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# β-alanine scavenging of free radicals protects mitochondrial function and enhances both insulin secretion and glucose uptake in cells under metabolic stress.

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Running title: β-alanine scavenging and mitochondrial function in metabolic disease

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Highlights

1) Presence of carnosine synthase in cells central to control of glucose homeostasis.
2) Effective scavenging of reactive species by β-alanine.
3) Protective action of β-alanine against metabolic stress-induced inhibition of mitochondrial function.
4) β-alanine preservation of normal cellular function, despite metabolic stress.



**SUMMARY**

Type 2 diabetes is a disease characterized by dysregulation of glucose homeostasis, with numerous diabetic complications attributable to the resulting chronic exposure of cells and tissues to elevated concentrations of glucose and fatty acids. This in part results from formation of advanced glycation end-products and advanced lipidation end-products that can form adducts with proteins, lipids, or DNA and disrupt their normal cellular function. There is, however, growing evidence that supplementation with the endogenous histidine-containing dipeptide, carnosine, or its rate-limiting precursor, β-alanine, can ameliorate aspects of metabolic dysregulation that occur in diabetes and related conditions. Here we investigated the scavenging potential of β-alanine in INS-1 pancreatic β-cells and C2C12 skeletal muscle myotubes, and show a significant reduction of >60% in reactive species that were generated by glucolipotoxic metabolic stress in both cell types following incubation with β-alanine for 5 days. Furthermore, β-alanine supplementation resulted in a protective action that helped prevent the damaging action of metabolic stress that otherwise leads to inhibition of mitochondrial function in both cell types. This in turn resulted in >60% preservation of insulin secretion and glucose uptake, in INS-1 cells and C2C12 cells

respectively, which would otherwise be inhibited by metabolic stress. This suggests potential therapeutic benefit to taking β-alanine supplements as an alternative to carnosine.

**Keywords:** β-alanine, reactive species scavenging, metabolism, mitochondrial respiration

**ABBREVIATIONS**

2-DG: 2-deoxy glucose

3-NT: 3-nitrotyrosine

4-HNE: 4-hydroxynoneal

AGE: advanced glycation end-product

ALE: advanced lipidation end-product

ATPGD1: ATP-grasp domain-containing protein 1

CARNS1: carnosine synthase

DCFDA: 2′,7-dichlorofuorescin diacetate

GLT: glucolipotoxic media

NO: nitric oxide

OCR: oxygen consumption rate

$ONOO^-$: peroxynitrite

RCS: reactive carbonyl species

T2D: type 2 diabetes

## INTRODUCTION

Type 2 diabetes (T2D) is a disease characterised by chronically elevated glucose levels, insulin resistance, and reduced insulin secretion from the pancreas. Alarmingly, the incidence of diabetes continues to grow, with the figure in 2045 projected to reach 693 million adults globally, an increase from 451 million adults in 2017 [1]. Similar increases in global levels of obesity have also been seen in the last 30 years [2], with obesity reported to account for 73.5% of the increase in diabetes mortality rates since 1990 [3]. However, whilst the causal link between obesity and diabetes is increasingly clear, the underlying cellular and molecular pathways driving T2D pathophysiology remain only partially understood.

Initial treatment strategies typically try to reduce obesity and T2D risk through reducing sedentary lifestyle and increasing daily exercise. However, whilst these are clear benefits to patients in terms of the long-term prognosis from successful implementation of these strategies [4,5], patient adherence often wanes over time. In this case, clinical observations of hyperglycaemia and dyslipidaemia have led scientists and clinicians to focus on T2D therapeutic strategies that are based on improved insulin secretion, increased cellular glucose uptake, or metabolic regulation as a means to control blood glucose levels. Whilst these are

valid strategies, even with good diabetes management, $HbA_{1C}$ levels are typically higher in individuals with diabetes than non-diabetic individuals. Consequently, whilst the rate of T2D disease progression can be significantly slowed with good management of blood glucose levels, nevertheless complications often still develop, meaning that additional therapeutic approaches are urgently needed.

Elevated glucose concentrations induce expression of the *NOS2* gene, resulting in elevated nitric oxide (NO) production. Superoxide and NO can then react together, leading to the generation of peroxynitrite ($ONOO^-$) [6,7]. The subsequent interaction of $ONOO^-$ and its derivatives with protein tyrosine residues results in the formation of 3-nitrotyrosine (3-NT) [8], which impacts upon protein steric and electronic properties and consequently alters protein functional capacity. Importantly, nitrotyrosine has been detected in plasma of patients with T2D, where it has been shown to correlate with plasma glucose concentrations [9] and is likely to contribute to diabetic complications [10]. Lipid peroxidation is an intermediate step in the generation of multiple other toxic species, with for instance 4-hydroxynoneal (4-HNE) resulting from oxidation of lipids containing polyunsaturated omega-6 acyl groups, such as arachidonic and linoleic groups. 4-HNE is also a relatively stable compound, and as such is one of the most extensively studied biomarkers of oxidative stress, with a concentration often reaching as high as micromolar concentrations in such instances [11].

Recent research has now started to focus on reactive carbonyl scavenging as a strategy to combat the effects of glucolipotoxic metabolic stress. Carnosine is a physiological histidine-containing dipeptide that was first reported to effectively scavenge reactive carbonyl species (RCS) in a mouse model of diabetes [12]. At the cellular level, carnosine scavenging has been shown to increase both insulin secretion and glucose uptake [13], and to prevent

damaging carbonyl adduction associated with metabolic stress in 65-90% of proteins, including those essential for stimulus-secretion coupling associated with insulin secretion and glucose uptake in INS-1 pancreatic β-cells, primary mouse islets, C2C12 myotubes, and human skeletal muscle [14]. Furthermore, promising data has shown a reduction in plasma glucose levels in obese and diabetic individuals who received regular dietary supplementation with carnosine [15-17].

Herein, we extend these studies to determine the effectiveness of β-alanine, a nutritional supplement and constituent amino acid component of the dipeptide carnosine, as a potential scavenger of reactive species. We also investigate its protective action on mitochondrial function in INS-1 pancreatic β-cells and C2C12 skeletal muscle myotubes under metabolic stress, and the functional impact of this to insulin secretion and glucose uptake.

## MATERIALS AND METHODS

### Materials

Antibodies were obtained from Abcam (Cambridge, UK) and Agilent Technologies (Santa Clara, CA, USA). Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or VWR International Ltd (Lutterworth, UK).

### INS-1 β-Cell Culture

INS-1 pancreatic β-cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) media, or RPMI media supplemented where indicated with 28mM glucose, 200μM oleic acid, and 200μM palmitic acid (GLT media) for 72h as detailed previously [18].

**C2C12 Skeletal Muscle Cell Culture**

C2C12 skeletal myoblasts were maintained in high glucose-DMEM supplemented with 10% (v/v) foetal bovine serum, 10% (v/v) heat inactivated newborn calf serum (Life Technologies, Paisley, UK), and 1% (v/v) penicillin-streptomycin (Life Technologies) in a humidified atmosphere with 5% $CO_2$ at 37$^{o}$C. Cells were switched to Dulbecco's Minimal Eagle's Medium (DMEM) supplemented with 2% (v/v) heat-inactivated horse serum (Life Technologies) for 7 days in order to facilitate myocytic differentiation. Cells were then incubated for a further 5 days ± GLT media (DMEM media with 11mM glucose; or DMEM media with 28mM glucose, 200μM oleic acid, 200μM palmitic acid) and β-alanine as indicated.

**Reactive Species Detection**

INS-1 and C2C12 cells were cultured for 5 days in standard tissue culture media, or media supplemented with 28mM glucose, 200μM oleic acid, and 200μM palmitic acid (GLT media). 10mM β-alanine was added and incubated for 1h. Cells were washed 3 times in Krebs-Ringer buffer (KRB), then 20μM 2',7'-dichlorofluorescin diacetate (DCFDA) loaded for 1h. Radical species detection was measured via fluorescence, with excitation at 495nm and emission at 530nm. Radical species are expressed as percentage change relative to control.

**Western Blotting**

INS-1 or C2C12 cells were lysed and protein separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose as described previously [18]. Protein was detected using anti-carnosine synthase (Abcam,

Cambridge UK) primary antibody and polyclonal goat anti-mouse horseradish peroxidase conjugated secondary antibody (Agilent Technologies, Santa Clara, CA, USA).

**Insulin Secretion**

INS-1 cells were treated as detailed previously [18], with KRB or KRB supplemented with secretagogue cocktail (13.5mM glucose, 1μM phorbol 12-myristate 13-acetate, 1mM isobutyl-methylxanthine, 1mM tolbutamide, 10mM leucine, 10mM glutamine) for 2h. Insulin secretion was determined using ELISA kit (Mercodia, Uppsala, Sweden).

**Glucose Uptake**

Following the indicated treatment, cells were serum-starved overnight in DMEM supplemented with 5mM glucose, then incubated for 1h in glucose-free DMEM +/-100nM insulin. Medium was replaced with PBS + 0.125mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30min, and then terminated by addition of stop buffer (0.4M HCl + 2% dodecyl trimethyl ammonium bromide). 2DG6P detection reagent was applied and data were acquired using a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany).

**Assessment of Mitochondrial Function**

One day prior to the assay, a Seahorse sensor cartridge (XFe24 Flux Assay Kit) was hydrated by filling each well with XF calibrant solution and placing in a non-$CO_2$ incubator at 37°C overnight. Cells were seeded at 30,000 per well in a 24-well Agilent Seahorse XF24 Cell Culture microplate, then grown, differentiated and treated for 3 days. On the day of the assay, media was removed, and cells washed twice with Seahorse XF-DMEM medium supplemented with 1mM pyruvate, 10mM glucose, and 2mM glutamine, then incubated at

37°C for 1h. During this incubation period the bioenergetic modulators oligomycin, FCCP, and rotenone/antimycin-A were loaded into the ports of the previously hydrated sensor cartridge, and the cartridge then loaded to the Seahorse XFe24 Analyzer for calibration and equilibration. Once completed, the utility plate was then replaced with the Seahorse plate containing the cells and the Mito Stress test run. Data was then obtained from the Seahorse XF Mito Stress Test Report Generator.

**Statistical Analysis**

Results are expressed as mean ± standard error of the mean (n = 3 or more independent experiments). Parameters were compared using one-tailed student t-test assuming equal variance, with statistical significance determined using an alpha value of 5%. A *p* value below 0.05 was considered to be statistically significant.



**RESULTS**

**Carnosine Synthase is Present in Pancreatic β-Cells and Skeletal Muscle Myotubes**

Carnosine is a dipeptide synthesised from the amino acids β-alanine and L-histidine. Thus, in order for β-alanine to be an effective therapeutic agent in the generation of carnosine action, this necessitates the anabolic enzyme, carnosine synthase, be present within target cells in order to facilitate synthesis of carnosine. We therefore determined whether INS-1 pancreatic β-cells, and/or C2C12 skeletal muscle myotubes, contained this key enzyme. As can be seen (Figure 1) both contain levels of enzyme that were readily detectable by Western blot. Furthermore, the level of this key enzyme was not significantly altered when the respective

cells were exposed for 5 days to tissue culture media containing glucolipotoxic (GLT: 28mM glucose, 200μM palmitic acid, 200 μM oleic acid) levels of metabolic stress.

**β-Alanine and Scavenging of Reactive Species**

Based on our previous studies investigating the mechanisms of action of carnosine to counteract metabolic stress in pancreatic β-cells and skeletal muscle [13,14], carnosine exerts a potent reactive species scavenging action in cells and tissues central to the control of glucose homeostasis. As can be seen (Figure 2), exposure of cells to metabolic stress (GLT) for 5 days resulted in a significant increase in reactive species generation relative to control. We therefore sought to determine whether β-alanine addition to INS-1 cells and C2C12 myotubes would result in a similar scavenging effect.

We previously investigated the effect of incubating cells with 10mM carnosine [13,14], a concentration selected based upon the physiological concentration that has previously been reported in skeletal muscle [19,20]. We therefore similarly incubated INS-1 cells and C2C12 myotubes with 10mM β-alanine here. When 10mM β-alanine was added to RPMI-1640 media for 1h in an attempt to scavenge reactive species, there was no significant change in INS-1 cell reactive species level (Figure 2A). However, when β-alanine was present for the entire 5-day duration of INS-1 cell exposure to metabolic stress (Figure 2B), the generation of reactive species was reduced from 157.4 ± 15.6% relative to control (GLT) to 118.6 ± 12.1% (GLT + Alanine). This represents a 67.6% reduction in metabolic stress-induced generation of reactive species. Similarly, when β-alanine was present for the entire 5-day duration of C2C12 myotube exposure to metabolic stress (Figure 2C), reactive species generation was reduced from 187.25 ± 15.33% relative to control (GLT) to 133.6 ± 14.2%

relative to control (GLT + Alanine). This represents a 61.5% reduction in metabolic stress-induced generation of reactive species.

**β-Alanine Preserves Mitochondrial Function in Cells Under Metabolic Stress**

We previously reported that a significant number of damaging AGE and ALE adduction events that are associated with metabolic stress were linked to proteins which function in stimulus-secretion coupling [14]. As many of these proteins function in mitochondria, we next sought to determine the impact of GLT and β-alanine supplementation on mitochondrial function. This was accomplished by employing a Seahorse Mito Stress Test kit (Agilent Tech., USA) and measuring the basal mitochondrial oxygen consumption rate (OCR), its maximal capacity, and the respiration associated with mitochondrial ATP generation, using a combination of compounds that specifically target components of the electron transport chain. INS-1 cells (Figure 3A) or C2C12 myotubes (Figure 4A) were incubated in either control or GLT media ± 10mM β-alanine for 5 days. All cells displayed robust mitochondrial respiration under control conditions (blue traces). However, both INS-1 cells (Figure 3A) and C2C12 myotubes (Figure 4A) showed a significant reduction in basal mitochondrial activity when exposed to metabolic stress (red traces). However, β-alanine supplementation resulted in a protective action that helped prevent this damaging GLT action (green traces).

Specifically, INS-1 cell oxygen consumption related to ATP production was reduced from 24.6 ± 0.2 pmol/min/μg to 14.2 ± 0.1 pmol/min/mg following exposure to GLT media. However, when β-alanine was present in the media for the entire 5-day exposure to metabolic stress, oxygen consumption related to ATP production was 23.8 ± 1.5 pmol/min/mg. This represents a 93.2% protection of oxygen consumption related to ATP production that would otherwise be lost following exposure of INS-1 cells to metabolic stress. Similarly, C2C12

myotube cell oxygen consumption related to ATP production was reduced from 6.6 ± 0.2 pmol/min/mg to 3.6 ± 0.3 pmol/min/mg following exposure to GLT media. However, when β-alanine was present in the media for the entire 5-day exposure to metabolic stress, oxygen consumption related to ATP production was 7.4 ± 0.5 pmol/min/mg. Thus, there was full protection of oxygen consumption related to ATP production that would otherwise have been lost following exposure of C2C12 myotubes to metabolic stress. Indeed, there was an 11.2% increase in oxygen consumption related to ATP production above control.

**Functional Impact of β-Alanine upon Insulin Secretion and Glucose Uptake**

Given the degree of protection to mitochondrial function provided by β-alanine in the face of glucolipotoxic metabolic stress in both cell types, we therefore went on to investigate how this might relate to the primary action of pancreatic β-cells and skeletal muscle myotubes, namely insulin secretion and insulin-stimulated glucose uptake. Cells were incubated +/- GLT +/- β-alanine in each case, then both insulin secretion and glucose uptake determined (Figure 5).

Control INS-1 cells showed a robust release of insulin in response to 2h incubation with a secretagogue cocktail (Figure 5A), so we proceeded to determine the effect of β-alanine on secretagogue-stimulated secretion. When cells were incubated for 5 days in GLT media, secretagogue-stimulated insulin secretion decreased from 5.3 ± 0.5 ng/μg cellular protein down to 3.8 ± 0.2 ng/μg cellular protein. The presence of 10mM β-alanine in the media for all 5 days of incubation, however, resulted in an increase in secretagogue-stimulated insulin secretion, such that the control value increased from 5.3 ± 0.5 ng/mg cellular protein up to 7.0 ± 0.6 ng/μg cellular protein, an increase of 31.3% relative to control. Furthermore, in the presence of GLT, secretagogue-stimulated insulin secretion increased from 3.8 ± 0.2 ng/μg

cellular protein up to 4.7 ± 05 ng/μg cellular protein, a 27.3% increase. This also represents a 60% reversal of GLT-associated inhibition of secretagogue-stimulated insulin secretion, back towards control values.

Insulin-stimulated glucose uptake in C2C12 myotubes followed a similar positive response to β-alanine (Figure 5B) as that detailed above for secretagogue-stimulated insulin secretion from INS-1 cells. Specifically, when cells were incubated for 5 days in GLT media, insulin-stimulated glucose uptake decreased from 158.9 ± 12.5% relative to control down to 69.9 ± 11.4%. However, when C2C12 myotubes were incubated in the presence of 10mM β-alanine for all 5 days of GLT treatment, this resulted in an increase in insulin-stimulated glucose uptake, such that the GLT value increased from 69.9 ± 11.4% up to 114.8 ± 16.8%, an increase of 64.2%. This also represents a 50.4% reversal of GLT-associated inhibition of insulin-stimulated glucose uptake, back towards control values.



**DISCUSSION**

Low bioavailability of β-alanine within cells and tissues has been reported to be the rate-limiting factor for carnosine synthesis [21]. Therefore, for β-alanine to be a potential therapeutic agent for the treatment of T2D and associated complications, it would first need to be taken up into cells and tissues central to the control of glucose homeostasis and then converted into carnosine. β-alanine has, however, already been shown to be taken up into cells by TauT, a $Na^+$ and $Cl^-$ dependent transmembrane transporter driven by $Na^+$ flux related to the action of the $Na^+/K^+$-ATPase pump [22]. This includes skeletal muscle [23]. We, therefore, sought to determine whether, once taken up into these cells, the enzyme required to convert it to carnosine was present. This would require the presence of carnosine synthase

(CARNS1); also referred to as ATP-grasp domain-containing protein 1 (ATPGD1). As can be seen in Figure 1, this key enzyme can be detected in both INS-1 cells and C2C12 myotubes by Western blot. Furthermore, its expression level does not change significantly following exposure of either cell type to metabolic stress, therefore suggesting that carnosine should likely be able to be synthesised once β-alanine is taken up into either cell type, and regardless of metabolic status. Furthermore, carnosine synthase also has a very high affinity for β-alanine [24,25], suggesting that upon entry into cells, synthesis to carnosine is likely to be rapid.

Previous preliminary studies have suggested that carnosine is able to reduce plasma glucose levels in obese and diabetic individuals [15-17]. Whilst there is more limited data for β-alanine, preliminary data suggests that supplementation with β-alanine can also lead to a reduction in fasting glucose, $HbA_{1C}$, and HOMA-IR, both in humans [26] and rodents [27]. Whilst carnosine appears to function through scavenging of reactive species [13] and prevention of damaging AGE and ALE protein adduction, thereby leading to preservation of mitochondrial function and stimulus-secretion coupling in pancreatic β-cells and skeletal muscle cells [14], the mechanistic basis for β-alanine action remains unknown. We therefore sought to determine the effect of *β*-alanine on reactive species scavenging, cellular respiration, and insulin secretion or glucose uptake respectively in INS-1 cells and C2C12 myotubes.

To determine whether the presence of extracellular β-alanine could lead to effective intracellular scavenging of reactive species, we incubated INS-1 cells with 10mM β-alanine for either 1h (Figure 2A) or 5 days in the presence or absence of glucolipotoxic metabolic stress (Figure 2B). Interestingly, we saw no protective effect of β-alanine against reactive

species when β-alanine was present for only the final 1h of a 5 day exposure to metabolic stress. By contrast, when present for the entire 5 days exposure, β-alanine was able to prevent formation of 67.6% of GLT-associated reactive species in INS-1 cells. Similarly, when present for the entire 5 days exposure, β-alanine resulted in a 61.5% reduction of GLT-associated reactive species in C2C12 myotubes. Whilst this is less effective than our previously reported protective action of 10mM carnosine in identical experiments in INS-1 cells and C2C12 myotubes [13], this might reflect a requirement to first enhance intracellular carnosine concentration sufficiently before being able to effectively scavenge reactive species. This could also explain why 1h treatment with 10mM β-alanine was seen to be ineffective, as opposed to 5 day treatment which significantly reduced metabolic stress-associated reactive species levels in both cell types (Figure 2). Nonetheless, this does suggest that, if taken regularly, β-alanine has the potential to similarly be used therapeutically to prevent the build up of damaging reactive species in cells under metabolic stress.

We next sought to determine how this protective scavenging action might impact on mitochondrial function in INS-1 cells (Figure 3) and C2C12 myotubes (Figure 4) exposed to metabolic stress. In the absence of β-alanine, incubation in GLT media resulted in a significant reduction in both mitochondrial and non-mitochondrial respiration. Treatment with β-alanine was beneficial to all measured parameters of respiration; in many cases function was restored to or above control values, even when cells were exposed to metabolic stress. This is consistent with a previously reported action of 800 μM β-alanine to significantly increase oxygen consumption in C2C12 myocytes [28]. Importantly, our data also indicates that β-alanine is able to preserve ATP production in the face of metabolic stress, an essential component required for both insulin secretion from pancreatic β-cells, and also GLUT4 vesicle translocation that facilitates glucose uptake into skeletal muscle.

Given the protective action of β-alanine on ATP production, which is otherwise severely inhibited in the presence of metabolic stress, we went on to determine the impact of β-alanine on cellular function central to the control of glucose homeostasis. Consistent with our previous findings [13,18], GLT inhibited secretagogue-stimulated insulin secretion from INS-1 cells and insulin-stimulated glucose uptake into C2C12 cells. In the presence of 10mM β-alanine however, we saw a 60% protection against GLT-associated inhibition of secretagogue-stimulated insulin secretion from INS-1 cells, and a 50.4% protection on insulin-stimulated glucose uptake into C2C12 myotubes. This protective action is, however, less robust than that which we previously reported for 10mM carnosine in similar INS-1 cell and C2C12 myotube experiments [13], possibly reflective of the more modest scavenging ability of β-alanine relative to carnosine at this concentration over this time period.

A further intriguing aspect of the results obtained concerns the significant increase in insulin secretion by control INS-1 cells (without GLT media) in the presence of 10mM ß-alanine. This effect is particularly interesting as it is present in conditions of maximum stimulation of the secretory capacity of INS-1 cells. As such, based on this result, one could potentially interpret this particular aspect of ß-alanine action as being unrelated to its scavenging action but, alternatively, to a "simple" action of stimulating secretion. This interpretation is supported by the observation that the increase in insulin secretion is quantitatively very similar in the second and fourth groups of Figure 5. Furthermore, we also previously published data showing a similar direct effect of carnosine on secretagogue-stimulated insulin secretion separate to its protective action against metabolic stress [13]. Given the wide-ranging nature of physiological actions of carnosine and β-alanine that have been reported in both health and disease [29] this would not be altogether surprising. This should therefore

form the basis for extensive further study. Nevertheless, the data presented here indicates potential therapeutic benefit to taking β-alanine supplements as an alternative to carnosine.

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**CONFLICT OF INTEREST**

CS has received funding to support a PhD studentship relating to the effects of carnosine on cardiac function from Natural Alternatives International; a company formulating and manufacturing customised nutritional supplements, including CarnoSyn β-alanine. The same company has also provided CS with supplements for other studies free of charge and has contributed to the payment of open access publication charges for some manuscripts on β-alanine supplementation. All other authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

MPB conducted experiments, analysed data, and helped prepare the manuscript.

CL conducted experiments, analysed data, and helped prepare the manuscript.

MJC conducted experiments, analysed data, and helped prepare the manuscript.

KH conducted experiments and analysed data.

CS contributed to study design and reviewed the manuscript.

MDT directed the study, analysed data, and prepared the manuscript.

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**FIGURE LEGENDS**

**Figure 1. Effect of metabolic stress on carnosine synthase expression.** INS-1 or C2C12 cells were incubated ± GLT for 5 days prior to protein lysis. Protein was quantified using

BCA assay and equal protein loaded to sample wells of a 10% acrylamide gel. Proteins were next separated using SDS-PAGE, then transferred to nitrocellulose membrane and immunoblotted using anti-carnosine synthase (CARNS1) antibody.

**Figure 2. Effect of β-alanine on metabolic stress-associated free radicals.** A) INS-1 cells were cultured in control or GLT media for 5 days. Cells were then incubated ± 10mM β-alanine for 1h, washed, and incubated in 20μM DCFDA for 1h prior to measurement. Reactive species were measured via fluorescence at excitation 495nm and emission 530nm. B) INS-1 cells were cultured in control or GLT media ± 10mM β-alanine for 5 days prior to the addition of 20μM DCFDA for 1h. Reactive species were measured via fluorescence with an excitation and emission of 495nm and 530nm respectively. C) C2C12 myotubes were cultured in control or GLT media for 5 days ± 10mM β-alanine and incubated in 20μM DCFDA for 1h prior to measurement. Reactive species were measured via fluorescence with an excitation and emission of 495nm and 530nm respectively. In each experiment reactive species are expressed as percentage change in comparison to control from 3 or more independent experiments. *$p<0.05$ **$p<0.005$.



**Figure 3. Effect of β-alanine on mitochondrial respiration in INS-1 pancreatic β-cells.** INS-1 cells were cultured in either standard media, or else media supplemented with GLT ± 10mM β-alanine. Cells were seeded in Seahorse XFe24 microplates using supplemented Seahorse XF DMEM and incubated for 24 hrs (last day of 5-day treatment) and OCR

measured from XFe24 Analyser. Each data point represents an OCR measurement, and figures are representative images from 3 or more independent experiments. *p<0.05

**Figure 4. Effect of β-alanine on mitochondrial respiration in C2C12 myotubes.** C2C12 myotubes were cultured in either standard media, or else media supplemented with GLT ± 10mM β-alanine. Cells were seeded in Seahorse XFe24 microplates using supplemented Seahorse XF DMEM and incubated for 24 hrs (last day of 5-day treatment) and OCR measured from XFe24 Analyser. Each data point represents an OCR measurement, and figures are representative images from 3 or more independent experiments. *p<0.05 **p<0.005 ***p<0.0005

**Figure 5. Effect of β-alanine on cellular function of INS-1 cells and C2C12 myotubes.** A) INS-1 cells were cultured for 5 days ± 10mM β-alanine. Insulin secretion was determined by ELISA assay following incubation ± secretagogue cocktail for 2h [(−) blue, (+) red], with data normalized to cellular protein content. B) C2C12 myotubes were cultured for 5 days ± 10mM β-alanine. Cells were serum-starved overnight in DMEM supplemented with 5mM glucose, then incubated for 1h in glucose-free DMEM+/−100nM insulin [(−) blue, (+) red]. Medium was replaced with PBS + 0.125mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30min. 2DG6P was detected using a luminometer. All data are expressed as means ± SEM of 3 or more independent experiments. *p<0.05 **p<0.005.