Toll-like receptor agonist induced changes in clonal rat BRIN-BD11 β-cell insulin secretion and signal transduction

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Abstract

Evidence for involvement of toll-like receptors (TLRs) (e.g. TLR4 and TLR2, whose agonists include lipopolysaccharides (LPS) and saturated fatty acids) in altered patterns of signalling in adipose, liver and muscle from animal models of insulin resistance and obesity has been published. We have now extended this area of research and have determined the effects of LPS on cell viability, insulin secretion, insulin signalling and metabolism in a clonal β-cell line. BRIN-BD11 β-cells were treated for 24 h with increasing concentrations of LPS. Chronic (24 h) and acute (20 min) insulin secretion, insulin content and parameters of cell metabolism and insulin signalling were determined. Incubation of BRIN-BD11 cells for 24 h in the presence of increasing concentrations of the TLR4 ligand LPS significantly decreased chronic (24 h) insulin secretion from 1.09±0.19 to 0.76±0.18 μg insulin/mg protein in the presence of 100 ng/ml LPS (P<0.05). There was no change in acute (20 min) stimulated insulin secretion or insulin content. Cell metabolism was not changed. Insulin receptor-β (IRβ) expression levels were increased significantly from 1±0.52 to 8.6±1.83 units (P<0.01), whereas calcineurin activity and Akt phosphorylation were significantly (P<0.01 and P<0.05 respectively) reduced in response to 24 h incubation in the presence of LPS. There was no change in IR substrate-1 protein expression or phosphorylation after 24 h. Further incubation for 24 h in the absence of LPS resulted in the recovery of chronic insulin secretion. The negative β-cell effects of LPS may contribute to hyperglycaemia in vivo.


Introduction

Toll-like receptors (TLRs) are a family of evolutionarily conserved receptors primarily involved in regulation of the immune system by sensing a range of chemicals produced by pathogens such as bacteria and viruses and activating immune cell responses (O’Neill 2006). TLR signalling has a number of effects on the activities of antigen presenting cells, including production of inflammatory cytokines and up-regulation of MHC products. TLR expression has been demonstrated on cells such as macrophages and dendritic cells (Atkinson 2008).

However, the expression has also been reported on numerous other cells throughout the body, including those from various tissues and organs and some cell lines (Vives-Pi et al. 2003, Tsukumo et al. 2007). Indeed, TLR4, TLR2 and the leucine-rich repeat MD-2 proteins have been detected in islet cells, thus suggesting that β-cells express a functional lipopolysaccharide (LPS) receptor (Vives-Pi et al. 2003). Evidence for involvement of innate immune product receptors, e.g. TLRs, in insulin resistance has been recently published (Tsukumo et al. 2007). Notably type 2 diabetes has been associated with chronic low-grade inflammation (Creely et al. 2007) in addition to the well-defined tissue insulin resistance (which is the result of changes in insulin receptor (IR) coupled signal transduction pathways in muscle, adipose tissue and liver, Tsukumo et al. 2007). As well as innate immune products such as LPS, adaptive immune factors such as pro-inflammatory cytokines (such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) can alter patterns of insulin signalling in these target tissues, resulting in resistance to the action of insulin (Tilg & Moschen 2008).

Recent work has implicated fatty acids as agonists for TLR4 and TLR2 and thus established a possible connection between altered patterns of insulin signalling in animal models of insulin resistance and obesity (Lee et al. 2003). In contrast to saturated fatty acids, polyunsaturated fatty acids inhibit this pathway (Poltorak et al. 1998, Lee et al. 2003, Tsukumo et al. 2007). Loss of function mutations in the TLR4 receptor had a beneficial effect on the insulin signalling pathways in adipose, muscle and liver tissues in mice and improved insulin action (Tsukumo et al. 2007). Human adipocytes were demonstrated to have inducible TLR4 and TLR2 pathways further supporting the role that adipose tissue may play in the regulation of inflammation (Vitseva et al. 2008).
Primary β-cells and some cell lines, such as HP62 and RINm5F, have been reported to express TLR2 and TLR4 (Vives-Pi et al. 2003); thus, we determined mRNA expression of the various TLRs in the rat β-cell line BRIN-BD11 and have determined the effects of LPS on β-cell insulin secretion and content, viability, metabolism and insulin signal transduction pathways.

While glucose and alanine are recognised as potent insulinotropic nutrients (Newsholme et al. 2006, McClenaghan 2007), glutamine is generally thought less important. However, glutamine metabolism in the β-cell may play multiple roles – 1) production of intracellular glutamate and glutathione, which acutely stimulates insulin secretion (Brennan et al. 2003), 2) stimulation of acyl-CoA production, which would be important for the amplification of insulin secretion, 3) chronic release of glutamate from the cell, therefore, inhibiting insulin secretion via interaction with ionotrophic and metabotropic glutamate receptors (Corless et al. 2006) and 4) up-regulation of calcineurin expression and activity, which may impact on pro-survival gene expression and NMDA receptor-dependent ion fluxes (Corless et al. 2006). Thus, a study, such as the one described here, of the effect of LPS on metabolism of insulinotrophic nutrients, should include determination of utilisation of glucose, alanine and glutamine.

BRIN-BD11 β-cells represent a useful model for such studies with stability in culture and well-characterised metabolic, signalling, insulin secretion and cell viability responses to glucose, amino acids and numerous other modulators of β-cell function (McClenaghan et al. 1996a,b, Chapman et al. 1999, McClenaghan & Flatt 1999, Brennan et al. 2002, 2003), and the present data extend characterisation of these cells and understanding of TLRs in the regulation of insulin secretion and signal transduction.

### Materials and Methods

#### Cell culture

BRIN-BD11 cells were cultured in RPMI 1640 media supplemented with 10% (v/v) foetal bovine serum and 0·1% antibiotics (100 U/ml penicillin and 0·1 mg/ml streptomycin). Cells (1·5 × 10⁶) were seeded in 24-well plates containing 1 ml media or 1·5 × 10⁶ cells were seeded in 6-well plates containing 5 ml media and allowed to adhere overnight, before treatment in the presence or absence of various concentrations of LPS (LPS from Escherichia coli was sourced from Autogen Bioclear, product no. thr-ebhps). A stock solution of LPS (5 mg/ml) was prepared utilising sterile PBS. Subsequent dilutions were prepared with standard RPMI 1640 culture media. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air using a Forma Scientific incubator.

#### RT-PCR

Total RNA was isolated using TRizol reagent (Invitrogen), according to the manufacturer’s recommendation. The Superscript Preamplification System II (Invitrogen) and oligo(dT) primer were used for the synthesis of cDNA. Gene-specific primers (Table 1) were used for the PCR, which utilised Taq polymerase (Applied Biosystems). The obtained PCR products were separated by EtBr supplemented agarose gel electrophoresis and visualised with u.v. light. PCR products were excised from the gel, purified and commercially sequenced (MWG Biotech, Edersberg, Germany). In all cases, the sequence returned matched the expected TLR sequence within 98% and in most cases, 100%.

### Table 1 PCR primer sequences for toll-like receptors (TLRs) in the rat

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<th>Sequence</th>
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Cell viability

Cell viability was determined by WST-1 analysis, a successor of the popular MTT assay, which has the benefit of being cheaper, faster and more convenient to use (Takamatsu 1998). Cell viability was determined using the cell proliferation reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells. Briefly, 100 µl cell suspension (containing $2 \times 10^5$ cells) was plated in each well of a 96-well plate. After overnight culture, to allow reattachment, the cells were incubated in the absence or presence of various concentrations of LPS or various concentrations of glutamine for 24 h. At the end of each experiment, the cell proliferation reagent WST-1 (10 µl) was added to each well, and the cells were incubated at 37 °C for either 0·5 or 1·5 h. $A_{450}$ was measured using a kinetic plate reader (Spectramax Plus; Molecular Devices, Sunnyvale, CA, USA).

Determination of metabolites

D-glucose, L-glutamine, L-glutamate and L-lactate concentrations were determined using the YSI 7100 Multiparameter Bioanalytical System. An aliquot of supernatant (either culture medium or Krebs–Ringer bicarbonate buffer) was removed from the tissue culture plate or flask, centrifuged for 5 min at 400 g and analysed. The linear range of each assay run was as follows: D-glucose (dynamic range of 0–25 g/l); L-lactate (0·05–2·7 g/l); L-glutamate (15–1460 mg/l); and L-glutamine (30–1169 mg/l). Nitric oxide concentrations in the culture media were determined using the Griess Reagent System (Promega, Medical Supply Co). The rate of nitrite production was expressed as μmol/mg protein per 24 h.

Insulin secretion

Cells were treated for 24 h in the absence or presence of various concentrations of LPS. The media was removed and stored for later insulin analysis. The cells were then washed with PBS, and acute insulin secretion was initiated after incubating the cells for 40 min in KRB pH 7·4 containing 1·1 mM glucose (in the absence of LPS), by subsequent stimulation for 20 min in KRB pH 7·4 containing 16·7 mM glucose plus 10 mM alanine (in the absence of LPS). Insulin secretion (acute and chronic) was determined using a Mercodia Ultrasensitive Rat Insulin ELISA kit (Uppsala, Sweden).

Insulin content

After 24-h incubation, the media was removed and cells were washed with sterile PBS. Intracellular insulin was extracted overnight at 4 °C from the monolayer cells with 1 ml/well of acid ethanol (1·5% HCl in 70% ethanol, Hamaguchi et al. 2003).

Western blot analysis

Protein was extracted using the RIPA buffer supplemented with protease inhibitor cocktail (Sigma P8340), phenylmethylsulphonyl fluoride (1 mM), NaF (1 mM), microcysteine (2 mM), Na3VO4 (100 mM) and benzamidine (10 mM). Protein concentration was determined with the BCA protein assay kit (Pierce products supplied by Fisher Scientific, Dublin, Ireland) and equivalent amounts of protein were subjected to SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheet. The sheet was blocked in 5% milk protein and incubated with polyclonal anti-IR substrate-1 (IRS1), p-IRS1, Akt, p-Akt (Akt/Thr$_{308}$ phosphorylation, a marker of Akt activation, Barry et al. 2009), IRβ, p42/44 or p-p42/44 (Santa Cruz, Heidelberg, Germany). The blots were washed and probed with HRP and visualised with SuperSignal West Pico Chemiluminescent Substrate (Pierce). GAPDH was used as a loading control.

Protein determination

Cell protein was extracted using RIPA lysis reagent containing 1× Halt Protease Inhibitor Cocktail (Thermo Scientific products supplied by Fisher Scientific, Dublin, Ireland). Cellular protein was determined using a BCA protein assay kit (Pierce, kit no. 23225), which utilises a modification of the biuret reaction.

Calcineurin activity

Calcineurin activity was determined by the Calcineurin Assay Kit (Calbiochem) following incubation of the cells for 24 h in the presence of LPS and in the presence or absence of either 2 or 10 mM l-glutamine.

Statistical analysis

The results are presented as mean ± S.E.M. Groups of data were compared using a Student’s unpaired t-test or ANOVA where appropriate. Differences were considered significant at a P value of $<0·05$ (indicated by *) or $0·01$ (indicated by **).

Results

Receptor expression

The mRNA expression of nine members of the TLR family was detected by RT-PCR in the BRIN-BD11 pancreatic β-cell line, including TLR4 and TLR2 (Fig. 1). PCR

![Figure 1](image-url)
products were excised from the gel, purified and commercially sequenced (MWG Biotech). In all cases, the sequence returned matched the expected TLR sequence within 98% and in most cases, 100%. We were unable to explain the presence of a small PCR product in lane 5.

Cell viability, insulin content and insulin secretion

Cell viability as determined by WST-1 analysis was not altered following 24 h exposure to various concentrations of LPS, 10–1000 ng/ml (Fig. 2). As WST-1 absorbance is proportional to the activity of mitochondrial dehydrogenases, our results indicate that LPS exposure did not result in an increase (or indeed decrease) in mitochondrial metabolism; thus, did not alter mitochondrial oxidative phosphorylation.

A significant \((P < 0.05)\) reduction in chronic (24 h) insulin secretion was provoked by chronic exposure to the TLR4 agonist LPS (24 h, Fig. 3A), but acute insulin secretion (20 min stimulation), tested after 24 h exposure to LPS, was not altered (Fig. 3B). BRIN-BD11 insulin content, determined after 24 h exposure to 10–1000 ng/ml LPS, was not altered (Fig. 3C). Incubation of treated cells for another 24 h, in the absence of LPS, demonstrated a recovery of chronic (24 h) insulin secretion (Fig. 3D). The higher levels of insulin secretion determined after 24 h, as described in Fig. 3D compared with Fig. 3A, may represent a cell culture-dependent elevation in chronic insulin secretion, related to the time spent in culture.

![Figure 2](https://example.com/fig2.png)

**Figure 2** BRIN-BD11 cells were incubated for 24 h in the presence of increasing concentrations of LPS. Relative cell viability was determined by the WST-1 assay. There was no loss of cell viability after incubation of BRIN-BD11 cells for 24 h in the presence of up to 1000 ng/ml LPS. Results are expressed as mean ± S.E.M., \(n = 3\).

![Figure 3](https://example.com/fig3.png)

**Figure 3** BRIN-BD11 cells were incubated for 24 h in the presence of increasing concentrations of LPS. In A, the media was removed and insulin concentration was determined by ELISA (Mercodia). Chronic insulin secretion (24 h) was significantly \((P < 0.05)\) reduced after culture in the presence of 100 or 1000 ng/ml LPS. Results are expressed as mean ± S.E.M., \(n = 3\) in duplicate. In B, after the media was removed, cells were incubated in Krebs buffer supplemented with 1-1 mM glucose for 40 min (in the absence of LPS) before washing, and then were subjected to 20 min exposure to Krebs buffer supplemented with 1-1 mM glucose (in the absence of LPS) or 16-7 mM glucose plus 10 mM alanine (in the absence of LPS). Acute nutrient-stimulated insulin secretion was not altered following 24 h incubation in the presence of LPS up to 1000 ng/ml; insulin was determined by ELISA (Mercodia). Results are expressed as mean ± S.E.M., \(n = 3\). In C, the BRIN-BD11 cells were incubated for 24 h in the presence of increasing concentrations of LPS. Intracellular insulin was extracted using acid–ethanol and concentration determined by ELISA (Mercodia). There was no change in insulin content after 24 h in the presence of increasing concentrations of LPS. Results are expressed as mean ± S.E.M., \(n = 3\). In D, the BRIN-BD11 cells were incubated for 24 h in the presence of increasing concentrations of LPS, before washing with PBS, and incubated for another 24 h in the presence of RPMI 1640 media after which the insulin secretion was determined. Results are expressed as mean ± S.E.M., \(n = 3\).
Nutrient consumption and product formation

There was no change in glucose, alanine or glutamine consumption (three key nutrients for pancreatic β-cells, Dixon et al. 2003) or lactate, glutamate or nitrite production after 24 h incubation in the presence of 10–1000 ng/ml LPS (results not shown), which indicated that cell metabolism was not altered by TLR signalling thus metabolic stimulus secretion coupling most likely remained intact.

Glutamine protection from LPS-induced inhibition of insulin secretion

After 24 h incubation in the presence of 1000 ng/ml LPS and 2 mM glutamine (standard tissue culture concentration), there was a significant (P<0.05) reduction in chronic insulin secretion (Fig. 4). However, the addition of 10 mM glutamine attenuated LPS-induced inhibition of insulin secretion (Fig. 4).

Calcineurin activity

Calcineurin activity determined after BRIN-BD11 cells were incubated for 24 h in the presence of 2 mM glutamine was significantly (P<0.01) reduced (by 42%) after incubation for 24 h in the presence of 1000 ng/ml LPS. Calcineurin activity was not elevated by the addition of 10 mM t-glutamine, a concentration that has previously been shown to promote calcineurin activity in tissue culture conditions (Corless et al. 2006; Fig. 5).

Insulin signalling

LPS significantly (P<0.01) increased IRβ expression at 100 and 1000 ng/ml over a 24 h incubation period (Fig. 6). By contrast, exposure to LPS resulted in a significant reduction in the level of Akt/Thr308 phosphorylation (activated Akt, P<0.05; Fig. 7). However, phosphorylated IRS1 and phosphorylated p42/44 levels were not significantly altered by LPS (Fig. 8).

Discussion

TLRs play a critical role in the activation of appropriate innate immune responses in mammals by recognising conserved pathogen-associated molecules such as LPS (Tsukumo et al. 2007). Indeed, 13 members of the TLR family have been identified in mammals and we have determined nine members in the rat β-cell line BRIN-BD11. While TLR2 is involved in the responses to a range of constituents of the cell walls of pathogens, TLR4 is a subclass that can be ligated by LPS and by non-bacterial agonists, such as saturated fatty acids (Tsukumo et al. 2007). Activation of TLR4 signalling at the cell surface induces activation of specific intracellular inflammatory pathways, which sensitive cells and tissues are related to the induction of insulin resistance due to suppression of insulin signalling pathways. Interestingly, TLR2 maps to the same chromosome region as the NOD mouse diabetes susceptibility gene Idd17 (Podolin et al. 1997), and the gene encoding TLR4 maps to chromosome 9q33 (Rock et al. 1998), which has also been mapped to an unnamed type 1 diabetes locus (Concannon et al. 2005). In a recent study, mice with a loss-of-function mutation in TLR4 (C3H/HeJ) were protected against the development of diet-induced obesity and insulin resistance and that, in isolated muscles from C3H/HeJ mice, there was a protection from saturated fatty acid-induced insulin resistance (Tsukumo et al. 2007). Saturated fatty acids are known to suppress insulin signalling in muscle (Pan et al. 1997).

High fat feeding has been reported to increase plasma LPS levels, while LPS infusion in normal fed mice caused a metabolic response similar to that of the high-fat fed mice (Cani et al. 2007). Additionally, endogenous LPS is continuously produced from the gut due to the degradation of intestinal flora (Drewe et al. 2001). LPS has been
reported to lower blood glucose levels in both humans and animals (Oguri et al. 2002), via enhanced systemic consumption of glucose, depletion of glucose from the liver and muscle and impaired hepatic gluconeogenesis (Sherry et al. 2007). There are no studies to date that have addressed the effects of LPS on in vitro cell culture insulin secretion, but one in vivo study has demonstrated positive correlation between serum LPS and fasting insulin levels in humans (Creely et al. 2007). We have now demonstrated that LPS can reduce chronic (24 h) insulin secretion in cell culture conditions, but subsequent acute (20 min) glucose plus amino acid-stimulated insulin secretion was not altered, nor was BRIN-BD11 insulin content. Nutrient consumption (glucose and glutamine) and metabolite production (glutamate, lactate and nitrite) were unaffected by LPS in the concentration range used in this study. This indicates that cell metabolism was not altered by TLR signalling, thus, metabolic stimulus secretion factor generation was not impaired.

Post-transplant diabetes mellitus has been linked to inhibition of calcineurin by immune system suppressors such as cyclosporin A (Heit et al. 2006) suggesting impairment of β-cell function by the drug. Calcineurin negatively regulates TLR-mediated activation pathways in macrophages by inhibiting the adaptor proteins MyD88, TRIF, TLR2 and TLR4 (Kang et al. 2007). Calcineurin inhibitors have been shown to induce NFkB activation in macrophages (Kang et al. 2007). The fact that calcineurin interacts with MyD88, TRIF, TLR2 and TLR4 was established by co-immunoprecipitation; however, the cause/effect relationship was not (Kang et al. 2007). We have demonstrated that calcineurin activity in BRIN–BD11 cells incubated for 24 h in the presence of LPS was reduced by 42%. Indeed, calcineurin may be an important signalling component in β-cells as it is Ca2+ sensitive (Lawrence et al. 2008) and will dephosphorylate important transcription factors such as NFAT (Heit et al. 2006). It has also been demonstrated that 10 mM Gln significantly increased calcineurin activity compared with 1 mM Gln in BRIN–BD11 β-cells (Corless et al. 2006). In the study reported here, incubation of BRIN-BD11 cells in 10 mM l-glutamine attenuated LPS suppressed chronic insulin secretion, but this effect was not due to restored calcineurin activity. There was no increase of NO production (determined by nitrite analysis) in BRIN-BD11 cells incubated with LPS. i.p. injection of LPS (10 mg/kg) for 48 h was recently demonstrated to significantly increase enterocyte apoptosis in rat intestines in vivo, and this was significantly attenuated by dietary glutamine (Kessel et al. 2008). Glutamine has also been implicated as a treatment for LPS-induced septic shock by reducing the expression of HSP70 and plasma concentrations of TNF-α and IL-6 in a rodent population (Jing et al. 2007).

Following insulin binding, the IR is autophosphorylated. The activated IR then phosphorylates IRS1 and related substrates. Activated IRS1 can activate PI3-kinase, which in turn activates PI3 kinase-dependent serine/threonine kinases and then PKB/Akt. PKB/Akt is involved in the regulation of multiple signalling processes, including regulation of glucose metabolism. Figure 6 BRIN-BD11 cells were incubated for 24 h in increasing concentrations of LPS. Western blot analysis was performed to determine the levels of IRβ protein expression; GAPDH was used as an expression control. There was a significant increase (*P<0.01) in IRβ expression levels after 24 h incubation in the presence of LPS. Results are expressed as mean±s.e.m., n=3.

Figure 6

![Image](image1.png)

Figure 6: BRIN-BD11 cells were incubated for 24 h in increasing concentrations of LPS. Western blot analysis was performed to determine the levels of IRβ protein expression; GAPDH was used as an expression control. There was a significant increase (*P<0.01) in IRβ expression levels after 24 h incubation in the presence of LPS. Results are expressed as mean±s.e.m., n=3.

Figure 7 BRIN-BD11 cells were incubated for 24 h in increasing concentrations of LPS. Western blot analysis was performed to determine levels of Akt and p-Akt (Thr308) protein expression. There was a small but significant (*P<0.05) reduction in phosphorylated Akt levels after 24 h incubation in the presence of 100–1000 ng/ml LPS. Results are expressed as mean±s.e.m., n=3.

Figure 7

![Image](image2.png)
metabolism, cell survival and proliferation and gene transcription. PKB/Akt is a pro-survival intracellular signalling molecule, known to be activated in the pancreatic β-cell by a number of different growth factors (Fiaschi-Taesch et al. 2007). PKB/Akt is activated by phosphorylation on Thr³⁰⁸ and Ser⁴⁷³. Activation of the PI3K/Akt pathway has been shown to be associated with increased cell survival and proliferation in rodent cells in vitro (Dickson & Rhodes 2004).

It is accepted that elevated glucose metabolism can stimulate insulin secretion in a process involving oxidative glucose metabolism, an elevation in the ATP/ADP ratio, closure of the K⁺ATP channels, membrane depolarisation and calcium influx, which results in insulin release (McClenaghan 2007). More recently, the involvement of the β-cell IR and its signalling pathway in secretion has been reported (Accili 2004). Site-specific inhibition of either the IR or IRS1 in β-cells has been shown to impair glucose sensing (Kulkarni et al. 1999a,b), implying that insulin resistance at the level of the insulin signalling cascade in the β-cell may play a role in the pathogenesis of type 2 diabetes.

Indeed, the observed decrease in phosphorylated (activated) Akt reported in this study may account for the inhibition of chronic (24 h) insulin secretion in culture conditions, while acute (20 min) stimulation was unaffected, probably due to the fact that acute stimulation is almost entirely dependent on metabolic stimulus secretion coupling. Conversely, overexpression of a constitutively active Akt in the mouse β-cell increased β-cell size and number, and total islet mass (Bernal-Mizrachi et al. 2001, Tuttle et al. 2001). A recently published study investigated insulin signalling in the livers of C3H/HeJ mice (which have a loss-of-function mutation in TLR4) and control mice (Tsukumo et al. 2007). They detected a 50% reduction in insulin-induced IRS1 tyrosine phosphorylation accompanied by a 30% reduction in IRS1 protein content and a 70% reduction in Akt phosphorylation in the livers of control mice fed a high-fat diet but not in C3H/HeJ mice fed the same diet. While we determined the effect on insulin signalling in response to LPS only, in β-cells, both LPS and saturated fatty acids may exert their effects through the TLR4 protein in vivo.

Furthermore, we were able to demonstrate an increase in IRβ protein level but a reduction in activated Akt in response to LPS, but IRS1 and p42/44 phosphorylation (and therefore activation) levels were unaltered.

In conclusion, TLR agonists such as LPS may suppress insulin secretion from the β-cell, which may compound the known detrimental effects of insulin resistance in vivo.

Declaration of interest

There is no conflict of interest in relation to any of the authors of this paper and publication in the Journal of Endocrinology.

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