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D-Alanine in the islets of Langerhans of rat pancreas

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ABSTRACT

Relatively high levels of D-alanine (D-Ala), an endogenous D-amino acid, have been found in the endocrine systems of several animals, especially in the anterior pituitary; however, its functional importance remains largely unknown. We observed D-Ala in islets of Langerhans isolated from rat pancreas in significantly higher levels than in the anterior/intermediate pituitary; specifically, 180 ± 60 fmol D-Ala per islet (300 ± 100 nmol/g islet), and 10 ± 2.5 nmol/g of wet tissue in pituitary. Additionally, 12 ± 5% of the free Ala in the islets was in the D form, almost an order of magnitude higher than the percentage of D-Ala found in the pituitary. Surprisingly, glucose stimulation of the islets resulted in D-Ala release of 0.6 ± 0.5 fmol per islet. As D-Ala is stored in islets and released in response to changes in extracellular glucose, D-Ala may have a hormonal role.

1. Introduction

D-Amino acids (D-AAs) are physiologically important but relatively low-abundant compounds present in all domains of life, including microorganisms [1] and animals [2,3]. D-AAs may be endogenously synthesized through enzymatic activity or obtained from exogenous sources. D-Alanine (D-Ala) has been observed in microorganisms and mollusks [4,5], crustaceans [6,7] and mammals [8,9], including humans [10]. One reported source in animals is intestinal microorganisms [11,12], which incorporate D-Ala into their cell walls [13,14]. In mammals, D-AAs have been found in the central nervous system [15,16] and a variety of organs, e.g., kidney [17,18]. In marine animals, D-Ala is found in tissues other than the nervous system, and may help to adjust their salinity tolerance [6,19]. However, its physiological role has not been determined in higher animals.

Although there appear to be no identified Ala racemases in higher animals, an Ala racemase cDNA clone has been isolated from prawn [20], and a study on crayfish demonstrated that this enzyme is PLP-dependent [21]. The racemase can convert L-Ala into D-Ala and *vice versa* [19,21,22]. It is not known if mammals have an Ala enzyme, but other D-AA racemases have been discovered. For example, the first neuronal D-aspartate (D-Asp) enzymes were only recently reported [23,24], even though the presence of D-Asp in neuronal tissue of animals has been known for much longer [25]. Of course, because intestinal microorganisms produce D-Ala, some D-Ala may be expected in mammals. While D-Ala formation is not fully understood, its catabolism through DAAO is well documented; some species express a DAAO subtype that has a higher affinity to D-Ala as a substrate among various D-AAs [26,27]. Regulation of D-Ala levels by DAAO in tissues and urine has been shown in a mutant mouse lacking DAAO [11,28]. Furthermore, significantly higher amounts of D-Ala have been observed in all brain regions of DAAO knockout mice and rats [8,29,30].

Significant levels of D-Ala are present in endocrine system organs [8,9]. D-Ala is localized to adrenocorticotropic hormone-secreting cells in the anterior pituitary (AP) [31] and to key players of blood glucose control, the insulin-secreting beta-cells in the pancreas [32]. Furthermore, concentrations of D-Ala in rat serum, pancreas and AP change with circadian rhythm [33], suggesting that further studies are needed to correlate D-Ala levels with glucose.

We measured D-Ala levels in the AP and individual islets of Langerhans, as well as D-Ala release from isolated islets. After analyte preconcentration, capillary electrophoresis (CE) with laser-induced fluorescence detection (LIF) was used to measure D-Ala levels, followed by several enzymatic approaches to validate the measurements. CE-LIF offers excellent mass detection limits for small-volume samples [34], enabling us to reliably measure and detect D-Ala and its glucose-dependent release.

2. Materials and methods

2.1. Reagents and buffers

Chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. Ultrapure deionized (DI) water was

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prepared using a Milli-Q filtration system (Millipore, Belford, MA). Phosphate-buffered saline (PBS) was made by dissolving 154 mM NaCl in 20 mM phosphate buffer (pH 7.4). Pyrophosphate buffer was made by mixing appropriate amounts of monobasic and dibasic pyrophosphate solutions to yield 44 mM pyrophosphate (pH 8.0). Acidified acetone was made by mixing acetone, water, and HCl (12 M) in a volume ratio of 40:6:1. Amino acids were stored as solutions dissolved in PBS except L-norleucine (L-Nle), which was in 100 mM borate (pH 9.5) buffer. Naphthalene-2,3-dicarboxaldehyde (NDA) and potassium cyanide (KCN) were purchased from Invitrogen (Grand Island, NY). For non-aqueous capillary electrophoresis (NACE) separations, formamide (FA), N-methyl formamide (NMF), hydroxypropyl γ-cyclodextrin (HP-γ-CD), (1S)-(+)-camphorsulfonic acid (CS), quinine (QN) and sodium acetate were used. For D-Ala peak identification, flavin adenine dinucleotide (FAD), DAAO from porcine kidney (8.2 U/mg solid), and catalase from bovine liver (10,000–40,000 U/mg protein, 34 mg protein/mL suspension) were dissolved in PBS. These solutions contained 5 mM FAD, 15 U/mL DAAO and 15.6–62.5 U/mL catalase, respectively. Dichloromethane was used for liquid–liquid extraction.

2.2. Sample preparation

Vertebrate animal use was approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana-Champaign. Adult male (3–4 months old) Sprague–Dawley rats (Harlan Laboratories, Madison, WI) were decapitated by a sharp guillotine. Pituitaries were surgically removed and placed into ice cold Modified Gey's balanced salt solution (mGBSS): 1.5 mM $CaCl_2$, 4.9 mM KCl, 0.2 mM KH_2PO_4, 11 mM $MgCl_2$, 0.3 mM $MgSO_4$, 138 mM NaCl, 27.7 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 25 mM HEPES, pH 7.2, adjusted with NaOH.

Islet isolation was performed according to Method II described in Roche's manual for Collagenase P [35] with some modifications. Briefly, the pancreas was injected through the common bile duct with 2 mL of 2 mg/mL collagenase P from *Clostridium histolyticum* (Clostridiopeptidase A, EC 3.4. 24. 3; Cat. No. 11 213 857 001, Roche Diagnostics, Indianapolis, IN) dissolved in Krebs–Ringer solution containing 115 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.56 mM $CaCl_2$, 1.2 mM $MgSO_4{\cdot}7H_2O$, 20 mM $NaHCO_3$, 16 mM HEPES, pH 7.5. The injected portion of the pancreas was surgically dissected, placed into the collagenase P solution and cut into small pieces. Incubation by shaking at 37 °C for 20–30 min followed. After multiple washes with Krebs–Ringer solution, islets and small pieces of acinar tissue suspension were deposited into a Petri dish for manual collection of the islets, performed under visual control (Fig. S1A). The quality of islet isolation was determined by visual examination of the collected islets and direct matrix-assisted laser/desorption ionization-mass spectrometry profiling of their peptide content (Fig. S1B) [36,37]. After additional washes with the Krebs–Ringer solution, 7–20 islets were placed into individual plastic Protein LoBind tubes (Eppendorf, Hauppauge, NY) filled with 0.4 mL of Krebs–Ringer solution and incubated for 30 min at 37 °C bubbled with a 95% O_2 and 5% CO_2 gas mixture. Half of the media was replaced with fresh Krebs–Ringer solution and incubated for an additional 30 min. Next, 200 μL of the media was collected and frozen in Protein LoBind tubes as background release samples; 200 μL of 33 mM 2 × stock D-glucose solution prepared with Krebs–Ringer buffer were added to the tubes containing islets, followed by a 40 min incubation. For the stimulated release samples, 200 μL of the solution was collected from each tube, and the remaining solution with islets was frozen in LoBind tubes. All solutions were stored at −80 °C until analysis. CE measurements were performed 1–2 days after sample collection.

2.3. Sample derivatization

For NDA derivatization, each authentic amino acid sample in PBS was mixed with an equal volume of NDA (10 mM in methanol), KCN (20 mM in borate buffer), L-cysteic acid (5 mM in PBS) and L-norleucine (L-Nle, 10 μM in borate buffer) solutions. The mixtures were allowed to react for 30 min in the dark at 20–24 °C, and then diluted with an equal volume of fluorescein (4 μM in DI water) solution to obtain N-substituted 1-cyanobenzo isoindole (CBI)-amino acids with 2 μM fluorescein and 1 μM CBI-L-Nle in preparation for CE measurement. Fluorescein was an internal standard and CBI-L-Nle was used to confirm efficient derivatization.

Islet homogenate samples were mixed with the NDA, KCN and L-Nle solutions at a 2:1:1:1 or 4:1:1:2 ratio. After a 30 min reaction in the dark at 20–24 °C, each mixture was diluted with an equal volume of fluorescein solution. After the derivatization and dilution steps, each derivatized sample represented a 5- or 10-fold dilution of the original solution with 2 μM fluorescein and 1 μM CBI-L-Nle added as internal standards.

Each group of isolated pituitaries was placed in acetone, homogenized and sonicated for 15 min at 20 °C, then centrifuged at 5900×*g* for 5 min at 4 °C to collect the supernatant, which was then dried using a Savant SpeedVac concentrator (Thermo, Milford, MA). Samples were re-suspended in borate buffer for NDA derivatization, performed in the same manner as described above.

Aliquots (200 μL) of releasate samples were preconcentrated by drying under vacuum and reconstituting in 2 μL of borate buffer. To determine the effects of glucose and/or salts from mGBSS on the reconstituted aliquots after derivatization, liquid–liquid extraction with dichloromethane [38] was performed, after which the dichloromethane was evaporated by vacuum and samples were reconstituted in 2 μL of borate buffer. Samples were mixed with NDA, KCN, L-Nle and fluorescein solutions in a volume ratio of 4:1:1:1:1 and incubated for 30 min in the dark at 20–24 °C. The derivatized mixtures containing 1 μM of L-Nle and 2 μM of fluorescein were analyzed with CE-LIF.

For D-Ala peak identification, samples were mixed with DAAO (15 U/mL), catalase (15.6–62.5 U/mL), FAD (5 mM) solutions, and PBS in a volume ratio of 2:1:1:1:1. After incubation for 1 h in a water bath at 37 °C, 1.5 μL of the DAAO-treated samples were mixed with 0.5 μL NDA and 0.5 μL KCN for analyte derivatization. After 30 min incubations in the dark at 20–24 °C, the derivatized samples were diluted with DI water or directly analyzed with CE-LIF depending on the levels of amino acids.

2.4. CE-LIF and non-aqueous CE (NACE) separation

Separations were performed using an automated P/ACE MDQ CE system equipped with LIF detection (Beckman Coulter, Brea, CA), coupled via a fiber optic cable (OZ Optics, Ottawa, ON, Canada) to an external diode laser 56ICS426 (Melles Griot, Carlsbad, CA) emitting 440 ± 8 nm. The laser power was adjusted to 3 mW at the output terminus of the optic cable to avoid damage to the photomultiplier tube (PMT). A band-pass filter of 490 ± 15 nm (Omega Optical, Brattleboro, VT), located prior to the PMT, was used to select the appropriate fluorescence emission band for detection. For separations, uncoated fused-silica capillaries (75 μm inner diameter; 360 μm outer diameter, Polymicro Technologies, Phoenix, AZ) were used. Total capillary length was 40 cm, with an effective length of 30 cm to the detection window. Capillaries were injected with 0.1 M NaOH at a pressure of 30 psi for 1 min and allowed to prime in NaOH for 25 min before initial use. Samples were introduced into the capillaries by pressure injection (0.5 psi for 5 s). The NACE separation buffer contained 40 mM HP-γ-CD, 80 mM CS, 80 mM QN, and 10 mM sodium acetate in FA. Separations were carried out by applying 27 kV of

normal polarity. Between each run, the capillaries were rinsed with NMF (5 min), 0.1% (w/v) $NaBH_4$ (aq) (5 min), 0.1 M NaOH (15 s), and separation buffer (1 min). The electropherograms were analyzed using Origin 8 software (Origin Lab Corp., Northampton, MA). For quantitative analysis, the analyte peak area was divided by the fluorescein peak area, and this normalized analyte peak area used to generate calibration curves.

3. Results

3.1. D-Ala peak identification

We optimized the separation of D-Ala from other cellular compounds as well as the other common D-amino acids, as detailed in the Supplementary material. Because D-Ala levels are much lower than L-Ala [39], baseline separation of Ala enantiomers is required for quantitative analysis (see Fig. S2). Even with an efficient separation from a complex tissue extract, overlapping peaks can be expected. While mass spectrometry can be used as a detector for CE [40–42], its effectiveness for chiral separations requiring surfactant-based chiral selectors is limited. Hence, we used LIF detection to confirm the peak identities via enzymatic degradation, similar to what we used to quantify D-Asp and D-Ser [43,44]. DAAO removes the D-Ala peak but does not change other analyte peaks, although in practice, several additional peaks may appear in the electropherogram from other compounds in the enzyme solutions. We confirmed our identifications using an authentic D-Ala standard. Enzymatic digestion of D-Ala caused the D-Ala peak to disappear in the NACE-LIF electropherograms (Fig. 1A). While DAAO eliminates the amino group of D-Ala and thus prevents the reaction with NDA (Fig. 1B), it did not prevent D-Ala derivatization, nor interfere with the D-Ala peak in the NACE separation at the concentrations of DAAO, catalase and FAD present in our derivatization mixtures. Moreover, it did not affect the CE injection (Fig. 1A); therefore, the DAAO-treated biological samples were directly derivatized and analyzed with CE-LIF.

3.2. D-Ala in islets of Langerhans and pituitary

Islets of Langerhans isolated from rat pancreas were analyzed with NACE-LIF. Between one to 26 islets were contained in each sample. First, islets were incubated in PBS, pyrophosphate buffer, or acidified acetone solution to determine which buffer was better for extraction of D-Ala without mechanical homogenization, which could lead to significant analyte loss due to the small size of islets (Fig. S1A). Additionally, to improve extraction, samples were sonicated for 3 min in an ice-bath. Our results did not show a significant difference between solutions for islet extraction and storage. Because the DAAO was dissolved in PBS, it was selected for further sample storage and analyte extraction.

Representative electropherograms of an NDA-derivatized islet sample, and one treated with DAAO, are shown in Fig. 2. Although the D-Ala peak in the islet sample was small, it was still detectable, and was eliminated by addition of DAAO. For D-Ala quantitation, the working curve was created using the linear equation, $y = 1.88 \times 10^{-3}x$, where y is the ratio of the D-Ala/fluorescein peak areas and x is the D-Ala concentration in the sample in nM. The limit of D-Ala detection was 2.0 nM, and the average D-Ala amount per islet was 180 ± 60 fmol ($n = 6$); D-Ala represents 12 ± 5% of the total amount of Ala (Table 1). Other signals originating from islet samples did not interfere with the D-Ala peak.

The amount of D-Ala in pituitary extracts was also measured with NACE-LIF. The AP and intermediate pituitary extracts contained average D-Ala of 9.50 ± 2.5 nmol/g of wet tissue ($n = 4$); the percentage of D-Ala was 1.7 ± 1% of the total Ala (Table 1).

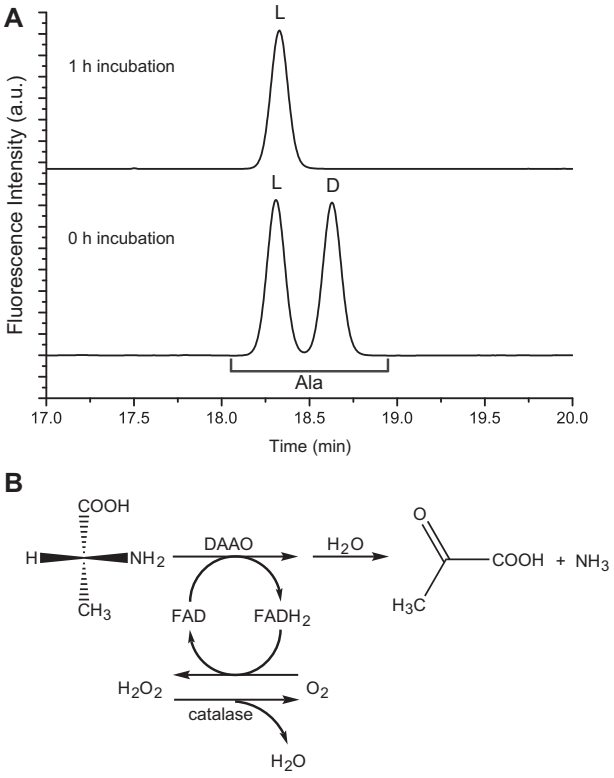

Fig. 1. Confirming the identity of a D-Ala peak using enzymatic oxidation. (A) Electropherograms of separated Ala enantiomers just after the addition of DAAO (bottom) and after 1 h incubation at 37 °C (top). The separation conditions are as described in Fig. S2. (B) Schematic of D-Ala oxidation by DAAO in the presence of the cofactor FAD, which is regenerated by the activity of catalase.

Meanwhile, D-Ala was not found in tissue extract from the posterior pituitary, demonstrating that D-Ala is localized in the AP and intermediate pituitary, in good agreement with previous reports [8,9,33].

3.3. D-Ala release from islets

Releasates from islets were collected at two time points, before D-glucose stimulation and 30 min after stimulation. D-Ala was found in releasate collected from isolated islets in D-glucose-containing mGBSS (stimulated sample) (Fig. 3), but not in the presence of mGBSS alone (control). The D-Ala peak was identified in the same sample with DAAO treatment and D-Ala spiking (Fig. 3), demonstrating that D-Ala was released from islets during D-glucose stimulation. The average D-Ala amount released was 0.6 ± 0.5 fmol per islet ($n = 5$).

4. Discussion

We describe an approach to measure D-Ala in islets of Langerhans and pancreas tissues, and islet releasates, using NACE separation. The amount of D-Ala was 180 ± 60 fmol of D-Ala per islet. Assuming that rat islets are spherical and 0.1 mm in diameter [45,46], D-Ala concentration in a single islet is roughly 340 μM. Given that the density of islets is close to 1.1 g/mL [47], the estimated D-Ala content is approximately 310 nmol/g per islet. The level of D-Ala in Wistar rat pancreas has been reported to reach 29 ± 5.0 nmol/g of wet tissue [9], varying between 7.5 and 65 nmol/g of wet tissue at different times of day [12,33]. Our measurements demonstrate that D-Ala concentration in islets is much higher than its average concentration in the entire pancreas

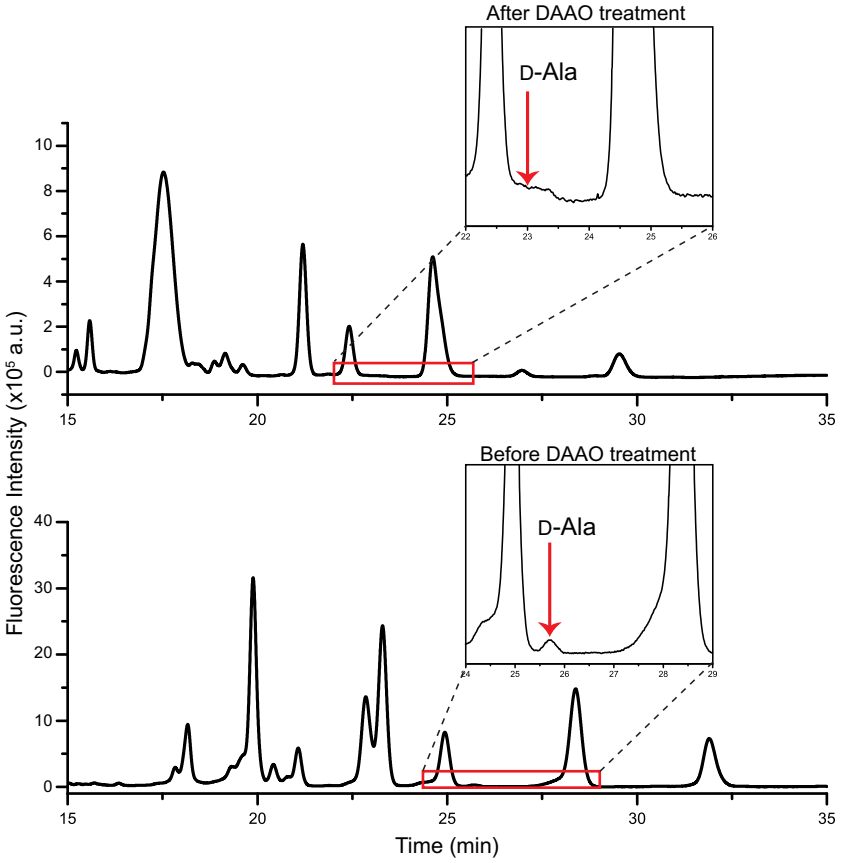

Fig. 2. NACE-LIF analysis of isolated islets demonstrating presence of D-Ala. Electropherograms of an islet sample without DAAO treatment (bottom) and the same sample after DAAO treatment (top) are shown. The D-Ala peak in the original sample disappeared after 1 h of DAAO digestion at 37 °C. The separation conditions are as described in Fig. S2.

Table 1
Amount of D-Ala in various rodent tissues.

Animal	Tissue	D-Ala amount*	% D-Ala**	Ref.
Sprague–Dawley rat	Islets in pancreas	178 ± 58 fmol/islet (309 ± 101 nmol/g islet)	12.4 ± 5.0	[a]
Sprague–Dawley rat	Islets in pancreas	0.55 ± 0.48 fmol release/islet (0.95 ± 0.78 nmol release/g islet)	(>50%; L-Ala was below LOD)	[a]
Sprague–Dawley rat	AP/intermediate pituitary	9.49 ± 2.52	1.7 ± 1.3	[a]
Sprague–Dawley rat	Posterior pituitary	n.d.[b]	n.d.[b]	[a]
Wister rat (nocturnal)	Pancreas	29.2 ± 5.0	1.3	[13]
Wister rat (nocturnal)	AP daytime (sleeping)	63.4 ± 13.2[c]	2.5[c]	[13]
Wister rat (nocturnal)	AP nighttime (waking)	22.4 ± 3.4	N/A	[13]
Mouse	Pituitary gland	29.1 ± 6.0	1.9	[12]
Wister rat (nocturnal)	Pancreas nighttime (waking)	7.5[d]	N/A	[39]
Wister rat (nocturnal)	Pancreas daytime (sleeping)	35[d]	N/A	[39]
Wister rat (nocturnal)	AP daytime (sleeping)	111.3	N/A	[39]
Wister rat (nocturnal)	AP nighttime (waking)	28.9	N/A	[39]
Wister rat (nocturnal, ddY/DAO$^+$)	Pituitary gland	10[d] (day, sleeping); 3.5[d] (night, waking)	0.36–0.13[e]	[17]
ICR rat (nocturnal, germ-free)	Pituitary gland	4.5[d] (day, sleeping); 3.5[d] (night, waking)	N/A	[17]
Wister rat (nocturnal, ddY/DAO$^+$)	Pancreas	65[d] (day, sleeping); 15[d] (night, waking)	1.1–0.33[e]	[17]
ICR rat (nocturnal, germ-free)	Pancreas	4.0[d] (day, sleeping); 6.5[d] (night, waking)	N/A	[17]

[a] Data obtained in this study.
[b] D-Ala was below the limit of detection (less than 0.45 nmol/g wet tissue).
[c] The values obtained from different experimental groups of rats.
[d] The values estimated from the figures in the cited publications.
[e] Calculated values.
* Shown in nmol/g wet tissue unless otherwise noted.
** (D-Ala)/(total Ala) in percentage; AP: anterior pituitary; n.d.: not detectable; N/A: data unavailable.

(Table 1), which is predominantly composed of exocrine acinar tissue. This suggests that D-Ala is specifically localized in the islets of Langerhans, in agreement with a prior immunohistochemical study [32] indicating that D-Ala is present in insulin-secreted beta-cells. Based on these results, we tested whether D-Ala was released upon application of glucose under conditions that would

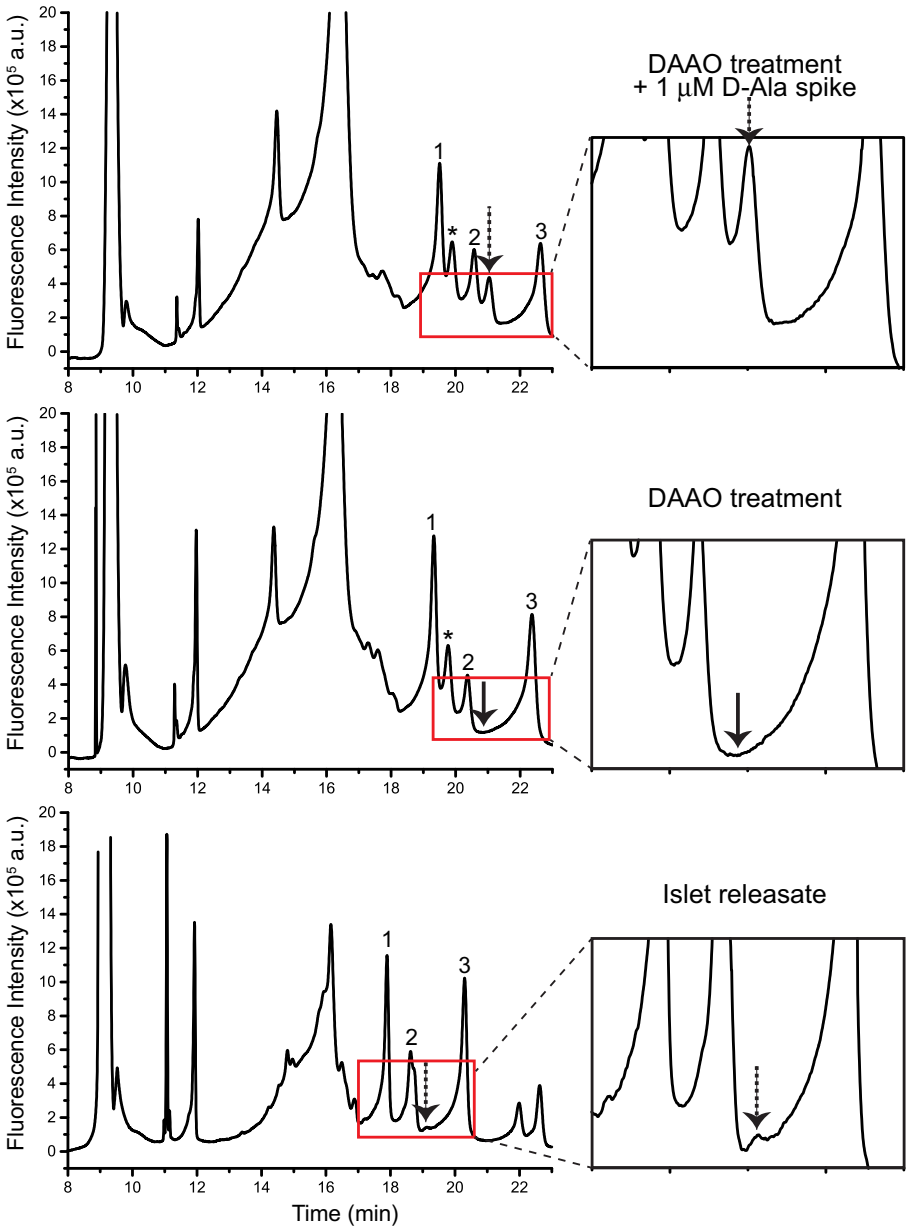

Fig. 3. D-Ala in islet releasate before/after stimulation with 16.5 mM D-glucose. Electropherograms acquired during analysis of the original releasate sample (bottom), the same releasate sample after DAAO treatment (middle), and after DAAO treatment and 1 μM CBI-D-Ala spike after derivatization (top). Dotted arrows indicate D-Ala peaks and solid arrows point towards the region of the electrochromatogram where the D-Ala peak typically presents. Enzymatic treatment produces additional signals (e.g., peaks denoted with *). Numbered peaks were used as reference points of migration time to determine the location of the D-Ala peak. These reference peaks were independent of DAAO treatment and bracketed the D-Ala peak.

cause insulin release from beta-cells. Our finding of glucose-stimulated D-Ala release from islets indicates that D-Ala is within the beta-cells and suggests it is located within the insulin-containing vesicles. On average, 0.6 ± 0.5 fmol of D-Ala was released from an islet, corresponding to 0.3% of the D-Ala in an entire islet. Therefore, a sufficient amount of D-Ala is stored in islets and released over a significant duration of time in response to changes in extracellular D-glucose concentration. Although further work is needed to determine the role of D-Ala, it is intriguing to speculate that it has a cell–cell signaling function as well as being involved in mechanisms of feeding and homeostasis.

Multiple studies have demonstrated that D-Ala is not synthesized within the mammal, but results from the intake of D-Ala from intestinal bacteria [12,32,33], and yet has such high local concentrations, suggests that an efficient and selective uptake mechanism exists for specific tissues. More difficult to understand is the previously reported inverse relationship between plasma insulin and D-Ala levels [33]. Of course, D-Ala may be released from other endocrine structures such as the pituitary, masking the relationship between these two compounds in islets. The possibility of other sources of D-Ala contributing to serum levels is supported by the observed rhythmic changes in D-Ala levels occurring in the AP during different times of day, with variable amounts reported, reaching 22–111 nmol/g of wet tissue [9,33]. Future work will explore the relationship between insulin release and D-Ala release, and determine its physiological effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.153.

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