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L-Arginine is essential for pancreatic β-cell functional integrity, metabolism and defense from inflammatory challenge

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Abstract

In this work, our aim was to determine whether L-arginine (a known insulinotropic amino acid) can promote a shift of β-cell intermediary metabolism favoring glutathione (GSH) and glutathione disulfide (GSSG) antioxidant responses, stimulus–secretion coupling and functional integrity. Clonal BRIN-BD11 β-cells and mouse islets were cultured for 24 h at various L-arginine concentrations (0–1·15 mmol/l) in the absence or presence of a proinflammatory cytokine cocktail (interleukin 1β, tumour necrosis factor α and interferon γ). Cells were assessed for viability, insulin secretion, GSH, GSSG, glutamate, nitric oxide (NO), superoxide, urea, lactate and for the consumption of glucose and glutamine. Protein levels of NO synthase-2, AMP-activated protein kinase (AMPK) and the heat shock protein 72 (HSP72) were also evaluated. We found that L-arginine at 1·15 mmol/l attenuated the loss of β-cell viability observed in the presence of proinflammatory cytokines. L-Arginine increased total cellular GSH and glutamate levels but reduced the GSSG/GSH ratio and glutamate release. The amino acid stimulated glucose consumption in the presence of cytokines while also stimulating AMPK phosphorylation and HSP72 expression. Proinflammatory cytokines reduced, by at least 50%, chronic (24 h) insulin secretion, an effect partially attenuated by L-arginine. Acute insulin secretion was robustly stimulated by L-arginine but this effect was abolished in the presence of cytokines. We conclude that L-arginine can stimulate β-cell insulin secretion, antioxidant and protective responses, enabling increased functional integrity of β-cells and islets in the presence of proinflammatory cytokines. Glucose consumption and intermediary metabolism were increased by L-arginine. These results highlight the importance of L-arginine availability for β-cells during inflammatory challenge.

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Introduction

Obesity, insulin resistance and type 2 diabetes (T2DM) are known to induce a proinflammatory state that, together with the adverse effects of hyperglycemia and hyperlipidemia, leads to the progressive dysfunction and demise of pancreatic β-cells (Newsholme *et al.* 2010). Among the factors involved in the reduction of β-cell viability is the local inflammatory process in the islets of Langerhans. This is induced by islet dendritic cell activation and infiltration by T-lymphocytes and macrophages, leading to microenvironmental changes, resulting in β-cell injury and death (Masters *et al.* 2010).

Destruction of β-cells during islet inflammation can be mediated by direct contact with activated macrophages and by exposure to soluble mediators secreted by dendritic cells, macrophages and T-lymphocytes, including cytokines, oxygen free radicals and nitric oxide (NO; Eizirik & Mandrup-Poulsen 2001). Indeed, overproduction of NO by β-cells themselves is one of the most significant mechanisms leading to β-cell dysfunction and death (Eizirik & Mandrup-Poulsen 2001). The production of NO is promoted by inflammatory cytokines which activate the transcription of β-cell inducible isoform of NO synthase (iNOS, encoded by the *NOS-2* gene), an enzyme whose expression is nuclear factor κB (NFκB)-driven and which uses L-arginine as substrate (Eizirik & Mandrup-Poulsen 2001). While excessive NO production within β-cells may trigger oxidative/nitrosative stress leading to cell death, NO also serves as an important β-cell stimulus–secretion coupling factor (Smukler *et al.* 2002, Newsholme *et al.* 2005, 2007*a*,*b*, 2010, Krause & de Bittencourt 2008). L-Arginine is recognized as one of the most powerful insulin secretagogues (Palmer *et al.* 1976) and while it can contribute to promotion of exocytosis via transport-associated membrane depolarization, there are

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possibly many other mechanisms by which it positively modulates insulin secretion. At specific concentrations, evidence suggests that NO is a physiological regulator of insulin secretion in β-cells (Spinas 1999, Smukler *et al.* 2002). The presence of an NFκB-dependent iNOS while beneficial at low concentration/activity, predisposes β-cells to development of oxidative/nitrosative stress at higher levels, because NO may inhibit metabolism, mitochondrial activity and generation of stimulus–secretion coupling factors (Krause & de Bittencourt 2008).

Macrophages can release, into the inflammatory islet microenvironment, the enzyme arginase (Murphy & Newsholme 1998), which splits L-arginine into urea and L-ornithine, thus avoiding its conversion into NO and favoring resolution of inflammation (Zhai *et al.* 2009). Interestingly, the depletion of L-arginine itself is sufficient to inhibit T-cell proliferation by the downregulation of the ζ-chain, the main signal transduction component of the T-cell receptor complex (Bronte & Zanovello 2005). It has also been demonstrated that β-cells possess a cytokine-inducible arginase activity (Stickings *et al.* 2002), which may account for reduction in NO synthesis under appropriate conditions. Although release of arginase by infiltrating macrophages may promote the resolution of inflammation, restriction of arginine availability in the islet microenvironment may be detrimental for β-cell metabolism, antioxidant defenses and insulin secretion (Newsholme *et al.* 2010). Interestingly, decreased plasma and intracellular concentrations of L-arginine have been reported in patients with type 2 diabetes (Pieper & Dondlinger 1997). L-Arginine also increased β-cell neogenesis and antioxidant defenses in rats treated with alloxan and aided recovery of endothelium-dependent relaxation in patients with type 2 diabetes (Pieper & Dondlinger 1997, Vasilijevic *et al.* 2007). Administration of L-arginine has also been reported to reduce adiposity in obese–diabetic humans, genetic and diet-induced obese rats as well as finishing pigs (McKnight *et al.* 2010).

To further investigate the importance of L-arginine for β-cell function, we determined changes in clonal β-cell insulin secretion, metabolism, redox status and integrity *in vitro* in response to manipulation of L-arginine concentration in tissue culture in the absence or presence of proinflammatory cytokines. Key experimental findings were subsequently further explored in heterogeneous (mixed endocrine cell) mouse islet incubations.

Materials and Methods

Culture of BRIN-BD11 pancreatic β-cells, proinflammatory cocktail challenge and measurement of insulin secretion at different L-arginine concentrations

The clonal rat insulin-secreting β-cell line BRIN-BD11 was chosen because its metabolic, signaling and secretory responses to glucose, amino acids and other stimuli have been extensively characterized (McClenaghan *et al.* 1996, McClenaghan & Flatt 1999, Brennan *et al.* 2003). BRIN-BD11 cells were maintained in culture overnight as described previously (Kiely *et al.* 2007). After washing with PBS, cells were incubated in fresh (L-arginine free) RPMI-1640 medium, supplemented with 11·1 mmol/l D-glucose, 2 mmol/l L-glutamine and different concentrations of L-arginine (0, 0·1, 0·25 and 1·15 mmol/l). Cells were incubated in the presence or absence of a non-lethal (with respect to cells cultured in normal culture medium) proinflammatory cytokine cocktail (interleukin 1β (IL1β) 0·3125 U/ml, tumour necrosis factor α (TNFα) 31·25 U/ml and interferon γ (IFNγ) 15·625 U/ml; Kiely *et al.* 2007). IL1β has been implicated in macrophage associated β-cell dysfunction typical of type 2 diabetes (Masters *et al.* 2010). The L-arginine concentration range tested was chosen to include absence (0 mM), the physiological range in human blood (0·1–0·25 mmol/l) and the normal concentration found in the RPMI-1640 culture medium (1·15 mmol/l L-arginine). After 24 h, an aliquot of the medium was removed for insulin assay or centrifuged at 16 000 ***g*** for 10 min at 4 °C for the later determination of metabolites. In addition, for the set of experiments designed to test the effect of 24 h culture in various concentrations of L-arginine in the absence or presence of proinflammatory cytokines on subsequent acutely stimulated insulin secretion, cells were cultured for 24 h as above, preincubated for 40 min at 1·1 mmol/l glucose and then acutely stimulated for 20 min in the presence of 16·7 mM glucose + 10 mM alanine. The latter combination has been shown to evoke a robust and reproducible insulin secretion response (Brennan *et al.* 2002, Kiely *et al.* 2007). Aliquots of incubation medium were taken for analysis of insulin using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Islet isolation and culture; viability and chronic (24 h) insulin secretion

Pancreatic islets were isolated from wild-type C57 black mice. Each pancreas was excised and inflated with a Liberase TL grade solution (Roche 1815032; 8 mg/ml) and chopped into small pieces. Digestion was initiated during sample incubation at 37 °C for 3 min with constant shaking. The digest was washed with 0·1% BSA Krebs solution (5·6 mM glucose) and the islets were sedimented by gentle centrifugation (500 ***g*** for 10 min at 4 °C) with Histopaque 1077 (Sigma–Aldrich). Islets were resuspended in Krebs buffer containing 0·1% BSA, individually picked and cultured for 24 h with RPMI-1640 culture medium (as above), in the presence or absence of a non-lethal proinflammatory cytokine cocktail (IL1β 0·3125 U/ml, TNFα 31·25 U/ml and IFNγ 15·625 U/ml; Kiely *et al.* 2007) at 0 or 1·15 mmol/l of L-arginine. Chronic (24 h) insulin release was determined using the Mercodia Ultrasensitive Mouse Insulin ELISA kit.

Cell viability measurements

The neutral red uptake assay provides a quantitative estimation of the number of viable cells based on their

ability to incorporate and bind the dye neutral red in lysosomes. BRIN-BD11 cells were exposed to different concentrations of L-arginine, with or without the proinflammatory cytokine cocktail as described earlier. After 2 h incubation in presence of neutral red (100 μg/ml), cells were washed with PBS followed by disruption with acid ethanol (alcohol/glacial acetic acid, 50:1v/v). Aliquots of the resulting solution were transferred to 96-well plates and absorbance at 540 nm was recorded using a microplate spectrophotometer (Molecular Devices SpectraMax Plus 384, Sunnyvale, CA, USA). This procedure was highly reproducible and comparible with other cytotoxicity tests (tetrazolium salts, enzyme release, or DNA content; Repetto *et al.* 2008). To determine cell membrane integrity (an indirect measurement of cell viability), a lactate dehydrogenase (LDH) release assay (Biovision, Dublin, Ireland) was used and the results expressed as percentage of islet LDH released.

There was good correlation between cell membrane integrity, mitochondrial function and level of apoptosis and necrosis in a clonal β-cell line exposed to proinflammatory cytokines (Kiely *et al.* 2007). Islet β-cells contain a relatively low content of LDH but this is clearly measurable using the Biovision kit (Mountain View, CA, USA).

Enzymatic determination of metabolites

Urea and nitrite production, glucose and glutamine consumption and production of glutamate and lactate plus intracellular glutamate concentration were determined as described previously (Brennan *et al.* 2002, Kiely *et al.* 2007).

Measurement of glutathione and glutathione disulfide content

BRIN-BD11 cells were seeded into six-well plates (1×10^6 cells/well), allowed to adhere overnight and then washed with PBS after which they were incubated for 24 h in fresh media as described earlier. Cells were then rinsed twice with PBS and disrupted in 200 μl of 5% (w/v) metaphosphoric acid on ice. After centrifugation (14 000 ***g***, 5 min at room temperature), cell lysates were spectrophotometrically (415 nm) assayed in a temperature-controlled microplate reader (Molecular Devices SpectraMax Plus 384) by a modification of the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)/glutathione disulfide (GSSG) reductase recycling method, using the *N*-ethylmaleimide conjugating technique for GSSG sample preparation (Krause *et al.* 2007). Samples (10 μl) were assayed in 105 μl final volume in 96-well polystyrene plates at 37 °C in the presence of 10 mM DTNB, 0·17 mM β-NADPH (dissolved in 0·5% (w/v) $NaHCO_3$ as a stabilizing agent) and 0·5 U/ml GSSG reductase (EC 1.6.4.2).

Superoxide production in BRIN-BD11 cells

Dihydroethidium (DHE) is widely used as a probe to measure superoxide $\left(O_2^{\cdot}\right)$. DHE is cell permeable and reacts with $\left(O_2^{\cdot}\right)$ to form ethidium, which in turn intercalates with DNA, providing nuclear fluorescence at an excitation wavelength of 525 nm and an emission wavelength of 590 nm (Benov *et al.* 1998). BRIN-BD11 cells were incubated (1×10^4 cells/100 μl per well) in a 96-well black plate (Corning, Inc. Costar 3603, Dublin, Ireland) allowed to adhere overnight. Cells were then washed with PBS and incubated in various conditions for 24 h as described earlier. Subsequently DHE was added to the culture medium for 30 min to allow for the reaction between the dye and the superoxide to reach completion. The fluorescence was then read (ex=525/em=590). The results were expressed as average of fluorescence intensity/milligram of protein per 24 h.

Preparation of protein extracts from BRIN-BD11 cells

BRIN-BD11 cells were lysed in 150 μl of RIPA lysis buffer (MSC, Dublin, Ireland) containing protease inhibitors. Cell lysates were transferred to fresh ice-cold microcentrifuge tubes and placed on a shaker at 4 °C for 15 min. The cells were then centrifuged at 14 000 ***g*** for 15 min at 4 °C. The supernatant fraction was transferred to a fresh tube and stored at −20 °C. Cellular protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA; kit no. 23225), which utilizes a modification of the biuret reaction.

Western blot analysis

Cells were seeded into six-well plates ($1{\cdot}5\times10^6$ cells/well), allowed to adhere overnight and then washed with PBS after which they were incubated in fresh media for 24 h, in various conditions as described earlier. Subsequently cells were lysed and equal amounts of BRIN-BD11 cell protein extracts were prepared and subjected to 10% SDS–PAGE then electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked in 5% (milk protein or BSA) and probed with polyclonal antibodies anti-NOS-2 and heat

Table 1 Pancreatic β-cell viability (%, by neutral red assay). Effect of 24 h culture in the presence of various concentrations of L-arginine on the viability of BRIN-BD11 cells in the absence or presence of proinflammatory cytokines. Results are presented as percentage of control values. Data are means±S.D. of three separate preparations

	Cytokine cocktail	
	−	+
Condition		
1·15 mM L-arginine	$100\pm8{\cdot}7^{\dagger,\ddagger}$	$96{\cdot}3\pm8{\cdot}1^{\dagger,\ddagger,\S}$
0·25 mM L-arginine	$94\pm3^{*,\dagger}$	$56{\cdot}3\pm10{\cdot}9^{\dagger,\|\|}$
0·1 mM L-arginine	$89\pm4{\cdot}6^{*,\dagger}$	$56{\cdot}1\pm20{\cdot}9^{\dagger,\|\|}$
No L-arginine	$61\pm4{\cdot}6^{*}$	$42{\cdot}1\pm4{\cdot}5$

$^{*}P<0{\cdot}05$ versus cytokine treatment at the same L-arginine concentration; $^{\dagger}P<0{\cdot}05$ vs 0 mmol/l L-arginine; $^{\ddagger}P<0{\cdot}05$ vs 0·1 mmol/l L-arginine; $^{\S}P<0{\cdot}05$ vs 0·25 mmol/l L-arginine; and $^{\|}P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

shock protein 72 (HSP72; Sigma–Aldrich); anti-AMP-activated protein kinase (AMPK), phosphorylated AMPK (AMPK-P; Cell Signalling Technologies, Denvers, MA, USA). The blots were washed and visualized with a HRP-based Supersignal West Pico chemiluminescent substrate (Pierce). Results of digitalized images were expressed as means ± S.D. using anti-GAPDH antibodies for GAPDH detection (Cell Signalling Technologies) as an expression control.

Statistical analysis

Following confirmation of a normal distribution through the use of repeated Shapiro–Wilks tests, paired and unpaired two-tailed Student's *t*-tests were used where appropriate to evaluate the statistical significance of differences between the group means and ANOVA was used for multiple comparisons. Data are presented as means ± S.D. and differences were considered significant at a P value of $<0{\cdot}05$.

Results

Effect of L-arginine on BRIN-BD11 cell viability in the absence or presence of a proinflammatory cytokine cocktail

The effects of 24 h culture in the presence of various concentrations of L-arginine on the viability of BRIN-BD11 cells in the absence or presence of proinflammatory cytokines was investigated using the neutral red assay. Consistent with the importance of L-arginine for the maintenance of β-cell function, viability of BRIN-BD11 cells decreased from a normalized value of 100–61% in the absence of the amino acid (Table 1). Loss of viability was more dramatic when the proinflammatory cytokine cocktail was added, reducing viability to 40% of control in the absence of L-arginine. While 'viability' measurements invariably determine loss of cell function, e.g. loss of plasma membrane integrity by LDH release, or reduced lysosomal function via binding of neutral red, or activation of enzymes associated with cell apoptosis, key cell structures may remain intact at the time of assay. Thus, BRIN-BD11 cells were still able to metabolize glutamine at high rates and maintain their GSSG/glutathione (GSH) ratio, even when neutral red binding capacity was severely reduced (see below).

Effect of L-arginine on GSH levels, intracellular glutamate levels, glutamate release and glutamine consumption in BRIN-BD11 cells in the absence or presence of a proinflammatory cytokine cocktail

The effects of 24 h culture in the presence of various concentrations of L-arginine on GSH levels, cellular redox state, glutamate levels, glutamate release and glutamine consumption of BRIN-BD11 cells in the absence or presence of proinflammatory cytokines was investigated. Since glutamine (and the immediate product of its metabolism, glutamate)

Table 2 Glutathione metabolism (mmol/mg protein) and redox state (GSSG/GSH (AU)) over 24 h incubation. Pancreatic β-cell glutathione metabolism. Effect of 24 h culture in the presence of various concentrations of L-arginine on glutathione levels and cellular redox state of BRIN-BD11 cells in the absence or presence of proinflammatory cytokines. Data are means ± S.D. of three separate preparations

Experimental condition	Total glutathione		GSSG		GSH		GSSG/GSH	
Cytokine cocktail	−	+	−	+	−	+	−	+
1·15 mM L-arginine	$1{\cdot}45\pm0{\cdot}19^{\dagger,\ddagger,\S}$	$1{\cdot}7\pm0{\cdot}18^{\dagger,\ddagger,\S}$	$0{\cdot}26\pm0{\cdot}029^{\dagger,\ddagger,\S}$	$0{\cdot}36\pm0{\cdot}06^{\dagger,\ddagger,\S}$	$0{\cdot}91\pm0{\cdot}14^{\dagger,\ddagger}$	$0{\cdot}96\pm0{\cdot}18^{\dagger,\ddagger}$	$0{\cdot}28\pm0{\cdot}02^{\dagger,\ddagger,\S}$	$0{\cdot}39\pm0{\cdot}13^{\dagger,\ddagger}$
0·25 mM L-arginine	$1{\cdot}21\pm0{\cdot}15^{\dagger,\ddagger,\|\|}$	$1{\cdot}18\pm0{\cdot}07^{\dagger,\ddagger,\|\|}$	$0{\cdot}19\pm0{\cdot}03^{\dagger,\ddagger,\|\|}$	$0{\cdot}22\pm0{\cdot}021^{\dagger,\ddagger,\|\|}$	$0{\cdot}828\pm0{\cdot}08^{\dagger,\ddagger}$	$0{\cdot}73\pm0{\cdot}09^{\dagger,\ddagger}$	$0{\cdot}23\pm0{\cdot}02^{\dagger,\ddagger}$	$0{\cdot}31\pm0{\cdot}06^{\dagger,\ddagger}$
0·1 mM L-arginine	$0{\cdot}49\pm0{\cdot}07^{\S,\|\|}$	$0{\cdot}5\pm0{\cdot}05^{\S,\|\|}$	$0{\cdot}107\pm0{\cdot}02^{\S,\|\|}$	$0{\cdot}12\pm0{\cdot}008^{\S,\|\|}$	$0{\cdot}28\pm0{\cdot}02^{\dagger,\S,\|\|}$	$0{\cdot}24\pm0{\cdot}05^{\dagger,\S,\|\|}$	$0{\cdot}37\pm0{\cdot}07^{\dagger,\ddagger,\|\|}$	$0{\cdot}37\pm0{\cdot}07^{\dagger,\ddagger,\|\|}$
No L-arginine	0·44 ± 0·02	0·44 ± 0·03	0·15 ± 0·01	0·15 ± 0·02	0·13 ± 0·007	0·13 ± 0·034	1·23 ± 0·35	1·19 ± 0·21

*$P<0{\cdot}05$ versus cytokine treatment at the same L-arginine concentration; †$P<0{\cdot}05$ vs 0 mmol/l L-arginine; ‡$P<0{\cdot}05$ vs 0·1 mmol/l L-arginine; §$P<0{\cdot}05$ vs 0·25 mmol/l L-arginine; and ||$P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

is important precursors for β-cell GSH synthesis (Brennan *et al.* 2003), both reduced and oxidized forms of GSH were determined as was the GSSG/GSH ratio in addition to intracellular glutamate levels, glutamate release plus glutamine consumption. The intracellular levels of reduced GSH increased substantially in the presence of L-arginine (Table 2) from 0·13±0·01 μmol/mg (0 mmol/l L-arginine) to 0·92 ±0·14 μmol/mg (1·15 mmol/l L-arginine). This greater than sixfold increase evoked by L-arginine was concentration-dependent (Table 2). Levels of GSH disulfide were also increased but to a much lesser extent. Intracellular glutamate did not change when cells were exposed to physiologic or high concentrations of L-arginine (Table 3). However, in the total absence of L-arginine, intracellular glutamate concentration was decreased by ∼50% indicating that a minimum concentration of L-arginine was essential for the maintenance of β-cell glutamate levels (Table 3). Glutamate release was increased approximately fourfold by cytokine exposure, but amounts of glutamate released were lowered when the L-arginine concentration was increased. With respect to glutamine consumption, an approximate fourfold increase (from 3·52±0·87 to 15·05±3·05 μmol/mg) was observed with all cytokine incubations. This was unchanged by manipulation of L-arginine concentration (Table 3).

Effect of L-arginine on chronic (24 h) or acute (20 min) insulin secretion, glucose consumption and lactate production by BRIN-BD11 cells in the absence or presence of a proinflammatory cytokine cocktail

The effects of 24 h culture in the presence of various concentrations of L-arginine on insulin secretion, glucose consumption and lactate production from BRIN-BD11 cells in the absence or presence of proinflammatory cytokines was investigated. As expected, L-arginine stimulated chronic (24 h) tissue culture insulin release (by 28%) from cells cultured at 1·15 mM compared with 0 mM L-arginine (928 μg/mg protein compared with 727 μg/mg protein, Fig. 1A). In the presence of proinflammatory cytokines, insulin secretion was blunted by at least 50% in all incubations (Fig. 1A). Glucose consumption and lactate production were augmented by proinflammatory cytokine challenge (Table 3). Interestingly, glucose consumption and lactate production were further increased at higher L-arginine concentrations. It is also important to note that L-arginine increased glucose consumption and lactate production in the absence of cytokines, indicating L-arginine is able to alter β-cell glycolytic metabolism to pyruvate, so promoting lactate production and pyruvate oxidation. In a separate experiment BRIN-BD11 cells were cultured for 24 h in various concentrations of L-arginine in the absence or presence of proinflammatory cytokines and then subsequently stimulated for 20 min with a potent insulinotropic nutrient mixture (glucose+alanine, Fig. 1B–E). Cells cultured for 24 h in the absence of L-arginine were associated with reduced GSH levels, an elevated GSSG/GSH ratio and reduced intracellular

Table 3 Pancreatic β-cell metabolites consumption/production (mmol/mg protein) over 24 h incubation. Effect of 24 h culture in the presence of various concentrations of L-arginine on glucose and glutamine consumption, lactate production, intracellular and extracellular glutamate from BRIN-BD11 cells in the absence or presence of proinflammatory cytokines. Data are means±S.D. of three separate preparations

Experimental condition	Glucose consumption		Lactate production		Glutamine consumption		Extracellular glutamate		Intracellular glutamate	
Cytokine cocktail	−	+	−	+	−	+	−	+	−	+
1·15 mM L-arginine	27·6±2*,†,‡,§	120±18†,‡	31±3*,†,‡,§	140±32†,‡	3·82±0·6*	14·07±4·08	2·46±0·4*,†	13·05±2·25†	12·8±0·7†	13·18±0·9†
0·25 mM L-arginine	19·01±4$^{*,\dagger,\|\|}$	123±40·6†,‡	19±1·3$^{*,\dagger,\ddagger,\|\|}$	106±24$^{\dagger,\|\|}$	3·16±0·71*	15·6±6·7	2·9±0·28*,†	16·48±3·42†	13·3±0·4†	13·12±0·4†
0·1 mM L-arginine	19·4±2$^{*,\dagger,\|\|}$	74·71±11$^{\S,\|\|}$	16·8±1$^{*,\dagger,\ddagger,\|\|}$	99·92±16$^{\dagger,\|\|}$	3·52±0·8*	15·04±3·05	2·96±0·46*,†	16·99±3·42†	12·6±0·2†	13·31±0·19†
No L-arginine	15·57±2·35*	71·15±8·5	10·24±1·77*	20·83±4·6	5·62±1·7*	17·97±3·01	4·15±0·4*	21·88±3·03	6·42±0·5	5·9±0·6

*$P<0·05$ versus cytokine treatment at the same L-arginine concentration; $^{\dagger}P<0·05$ vs 0 mmol/l L-arginine; $^{\ddagger}P<0·05$ vs 0·1 mmol/l L-arginine; $^{\S}P<0·05$ vs 0·25 mmol/l L-arginine; and $^{\|}P<0·05$ vs 1·5 mmol/l L-arginine.

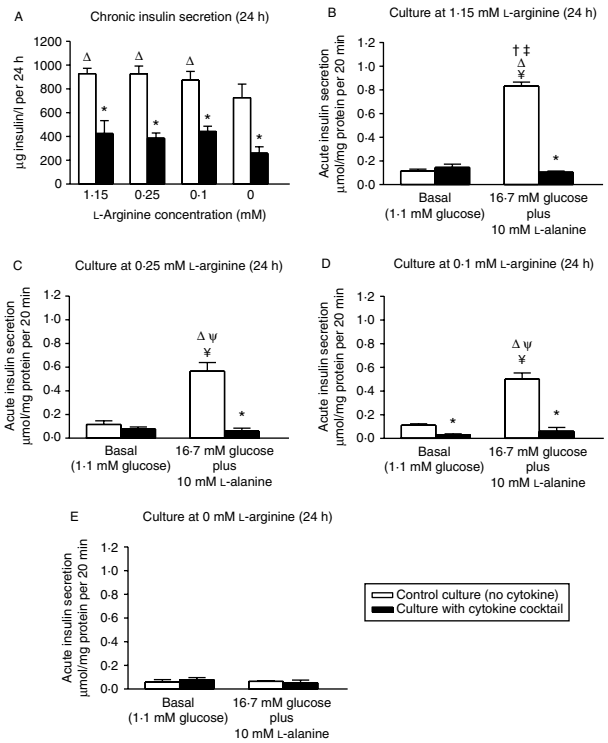

Figure 1 Effect of 24 h culture in the presence of various concentrations of L-arginine in the absence or presence of proinflammatory cytokines on chronic insulin secretion and subsequent 20 min acute nutrient stimulation of insulin secretion by BRIN-BD11 cells. (A) Chronic insulin secretion. 24 h pre-incubation in 1·15 mmol/l L-arginine (B); 24 h preincubation in 0·25 mmol/l L-arginine (C); 24 h preincubation in 0·10 mmol/l L-arginine (D); and 24 h preincubation in 0 mmol/l L-arginine (E). Cytokines were added to the 24 h culture where indicated. Data are means±S.D. of three separate preparations. Significance: $^{*}P<0{\cdot}05$ versus cytokine treatment at the same L-arginine concentration; $^{\Delta}P<0{\cdot}05$ vs 0 mmol/l L-arginine; $^{¥}P<0{\cdot}05$ versus respective basal; $^{‡}P<0{\cdot}05$ vs 0·1 mmol/l L-arginine; $^{†}P<0{\cdot}05$ vs 0·25 mmol/l L-arginine; and $^{\Psi}P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

glutamate (a key stimulus secretion coupling factor) as described earlier, which contribute to attenuation of acute glucose+alanine stimulated insulin secretion (Fig. 1E). This increase of glucose metabolism in cells incubated in the absence of cytokines was associated with enhanced insulin release from cells acutely stimulated with glucose+alanine (Fig. 1B–D). However, addition of cytokine cocktail potently attenuated nutrient-stimulated insulin secretion, an effect that has been previously reported (Kiely *et al.* 2007).

Effect of L-arginine on NOS-2 expression, production of nitrite, urea and superoxide by BRIN-BD11 cells in the absence or presence of a proinflammatory cytokine cocktail

The effects of 24 h culture in the presence of various concentrations of L-arginine on NOS-2 expression, nitrite, urea production and superoxide generation in BRIN-BD11 cells in the absence or presence of proinflammatory cytokines was investigated. Since proinflammatory cytokines are likely to induce oxidative stress by the generation of oxygen- and nitrogen-based free radicals, iNOS expression, NO and superoxide production were examined. Cytokine addition resulted in a large increase in iNOS protein levels (Fig. 2). This increment was dependent on extracellular L-arginine and showed clear concentration-dependency. iNOS was not detectable in the absence of the cytokine cocktail. As a consequence greater amounts of NO were detected (by the production of nitrite) in proportion to the extracellular L-arginine concentration (Fig. 2C). Urea levels were determined in the absence or presence of cytokines at all L-arginine concentrations (Fig. 2D), but while cytokines increased urea production this was not altered by L-arginine concentration. L-arginine availability (with the exception of zero concentration) was inversely proportional to superoxide production (Fig. 2E) perhaps as a result of the effect of the lack of L-arginine availability on the uncoupled iNOS phenomenon (Wu & Meininger 2009).

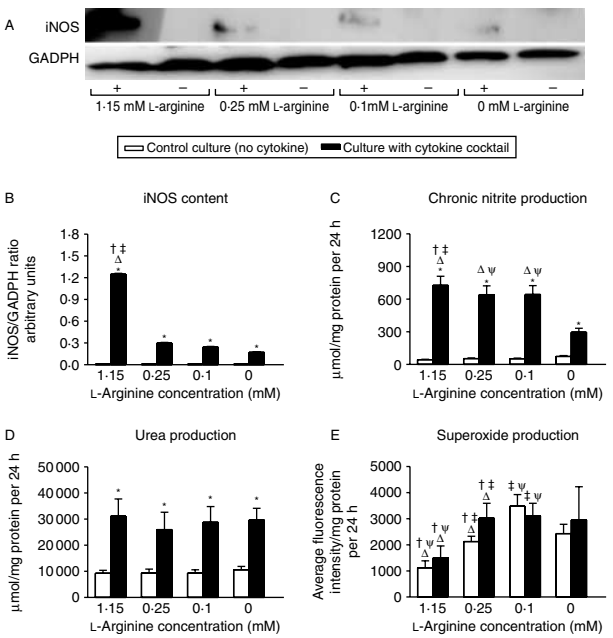

Figure 2 Effect of 24 h culture in the presence of various concentrations of L-arginine on NOS-2 expression, nitrite, urea production and superoxide generation in BRIN-BD11 cells in the absence or presence of proinflammatory cytokines. NOS-2 expression (A and B) is shown relative to GAPDH as control. Parts of the same representative gel are mounted and shown for each immunoblot. Results are shown for (C) NO production evaluated by measuring nitrite, (D) urea production and (E) superoxide production. Data are means±S.D. of three separate preparations. Significance: $^{*}P<0{\cdot}05$ versus cytokine treatment at the same L-arginine concentration; $^{\Delta}P<0{\cdot}05$ vs 0 mmol/l L-arginine; $^{‡}P<0{\cdot}05$ vs 0·1 mmol/l L-arginine; $^{†}P<0{\cdot}05$ vs 0·25 mmol/l L-arginine; and $^{\Psi}P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

Effect of L-arginine on the phosphorylation state and protein levels of AMPK and protein levels of HSP72 in the absence or presence of a proinflammatory cytokine cocktail

Since β-cell metabolism is dependent on regulation by the activity of key enzymes such as AMPK (Kiely *et al.* 2007, Newsholme *et al.* 2007*b*), protein levels of AMPK and its AMP-P were determined following 24 h incubation in various concentrations of L-arginine in the absence or presence of proinflammatory cytokines. L-Arginine evoked concentration-dependent activation of AMPK (as determined by phosphorylation status) that was further enhanced by the addition of the cytokine cocktail, at the higher concentrations of the amino acid (Fig. 3). HSP72, a protein involved in stress adaptation in all cell types (Krause & Rodrigues-Krause 2011), was also investigated. L-Arginine increased HSP72 levels in a dose-dependent manner but, with the exception of the 1·15 mM L-arginine condition, cytokine exposure did not significantly change HSP72 content (Fig. 3).

Effect of L-arginine on LDH release and chronic (24 h) insulin secretion from mouse islets in the absence or presence of a proinflammatory cytokine cocktail

To confirm validity of observations using BRIN-BD11 cells reported above, experiments were made using mouse islets as primary cell model. The effect of 24 h culture in the presence of either 0 or 1·15 mmol/l L-arginine on cell viability and chronic insulin secretion of mouse islets after 24 h incubation

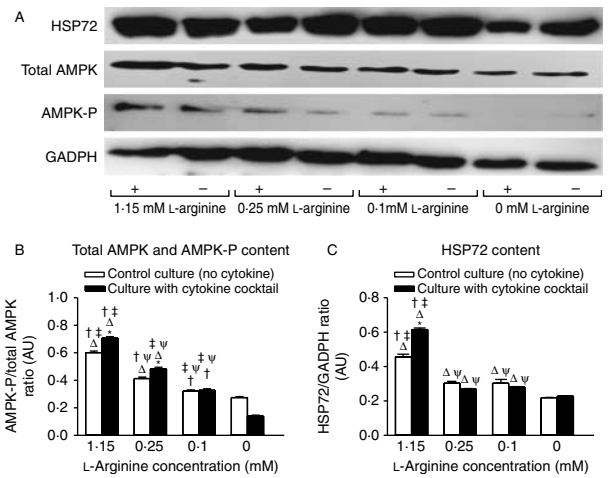

Figure 3 Effect of 24 h culture in the presence of various concentrations of L-arginine on the expression of AMPK and its AMPK-P and also the levels of HSP72 in BRIN-BD11 cells in the absence or presence of proinflammatory cytokines. Results are expressed as mean ± S.D. of at least three separate preparations in duplicate using GAPDH as an expression control. Parts of the same representative gel are mounted and shown for each immunoblot. Data are means ± S.D. Significance: $^{*}P<0{\cdot}05$ versus cytokine treatment at the same L-arginine concentration; $^{\Delta}P<0{\cdot}05$ vs 0 mmol/l L-arginine; $^{\ddagger}P<0{\cdot}05$ vs 0·1 mmol/l L-arginine; $^{\dagger}P<0{\cdot}05$ vs 0·25 mmol/l L-arginine; and $^{\Psi}P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

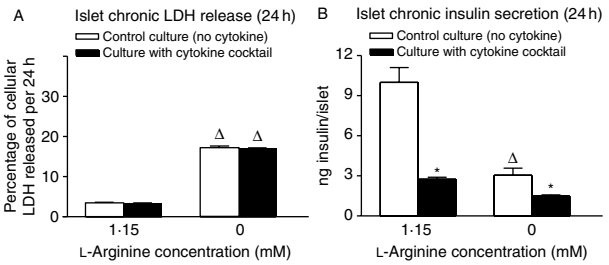

Figure 4 Effect of 24 h culture in the presence of either 0 or 1·15 mM L-arginine on membrane integrity as assessed by LDH release (A) and chronic (24 h) insulin secretion (B) of mouse islets in the absence or presence of proinflammatory cytokines. Cellular LDH release results are presented as percentage of cellular LDH released over 24 h of incubation according to the manufacturer's instructions (Biovision, Dublin, Ireland) following incubation in the presence of either 0 or 1·15 mmol/l. Insulin secretion results are expressed as nanograms insulin released/islet for insulin secretion. Results are expressed as mean ± S.D. of three separate preparations. Significance: $^{*}P<0{\cdot}05$ for control versus cytokine treatment at the same L-arginine concentration and $^{\Delta}P<0{\cdot}05$ vs 1·15 mmol/l L-arginine.

in the absence or presence of proinflammatory cytokines were investigated. Islets incubated in media lacking L-arginine were associated with a significant increase in LDH release compared with islets incubated in the presence of 1·15 mM L-arginine (Fig. 4A). This finding, together with the results on islet function (chronic insulin secretion) confirmed that L-arginine is essential for viability of primary islet cells as well as clonal β-cells. Chronic (24 h) insulin release from cultured islets was also significantly attenuated (by ~70%) in the absence of L-arginine, an effect that was also evident in the presence of inhibitory cytokine cocktail irrespective of the absence or presence of L-arginine (Fig. 4B).

Discussion

L-Arginine is synthesized from glutamine, glutamate and proline via the intestinal-renal axis in humans and most other mammals (Wu *et al.* 2009). It is essential for metabolism and function of multiple body organs, with decreased plasma and cellular levels reported in type 2 diabetic subjects (Pieper & Dondlinger 1997). Since the amino acid is the precursor of NO that serves as a key cell signaling molecule, restriction in the availability of L-arginine is likely to contribute to derangements in the secretion and action of insulin (Newsholme *et al.* 2010).

While excessive NO production can trigger oxidative/nitrosative stress and is undoubtedly a key contributor to β-cell death (Palmer *et al.* 1976, Newsholme *et al.* 2005, 2007*a*,*b*, 2010, Michalska *et al.* 2010), evidence now suggests that lesser amounts of NO, produced by the NFκB-regulated iNOS, serve as an important coupling factor in insulin-secreting cells (Palmer *et al.* 1976, Newsholme *et al.* 2005, 2007*a*,*b*, 2010, Michalska *et al.* 2010). We have demonstrated herein that L-arginine is an important stimulator of β-cell glucose consumption and intermediary metabolism. Such

actions lead to increased insulin secretion, enhanced antioxidant and protective responses with greater functional integrity when challenged with proinflammatory cytokines. Given that insulin-secreting cells have very low expression levels of antioxidant enzymes such as catalase and GSH peroxidase (Lenzen *et al.* 1996, Tiedge *et al.* 1997), β-cells are particularly prone to chemical stress in the diabetogenic or inflammatory environment typical of type 1 and possibly type 2 diabetes (Rocie *et al.* 1997, Santini *et al.* 1997, Kiely *et al.* 2007, Newsholme *et al.* 2007*a*, Michalska *et al.* 2010). The novel finding that L-arginine increased glucose consumption and lactate production in pancreatic β-cells, both in the presence or absence of cytokines, indicates that L-arginine is able to promote β-cell glycolytic flux in a concentration-dependent manner. Because glycolysis is essential to produce ATP (for stimulus–secretion coupling) and other energy-dependent process such as protein and GSH synthesis, this cellular response may be essential during inflammation of the pancreatic islet. The molecular mechanisms of the effect of L-arginine on glycolysis remains unknown but may be mediated via interaction at key regulatory points such as GLUT2, glucokinase, or phosphofructokinase 1. As T2DM patients are associated with lower levels of plasma L-arginine then the reduced availability of this amino acid could, at least in part, explain the reduced insulin secretory response during metabolic challenge *in vivo* (Salehi *et al.* 2010).

These positive actions of L-arginine on viability, antioxidant status and insulin secretion are likely to reflect, in large part, the importance of GSH and the GSH reductase/peroxidase systems as the main line of antioxidant defense in β-cells. To maintain GSH, β-cells may either regenerate it from GSSG via a GSSG reductase catalyzed reaction or synthesize it, *de novo*, through the concerted action of γ glutamylcysteine synthetase and GSH synthetase, which are ATP-consuming enzymes (see Fig. 5 for metabolic scheme). Regeneration of GSH from GSSG, which utilizes NADPH as a cofactor but does not require ATP, is metabolically less expensive than the *de novo* synthesis from the constituent amino acids. However, pentose phosphate shunt activity is relatively low in β-cells (Droge 2002), which is exacerbated by the high flux of glucose directed toward ATP production (Krause & de Bittencourt 2008). Therefore, β-cell NADPH can be obtained from the cytosolic malic enzyme, capable of converting malate to pyruvate with the concomitant production of NADPH from $NADP^+$ (MacDonald 1995). *De novo* GSH synthesis, on the other hand, is completely dependent on the supply of glutamate, not only because this amino acid is a constituent of the GSH molecule, but is also an amino acid donor in the synthesis of serine, which subsequently can be converted to glycine, via a reaction requiring tetrahydrofolate. In fact, previous reports from our laboratories have highlighted the importance of glutamine and alanine (which give rise to glutamate) for GSH generation, insulin secretion and protection against proinflammatory cytokines (Brennan *et al.* 2002, 2003, Cunningham *et al.* 2005).

The observation reported herein, that L-arginine is able to increase β-cell GSH synthesis, regardless of the presence or absence of proinflammatory cytokines, sheds light on an as yet unappreciated facet of β-cell metabolism, namely that L-arginine could be a precursor of GSH via glutamate generation (as L-arginine enhanced glutamate production and decreased glutamate release). This scenario is illustrated in a metabolic scheme depicted in Fig. 5. Increasing L-arginine availability allows for an increase in metabolic flux that produces glutamate from L-arginine by coupling production of L-ornithine to L-glutamate formation via pyrroline-5-carboxylate dehydrogenase and ornithine aminotransferase as described previously (Malaisse *et al.* 1989). This latter pathway may be important when L-glutamine or L-alanine availability is compromised. Inclusion of 0·25 mmol/l L-arginine in culture was sufficient to provide redox protection, as estimated by GSSG–GSH ratio. This suggests that L-arginine is an essential source of L-glutamate for GSH synthesis and maintenance of redox state during periods of inflammatory stress. Superoxide production evoked by proinflammatory cytokines was significantly reduced at higher L-arginine levels (Fig. 5).

The activation of the iHSP70 is *sine qua non* for the promotion of tissue repair, since the expression of this chaperone confers cytoprotection and also exerts anti-inflammatory effects (Krause & Rodrigues-Krause 2011). The increase in the levels of HSP72 induced by L-arginine in β-cells could be one of the key mechanisms mediating the observed protection against stress and inflammation. Thus, the higher level of HSP72 together with the increment in GSH synthesis induced by L-arginine may provide effective β-cell protection against inflammatory insult.

The stimulation of insulin release by L-arginine involves transport of the cationic amino acid into β-cells (via the amino acid transporter mCAT2A). This leads to direct membrane depolarization, activation of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, elevation of intracellular Ca^{2+} and discharge of insulin by exocytosis (Newsholme *et al.* 2005). The finding that L-arginine significantly increased β-cell glucose consumption is a novel finding and suggests that L-arginine promotes diversion of glucose carbon from mitochondrial oxidation toward the formation of NADPH via the cytosolic malic enzyme, so requiring that glucose-derived-malate is transported from the mitochondrial matrix to the cytosol. Indeed, we believe that, in the presence of L-arginine, glutamate can be generated from both L-arginine and glucose (via 2-oxoglutarate formation and transamination of glutamate) and is subsequently utilized for GSH synthesis (Fig. 5). However, in an early study, oxidation of L-arginine was barely detectable in the presence or absence of glucose, with the authors concluding that the amino acid does not give rise to oxidative intermediates (Hellman *et al.* 1971). This is entirely in agreement with our findings, as L-arginine conversion to glutamate and GSH does not require oxidation of L-arginine carbon, thus does not directly result in stimulation of ATP synthesis. However, the

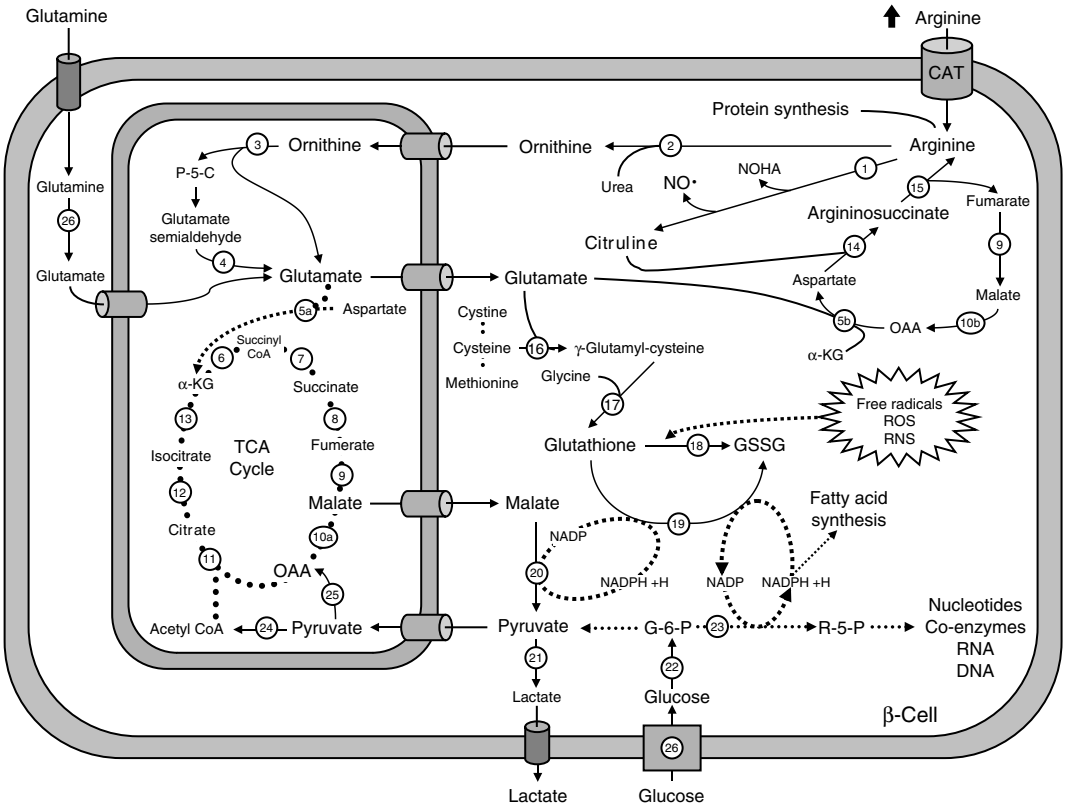

Figure 5 Scheme depicting major pathways relating to L-arginine metabolism in pancreatic β-cells. Entry of L-arginine into β-cells occurs via cationic transporters (CAT) in the plasma membrane and can be enhanced by cytokines. Once inside the cell L-arginine can be metabolized by iNOS to produce nitric oxide and citrulline and/or by arginase producing ornithine and urea. Ornithine can be converted subsequently in a sequence of reactions to pyrroline-5-carboxylate (P5C), glutamate semialdehyde and finally to glutamate. The glutamate produced is used for the synthesis of glutathione to enhance cell protection. Under normal secretagogue-induced insulin release, both NO and GSH are obligatory intermediates. Accordingly, β-cells have an intricate iNOS-centered machinery to produce NO, which potentiates insulin secretion physiologically. At the same time, β-cells utilize glutamate-derived GSH to maintain redox status needed to allow secretion and to avoid a possible NO-mediated cytotoxicity. L-Arginine is an established insulin secretagogue that depolarizes the cell membrane by virtue of its positive charge, leading to influx and elevation of intracellular Ca^{2+}. On the other hand, L-arginine increases β-cell metabolism leading to increments in glucose consumption, NADPH and lactate. Lack of L-arginine will result in decreased metabolism and reduced glutathione production, leading to a fragile state against stress factors such as inflammation and free radicals. Low L-arginine availability would also result in decreased ATP production, blunting the insulin secretion. Enzymes/reactions: 1, iNOS (inducible nitric oxide synthase); 2, arginase; 3, ornithine aminotransferase; 4, pyrroline-5-carboxylate dehydrogenase; 5, aspartate-aminotransferase (a: mitochondrial and b: cytosolic); 6, α-ketoglutarate dehydrogenase; 7, succinate thiokinase; 8, succinate dehydrogenase; 9, fumarase; 10a, malate dehydrogenase; 10b, cytosolic malate dehydrogenase; 11, citrate synthase; 12, aconitase; 13, isocitrate dehydrogenase; 14, argininosuccinate synthase; 15, argininosuccinate lyase; 16, γ-glutamylcysteine synthetase; 17, glutathione synthetase; 18, glutathione peroxidase; 19, glutathione reductase; 20, malic enzyme; 21, lactate dehydrogenase; 22, glucokinase; 23, pentose phosphate pathway; 24, pyruvate carboxylase; 25, pyruvate dehydrogenase; 26, GLUT2; 27, glutaminase.

L-arginine-dependent stimulation of glucose consumption may lead to elevated ATP levels. Thus, the long-accepted view that L-arginine exerts a stimulatory effect on insulin secretion in the presence of glucose, simply due to membrane depolarization induced by transport of a positively charged amino acid across the membrane must be revised. We propose that L-arginine exerts its positive effects via a combination of membrane depolarization, its metabolism to NO (and citrulline) or ornithine (and urea) and subsequent conversion to glutamate and GSH as well as an enhancement of glucose metabolism, so contributing to both stimulus–secretion coupling and antioxidant status. The fact that proinflammatory cytokines can impair insulin secretion even in the presence of high concentrations of L-arginine may indicate that non-lethal concentrations of proinflammatory cytokines shift β-cell metabolism away from energy generation and stimulus–secretion coupling and toward a catabolic state which may be related to cell defense (Kiely *et al.* 2007), as indicated by the GSH metabolism changes reported here.

The significance of AMPK in β-cell stimulus–secretion coupling is as yet unclear (Newsholme *et al.* 2007*b*), but L-arginine enhanced the level of AMPK-P in BRIN-BD11 cells (Fig. 3), thus favoring fatty acid oxidation. Interestingly,

L-arginine supplementation in Zucker diabetic fatty rats, resulted in powerful activation of HO-3, AMPK and PGC-1α (PPARγ coactivator 1α), which would be expected to increase mitochondrial biogenesis and increase oxidative metabolism in skeletal and cardiac muscle, brain, liver and adipose tissue (Fu *et al.* 2005). It has been suggested that changes in AMPK activity may also contribute to β-cell functional integrity (Wang *et al.* 2007, Nyblom *et al.* 2008). Thus, activation of AMPK by L-arginine may not only promote fatty acid oxidative metabolism thereby driving ATP production, but it may also serve to counter β-cell glucolipotoxicity.

In summary, L-arginine exerts a broad spectrum of beneficial effects on clonal β-cells and isolated islets in addition to simple membrane depolarization and triggering of insulin secretion. The novel findings reported in this paper suggest an important role of L-arginine in promotion of GSH synthesis and antioxidant defense that may encourage the development of novel strategies for the protection of β-cells against chemical/immune insult and diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M S K completed all experiments described in this manuscript. M S K and P N co wrote the manuscript. N H M, P R F, P I H and C M provided experimental advice and helped with manuscript revision. N M was responsible for the presentation style of all figures. C M and P N were responsible for grant support with respect to TSR: strand III – Core Research Strengths Enhancement Scheme (Ireland).

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