

Prolonged L-alanine exposure induces changes in metabolism, Ca²⁺ handling and desensitization of insulin secretion in clonal pancreatic beta-cells

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Summary

Acute insulin-releasing actions of amino acids have been studied in detail, but comparatively little is known about the beta-cell effects of long-term exposure to amino acids. This study examined the effects of prolonged exposure of beta cells to the metabolizable amino acid, L-alanine. Basal insulin release or cellular insulin content were not significantly altered by alanine culture, but acute alanine-induced insulin secretion was suppressed by 74% ($p < 0.001$). Acute stimulation of insulin secretion with glucose, KCl or KIC following alanine culture was not affected. Acute alanine exposure evoked strong cellular depolarization after control culture, while area-under-the-curve (AUC) analysis revealed significant ($p < 0.01$) suppression of this action after culture with alanine. Compared with control cells, prior exposure to alanine also markedly decreased ($p < 0.01$) the acute elevation of $[Ca^{2+}]_i$ induced by acute alanine exposure. These diminished stimulatory responses were partially restored after 18 h culture in the absence of alanine, indicating reversible amino acid-induced desensitization. ^{13}C NMR spectra revealed that alanine culture increased glutamate labelling at position C4 (by 60%, $p < 0.01$), as a result of an increase in the singlet peak, indicating increased flux through pyruvate dehydrogenase. Consistent with this, protein expression of pyruvate dehydrogenase kinases, PDK2 and PDK4, was significantly reduced. This was accompanied by a decrease of cellular ATP ($p < 0.05$), consistent with diminished insulin-releasing actions of this amino acid. Collectively, these data illustrate the phenomenon of beta-cell desensitization by amino acids, indicating that prolonged exposure to alanine can induce reversible alterations to metabolic flux, Ca^{2+} handling and insulin secretion.

Introduction

Insulin secretion from pancreatic beta-cells is tightly regulated by circulating glucose, and modulated by a number of physiological and pharmacological factors [1-4]. Amino acids represent an important class of modulators of pancreatic beta-cell function, and specific individual or mixtures of amino acids regulate insulin secretion both *in vitro* and *in vivo*. Pancreatic beta-cells are equipped with a range of specific amino acid transporters many of which are Na⁺ dependent [5, 6]. Three principal modes of insulinotropic action of amino acids have been characterized [7, 8]. Firstly, direct membrane depolarization resulting from beta-cell transport of cationic amino acids (such as arginine) promotes Ca²⁺-channel opening and insulin release. Other metabolizable amino acids (such as leucine) can increase intracellular ATP evoking closure of ATP-sensitive K⁺ channels (K_{ATP} channels), membrane depolarization and Ca²⁺ influx stimulating insulin release. In addition to these mechanisms, certain amino acids including alanine may stimulate insulin release through both metabolism and as a direct result of the membrane depolarizing actions of Na⁺ co-transport triggering Ca²⁺ influx and ultimately insulin release. While the insulinotropic effects of arginine and leucine have long been known [9-11], the importance of alanine in acute regulation of beta-cell metabolism and function has only been appreciated more recently [12-14]. Of particular note, is the positive effect of the abundant amino acid alanine on glucose metabolism and insulin secretion [12] suggesting that this amino acid may have a significant impact on beta-cells.

While acute exposure to nutrients and other insulinotropic agents exert positive beta-cell actions, prolonged exposure may induce desensitization and other detrimental effects [1, 3, 4, 15]. Glucose is the principal physiological regulator of insulin release but chronic exposure to high levels of this sugar is associated with beta-cell deterioration, so-called glucose desensitization and glucotoxicity perhaps involving oxidative stress [16-18], but this has been questioned [19, 20]. Similarly, free fatty acids act as important signalling molecules and beta-cell fuels, enhancing insulin release, but long-term exposure can induce beta-cell lipotoxicity particularly in the presence of high glucose levels [21]. A recent metabolomic analysis of urine from type 2 diabetic subjects and animals models showed that the main metabolic disturbances occurred in amino acid metabolism with an increased excretion of glutamine, glutamate, alanine, taurine and ornithine for human subjects [22]. The long-term consequences of such altered amino acid concentrations on the pancreatic beta cell has received little attention to date.

A recent study indicated that prolonged exposure to the amino acid, alanine, induced up-regulation of gene expression of certain metabolic and signal transduction elements coupled with enhanced protection against pro-inflammatory cytokine-induced apoptosis [23]. Notably, these preliminary studies indicated an alteration in beta-cell responsiveness subsequent amino acid-induced stimulation [23]. This observation prompted the present study examining in more detail the nature of the demise in insulin secretion and beta-cell function following prolonged alanine exposure, demonstrating induction of reversible alterations to metabolic flux, calcium handling and insulin secretion.

Materials and methods

Reagents

[3-¹³C]alanine was obtained from Goss Scientific (Great Baddow, Essex, UK). ¹²⁵I-bovine insulin was purchased from Amersham Biosciences (Bucks, UK). All other chemicals were obtained from Sigma-Aldrich Chemical Company (Poole, UK) and BDH chemicals (Poole, UK). Culture media and foetal bovine serum were obtained from Gibco (Glasgow, UK). Membrane potential kit and calcium assay kit were purchased from Molecular Devices (Sunnyvale, CA, USA).

Cell culture and treatment with L-alanine for NMR studies

Experiments utilized glucose- and amino acid-responsive clonal pancreatic BRIN-BD11 beta-cells [4, 24]. These cells have proven to be particularly useful as model beta-cells for studies involving NMR based experiments which require substantial cellular mass [12, 25]. The origin of BRIN-BD11 cells are described elsewhere [4, 26]. These cells provide an appropriate beta-cell model as evidenced by studies of insulin secretion [4, 24], electrophysiology, Ca²⁺ handling [27] and cellular defense [4]. In addition, signalling, insulin secretory and cell viability responses to glucose, amino acids as well as other stimuli have been well characterized [26-29]. BRIN-BD11 cells were grown and maintained in RPMI-1640 tissue culture medium with 10% (v/v) foetal bovine serum, 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose in a 37 °C in a humidified atmosphere of 5% CO₂ and 95% air as described previously [26].

For experiments investigating prolonged exposure to L-alanine, cell monolayers were maintained in T175cm² flasks (Greiner, Germany) and treated for 18 h in the presence of 10 mmol/l L-alanine. A concentration of 10 mmol/l was used as this provides a robust and reproducible stimulus for insulin secretion experiments [30]. For comparison cells were grown in parallel in the absence of L-alanine (Standard culture; Control). Cells were then washed with phosphate buffered saline and pre-incubated at 37 °C for 20 min in Krebs-Ringer Bicarbonate (KRB) buffer comprising 115 mmol/l NaCl, 4.7 mmol/l KCl, 1.28 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄·7H₂O, 10 mmol/l NaHCO₃, 5 g/l bovine serum albumin (pH 7.4) supplemented with 1.1 mmol/l D-glucose. This was followed by 1 h incubation with KRB test buffer containing 1.1 mmol/l glucose and 10 mmol/l [3-¹³C]alanine and aliquots of test buffer was removed and stored at -20 °C for further analysis. After test exposures metabolites were extracted from cells using a perchloric acid extraction procedure. Briefly, cells were washed with ice-cold phosphate-buffered saline, perchloric acid (6%) added and cells scraped from tissue culture flasks. Extracts of six culture flasks (approximately 10⁸ cells) were pooled, centrifuged at 200 g and resulting supernatants neutralised with KOH (5 M and 0.1 M solutions) before soaking pellets overnight in 0.1 M NaOH. The protein concentration was determined using the Lowry method [31] and neutralised supernatant was centrifuged again (at 200 g), and supernatant treated with Chelex-100 resin before lyophilization. Each experiment was carried out on at least three independent cultures of the BRIN-BD11 cells. Lyophilised cell extracts were dissolved in 3 ml potassium phosphate buffer (100 mmol/l; pH 7.0), centrifuged and supernatant carefully removed before addition of 10% D₂O and pH checked and adjusted where necessary with 0.1 M NaOH or 0.1 M HCl. NMR analyses were then performed as detailed below.

NMR analyses

For ¹³C NMR experiments, an insert containing 5 % v/v dioxane in water was used as an external signal intensity reference. A solution of L-alanine, L-glutamate, lactate and D-glucose, each at a concentration of 100 mmol/l, was prepared and used to quantitate concentrations of metabolites in the ¹³C spectra. Proton decoupled ¹³C spectra were acquired on a Bruker DRX 500 spectrometer using a 10 mm broadband probe. Typically spectra were acquired with 32 K data points using 90° pulses, 260 ppm spectral width, 2.5 s relaxation delay and 12000 - 20000 scans. Spectra were recorded at 25 °C.

Chemical shifts were referenced to tetramethylsilane at 0 ppm. Data were processed with no zero filling using Bruker WINNMR software and exponential multiplications with 2 Hz line broadening were performed. Assignments of intermediate metabolites were made by comparison with chemical shift tables in the literature or by addition of 100 mmol/l of unlabelled amino acid. The amount of ^{13}C in each resonance was evaluated by integration of the extract peaks and the corresponding peaks in the standard sample relative to the dioxane signal. Corrections for the natural abundance signal were made. In the case of the aspartate peaks the amount of ^{13}C was estimated by use of the integrals and the known dioxane concentration. The contribution of the individual isotopomers was assessed using the deconvolution routines in WINNMR. The absolute enrichments of the L-glutamate were related to the glutamate concentration in the extracts to give the specific enrichments.

Determination of insulin release and cellular content

Insulin release was determined from BRIN-BD11 cells monolayers. Briefly, cells were harvested, resuspended in culture medium and seeded in each well of 24-well multiplates at a density of 1.5×10^5 cells per well. After overnight incubation and attachment at 37 °C, standard RPMI-1640 tissue culture medium was replaced with standard culture media (no L-alanine) or culture media supplemented with 10 mmol/l L-alanine and cells returned to culture. After 18 h, culture media was removed from each well and replaced with 1 ml KRB buffer supplemented with 0.1 % (w/v) bovine serum albumin and 1.1 mmol/l D-glucose, for a 20 min pre-incubation at 37 °C. At the end of the pre-incubation period, KRB pre-incubation buffer was removed and replaced with 1 ml of KRB test buffer supplemented with 1.1 mmol/l glucose and 10 mmol/l L-alanine as indicated in the Figures. After 60 min incubation, aliquots of test buffer were removed from each well and stored at -20 °C. Cellular insulin was extracted by addition of 1 ml of ice-cold acid-ethanol solution (75% v/v ethanol, 1.5% v/v concentrated HCl). Insulin was measured from each sample by dextran-charcoal radioimmunoassay [32], using guinea-pig anti-porcine insulin serum, and rat insulin standard.

Assessment of cellular integrity following alanine culture

Following culture in the absence or presence of alanine, cellular integrity was assessed by means of either the neutral red or MTT (dimethyl thiazolyl blue tetrazolium bromide) assay. For the neutral red assay 2×10^4 cells were seeded into each well of 96 well plates and cultured for 18 h in the absence or presence of 10 mmol/l alanine. Cells were then exposed to 100 μl of neutral red solution (PBS, 0.05 mg/ml neutral red) for 2 h, and after washing with PBS, 100 μl of glacial acetic acid solution (50 ml ethanol, 1 ml glacial acetic acid, 49 ml dH₂O) was added to each well, plates were shaken for 15 min for cell lysis and neutral red release from cells. Absorbance from each well was then recorded at 540 nm and compared to control (culture in absence of 10 mmol/l alanine) and viability expressed as a percentage relative to control. For MTT analyses, 2×10^4 cells were seeded into each well of 96 well plates and cultured for 18 h in the absence or presence of 10 mmol/l alanine before incubation with 100 μl MTT solution (1 : 10 dilution of the 5 mg/ml stock - MTT in RPMI without phenol red) at 37 °C for 30 min. MTT solution was then removed and cells lysed with 100 μl DMSO (dimethylsulphoxide). Optical densities were recorded at 562 nm with a reference wavelength of 650 nm and cell viability expressed as a percentage relative to control.

ATP and protein expression determinations

For ATP and protein measurements the cells were seeded in 6 well plates (1×10^6 cells) and allowed to form monolayers overnight. Cells were then exposed to culture in the absence or presence of 10 mmol/l L-alanine for 18 h followed by a 1 h incubation in KRB buffer, as described earlier. Following incubation cells were lysed with a somatic cell ATP-releasing reagent and placed on ice [33]. Intracellular ATP was measured using a luciferin/luciferase-based assay (Biaffin GmbH) ice [33].

Protein expression was examined using Western blotting where cells were washed with ice cold PBS before lysis in ripa buffer (0.5 M Tris-HCl pH7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 nM EDTA). Samples were centrifuged at 12,000 g for 15 min at 4 °C, supernatant collected and total protein determined using a BCA assay. Samples were then subjected to SDS-PAGE electrophoresis using a 12.5 % resolving gel after which resolved proteins were transferred to nitrocellulose membranes and blocked for 1 h at room temperature with Tris-buffered saline supplemented with 0.1 % Tween and 5 % non-fat powdered milk. Nitrocellulose blots were incubated for 2 h with the pyruvate dehydrogenase kinase-2 (PDK-2) polyclonal antibody (Abgent) or 4 h with the PDK-4 polyclonal antibody, washed and incubated with horseradish peroxidase (Santa Cruz Biotechnology). Bound antibody was visualised by using ECL according to the manufacturers instructions (Pierce, Rockford, IL, USA). Equal loading was varied by analysis of total c-Jun N-terminal kinase (JNK) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The blots were then exposed to Hyperfilm and bands quantified by scanning densitometry.

Semi-quantitative analyses of PDK-2 and PDK-4 gene expression

For semi-quantitative detection of PDK-2 and PDK-4 mRNA levels, cells were grown and maintained in 6-well plates. Total RNA was extracted using the thiocyanate-phenol-chloroform method using Tri reagent according to the manufacturers protocol (Molecular Research Centre Inc., Cincinnati). Complementary DNA (cDNA) was synthesized and amplified by PCR using primers for PDK-2, PDK-4 and 18S (housekeeping gene). The following primer sequences were used: PDK-2 forward-AGTTCAGTGCCTGGTC, PDK-2 reverse-GTTGGTGGCATTGACTTCT, PDK-4 forward-CGTCGCCAGAATTAAAGCTC, PDK-4 reverse-TAACCAAACCAGCCAAAGG. PCR was carried out according to the following protocol: 2 min denaturing step at 94 °C followed by 25-30 cycles of amplification (45 s denaturation at 94 °C, 45 s annealing at 60 °C, 45 s extension at 72 °C) and a final extension step of 2 min at 72 °C. PCR products were analysed on ethidium bromide-stained agarose gels, photographed under UV transillumination and data expressed relative to housekeeping gene 18S.

Determination of metabolite levels

Alanine concentrations were determined in the media following the incubation periods using an enzymatic-based reaction coupled to the production of NADH. Total glutamate and lactate levels were determined using a YSI 7100 amino acid analyzer.

Measurement of membrane potential and intracellular calcium

Membrane potential and intracellular calcium ($[Ca^{2+}]_i$) were determined using monolayers of BRIN-BD11 cells [34]. Cells were seeded into 96-well black-walled, clear bottom microplates at a density of 1.0×10^5 cells per well and allowed to attach overnight, before replacing culture media with either standard culture media or 10 mmol/l alanine-supplemented culture media. After 18 h culture, media in each well was replaced with 100 μ l of KRBB and monolayers incubated for 20 min after which 100 μ l of either FLEX membrane potential assay kit [35] or FLEX calcium assay kit (Molecular Devices, CA, USA), was added to wells at 37 °C as previously described [34]. Fluorometric data were acquired using the FLEXstationTM scanning fluorometer and integrated fluid transfer workstation (Molecular Devices, CA, USA). The cells were exposed to excitation light from a xenon-arc flashlamp at a wavelength of 530 nm (membrane) or 485 nm (calcium) and subsequent fluorescence emission measured at 565 nm (membrane) or 525 nm (calcium) using a bottom read mode. Emission cut-off filters were set at 550 nm for membrane potential or 515 nm for $[Ca^{2+}]_i$.

Statistical analysis

Results are expressed as means \pm SEM. Statistical significance was evaluated by using one-way ANOVA.

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Results

Effects of prolonged exposure to alanine on cellular integrity, insulin secretory responses and cellular insulin content

Analysis of cellular integrity, by neutral red and MTT assays, following culture of cells in the absence or presence of 10 mmol/l alanine revealed that alanine culture did not significantly alter cellular integrity compared with standard culture (Figure 1). The insulin-secretory responses to 10 mmol/l alanine after 18 h culture in the absence or presence of 10 mmol/l alanine are given in Figure 2A. As illustrated, while prior exposure to 10 mmol/l alanine did not significantly alter basal insulin release at 1.1 mmol/l glucose, alanine culture markedly suppressed the subsequent stimulatory effect of acute addition of alanine compared with control cells (74% reduction, $p < 0.001$). This alanine-induced demise in insulin output was partially restored ($p < 0.01$) after a further 18 h recovery period involving culture in absence of alanine. As shown in Figure 2B, 18 h exposure to 10 mmol/l alanine in culture did not significantly affect insulin content compared with control cells exposed to standard culture medium, and there was evidence of enhanced content after an 18 h recovery period. Taken together, these data indicate alanine-induced beta-cell desensitization.

It was also of interest to assess if the observed desensitization was specific to beta-cell alanine responsiveness, by assessing the impact of prolonged exposure to alanine on subsequent responses to a depolarizing concentration of KCl or other key nutrient secretagogues (Figure 3). Alanine culture did not alter the marked 6.7-fold ($p < 0.001$) acute insulinotropic action of 30 mmol/l KCl (Figure 3A). Similarly, the insulin-releasing effects of acute stimulatory 16.7 mmol/l glucose, 10 mmol/l mannose or 30 mmol/l KIC were not affected by alanine culture (Figure 3B). To probe the alanine-induced desensitization mechanism further cells were cultured with the non-metabolizable amino acid analogue aminoisobutyric acid (AIB) which shares the same transport mechanism as alanine (Figure 4). As with alanine culture, prolonged exposure to AIB in culture did not alter basal insulin release at 1.1 mmol/l glucose. Interestingly, alanine culture also served to significantly reduce subsequent responsiveness to AIB (by 44%, $p < 0.05$), although not to the same extent as the reduction in alanine-induced insulin output (Figure 4). Likewise, AIB culture served to decrease alanine-induced insulin release (by 71%, $p < 0.001$) and the insulinotropic response to AIB (by 63%, $p < 0.01$) (Figure 4). While alanine or AIB culture also reduced serine-induced insulin secretion compared with standard culture ($p < 0.01$) (Figure 4).

Effects of prolonged exposure to alanine on membrane potential and intracellular calcium

The impact of culture with 10 mmol/l alanine on subsequent membrane potential and $[Ca^{2+}]_i$ responses to acute stimulation with alanine (10 mmol/l) are illustrated in Figure 5. As shown, after standard culture in the absence of alanine, BRIN-BD11 cells demonstrated a marked membrane depolarization, but area-under-the-curve (AUC) analysis revealed that this increase in membrane potential was significantly ($p < 0.01$) suppressed in cells exposed to alanine culture (Figure 5A), although membrane potential at 1.1 mmol/l glucose was not altered (data not shown). The suppressed depolarization response to acute stimulation with 10 mmol/l alanine was partly reversed after an 18 h recovery period consistent with a partial restoration of insulin release.

Corresponding to the membrane depolarizing effect of alanine, after standard culture, acute stimulation with 10 mmol/l alanine caused a notable rise in $[Ca^{2+}]_i$ consistent with the insulin-releasing actions of this amino acid (Figure 5B). However, AUC analysis also demonstrated a significant ($p < 0.01$) reduction in the 10 mmol/l alanine-induced rise in $[Ca^{2+}]_i$ following alanine culture (Figure 5B). At 1.1 mmol/l glucose $[Ca^{2+}]_i$ levels were similar following culture in presence or absence of alanine (data not shown). Again, the reduced rise in $[Ca^{2+}]_i$ following alanine culture was partially

restored by 18 h recovery in culture medium in the absence of alanine, consistent with the view that the alanine-induced beta-cell suppression was a reversible desensitization.

Effects of prolonged exposure to alanine on subsequent alanine metabolism

Following incubation with [$3\text{-}^{13}\text{C}$]alanine, the end products included glutamate labelled at positions C2, C3 and C4 and aspartate labelled at position C2 and C3 (Figure 6). The glutamate C4 peak consisted predominantly of a singlet peak due to enrichment at the C4 position only (Figure 6A). A small doublet peak was also present, which was due to glutamate labelled at position C3 and C4 (Figure 6A). Following 18 h alanine culture there was a significant increase in glutamate labelled at position C4 but not at position C2 and C3 (Figure 6A and Figure 6B). Closer examination of the C4 peak revealed that the C4 singlet (C4S) increased significantly from 18.2 ± 1.9 to 29.6 ± 1.7 nmol/mg protein ($P < 0.005$). The singlet at C4 (C4S) can only be labelled by entry via pyruvate dehydrogenase (PDH), indicating increased flux through PDH following prolonged alanine exposure [36].

Calculation of the specific enrichment showed that there was no change for glutamate position C2 and C3 (Figure 6C). However, the percentage enrichment increased in position C4 significantly (Figure 6C). The amount of ^{13}C labelled alanine in the extracts did not significantly change following prolonged exposure to alanine (0.53 ± 0.01 $\mu\text{mol/mg}$ protein for control conditions and 0.49 ± 0.04 $\mu\text{mol/mg}$ protein for alanine culture). Interestingly, lactate release did not change significantly from control conditions during the 1 h incubation (25.7 ± 1.8 nmol/mg protein for control conditions, and 22.5 ± 3.6 nmol/mg protein for alanine culture). Furthermore, the amount of L-alanine remaining in the media at the end of the 1 h incubation was not significantly different following prolonged culture with L-alanine (17.1 ± 3.1 $\mu\text{mol/mg}$ protein for control conditions, and 11.7 ± 4.0 $\mu\text{mol/mg}$ protein for alanine culture).

Intracellular ATP levels were measured in parallel experiments at the end of the 1 h incubation and under control conditions the ATP concentration was 5.8 pmol/ 10^4 cells. Alanine culture significantly ($p < 0.05$) reduced the ATP concentration by 28 % to 4.2 pmol/ 10^4 cells.

Effects of prolonged exposure to alanine on PDK-2 and PDK-4 gene and protein expression

18 h exposure of BRIN-BD11 cells to alanine in culture resulted in a reduction in pyruvate dehydrogenase kinase-2 (PDK-2) protein expression as evaluated by Western blot analysis (Figure 7A,B). Consistent with this alteration in protein expression, semi-quantitative reverse transcription-PCR analysis of PDK-2 gene expression was also suppressed by alanine culture (Figure 7C). Similarly, as shown in Figure 8A, alanine culture resulted in reduced pyruvate dehydrogenase kinase-4 (PDK-4) protein expression and mRNA levels of PDK-4 (Figure 8B). These observations are consistent with the altered flux through PDH observed by the NMR experiments.

Discussion

Prolonged exposure of beta cells to glucose, fatty acids and insulinotropic drugs has been shown to induce beta-cell desensitization to subsequent acute stimulation [1, 15, 21]. Most recently, the effects of prolonged (24 h) exposure to alanine or glutamine on gene expression has been reported, revealing an alteration of 66 or 166 genes, respectively, with a large functional core related to signalling and metabolism [23, 37]. These studies also indicated that prolonged alanine exposure may confer protection against cytokine-induced beta-cell apoptosis. However, while the detrimental actions of glucose and lipids on beta-cell function are well characterized the consequences of prolonged amino acid exposure on beta-cell metabolism, insulin secretion and function has been largely neglected,

prompting the present studies. The importance of the present study is emphasised by the fact that alanine is quantitatively the second most abundant amino acid in blood and extracellular tissues *in vivo*.

Following 18 h culture with alanine there was a marked impairment to subsequent characteristic potent insulin-releasing actions of this amino acid. Alanine culture did not however significantly diminish basal insulin release or cellular insulin content. Furthermore, when cells exposed to alanine for 18 h were cultured for a further 18 h in the absence of this amino acid, there was a partial restoration of alanine-induced insulin release. This would indicate a reversible desensitization as previously reported with other insulinotropic agents [15, 38, 39]. Consistent with a specific alanine-induced amino acid desensitization, responses to depolarizing concentration of KCl, stimulatory glucose, the insulinotropic hexose sugar mannose, or the 2-keto acid KIC were not affected by chronic alanine culture.

The mode of action by which alanine stimulates insulin release involves both co-transport into cells with Na⁺ and rapid intracellular metabolism to generate ATP and other metabolic signalling factors [12-14, 40, 41]. The insulinotropic response to a non-metabolizable amino acid analogue of alanine, AIB was significantly reduced following alanine culture albeit to a lesser extent than alanine-induced insulin output. Similarly, AIB culture significantly impaired both alanine- and AIB-induced insulin release which would further indicate desensitization of the amino acid – Na⁺ co-transport system. However, it should be noted the acute uptake of alanine did not change following prolonged culture with alanine as indicated by the amount of ¹³C labelled alanine in the extracts. In the present studies, alanine induced a rapid membrane depolarization, reaching a maximum peak around 30 sec and this response was markedly decreased in cells cultured for 18 h with alanine. The depolarizing action of alanine is consistent with acute effects of this and other amino acids co-transported with Na⁺ [40]. Such amino acids evoke membrane depolarization and increase Ca²⁺ influx, while metabolizable amino acids (including L-alanine) will additionally increase intracellular ATP concentration and other signalling factors, prompting K_{ATP} channel closure and insulin exocytosis [30]. Indeed, the initial rapid rise in membrane potential observed here indicates potent membrane depolarizing actions, largely attributable to Na⁺ co-transport and accumulation, while the sustained plateau likely reflects the actions of cellular metabolic signalling factors including ATP acting through K_{ATP} channels and other signal elements to modulate [Ca²⁺]_i and insulin release [40].

Consistent with other studies, acute exposure to alanine resulted in a rapid rise in intracellular Ca²⁺ concentration [34, 42]. However, in cells cultured with alanine the maximal [Ca²⁺]_i peak was markedly reduced correlating with suppressed membrane depolarization, an effect which was partially reversed following an 18 h recovery period, again suggesting non-permanent alanine-induced desensitization with prolonged exposure. While the rise in [Ca²⁺]_i reflects co-transport with Na⁺ and metabolic ATP generation, the decreased response following prolonged alanine exposure prompted additional studies investigating L-alanine metabolism and ATP generation in cells cultured with L-alanine.

To explore the relevance of metabolic changes to the diminished insulin-secretory responses to alanine following prolonged alanine exposure, metabolism was traced using ¹³C NMR analyses. The authors have previously utilized this approach to characterize nutrient metabolism taking advantage of cultured pancreatic BRIN-BD11 beta cells to enhance understanding of complex metabolic pathways [12, 25, 43, 44]. The present studies revealed a significant increase in flux through PDH following 18 h alanine culture, indicated by an increase in the C4S peak of glutamate. Interestingly, this increased flux through PDH should subsequently lead to an increased NADH concentration and subsequently enhanced ATP production. However, alanine culture resulted in a small but significant decrease in ATP concentration, despite a notable increase in oxidative metabolism. Nevertheless as ATP is an important modulator of the depolarizing and Ca²⁺-dependent insulin-releasing actions of alanine, this observation is consistent with data arising from other components of this study. It should be noted that the changes in ATP levels did not affect the viability of the cells.

Activity of the beta-cell Na^+/K^+ pump has been reported to consume 75-80 % of basal energy production in these cells [45], and thus is it possible that the decrease in cellular ATP may be related to enhanced Na^+/K^+ pump activity following prolonged alanine exposure. A study by Elmi and co-workers investigated the relationship between islet ATP content and pump activity [46], demonstrating that pump inhibition with ouabain did not adversely affect ATP levels during acute incubations. However, on longer incubation a very different pattern emerged with decreased islet ATP concentration corresponding to reduced glucose oxidation, opposing the expected outcome of inhibition of the ATP consuming Na^+/K^+ pump. Moreover, diazoxide has been demonstrated to reduce the basal activity of the pump and elevate islet ATP content [47], but the opposite has also been reported [48], making it difficult to draw clear conclusions on the relationship between ATP concentration and the activity of the Na^+/K^+ pump. Considering that alanine is present in both our control and pre-cultured conditions and that acute alanine uptake did not change significantly it is unlikely that the reduction in ATP at the end of the 1 hr incubation is due to increased activity of the Na^+/K^+ pump. However, it is possible that the observed alanine-induced decrease of ATP despite increased oxidative metabolism could result from an uncoupling of ATP production in mitochondria resulting from reactive oxygen species generated as a by-product of oxidative metabolism.

Given the observation that prolonged alanine exposure resulted in an increased flux through PDH it was of interest to examine the expression of major inhibitory pyruvate dehydrogenase kinases (PDK-2 and PDK-4). It should be noted that regulation of PDH activity is complex and that the PDKs represent only part of the regulation of PDH activity [49]. PDKs control PDH activity through phosphorylation, causing reduced enzyme activity [50]. Notably, several reports have linked altered PDH activity with a decline in beta-cell function, and expression of PDK-1, PDK-2 and PDK-4 has been reported in pancreatic beta-cells [50, 51]. Phosphorylation of PDH occurs at three specific sites and PDK-2 exhibiting the highest activity at site 1 while PDK-4 has the highest activity towards site 2 [49]. The present data demonstrating down-regulation of PDK-2 and PDK-4 mRNA levels following alanine-culture, using semi-quantitative reverse transcription PCR are entirely consistent with the observed increased flux through PDH. Prolonged alanine culture also down-regulated PDK-2 and PDK-4 protein expression, again being consistent with an increased flux through PDH. Interestingly, a similar downregulation of PDK-4 was reported following exposure to high glucose [51] and up-regulation of PDK-4 has been reported to lower PDH activity during starvation [50], allowing entry of acetyl-CoA derived from fatty acids.

Collectively, the present data demonstrate alanine-induced downregulation of important PDK isoforms in beta-cells, resulting in the observed increase in PDH activity / flux, which has the knock-on effect of reducing TCA cycle entry via PC and reduced entry of acetyl-CoA from fatty acid oxidation. Consequently, as PC flux is important for fuel-driven insulin secretion [52-54], the decreased flux through PC may at least partly account for the reduction in insulin secretion following prolonged alanine exposure. Further studies including a timecourse of the response are required to explore these possibilities, to test whether desensitization observed here with alanine and related AIB extends to other functionally important amino acids such as leucine and glutamine, and to evaluate the role of reactive oxygen species and other metabolic intermediates in amino acid-induced beta-cell desensitization. Notably, however the present studies clearly demonstrate that prolonged exposure to alanine can induce beta-cell desensitization, impacting on cellular metabolism and amino acid regulation of cellular metabolic and ionic flux.

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Legends to Figures

Figure 1. The effects on cellular integrity of 18 h culture of 10 mmol/l alanine compared to control culture were assessed by neutral red assay as shown in Fig A. and by MTT assay as shown in Fig B. BRIN-BD11 cells were cultured in the presence or absence of 10 mmol/l alanine for 18 h and cell viability was assessed using the MTT and Neutral red assay. Values are expressed as a percentage of the control.

Figure 2.

Effects of prolonged exposure to alanine on subsequent alanine-induced insulin release (A) or cellular insulin content (B). BRIN-BD11 cells were cultured for 18 h in the absence (standard culture medium) or presence of 10 mmol/l alanine. An additional group examined the effects of a further 18 h incubation (recovery) on cells exposed to alanine using standard culture medium. For insulin secretion experiments (A), cells were pre-incubated (20 min) in buffer containing 1.1 mmol/l glucose, and the insulinotropic actions of 10 mmol/l alanine at 1.1 mmol/l glucose were tested during a 60 min incubation period. For examination of insulin content (B), after culture acid-ethanol extractions were performed prior to insulin radioimmunoassay analysis. Values are mean \pm SEM (n=6). **p<0.01, ***p<0.001 compared with effects in the absence of alanine (None). Δ p<0.05, $\Delta\Delta$ p<0.001 compared with effect after standard culture. Ψ p<0.01, $\Psi\Psi$ p<0.01 comparing effect following 18h recovery with effect after alanine culture.

Figure 3. Effects of prolonged exposure to alanine on insulin secretion in response to a depolarizing concentration of KCl (A) or stimulatory concentrations of glucose and other nutrient secretagogues (B). BRIN-BD11 cells were cultured for 18 h in the absence (standard culture medium) or presence of 10 mmol/l alanine then exposed for 1 h to the test agents. Cells were preincubated (1 h) in buffer containing 1.1 mmol/l glucose and the insulinotropic actions of 30 mmol/l KCl, 16.7 mmol/l glucose, 10 mmol/l mannose or 30 mmol/l 2-ketoisocaproic acid (KIC) were tested during a 60 min incubation. Fig (A) Value are mean \pm SEM (n=6). ***p<0.001 compared with effects in the absence of addition (None). Fig (B) Value are mean \pm SEM (n=6). *p<0.05 compared with effects of standard control culture.

Figure 4. Effects of prolonged exposure to either 10 mmol/l alanine or 10 mmol/l 2-aminoisobutyric (AIB) on subsequent insulin responses to amino acid test agents. BRIN-BD11 cells were cultured for 18 h in the absence (standard culture medium) or presence of 10 mmol/l alanine or 10 mmol/l AIB then exposed for 1 h to the test agents. Cells were preincubated (1 h) in buffer containing 1.1 mmol/l glucose and the insulinotropic actions of 10 mmol/l alanine, 10 mmol/l AIB or 10 mmol/l serine were tested during a 60 min incubation. Value are mean \pm SEM (n=6). *p<0.05, ***p<0.001 compared with effects in the absence of addition (None). Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 compared with effects after standard culture.

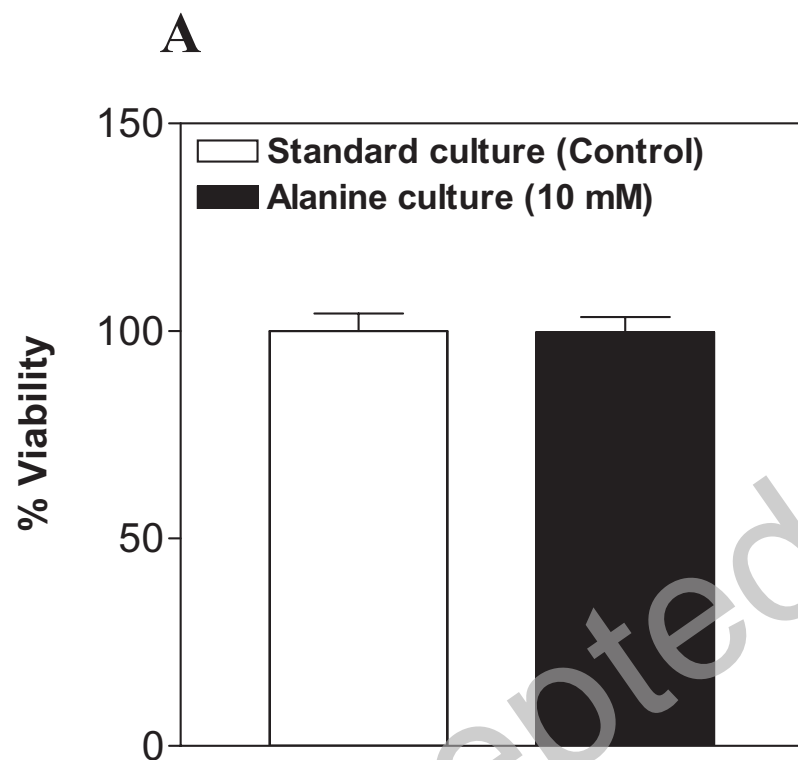
Figure 5. (A) Effects of prolonged exposure to alanine on alanine-induced changes in membrane potential in BRIN-BD11 cells at 1.1 mmol/l glucose. Each data point represents mean \pm SEM (n=6). Area-under-the-curves (AUCs) for standard culture or alanine culture were 6704000 \pm 388324 and 5077334 \pm 304649, respectively. (B) Effects of prolonged exposure to alanine on alanine-induced changes in intracellular Ca²⁺ in BRIN-BD11 cells at 1.1 mmol/l glucose. Each data point represents mean \pm SEM (n=6). Area-under-the-curves (AUCs) for standard culture or alanine culture were 573527 \pm 28720 and 374636 \pm 47437, respectively.

Figure 6. (A) ^{13}C NMR spectra of extracts of BRIN-BD11 cells incubated with 10 mmol/l L-[3- ^{13}C]alanine for 1 h following alanine culture (i) compared with standard culture (ii). (B) The amount of glutamate and aspartate specifically labelled during 1 h incubation with L-[3- ^{13}C]alanine after alanine culture or standard (control) culture. (C) Percentage enrichments of glutamate C2, C3 and C4 during 1 h incubation with L-[3- ^{13}C]alanine after alanine culture or standard (control) culture. Values are mean \pm SEM (n=3). *p<0.05 compared with control.

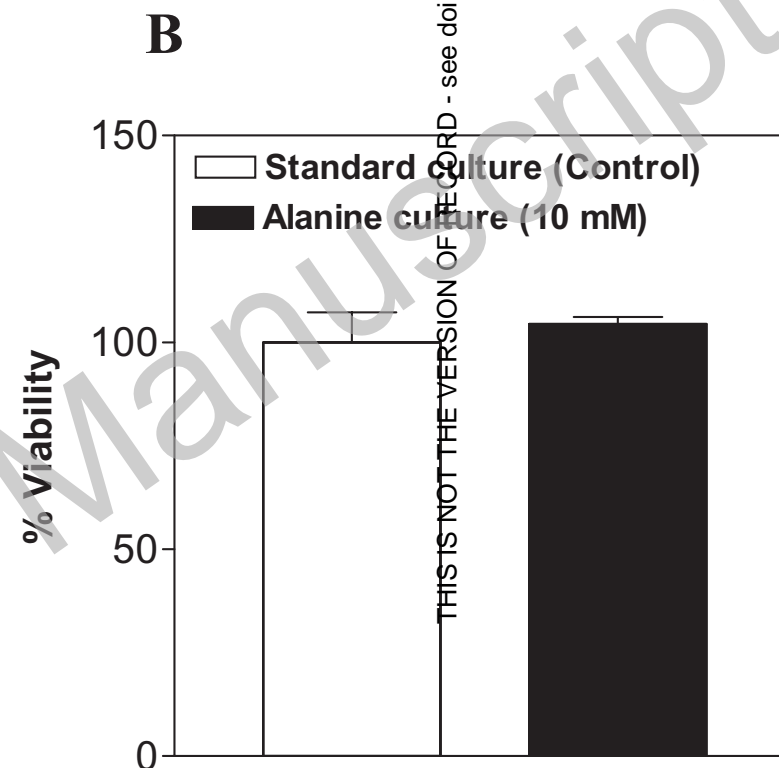
Figure 7. Effects of prolonged exposure to alanine on PDK-2 protein and gene expression. BRIN-BD11 cells were cultured in the absence or presence of 10 mmol/l alanine and additionally exposed for 1 h to 10 mmol/l alanine before analyses. (A) Western blot analysis of PDK-2 protein expression. (B) Characterization of PDK-2/JNK ratio to parallel Western blot analysis. (C) Characterization of PDK-2/18s mRNA levels (arbitrary units) detected by semi quantitative reverse-transcription-PCR. Values are mean \pm SEM (n=5) of target protein or gene over housekeeping protein (JNK) or gene (18 S). *p<0.05 compared with standard culture (control).

Figure 8. Effects of prolonged exposure to alanine on PDK-4 protein and gene expression. BRIN-BD11 cells were cultured in the absence or presence of 10 mmol/l alanine and additionally exposed for 1 h to 10 mmol/l alanine before analyses. (A) Characterization of PDK-4/GAPDH ratio by Western blot analysis. (B) Characterization of PDK-4/18s mRNA levels (arbitrary units) detected by semi quantitative reverse-transcription-PCR. Values are mean \pm SEM (n=5) of target protein or gene over housekeeping protein (GAPDH) or gene (18 S). *p<0.05, **p<0.01 compared with standard culture (control).

Figure 1



(Neutral red assay)



(MTT assay)

Figure 2

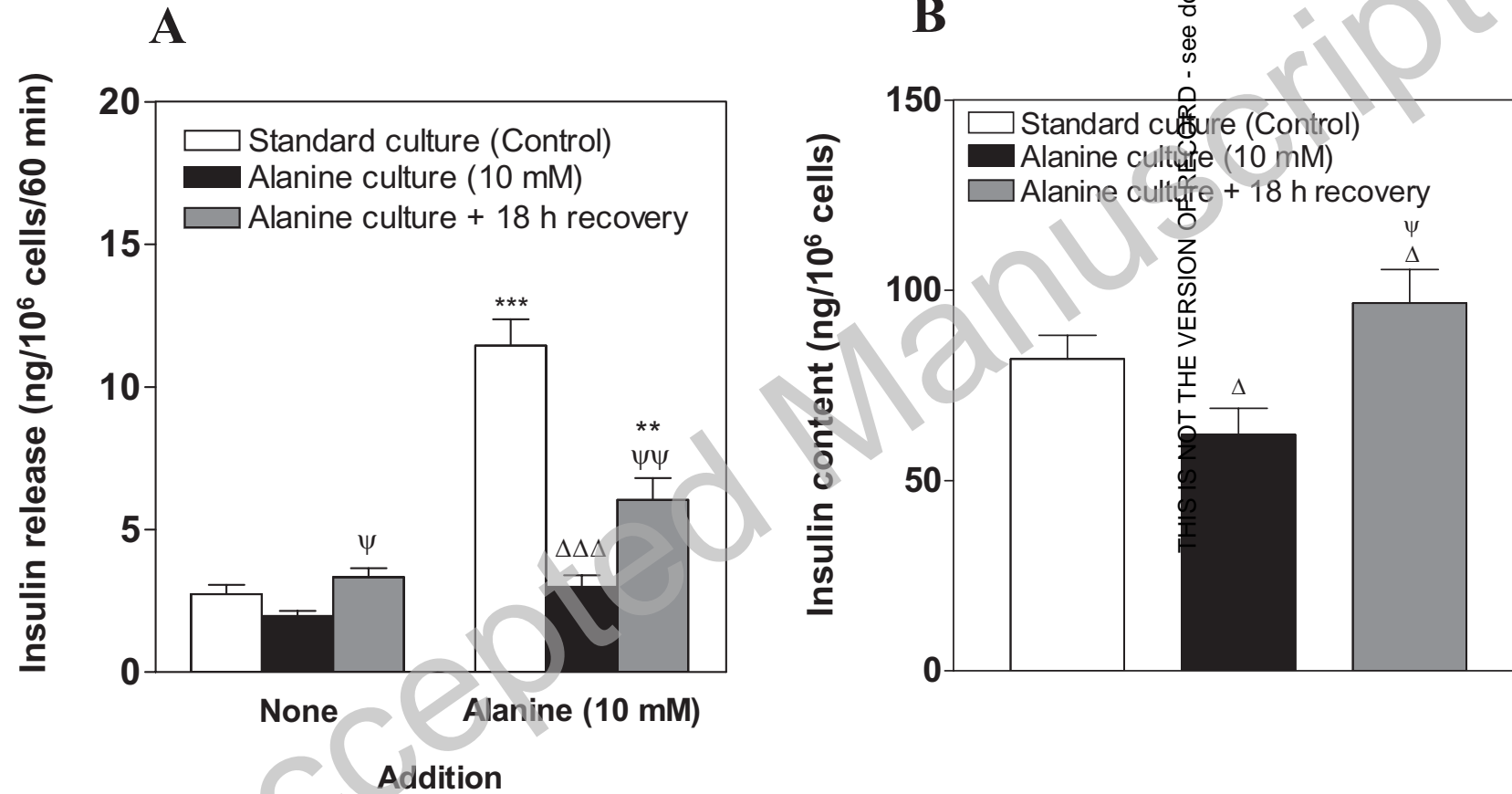


Figure 3

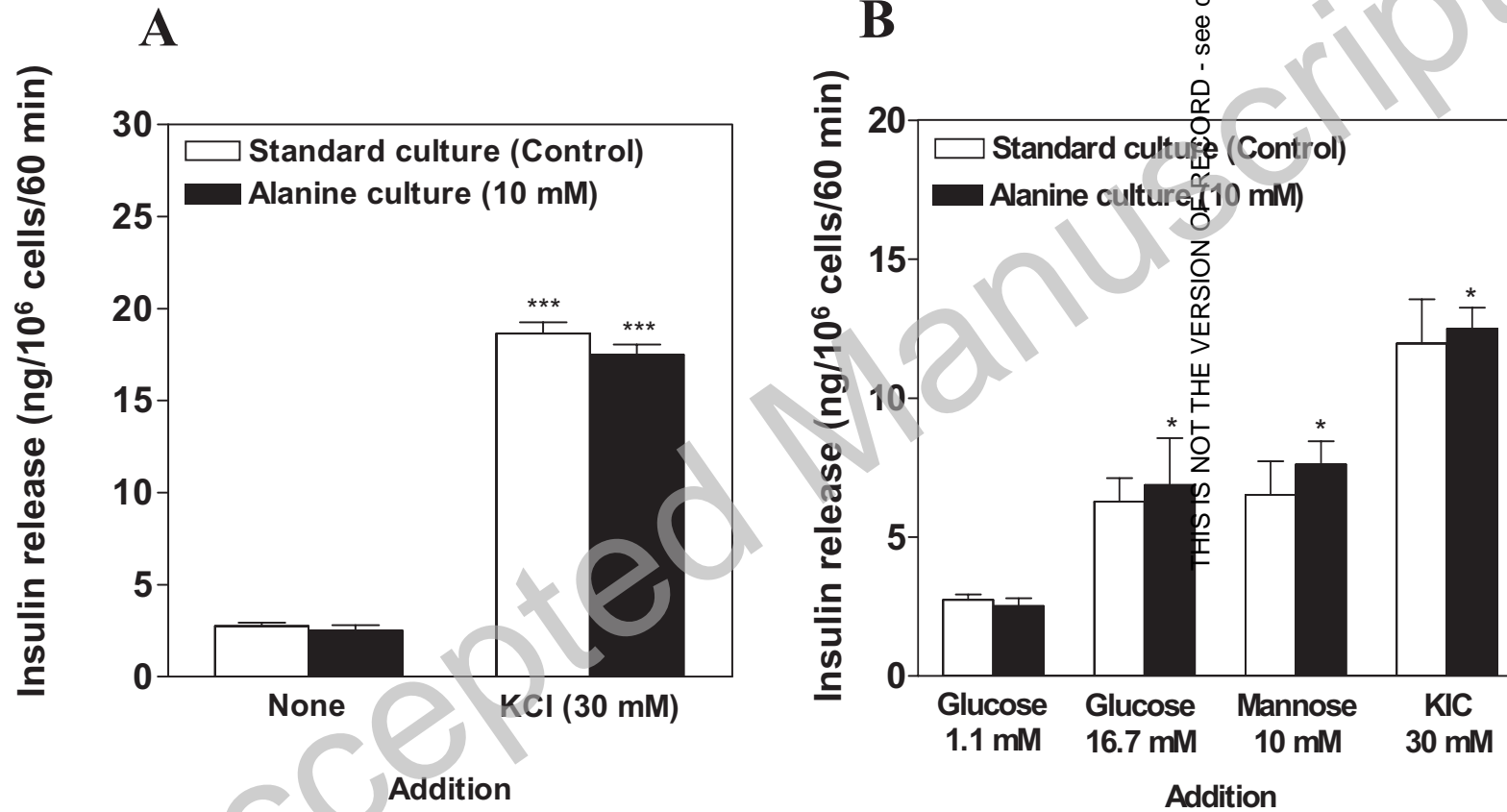


Figure 4

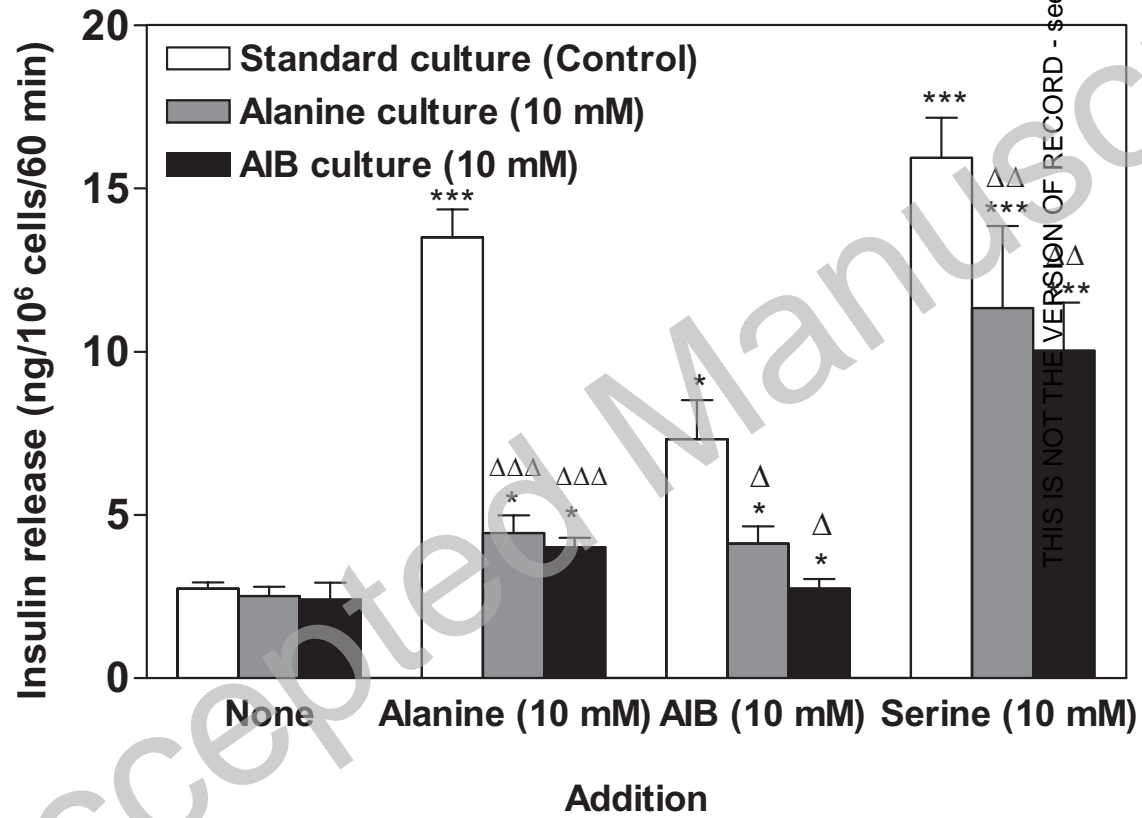


Figure 5

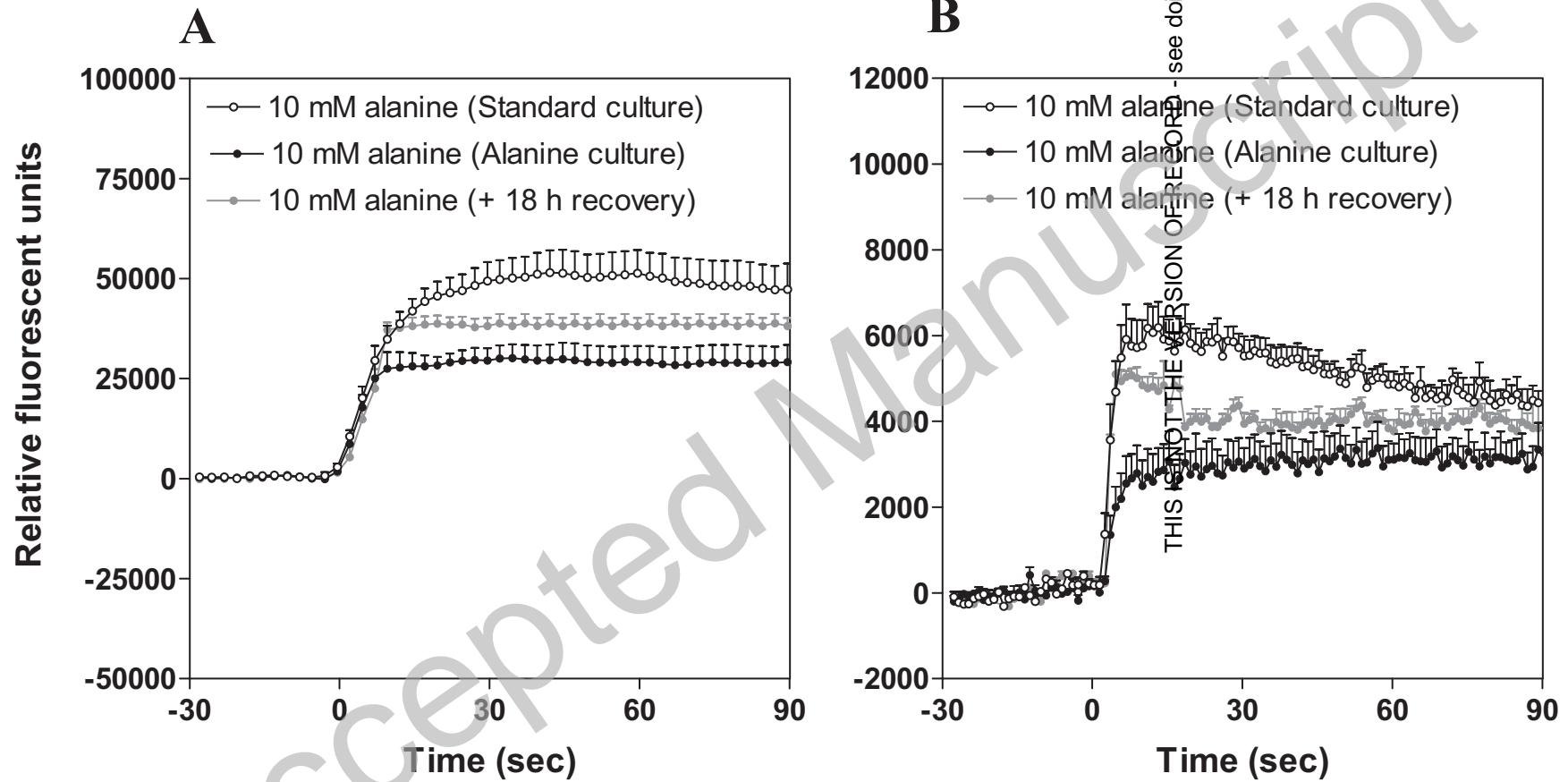
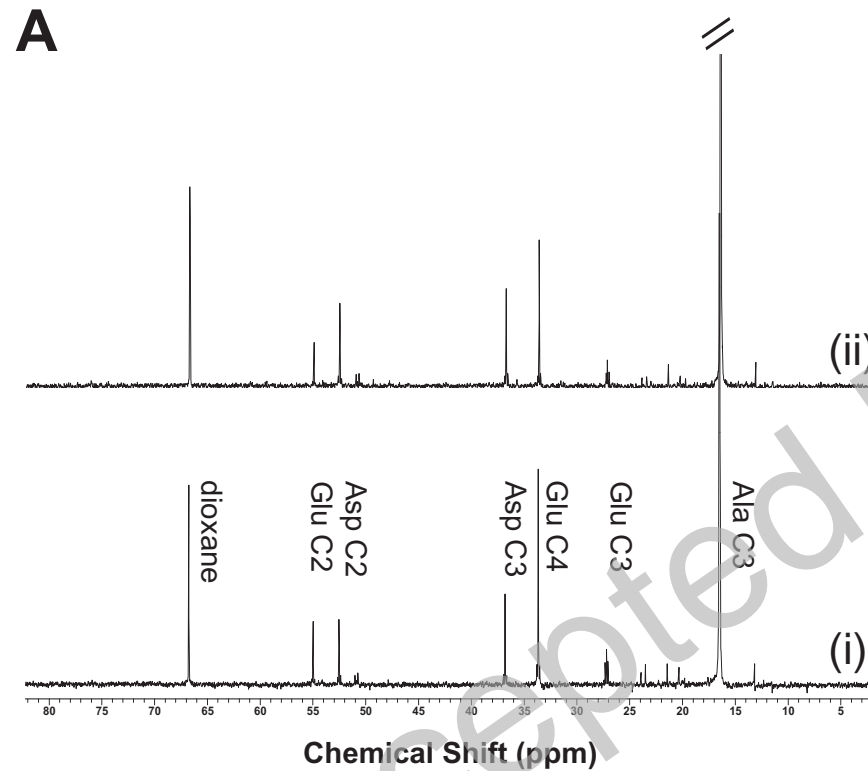


Figure 6



B Glu and Asp labeled from [3-¹³C]Alanine (nmol/mg protein)

	Control	Alanine culture
Glu C2	8.5 ± 1.6	11.1 ± 1.0
Glu C3	7.3 ± 1.1	8.2 ± 0.6
Glu C4	23.0 ± 2.2	37.1 ± 1.8 *
Asp C2	10.0 ± 0.8	8.6 ± 0.9
Asp C3	10.0 ± 1.0	9.4 ± 0.8

C Enrichments of Glutamate C2, C3 and C4 (%)

	Control	Alanine culture
Glu C2	12.9 ± 1.1	13.2 ± 0.5
Glu C3	11.2 ± 0.4	9.8 ± 0.6
Glu C4	35.8 ± 1.5	44.4 ± 2.5 *

Figure 7

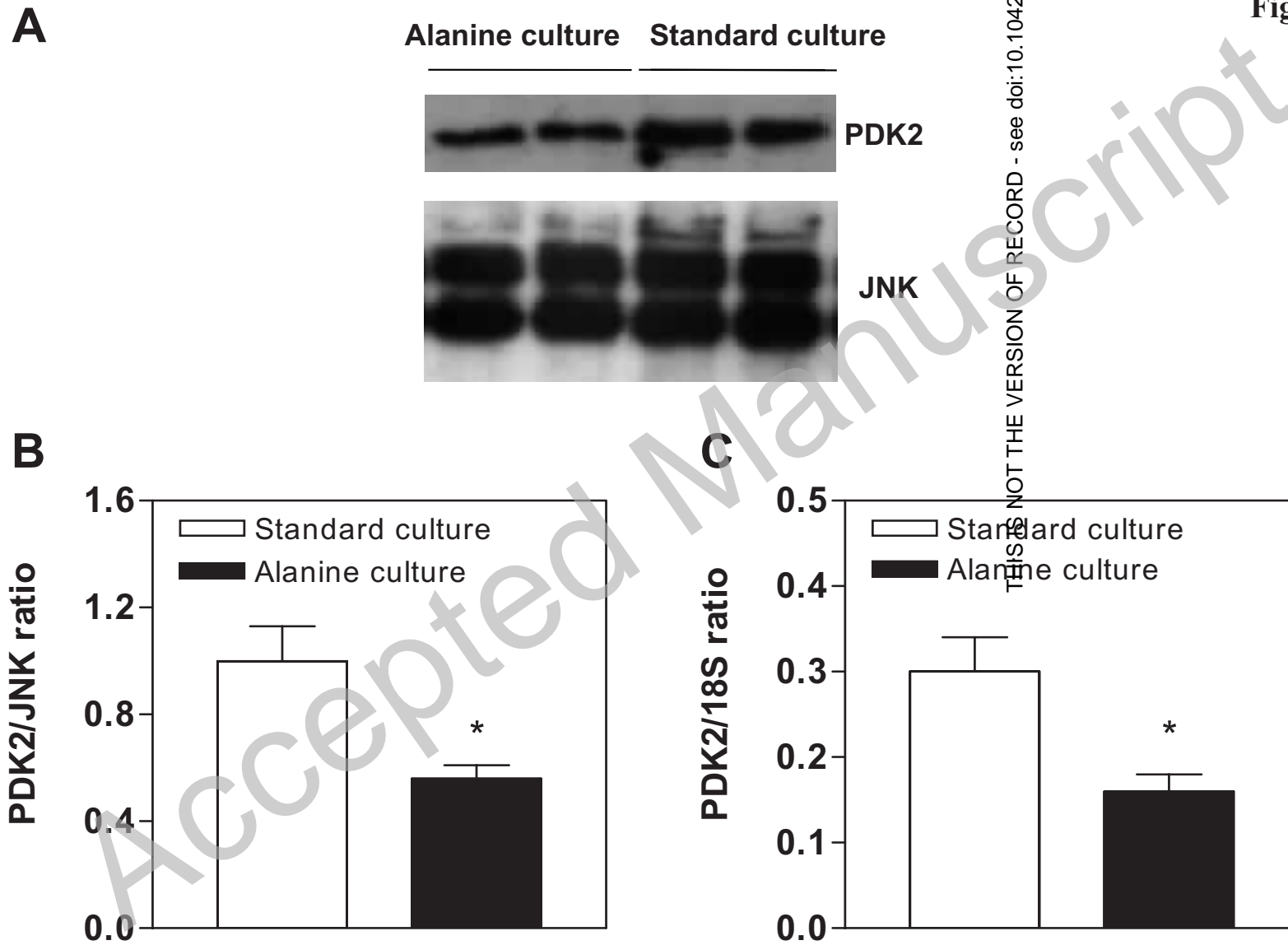


Figure 8

