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Relations between Glucose and D-Amino Acids in the Modulation of Biochemical and Functional Properties of Rodent Islets of Langerhans

Cindy J. Lee, Dong-Kyu Lee, I-An Wei, Tian A. Qiu, Stanislav S. Rubakhin, Michael G. Roper, and Jonathan V. Sweedler*

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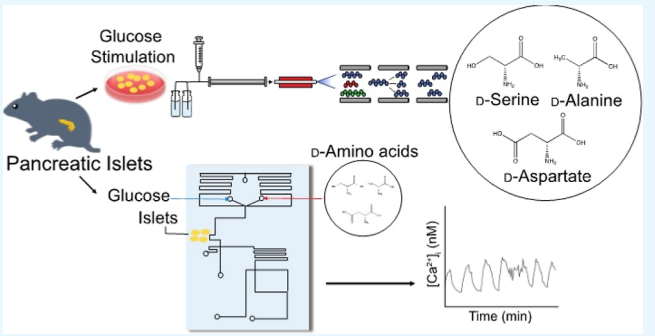
ABSTRACT: The cell-to-cell signaling role of D-amino acids (D-AAs) in the mammalian endocrine system, particularly in the islets of Langerhans, has drawn growing interest for their potential involvement in modulating glucose metabolism. Previous studies found colocalization of serine racemase [produces D-serine (D-Ser)] and D-alanine (D-Ala) within insulin-secreting beta cells and D-aspartate (D-Asp) within glucagon-secreting alpha cells. Expressed in the islets, functional *N*-methyl-D-aspartate receptors are involved in the modulation of glucose-stimulated insulin secretion and have binding sites for several D-AAs. However, knowledge of the regulation of D-AA levels in the islets during glucose stimulation as well as the response of islets to different levels of extracellular D-AAs is limited. In this study, we determined the intracellular and extracellular levels of D-Ser, D-Ala, and D-Asp in cultures of isolated rodent islets exposed to different levels of extracellular glucose. We found that the intracellular levels of the enantiomers demonstrated large variability and, in general, were not affected by extracellular glucose levels. However, significantly lower levels of extracellular D-Ser and D-Ala were observed in the islet media supplemented with 20 mM concentration of glucose compared to the control condition utilizing 3 mM glucose. Glucose-induced oscillations of intracellular free calcium concentration ($[Ca^{2+}]_i$), a proxy for insulin secretion, were modulated by the exogenous application of D-Ser and D-Ala but not by their L-stereoisomers. Our results provide new insights into the roles of D-AAs in the biochemistry and function of pancreatic islets.



INTRODUCTION

D-Amino acids (D-AAs) are endogenous molecules found throughout the mammalian central nervous and endocrine systems.[1] Due to relatively low levels compared to their L-counterparts and difficulties in detection and functional characterization, endogenous D-AAs were recognized as enigmatic molecules in animals. Significant progress in analytical method development and functional characterization approaches allowed the unveiling of the physiological roles for certain D-AAs [e.g., D-serine (D-Ser)] including their involvement in cell-to-cell signaling.[2−4] Relatively higher levels of D-AAs in endocrine systems aided a number of research projects summarized in a recent review published by Chieffi Baccari et al.[5] One endocrine structure of interest is the islets of Langerhans where multiple distinct D-AAs and related enzymes have been detected.[6−8]

Islets of Langerhans are clusters of cells that express and release hormones involved in the regulation of multiple biological functions, including glucose metabolism. In islets, serine racemase,[6] the enzyme that converts L-serine (L-Ser) to D-Ser, and D-alanine (D-Ala)[7] are colocalized within insulin-secreting beta cells, while D-aspartate (D-Asp)[8] is localized within glucagon-secreting alpha cells. Importantly, both D-Ser and D-Ala are known coagonists binding to the GluN1 subunits of the *N*-methyl-D-aspartate receptor (NMDAR), a functionally important glutamate-gated ion channel in different organs such as the brain that is involved in its normal functioning as well as a variety of neurological disorders.[9] D-Asp, on the other hand, acts as an agonist of the GluN2 subunit of the NMDAR.[10,11]

The presence of NMDARs beyond the central nervous system is of growing research interest; for example, NMDARs have been detected in cultured beta cell lines[12] and isolated islets where beta cells constitute the majority of the cellular population.[13] The islet NMDARs are involved in mediating leptin signaling[14] as well as mechanisms of beta cell apoptosis

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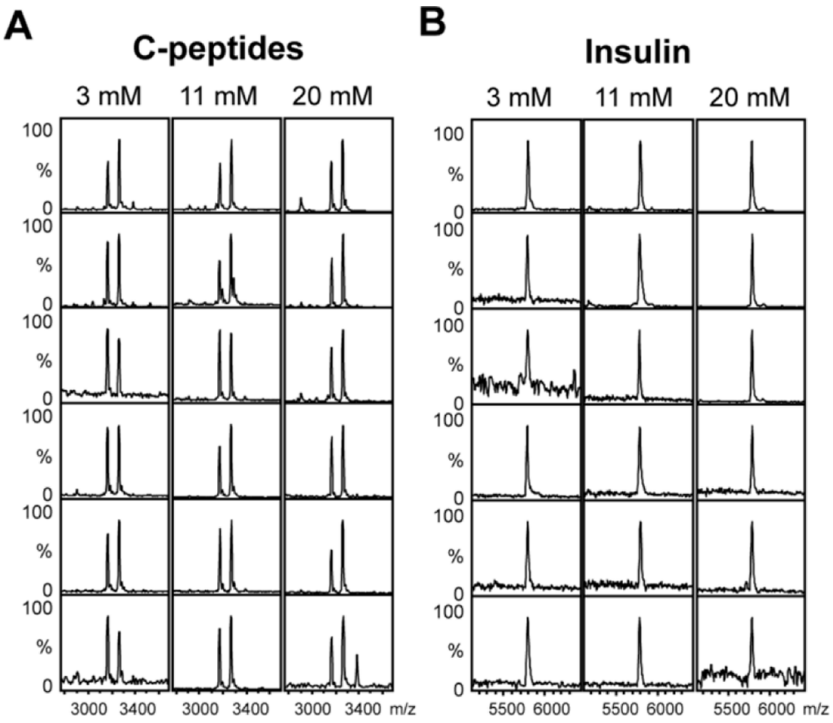

Figure 1. Representative MALDI-TOF MS spectra showing detection of insulin prohormone-related peptides in extracellular environments of 6 islet populations prepared from the pancreata of individual rats and stimulated with glucose. Three different extracellular glucose concentrations were tested. (A) Detection of two insulin C-peptides (m/z 3161 and m/z 3259); (B) detection of insulin (m/z 5800).

and dysfunction.[15] Modulation of NMDAR activity can also affect glucose-stimulated insulin secretion (GSIS).[16] The inhibition of NMDARs in mouse and human islets not only enhances GSIS but also increases, synchronizes, and stabilizes the beta cell activity.[17−19] However, the introduction of D-AAs, specifically D-Ser, suppressed GSIS upon chronic, high-dose supplementation[20] yet improved GSIS upon acute, low-dose supplementation.[21] This suggests a unique role of D-AAs in islet function. Together, the presence of D-AAs and NMDARs in pancreatic islets and their observed functional interactions suggest several functions of the relevant D-AAs.

In this study, we explored the potential functional roles of D-Ser, D-Ala, and D-Asp in islet physiology, especially in relation to islet regulation of glucose homeostasis, using rodents (i.e., rats and mice) as models. We assessed the effects of various levels of extracellular glucose [3 mM (basal), 11 mM (half-maximal), and 20 mM (maximal)] on the intracellular and extracellular levels of D-AAs in cultures of isolated rat islets in vitro. The influence of D-AAs on the intracellular calcium concentration ($[Ca^{2+}]_i$) in isolated mouse islets was also examined using Fura PE3 AM and fluorescence microscopy, a measurement often used for indirect monitoring of insulin secretion. Our study, therefore, allows us to evaluate the actions of D-AAs on rodent islet biochemistry and physiology.

RESULTS

Verification of Insulin Release from Cultures of Isolated Rat Pancreatic Islets by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Isolated rat islets were cultured overnight in RPMI 1640 media and then washed and stimulated with Krebs−Ringer buffer supplemented with different glucose concentrations. In these experiments, islets were first visually inspected under a light microscope to verify their healthy morphology. Additionally, to ensure the retaining of islet function during culturing, an aliquot of glucose-containing Krebs−Ringer solution collected after 30 min of islet stimulation was analyzed directly by MALDI-TOF MS as shown in Figure 1 for the detection of secreted insulin and insulin C-peptides, which are produced with insulin in islet beta cells and can be used as markers to assess insulin secretion.[22] In vitro GSIS is one of the commonly used methods to assess islet function.[23,24] For our study, MALDI-TOF MS was used for peptide detection due to its simplicity, rapid analysis, tolerance to complex mixtures, and high information content.

In Figure 1B, insulin ($m/z \sim 5800$) was detected in every glucose-containing Krebs−Ringer solution added postculture and sampled after 30 min of incubation. Two insulin C-peptides (signals at m/z 3161 and m/z 3259 for insulin 2 C-peptide and insulin 1 C-peptide, respectively[25]) were also observed (Figure 1A). MALDI-TOF MS analysis without internal standards is not a quantitative method as used here.[26] Therefore, our MALDI-TOF MS data indicate that rat islets were functional after culturing and glucose stimulation but did not directly indicate the levels of insulin-related peptides in the culture medium. Glucose concentration-dependent insulin release from rodent islets in vitro is well studied and reported.[27]

Assessment of D-AAs Levels by Liquid Chromatography−Mass Spectrometry-Multiple Reaction Monitoring. Chiral LC−MS/MS-MRM with precolumn derivatization using Nα-(2,4-dinitro-5-fluorophenyl)-L-valinamide (FDVA) was employed for multiplex analysis of intercellular D-Ser, D-Ala, and D-Asp and in islet extracellular environments. Table S1 shows the LC−MS/MS-MRM transitions used in this study. MRM follows not only precursor ions but also their specific fragment ions. Together with known retention times, MRM allows high confidence in analyte identification, as well as

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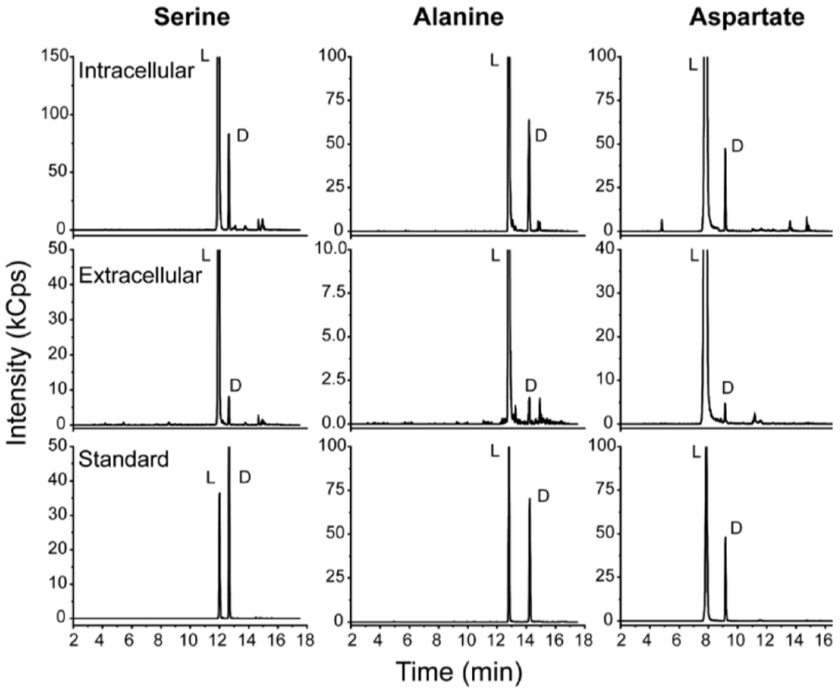

Figure 2. Representative mass chromatograms for the LC−MS/MS-MRM detection of D/L-Ser, D/L-Ala, and D/L-Asp. (Top) Islet analyte extracts−intracellular content; (middle) extracellular environment; and (bottom) analyte standards.

quantification. Representative chromatograms of FDVA-derivatized AAs in the extracted rat islets and extracellular environment, along with standards, are shown in Figure 2. Despite the relatively small D-AA signals detected in the islet extracellular environment samples, they were quantifiable using the calibration curves described by the following linear equations: $y = 237303x - 715.63$, $y = 389697x - 6515.5$, and $y = 456246x - 50271$ for D-Ser, D-Ala, and D-Asp, respectively, where y is the analyte peak area and x is the amount of the analyte injected. All R^2 values were greater than 0.997.

Intracellular Levels of D-AAs in In Vitro Cultures of Rat Pancreatic Islets and Their Relations to Extracellular Glucose Levels. Using LC−MS/MS-MRM, the intracellular levels of D-Ser, D-Ala, and D-Asp were measured in rat pancreatic islets that were overnight incubated and then exposed to different extracellular glucose levels. Overnight culturing allows pancreatic islets to establish steady-state conditions after isolation procedures which include enzymatic pancreas digestion.[23] Figure 3A summarizes data on the intracellular levels of each targeted D-AA determined by the analysis of the analyte extracts of islet populations. These populations were exposed to 3, 11, and 20 mM of extracellular glucose. Data obtained with 3 mM glucose represent the control, demonstrating basal levels of D-AAs. Figure 3B shows the amounts of intracellular D-AA normalized by the total protein content determined from each extract of the islet population. The average intracellular amounts of D-AAs at the basal glucose level were as follows: 127.2 ± 93.5 fmol/μg protein (D-Ser), 53.6 ± 33.9 fmol/μg protein (D-Ala), and 62.8 ± 31.2 fmol/μg protein (D-Asp). The paired data of control 3 and 20 mM glucose in Figure 3B indicate the differences in D-AA amounts in islet populations isolated from the same animal. No significant differences in intracellular D-AA levels were found between data sets obtained from islets exposed to different glucose concentrations, regardless of the normalization methods used. Similar patterns as D-AAs were observed for intracellular L-amino acids (L-AAs) (Figure 3C,D). Expectedly, higher levels of intracellular L-AAs than of D-AAs were observed. However, data variability suggests that an increase in sample size may address whether different pancreatic islet populations exist. Tests of the effects of glucose levels on pancreatic islet D-AA content without overnight culturing demonstrated similar outcomes to the described above in vitro experiments. No significant change in either D-AA or L-AA levels was observed with such an experimental timeline (Figure S1A−D).

Levels of D-AAs in Islet Extracellular Environments in In Vitro Cultures of Rat Pancreatic Islets and Their Relations to Extracellular Glucose Levels. Aliquots of islet extracellular environments (i.e., different levels of glucose-containing media after 30 min of islet stimulation following overnight culturing and washing) were collected for the D-AA measurements (Figure 4A−D). About 100 islets were used for each measurement. Unlike intracellular D-AA levels, the levels of D-Ser and D-Ala detected in extracellular environments of the islets were significantly lower by 80 and 30%, respectively, in cases of 20 mM glucose incubation compared to the control 3 mM glucose exposure (Figure 4A). The average D-AA amounts present in environments containing 20 mM glucose were 0.3 ± 0.2 fmol per islet (D-Ser), 0.3 ± 0.1 fmol per islet (D-Ala), and 1.8 ± 0.1 fmol per islet (D-Asp) (Figure 4B). In the case of L-AAs, levels of all three L-AAs were significantly different in islet environments containing 20 and 3 mM glucose with 90, 60, and 70% reduction in 20 mM glucose for L-Ser, L-alanine (L-Ala), and L-aspartate (L-Asp), respectively (Figure 4C). L-Ser was the only analyte that demonstrated significant differences between data sets obtained for both the 11 and 20 mM glucose concentrations. In Figure S2, both ratios of D-Ala and D-Asp (% D = D/(D + L) × 100) were significantly higher in environments containing 20 mM glucose compared with the controls measured at 3 mM glucose. This

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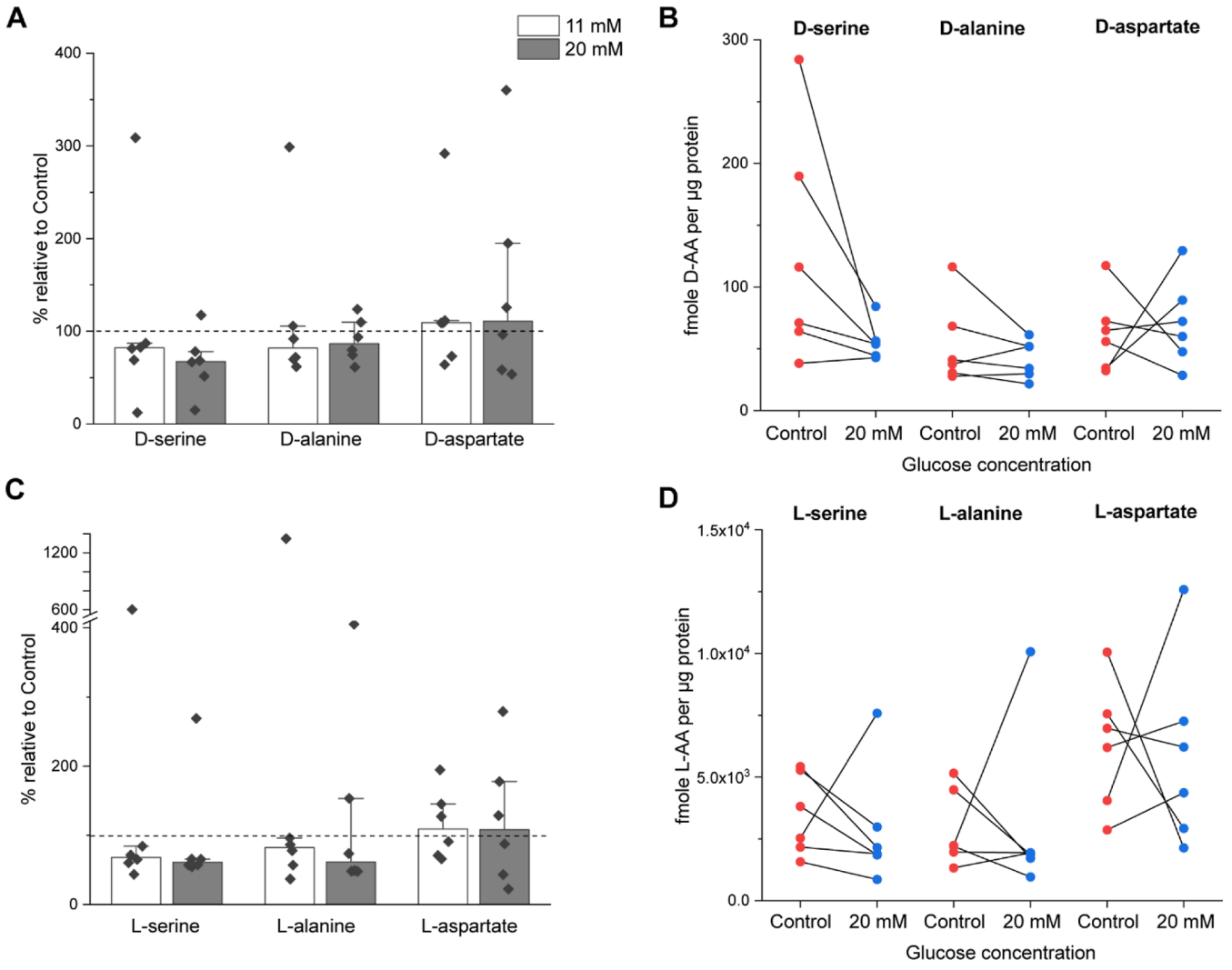

Figure 3. Intracellular levels of AAs in rat pancreatic islets after overnight culturing and exposure to different concentrations of glucose. (A,C) Levels of intracellular D/L-AAs in populations of rat islets exposed to 11 and 20 mM extracellular glucose normalized to the controls (3 mM glucose in extracellular media). (B,D) d/L-AA levels in pairs of populations of islets obtained from the same animal. These populations were exposed to an extracellular medium containing 3 mM (control) or 20 mM (stimulation). The data points obtained from islet populations of the same individual are connected by a solid line. Median along with interquartile ranges as error bars are plotted. Dashed line = control level (100%), n = 6 individual rats.

demonstrated distinct changes in L-Ala and L-Asp levels compared to those of their corresponding D-AAs. However, when islets were glucose-stimulated without overnight culturing, we did not observe a significant difference in the levels of D/L-AAs in extracellular environments of rat islets (Figure S3A−D), which could be caused by islets not having enough time to (re)equilibrate to their resting state after the relatively harsh islet isolation process.

Effects of Exogenous D-AAs on Mouse Islet Intracellular Calcium Oscillations. To assess the effect of exogenous D-AAs on rodent islet function, $[Ca^{2+}]_i$ within mouse islets was monitored using fluorescence microscopy which is used as an indirect measurement of insulin secretion activity.[28] Since significant differences in the levels of D-AAs in islet environments were observed in 20 mM glucose compared to 3 mM (Figure 4), D-AA induced changes in $[Ca^{2+}]_i$ were investigated in the presence of 20 mM glucose, as shown in Figure 5. During exposure to 100 μM D-Ser or D-Ala, the periods of Ca^{2+} oscillations, the time between the beginnings of two consecutive oscillations, were significantly increased (Figure 5A,B,D), but not during delivery of their L-AA counterparts (Figure S4A,B). Lower levels of exogenous D-Ser or D-Ala (i.e., 20 μM), as well as their L-AA enantiomers, did not modulate Ca^{2+} oscillations. D-Asp, up to 100 μM, did not induce a change in Ca^{2+} oscillations (data are not shown), but longer plateau fractions were induced by 500 μM D-Asp (Figure 5C); however, the application of 500 μM L-Asp resulted in a similar change in the Ca^{2+} oscillation periods (Figures 5D and S4C).

DISCUSSION

In recent years, there has been an increasing interest in pancreatic NMDARs, which can be characterized as a potential drug target for diabetes treatment.[17] Here, we further our understanding of pancreatic NMDARs by exploring the endogenous concentrations and roles of D-AAs, enigmatic cell-to-cell molecules that modulate NMDAR activity. To do so, quantitative analyses of the levels of D-Ser, D-Ala, and D-Asp inside and in the extracellular environments of rat islets in

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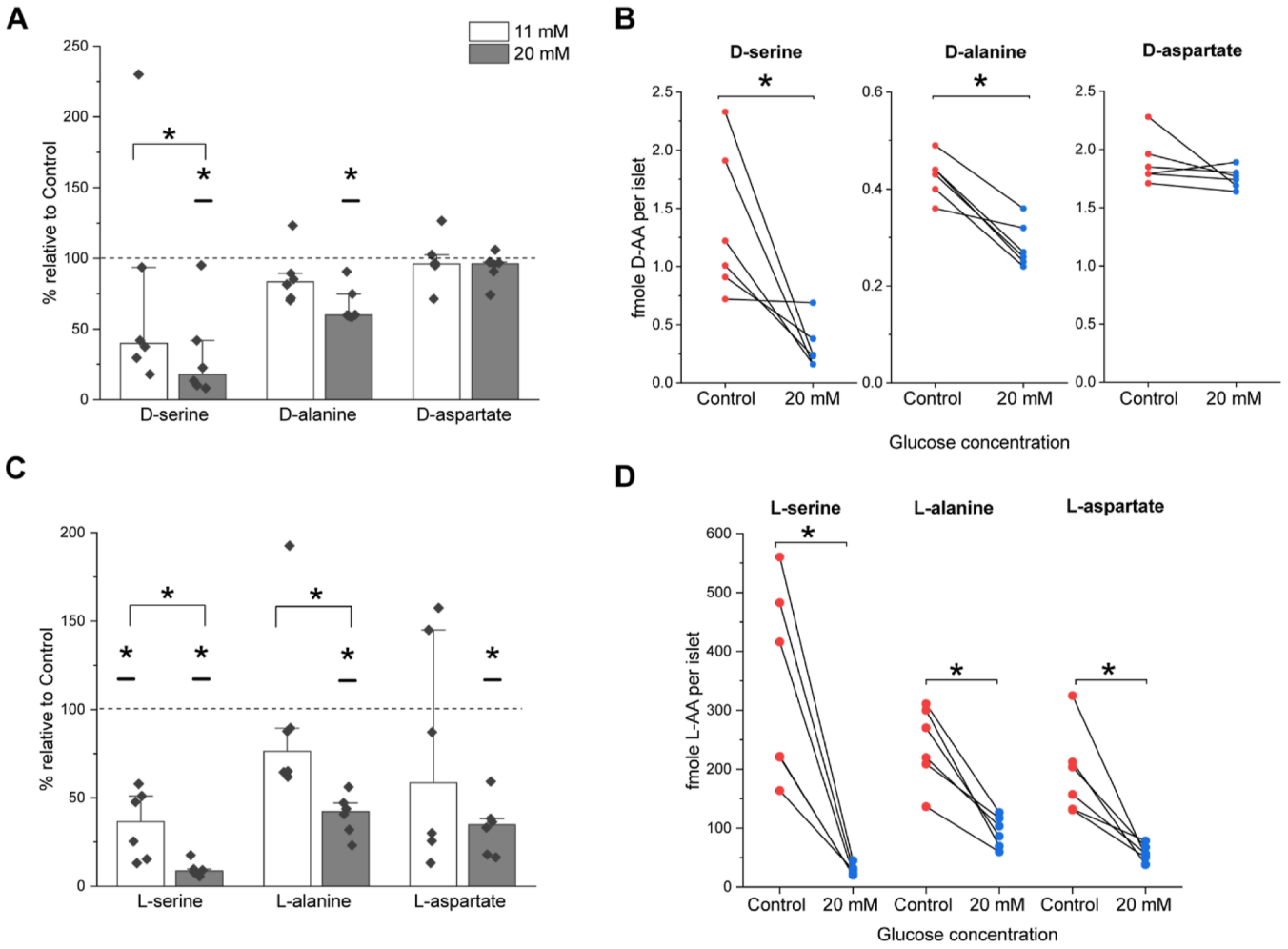

Figure 4. Levels of AAs detected in the islet extracellular environment after overnight islet culturing and islet exposure to different concentrations of glucose. (A,C) Normalized to the control (3 mM glucose) levels of D/L-AA in a rat islet environment after sample treatment with 11 and 20 mM glucose. (B,D) D/L-AA levels observed in extracellular environments of pairs of rat islet samples obtained from the same animal and exposed to either 3 mM or 20 mM glucose. Data points obtained from populations of islets collected from the same animal are connected by a solid line. Median along with interquartile ranges as error bars are plotted. Dashed line = control level (100%), $n = 6$ individual rats, $^{*}p < 0.05$, $\underline{^{*}p} < 0.05$ compared to the control.

different functional states were determined. GSIS buffers containing different concentrations of glucose were used to mimic effects from various diets and uncover the potential roles of D-AAs in the modulation of islet physiological activities. Our in vitro D-AA analysis in Figure 3 shows that intracellular amounts of all three D-AAs, as well as L-AAs, were independent of environmental glucose levels. It is not surprising that L-AAs maintain their intracellular levels (Figure 3C,D) since islets are incubated in nutrient-rich media and certain AAs, such as L-Ser, L-Ala, and L-Asp, are also derived from cellular metabolic processes, such as glycolysis and TCA cycle.[29] Additionally, signaling pathways that are involved in islet homeostasis, like mechanistic target of rapamycin complex 1 (mTORC1) and general control nonderepressible 2/activating transcription factor 4 (GCN2/ATF4), sense and maintain AA levels.[30−32]

Our results show that differences in environmental glucose levels did not affect the average D-AA concentrations in pancreatic islets (Figure 3A,B). However, there were cases of individual islet cultures that demonstrated lower D-AA contents in the presence of 20 mM glucose. This observation indicates the presence of animal- or islet-preparation-specific responses to higher glucose in the extracellular contents. There are a number of mechanisms regulating the D-AA levels within islets. For example, serine racemase that produces D-Ser is expressed in islets,[6] so the islet's ability to replenish D-Ser and regulate its levels is expected. In addition to the synthesis, sodium-dependent neutral amino acid exchanger (ASCT2) that has been shown to transport D-Ser in the central nervous system was also detected in rodent islets,[33,34] along with islet glutamate transporters shuttling D-Asp.[35] The regulation of D-Ala in islets, however, is more enigmatic as an enzymatic system to produce it has yet to be reported in mammals[9] and reports describe that rodents lacking a gut microbiome have greatly reduced D-Ala levels.[36] Without a mechanism to replenish released D-Ala, it is difficult to understand how D-Ala levels remain relatively constant in isolated islets, and further experiments are needed.

Higher levels of all three D-AAs in the pancreas were found in rodents without D-amino acid oxidase (DAAO) or D-aspartate oxidase (DDO),[5,37] the degradation enzymes for nonacidic D-AAs and acidic D-AAs, respectively. However, DAAO and DDO have not been reported in islets. A further study of the mechanisms of synthesis, transport, and

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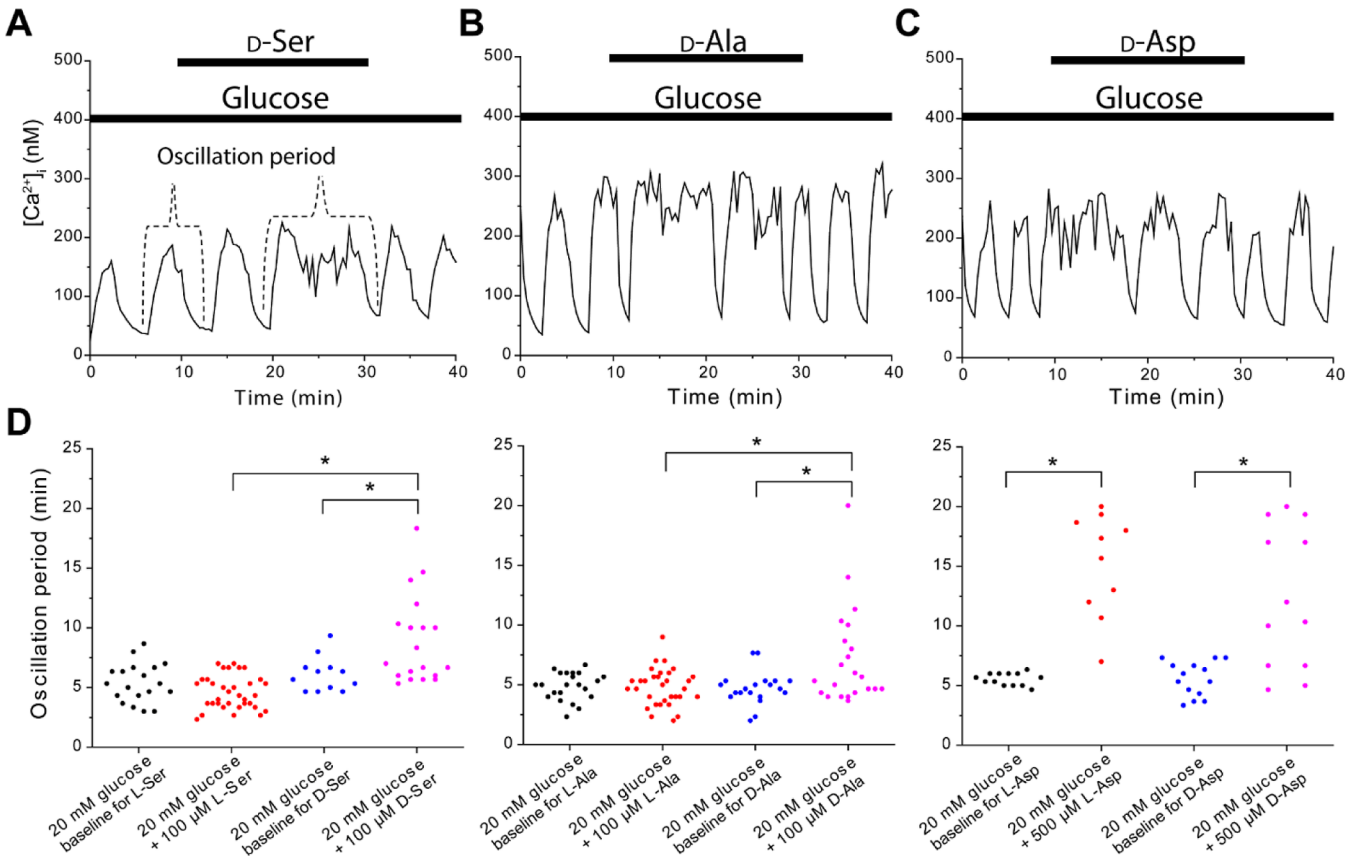

Figure 5. Mouse islet oscillations of $[Ca^{2+}]_i$. (A–C) Representative traces of $[Ca^{2+}]_i$ oscillations in isolated mouse pancreatic islets exposed to 20 mM glucose or mixtures of 20 mM glucose and (A) 100 μM D-serine, (B) 100 μM D-alanine, and (C) 500 μM D-aspartate. The horizontal bars above the plots show the duration of the exposures. (D) Individual oscillation periods for each islet tested during the conditions shown (n = 8 islet trace for each treatment). *p < 0.05.

degradation of these D-AAs in islets is necessary to understand the observed unchanged intracellular D-AA levels in islets in vitro.

As shown in Figure 4A,B, significantly lower levels of D-Ser as well as D-Ala in islet extracellular environments were measured with increasing glucose levels from the basal glucose level of 3 mM. Both D-Ser and D-Ala are present in islet beta cells and after release, they may bind to the extracellular glycine modulatory site of GluN1-containing NMDARs.[9,38,39] Our findings, here, differ from those by Ota et al.[40] where glucose (16.5 mM) induced a release of D-Ala in rat islets. The discrepancy in islet response may be due to differences in islet stimulation conditions; Ota et al.[40] adopted harsher stimulation by exposing freshly isolated islets to high levels of glucose following the incubation without any glucose. Nonetheless, despite the difference in the trend of D-Ala release, the levels in extracellular environments between the two studies were within the same order of magnitude (0.6 ± 0.5 vs 0.3 ± 0.1 fmol per islet).

Corroborating our results, Morikawa et al.[41] previously reported an inverse relationship between the amounts of D-Ala in the pancreas and plasma insulin levels, although their measurements sampled the acinar pancreatic tissue as well. Our results indicate that pancreatic islets, after overnight culturing to (re)equilibrate to the resting state, have opposing dynamics between extracellular levels of these two D-AAs and insulin release in vitro. This suggests that the D-AAs may not be contained in the same vesicles as the insulin. Other possibilities include D-AAs localizing to the smaller vesicles within the beta cells[42] or using a distinct mechanism of secretion such as via an amino acid transporter as reported within the nervous systems; interestingly, many of the same transporters are expressed in the pancreas.[33,43,44] As NMDARs have been suggested to act in an inhibitory manner in GSIS, as evidenced by GluN1 knockout islets and the application of inhibitor MK-801,[17,18] there may be negative feedback loops within islets to secrete less D-Ser and D-Ala from beta cells upon increasing glucose levels to minimize the activation of the NMDARs.

On the other hand, the extracellular levels of D-Asp, which is localized in alpha cells and acts on the glutamate site (GluN2) of NMDAR,[45] remained consistent, despite the increase in glucose levels (Figure 4A,B). Although D-Asp has been found in beta cells at low amounts, most D-Asp is located in glucagon-secreting alpha cells.[8,46] Glucagon release is inhibited by the increase of extracellular glucose and insulin levels.[47] This again suggests that D-Asp is stored/released in a distinct manner than glucagon. Interestingly, Ogunkunle et al.[48] recently reported reduced levels of glutamate in the releasates of islets stimulated with 20 mM glucose compared to 3 mM glucose using LC–MS/MS. Since glutamate is reported to be predominately secreted from the alpha cells together with glucagon,[18] different mechanisms of D-Asp and glutamate secretion in alpha cells are further expected.

Our bulk measurements of islet environments (Figure 4B) only partially represent the true local concentrations of D-AAs

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present in the extracellular spaces separating islet cells and involved in the modulation of NMDAR activity in beta cells. Therefore, we assessed the impact of exogenous D-AAs by monitoring free $[Ca^{2+}]_i$ in isolated mouse islets (Figure 5). Oscillations of this intracellular second messenger are widely hypothesized to be due to the oscillatory nature of glycolysis.[49] Notably, oscillations of insulin release, which are essential for the maintenance of euglycemia,[50] coincide with the free $[Ca^{2+}]_i$ oscillations in islets and are involved in mechanisms of Ca^{2+}-dependent vesicular release of hormones.[28,51] Our results of an increased plateau fraction in Ca^{2+} oscillations after exogenous delivery of D-Ser, D-Ala, or D-Asp, therefore, are consistent with a potential autocrine or paracrine role of secreted D-AAs, especially D-Ser and D-Ala, in modulating GSIS via islet NMDARs. The observed actions specific to D-Ser and D-Ala on the changes in Ca^{2+} oscillations also align with the fact that these D-AAs show greater potency than their L-enantiomers for the activation of NMDARs.[9] In the case of D-Asp, changes in oscillation were induced by both Asp enantiomers. The EC_{50} values of D-Asp and L-Asp for the glutamate site of NMDARs are higher than those of D-Ser and D-Ala for the glycine site of NMDARs,[52] which explains the higher concentrations of D-Asp required in our study to modulate oscillation periods. The mechanism of insulin secretion in relation to NMDARs in pancreatic islets, however, remains unclear due to the divergent findings described by previous reports.[12,17,20,21] Some parameters, for example, concentrations of D-AAs, glucose levels tested, and rodent strain differences, are likely responsible for observed differences. Future studies involving an additional NMDAR activator (e.g., NMDA), a competitive antagonist of the D-AA binding site of NMDARs, or real-time direct monitoring of insulin upon the introduction of exogenous D-AAs may further reveal the mechanism of how D-AAs affect the GSIS through NMDARs.

Besides D-AAs, pancreatic islet environments containing 20 mM glucose also exhibited significantly lower amounts of L-Ser, L-Ala, and L-Asp in comparison to environments supplemented with 3 mM glucose. Our findings aligned with previous reports where decreased AA concentrations and secretion rates were observed with increased levels of glucose.[48,53−55] One possible reason for this observation is that while islets maintain the homeostatic L-AA levels intracellularly (Figure 3C,D), secretion of L-AAs is suppressed to possibly involve a larger fraction of L-AAs in mechanisms responsible for increases in glucose metabolism and insulin secretion, as evidenced by L-Ser[56] and L-Ala.[57] Furthermore, AA reuptake by cells may be enhanced during high beta cell activity. Although our direct measurement of intracellular L-Ala showed no changes on average, Dixon et al.[57] demonstrated increased L-Ala consumption in islets after glucose introduction based on the measurement of NADH, a byproduct of pyruvate formation. This supports our hypothesis that the usage of L-AAs is indeed increased within the islets. Overall, more work is still required to elucidate the roles of D-AAs, along with L-AAs, in pancreatic islets. However, our findings of intracellular and extracellular environmental levels of D-AAs, as well as their exogenous application, in rodent islets support the autocrine or paracrine signaling roles of D-AAs in islet function in glucose metabolism.

CONCLUSIONS

We initially hypothesized that D-AAs located in islets are largely stored within the insulin- or glucagon-containing vesicles and are coreleased upon changes in extracellular glucose concentrations. Nevertheless, the observed decrease in the analyte amounts (i.e., D-Ser and D-Ala) in the islet environment and their homeostatic intracellular levels with elevated glucose levels suggest potential negative feedback within islets during the GSIS decreasing D-AA release and thus reducing the NMDAR activity in beta cells. Although further work is needed to validate the mechanisms of D-AA regulation of glucose homeostasis in islets, our work, here, demonstrates the simultaneous changes in intracellular and extracellular levels of D-AAs in rodent islets upon glucose stimulation, suggesting again a cell-to-cell signaling role of these enigmatic molecules.

METHODS

Materials and Chemicals. All materials and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher Scientific (Hampton, NH, USA), or Thermo Fisher (Waltham, MA, USA) unless stated otherwise. Collagenase P for mouse islet isolation was purchased from Roche Diagnostics (Indianapolis, IN, USA). For fabrication of the microfluidic device, poly(dimethylsiloxane) (PDMS) and curing agent from Dow Corning (Midland, MI, USA) and SU-8 photoresist from Kayaku Advanced Materials (Westborough, MA, USA) were used. All solutions were prepared with LC−MS or ultrapure deionized (DI) water (Millipore, Bedford, MA, USA).

Isolation of Rodent Pancreatic Islets. *Rat Sampling and Isolation of Rat Pancreatic Islets.* All animal-related procedures were performed according to the animal use project approved by the Institutional Animal Care and Use Committee (IACUC, University of Illinois, Urbana−Champaign) as well as local and federal regulations. Adult male (3−4 months old) Sprague−Dawley rats (Envigo, Madison, WI, USA) were euthanized using CO_2 asphyxia and then perfused transcardially with cold (4 °C) modified Gey's balanced salt solution (mGBSS) containing the following: 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, and 25 mM HEPES, pH 7.2. This step allows for blood removal and quickly chills the animal's body. A total of 10 rats were used for this experiment.

A protocol from Zmuda et al.[58] was adapted for the isolation of rat pancreatic islets. The pancreas was intraductally loaded with 3 mL of digestion buffer containing 0.53 Wünsch unit/mL (equivalent to 0.1 mg/mL) of Liberase TL dissolved in Hank's balanced buffer solution (HBSS) without calcium and magnesium (GIBCO, cat no. 14185-052) supplemented with 5 mM calcium chloride and 25 mM HEPES. The addition of 0.1 mg/mL Deoxyribonuclease I (DNase I) to this mixture eliminated sticky nucleic acid spilled out from digested tissue cells. The perfused pancreas was placed in the same digestion buffer and warmed to 37 °C for 15 min. After digestion was over, 10 mL of cold HBSS with 0.2% bovine serum albumin (w/v, HBSS-BSA) was added to the tube and the tubes were vigorously shaken to dissociate the tissue. After centrifugation at 300*g* for 3 min, the supernatant was removed. This HBSS-BSA solution addition, resuspension, centrifugation, and supernatant removal procedure was repeated 3 times. At the

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final resuspension, the supernatant was poured off by inverting the tube, and the inside of the tube was wiped with a paper towel to maximally remove the residual buffer. Subsequently, cell pellets were resuspended in 5 mL of Histopaque 1077 with gentle vortexing; 4 mL of HBSS was carefully added on top of the resulting islet and acinar tissue suspension, forming two zones divided by the sharp interface. This sample was centrifuged at 900*g* for 15 min. The islets migrated to the Histopaque/HBSS interface were collected by Pasteur pipet and filtered through a 70 μm cell strainer to clear out the cells and cell debris. After washing rat islets with HBSS 3 times, they were moved either to the Petri dish with cold HBSS-BSA, followed by isolating islets for overnight culture, or to the Petri dish with cold modified Krebs−Ringer solution (125 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 25 mM HEPES at pH 7.4) with basal glucose level (3 mM) for GSIS right after islet collection as described further in section Glucose Stimulation. Rat islets were manually picked, counted, and transferred to the other Petri dish using a 10 μL pipet.

Isolation and Culture of Mouse Pancreatic Islets. Mouse pancreatic islets were obtained from 25 to 40 g male CD-1 mice (Charles River Laboratories Internal, Wilmington, MA, USA) by a previously described method.[59] The islet isolation protocol was approved by the Florida State University Animal Care and Use Committee (protocol #202000078). The isolated mouse islets were incubated at 37 °C and 5% CO_2 in RPMI 1640 medium (Corning, Corning, NY, USA) supplemented with 11 mM glucose, L-glutamine, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL gentamicin. Mouse islets were used within 4 days after isolation. Prior to each experiment, 1 μL of 5 mM Fura PE3 AM in DMSO and 1 μL of Pluronic F-127 in DMSO were added to 1.998 mL of RPMI 1640 medium for a final concentration of a 2.5 μM Fura PE3 AM mixture. Mouse islets were incubated in this 2 mL solution at 37 °C and 5% CO_2 for 40 min and were manually loaded into a microfluidic device after the incubation as described in section Free Intracellular Calcium Level Measurements after D-AA Stimulation.

Glucose Stimulation. Harvested rat islets (about 300 islets per rat split into populations of ∼100 islets per treatment) were cultured overnight in RPMI 1640 media with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) or stabilized in prewarmed Krebs−Ringer buffer with basal glucose for 30 min. They were incubated at 37 °C in a surrounding atmosphere containing 5% CO_2. Islets after overnight culture in RPMI media were washed with Krebs−Ringer buffer containing 3 mM glucose 3 times and kept in this solution for 30 min before exposure to glucose. The islets from the same rat were transferred to 100 μL of prewarmed glucose-supplemented Krebs−Ringer solutions containing either 3, 11, or 20 mM glucose. Such preparations were placed into a 37 °C incubator for 30 min. A 90 μL aliquot of each media was collected, dried in a SpeedVac, and stored at −80 °C until the extracellular D-AA measurement, while 1 μL was put onto the MALDI metal plate to detect extracellular insulin and insulin C-peptides. Islets for intracellular measurement were directly transferred to 500 μL of LC−MS grade methanol located in the Precellys lysing kits (CK14 2 mL, Bertin Corp., Rockville, MD, USA) and stored at −80 °C until analysis.

Detection of Insulin and C-Peptides in Islet Extracellular Environment. An ultrafleXtreme mass spectrometer equipped with a Smartbeam II laser (Bruker Corp., Billerica, MA, USA) was used to detect C-peptides (m/z 3161 for insulin 2 C-peptide and m/z 3259 for insulin 1 C-peptide) and insulin ($m/z \sim 5800$) signals.[25] Bruker calibration standard II plus human insulin (MP Biomedicals, Irvine, CA, USA) or the rat islet releasates were mixed with the same volume of 2,5-dihydroxybenzoic acid (DHB) solution (20 mg/mL in 50% acetonitrile +0.1% trifluoroacetic acid) and put on the MTP 384 target plate with polished steel (Bruker) before analysis. Analyte detection in the m/z range of 1000−7000 was performed in linear mode and positive polarity. Mass spectrometer parameters were set as the following: ion source 1, 25 kV; ion source 2, 22.65 kV; lens, 5 kV; pulsed ion extraction 350 ns; detector gain, 2.7 kV. Data acquisition and processing were performed with FlexControl 3.4 and FlexAnalysis 3.4 (Bruker).

AA Extraction from Rat Islets. The methanol and water used for AA extraction were LC−MS grade. 500 μL of water was added to rat islets stored in the Precellys lysing kit at −80 °C. The islet samples were shaken 3 times for 20 s at 6800 rpm with 30 s pauses in between using a Precellys Evolution homogenizer (Bertin Corp.). The supernatant was collected, mixed with chloroform in a 4:1 ratio (v/v), vortexed, and centrifuged at 10,000*g* for 5 min to precipitate proteins. The top aqueous layer was collected and dried using a SpeedVac. 1000 μL of methanol/water in a 1:1 ratio (v/v) was added to the remaining in the lysing kit to repeat the homogenization and protein removal. The aqueous layer was combined with the previously dried AA extract, evaporated to dryness in a SpeedVac, and stored at −80 °C until LC−MS/MS analysis. The protein pellet was dried separately in a SpeedVac and dissolved in 200 μL of 4% SDS (w/v) in 25 mM NaOH for total protein quantification in islet samples using the Micro BCA Protein Assay Kit.

D-AA Measurements. *Modified Marfey's Derivatization.* All solvents used for LC−MS/MS analysis were of LC−MS grade. Rat islet extract and extracellular environment samples were reconstituted in 0.5 M $NaHCO_3$ solution. Standards containing D/L-Ser, D/L-Ala, and D/L-Asp ranging from 0.1 to 50 μM for D and 0.1−400 μM for L were also prepared in 0.5 M $NaHCO_3$ solution to generate calibration curves for quantitative analysis. The AAs in samples and standards were precolumn derivatized with *Nα*-(2,4-dinitro-5-fluorophenyl)-L-valinamide (FDVA) [1 mg/mL in acetonitrile (ACN)], a modified Marfey's reagent, in a 1:1 ratio (v/v). The mixture was then incubated in an oven at 60 °C for 3 h. After derivatization, the sample mixtures were dried in a SpeedVac.

Sample Desalting. Pierce Peptide Desalting Spin Columns were used to desalt and preconcentrate the FDVA-derivatized samples for subsequent LC−MS/MS analysis. The manufacturer's instructions with some modifications were followed. In brief, the spin columns were first conditioned using ACN and then 0.1% TFA in water. The dried samples were reconstituted in 150 μL of 0.1% TFA in water and loaded onto the column. For maximum binding of FDVA-derivatized AAs, the sample flowthrough was reloaded onto the same column a total of 3 times before discarding it. The columns were then washed with 0.1% TFA in water. To elute desalted analytes, 200 μL of 50% ACN, 0.1% TFA solution was loaded twice. Then, 200 μL of 80% ACN, 0.1% TFA solution followed by 150 μL of 80% ACN, 0.1% TFA solution (one each) was used to collect the eluate into the same tube. The eluate was dried in a SpeedVac and subjected to LC−MS/MS analysis.

LC−MS/MS. Dried samples reconstituted in loading buffer (95% LC mobile phase A, 5% ACN) and FDVA-derivatized

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standards diluted 40-fold in loading buffer were analyzed using an Elute UHPLC module coupled with a Bruker EVOQ Elite Triple Quadrupole Mass Spectrometer (Bruker). A reversed-phase Kinetex phenyl-hexyl HPLC column [2.6 μm particle size, 100 Å pore size, 100 mm length × 2.1 mm inner diameter (Phenomenex, Torrance, CA, USA)] with mobile phase A: 25 mM ammonium formate; mobile phase B: methanol; and flow rate 300 μL/min was used. The mass spectrometer equipped with an electrospray ionization source was operated under the following conditions: spray voltage for negative mode, −3500 V; cone temperature, 250 °C; cone gas flow, 20, heated probe temperature, 400 °C; probe gas flow, 45; and nebulizer gas flow, 50. Each analyte retention time and parent and product fragmentation parameters were established from the corresponding FDVA-derivatized standards using the MRM mode. The resulting chromatograms were processed and analyzed by a Bruker MS Data Review (Bruker). The data were normalized either relative to the analyte amount found in samples treated with basal glucose level (3 mM) or absolute in terms of the total protein amounts (islet intracellular) or per islet (extracellular environment).

Free Intracellular Calcium Level Measurements after D-AA Stimulation. *Microfluidic Device and Optical System.* The PDMS-glass hybrid device was fabricated using soft lithography with SU-8 2075 photoresist adapted from the previous study.[60] All channels were 250 × 40 μm (width × height). Two 60 mL syringes were used to hold the perfusion solutions: one syringe contained 20 mM glucose and the other contained 20, 100, or 500 μM L-AA or D-AA dissolved in 20 mM glucose. Both solutions were made in a balanced salt solution (pH 7.4) that consisted of 125 mM NaCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.9 mM KCl, 25 mM tricine, and 1 mg mL^{-1} of BSA. The syringes were connected to the perfusion inlets on the microfluidic device. The relative height of these two syringes caused different flow rates of the two solutions resulting in a final concentration of amino acid perfused into the islet chamber.[60,61] In each experiment, a group of 3−6 mouse islets was held in a 0.6 mm diameter chamber, where solution exchange occurred. The temperature was maintained at 36 ± 0.5 °C using polyimide flexible heaters sandwiched with a thermocouple as described elsewhere.[62]

Calcium Measurements. For fluorescence imaging, a xenon arc lamp equipped with a filter wheel and shutter (Sutter Instruments, Novato, CA) containing 340 ± 5 and 380 ± 5 nm filters (Omega Optical 340AF15 and 380AF15, Brattleboro, VT, USA) was focused onto the islet chamber using a 10×, 0.5 NA microscope objective. Fluorescence emission was collected with the same objective, passed through a dichroic mirror and a 510 ± 84 nm emission filter (Semrock, Rochester, NY, USA), and detected by a CCD camera (QImaging, Surrey, BC, Canada). The images of all islets were collected every 20 s with 150 ms exposure for each excitation wavelength. The ratio of fluorescence emission at 510 nm after excitation at 340 and 380 nm was collected to calculate free $[Ca^{2+}]_i$ using predetermined calibration values by reported methods.[63]

To quantify the period of calcium oscillations, we measured the time between the beginnings of two consecutive oscillations was measured. An oscillation was defined as when $[Ca^{2+}]_i$ elevated above a threshold level until dropping below it. The threshold level for each experiment was determined as the average free $[Ca^{2+}]_i$ during the nadirs of oscillations during the 20 mM glucose period before treatment plus ten times the standard deviation.

Statistics. The sample size of individual rats used in each experiment was $n = 6$ for overnight culturing conditions and $n = 4$ for without overnight culturing, respectively. To compare the effect of extracellular glucose stimulation to that of the basal level on islets from the same rat, a paired sample Wilcoxon signed rank test, a nonparametric test, was applied, as removing outliers may result in a loss of information from the same individual. Furthermore, $n = 10$ individual mice were used for mouse islet isolation and overnight culturing. Mann−Whitney U test was used to evaluate the effect of exogenous L-AA or D-AA on free $[Ca^{2+}]_i$ in mouse islets. Statistical analyses were performed using Origin Pro (OriginLab Corp., Northampton, MA, USA). Significant differences were assessed at $p < 0.05$.

■ ASSOCIATED CONTENT

Data Availability Statement

All processed data supporting the conclusions are presented in the main text and Supporting Information. All original data are available and can be shared upon request by contacting the corresponding author.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05983.

Table S1: LC−MS/MS-MRM quantifier and qualifier transition channels built using FDVA-derivatized amino acids. Figure S1: intracellular levels of AAs in rat pancreatic islets evaluated after their isolation and without overnight culturing. Figure S2: the differences in D-AA ratios in islet extracellular environments after overnight culturing, culture media washing, and exposure to different levels of extracellular glucose. Figure S3: levels of AAs detected in extracellular environments of freshly isolated islets containing different concentrations of glucose. Figure S4: representative traces of $[Ca^{2+}]_i$ oscillations in mouse islets exposed to 20 mM glucose and each L-AAs (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Jonathan V. Sweedler − *Department of Chemistry and the Beckman Institute, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, United States;* orcid.org/0000-0003-3107-9922; Email: jsweedle@illinois.edu

Authors

Cindy J. Lee − *Department of Chemistry and the Beckman Institute, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, United States;* orcid.org/0000-0002-4707-3755

Dong-Kyu Lee − *Department of Chemistry and the Beckman Institute, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, United States;* Present Address: College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea; orcid.org/0000-0003-2355-8560

I-An Wei − *Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States*

Tian A. Qiu − *Department of Chemistry and the Beckman Institute, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, United States;* Present Address: Department

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of Chemistry, Michigan State University, East Lansing, MI 48824 United States.; orcid.org/0000-0002-7254-9233

Stanislav S. Rubakhin − *Department of Chemistry and the Beckman Institute, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, United States;* orcid.org/0000-0003-0437-1493

Michael G. Roper − *Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c05983

Author Contributions

Cindy J. Lee: Writing—Original Draft, Investigation, Formal analysis; **Dong-Kyu Lee**: Investigation, Writing—review and editing; **I-An Wei**: Writing—Original Draft, Investigation, Formal analysis; **Tian A. Qiu**: Conceptualization, Writing—Original Draft; **Stanislav S. Rubakhin**: Resources, Writing—review and editing; **Michael G. Roper**: Supervision, Conceptualization, Writing—review and editing; **Jonathan V. Sweedler**: Supervision, Conceptualization, Writing—review and editing.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AA, amino acid; ACN, acetonitrile; D-AA, D-amino acid; D-Ala, D-alanine; D-Asp, D-aspartate; D-Ser, D-serine; FDVA, *Nα*-(2,4-dinitro-5-fluorophenyl)-L-valinamide; GSIS, glucose-stimulated insulin secretion; $[Ca^{2+}]_i$, intracellular calcium concentration; L-AA, L-amino acid; L-Ala, L-alanine; L-Asp, L-aspartate; LC−MS/MS, liquid chromatography−mass spectrometry; L-Ser, L-serine; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRM, multiple reaction monitoring; NMDAR, *N*-methyl-D-aspartate receptor

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