptides with confirmed GLP-1-releasing effect in GLUTag cells

Dipeptides showing significant increase in GLP-1 secretion effect are highlighted by black cells.

Fig. 5. GLP-1 secretory response to various combinations of amino acids composing selected dipeptides, in GLUTag cells

GLUTag cells were exposed to amino acid solutions for 60 min. (A) Each amino acid solution had a concentration of 20 mM. For example, F+Y was prepared to have both of amino acid at 20 mM of Phe (3.3 mg/mL) and Tyr (3.6 mg/mL). (B) Gly at 20 mM, Trp at 20 mM and their mixture. (C) Lys at 20 mM, Ala at 20 mM and their mixture. Values are means ± SEM expressed as GLP-1 levels relative to the blank control (n=3). Asterisks indicate significant differences compared to the blank control ($P<0.05$, Dunnett's test). Bars not sharing the same alphabet indicate significantly different ($P<0.05$, Tukey's HSD test).

Fig. 6. GLP-1 secretory responses (A) and LDH release to 6 tripeptides composed of Trp (W),

Tyr (Y), and Phe (F) in GLUTag cells

GLUTag cells were exposed to peptide solutions for 60 min.Tripeptides were examined at 1 mM (0.5 mg/mL) and 5 mM (2.6 mg/mL). Values are means ± SEM expressed as GLP-1 (A) and LDH (B) levels relative to blank control (n=3). Bars not sharing the same alphabet indicate significantly different ($P<0.05$, Tukey's HSD test).

Fig. 1

N-terminal

C-terminal

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
A	0.69	1.85	1.03	0.60	0.53	1.28	0.42	1.52	0.70	1.03	0.33	0.58	0.17	0.51	0.38	0.81	0.76	0.66	0.58	A
D	0.97	0.99	0.97	0.47	0.54	0.72	0.42	0.69	0.90	1.04	0.06	0.19	0.17	0.10	0.25	0.20	0.37	1.01	0.52	D
E	0.64		0.89	0.56	2.10	0.34	0.84	0.81	0.69	0.96	0.69	0.71	0.23	0.27	0.14	1.08	0.46	0.51	0.29	E
F	0.57	1.60		0.71	0.78	0.46	0.28	1.66	0.43	0.69	1.07	1.11	1.18	1.22	0.20	0.36	0.57	1.86	0.91	F
G	0.56	0.97	0.33	0.54	0.92			1.04	0.70	0.81	0.72	0.25	1.68	0.97		0.96	0.69	0.59	0.90	G
H	0.61	1.23	1.46	0.81	1.41	0.48	0.50	1.04	0.64	1.35	0.78	0.60	0.72	0.92	0.67	0.42	0.56	0.74	0.80	H
I	0.56		0.45	0.63	1.34	0.49	0.48	0.73	0.69	1.17	7.65	0.65	0.90	0.63	0.26	0.50	0.61	0.47	0.58	I
K	0.80	1.20	0.34	0.50		1.44	0.65	0.91		0.88	0.45	1.36	1.52	0.67	0.60	0.66	1.16	0.60	15.00	K
L	1.14	0.72	2.95	0.54	0.97	1.23	0.62	1.15	0.43	0.86	0.99	0.91	1.92	0.40	0.59	1.24	1.01	1.02	0.80	L
M	0.77	1.42	0.50	0.65	0.66	1.24	0.68	0.93	0.56	0.88	0.72	3.23	1.41	0.31	0.39	0.76	0.84	1.00	0.42	M
N		2.63	0.54	0.71	0.84	1.00	0.61	0.87	0.79	1.17	0.41	1.41	0.92	0.47	0.47	0.41	0.74	2.47	0.81	N
P	1.32	1.35	0.93	0.85	0.73	0.72	0.58	1.34			1.03	0.74	0.23	1.19	0.47	0.21	0.62	0.60	0.92	P
Q	1.02	0.67	0.27	0.47	0.85		0.58	0.71		1.41	0.75	0.48	1.28		0.61	0.42	0.68	0.74	0.95	Q
R	0.77	0.75	0.50	1.02	1.33	0.41	0.45	1.36		0.86	0.50			0.19	0.35	0.46	0.57	0.75	0.64	R
S	0.84	1.46	0.35	0.55	1.04	0.97	0.70	0.91		0.94		0.70	0.61	0.92	0.46	0.52	0.80	0.76	1.07	S
T	0.72	0.93	1.04	0.42	0.67	0.82	0.52	0.93	0.97	0.77	0.29	0.82	0.53	0.18	0.42	0.66	0.65	0.84	1.18	T
V	0.74	1.87	0.30	0.54	0.46	0.38	0.60	1.11	0.45	0.82	0.35	0.79	0.55	0.36	0.48	0.36		0.86	1.24	V
W	0.83	0.74	0.33	1.26	0.65	0.87	0.46	0.79	0.47	1.00	0.69	1.32	0.59	0.70	0.86	0.87	0.91	1.34	1.45	W
Y	0.91	0.41	0.52	2.38	0.97	0.75		1.07	0.67	0.78	0.46	1.39	4.91		1.04	0.14	2.00	3.05	0.82	Y
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	

Fig. 2

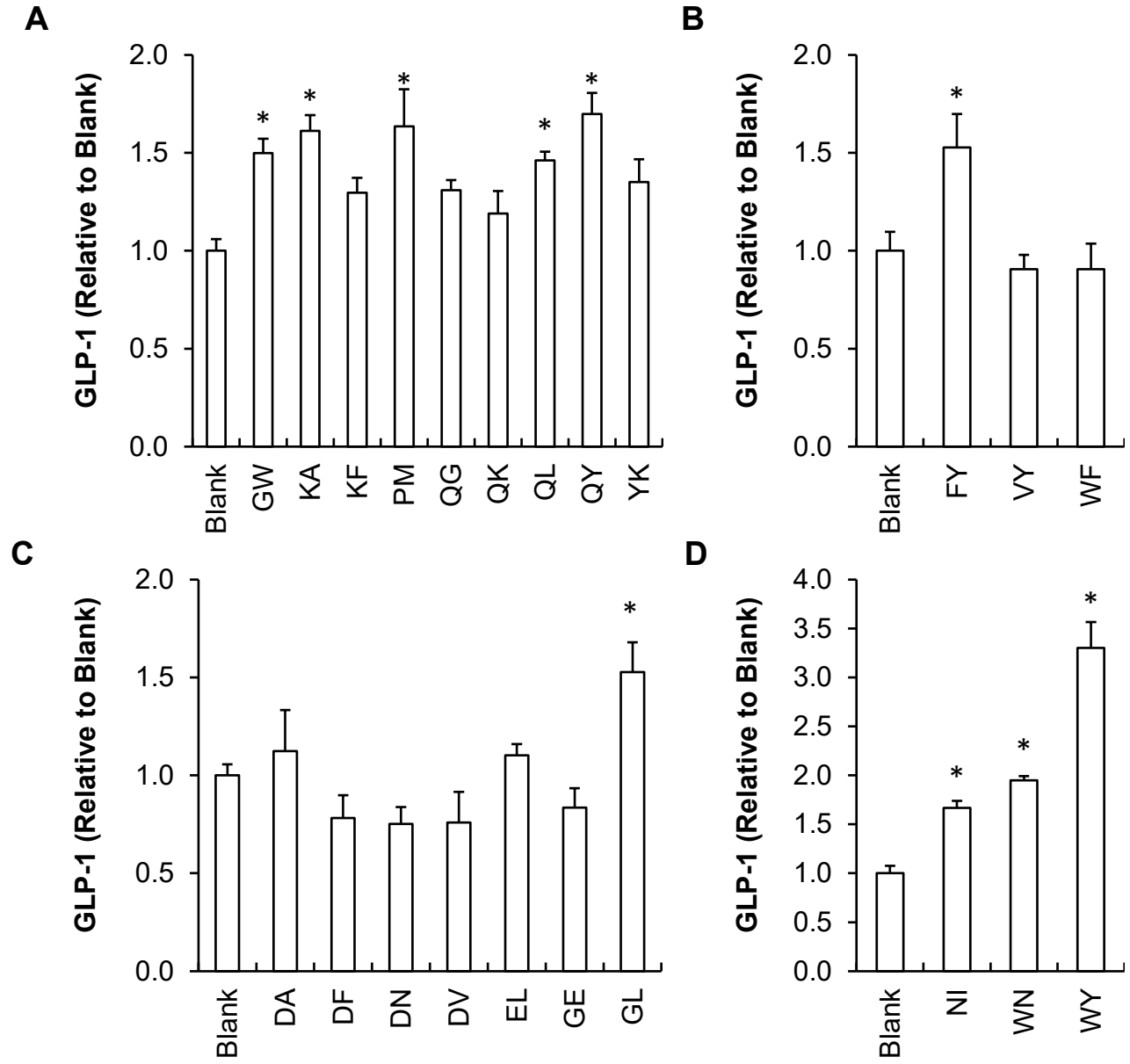

A
GLP-1 (Relative to Blank)
2.0
1.5
1.0
0.5
0.0
Blank
GW
KA
KF
PM
QG
QK
QL
QY
YK
B
GLP-1 (Relative to Blank)
Blank
FY
VY
WF
C
GLP-1 (Relative to Blank)
Blank
DA
DF
DN
DV
EL
GE
GL
D
GLP-1 (Relative to Blank)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Blank
NI
WN
WY

Fig. 3

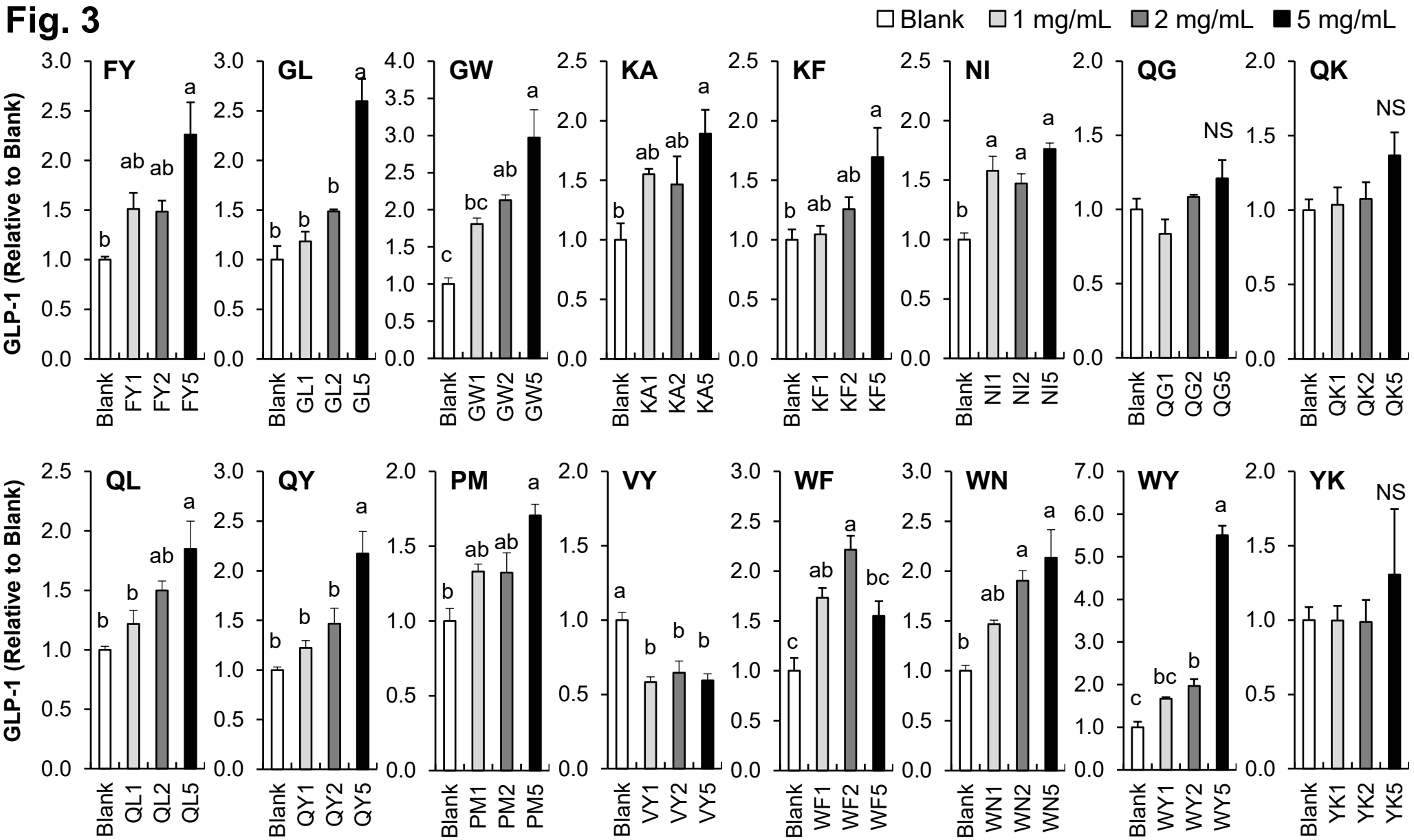

Blank
1 mg/mL
2 mg/mL
5 mg/mL
GLP-1 (Relative to Blank)
FY
GL
GW
KA
KF
NI
QG
QK
QL
QY
PM
VY
WF
WN
WY
YK
NS
Blank
FY1
FY2
FY5
GL1
GL2
GL5
GW1
GW2
GW5
KA1
KA2
KA5
KF1
KF2
KF5
NI1
NI2
NI5
QG1
QG2
QG5
QK1
QK2
QK5
QL1
QL2
QL5
QY1
QY2
QY5
PM1
PM2
PM5
VY1
VY2
VY5
WF1
WF2
WF5
WN1
WN2
WN5
WY1
WY2
WY5
YK1
YK2
YK5

Fig. 4

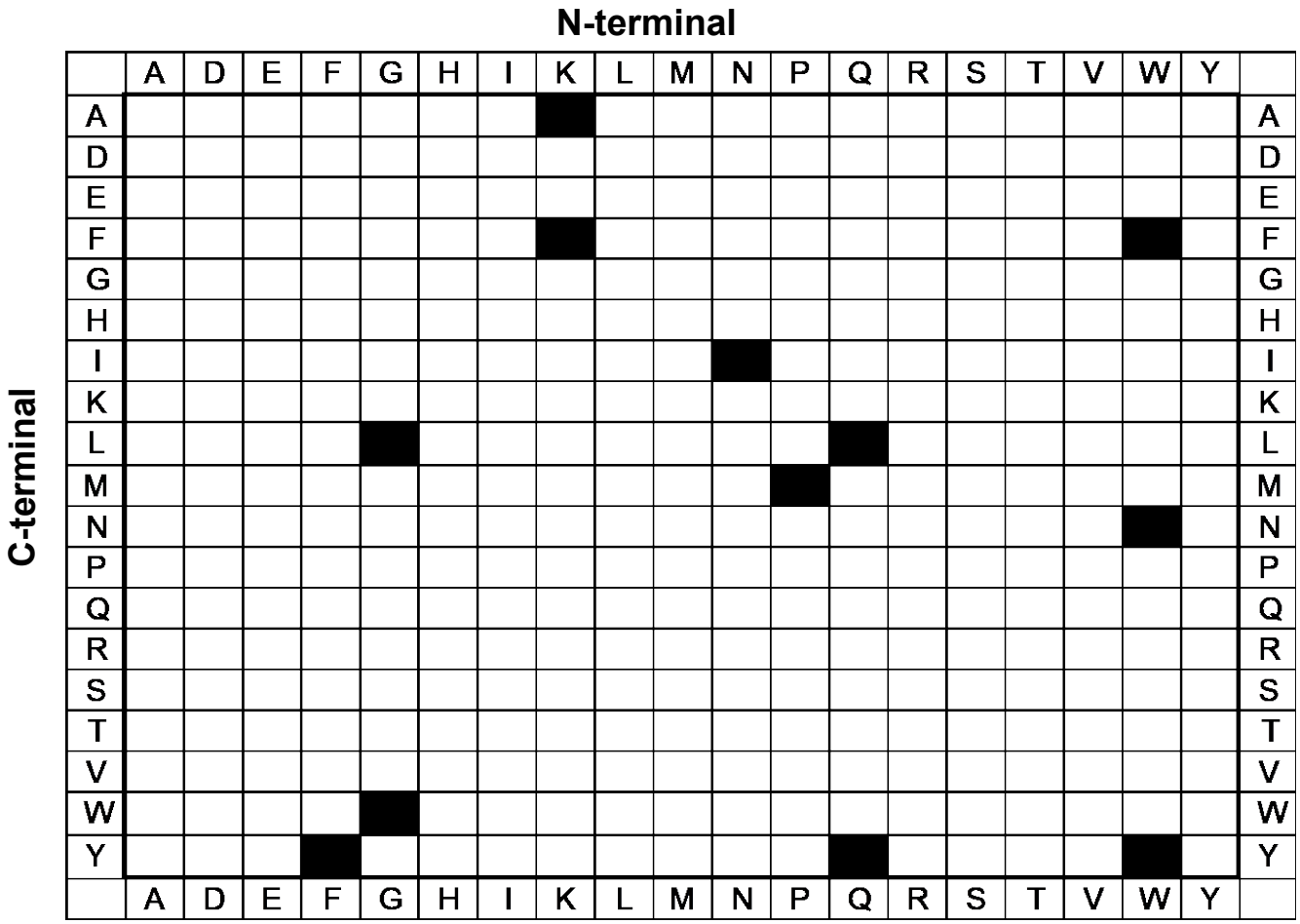


Fig. 5

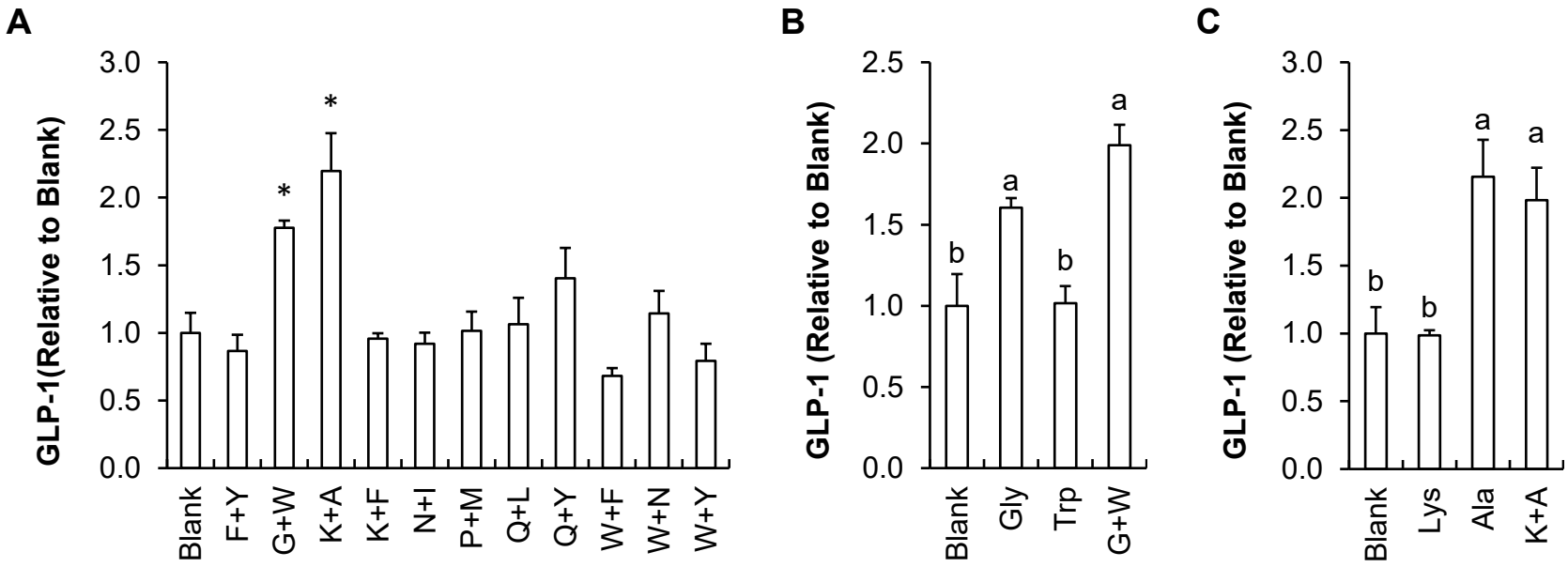
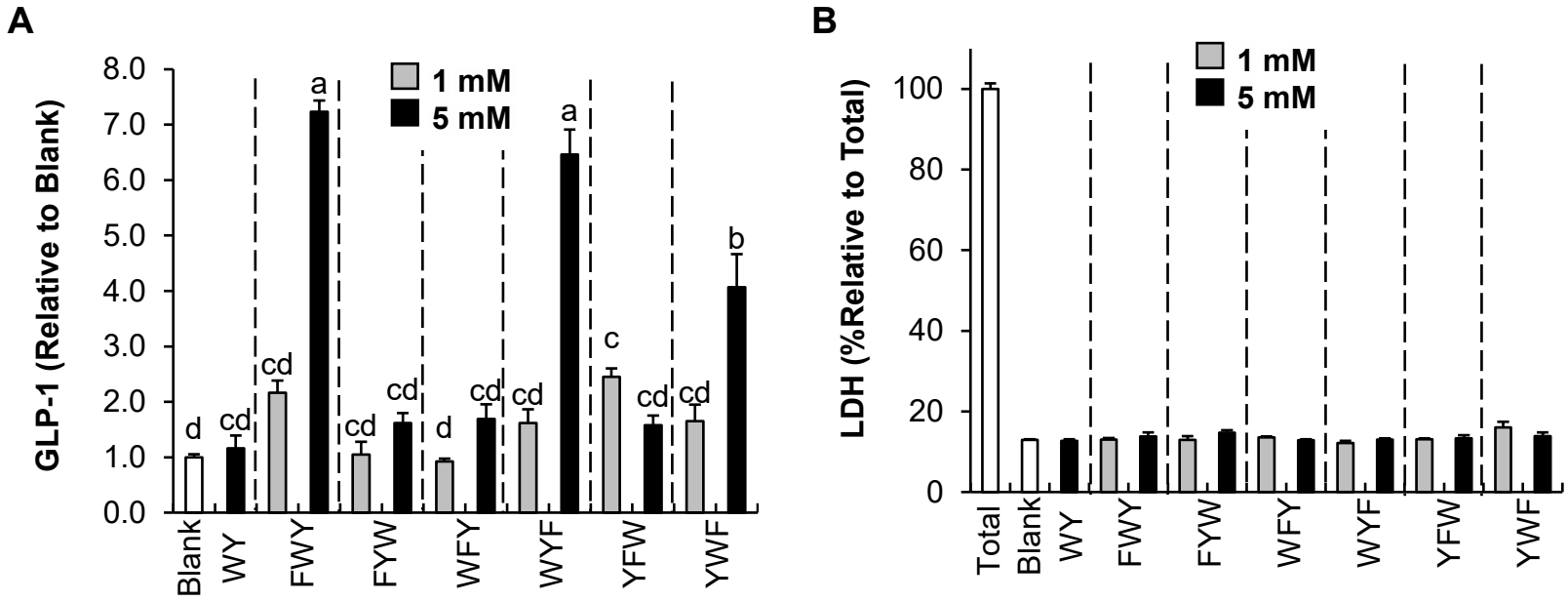

A
GLP-1(Relative to Blank)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Blank
F+Y
G+W
K+A
K+F
N+I
P+M
Q+L
Q+Y
W+F
W+N
W+Y
B
GLP-1 (Relative to Blank)
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
a
Blank
Gly
Trp
G+W
C
GLP-1 (Relative to Blank)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
b
a
a
Blank
Lys
Ala
K+A

Fig. 6


A
GLP-1 (Relative to Blank)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1 mM
5 mM
d
cd
cd
a
cd
cd
d
cd
cd
a
c
cd
cd
b
Blank
WY
FWY
FYW
WFY
WYF
YFW
YWF
B
LDH (%Relative to Total)
100
80
60
40
20
0
1 mM
5 mM
Total
Blank
WY
FWY
FYW
WFY
WYF
YFW
YWF