Visfatin regulates insulin secretion, insulin receptor signalling and mRNA expression of diabetes-related genes in mouse pancreatic β -cells

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Abstract

The role of the adipocyte-derived factor visfatin in metabolism remains controversial, although some pancreatic β -cell-specific effects have been reported. This study investigated the effects of visfatin upon insulin secretion, insulin receptor activation and mRNA expression of key diabetes-related genes in clonal mouse pancreatic β -cells. β -TC6 cells were cultured in RPMI 1640 and were subsequently treated with recombinant visfatin. One-hour static insulin secretion was measured by ELISA. Phospho-specific ELISA and western blotting were used to detect insulin receptor activation. Real-time SYBR Green PCR array technology was used to measure the expression of 84 diabetes-related genes in both treatment and control cells. Incubation with visfatin caused significant changes in the mRNA expression of several key diabetes-related genes, including marked up-regulation of insulin (9-fold increase), hepatocyte nuclear factor (HNF)1 β (32-fold increase), HNF4 α (16-fold increase) and nuclear factor κ B (40-fold increase). Significant down-regulation was seen in angiotensin-converting enzyme (-3.73 -fold) and UCP2 (-1.3 -fold). Visfatin also caused a significant 46% increase in insulin secretion compared to control ($P < 0.003$) at low glucose, and this increase was blocked by co-incubation with the specific nicotinamide phosphoribosyltransferase inhibitor FK866. Both visfatin and nicotinamide mononucleotide induced activation of both insulin receptor and extracellular signal-regulated kinase (ERK)1/2, with visfatin-induced insulin receptor/ERK1/2 activation being inhibited by FK866. We conclude that visfatin can significantly regulate insulin secretion, insulin receptor phosphorylation and intracellular signalling and the expression of a number of β -cell function-associated genes in mouse β -cells.

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Introduction

Type 2 diabetes (T2D) is a condition with a multifactorial aetiology. Obesity is one well-established risk factor for T2D, with the prevalence of obesity being $>90\%$ in sufferers of T2D in some western societies (Astrup & Finer 2000). Although the precise mechanisms by which increased adiposity predisposes so strongly for T2D are not fully understood, one emerging paradigm is an involvement of adipose tissue-secreted molecules commonly referred to as adipokines. These molecules include hormones, growth factors and cytokines such as resistin, visfatin, adiponectin, retinol-binding protein 4, leptin and tumour necrosis factor- α (Ahima 2006), which have been implicated in the modulation of both insulin sensitivity and β -cell function (Brown *et al.* 2002, 2007, Trayhurn & Wood 2005). Research has shown that the majority of these molecules circulate in increased concentrations in obese individuals.

One of the more recently described adipokines is visfatin (Berndt *et al.* 2005). Visfatin (also referred to as pre-B cell colony-enhancing factor) was initially reported as having insulin-mimetic properties. Subsequent studies have identified visfatin as a nicotinamide phosphoribosyltransferase (NAMPT) capable of producing nicotinamide mononucleotide (NMN), a precursor for the metabolic co-factor NAD⁺ (Kim *et al.* 2006). The circulating levels of visfatin have been reported to be elevated in both obesity and T2D (Li *et al.* 2006, Jin *et al.* 2008), suggesting that in these metabolic disorders, the pancreatic β -cell may be exposed to elevated visfatin levels. As the pancreatic β -cell is known to express the insulin receptor (Muller *et al.* 2006) and the insulin receptor is known to have an essential role in regulating β -cell function (Otani *et al.* 2004), any compound such as visfatin which may interact with insulin signalling could potentially elicit significant regulatory effects.

Interestingly, previous studies have given conflicting results regarding a role for visfatin in the β -cell with visfatin being reported to be not only a positive regulator of pancreatic β -cell function (Revollo *et al.* 2007) but also a negative one (Lopez-Bermejo *et al.* 2006), suggesting that the role of visfatin in diabetes is yet to be clearly elucidated.

This study therefore aimed to clarify the actions of visfatin in the pancreatic β -cell by investigating not only its effects on insulin secretion and insulin receptor phosphorylation, but also its regulation of the expression of a panel of diabetes-related genes in mouse pancreatic β -cells.

Materials and methods

Cell culture and treatment

All materials were purchased from Sigma unless otherwise stated.

β -TC6 cell line (BTC) cells were purchased from ATCC (LGC Promochem, Teddington, UK). Cells between passages 30–38 were cultured in standard 11 mmol/l glucose RPMI-1640 (supplemented with 2 mmol/l L-glutamine, 50 IU/ml penicillin and 50 μ g/ml streptomycin, and 10% foetal bovine serum) prior to treatment.

For experimental treatments, cells were seeded into 6-well plates (gene and protein expression) or in 96-well plates (for insulin secretion, extracellular signal-regulated kinase (ERK)1/2 activation and cell viability assays). Cells were serum starved for 4 h and were subsequently incubated with serum-free RPMI containing either recombinant visfatin (Axxora, Nottingham, UK) NMN, U0126 (Promega) or FK866 (AxonChem Groningen, Netherlands) for appropriate time courses.

PCR array analysis and real-time PCR

For gene expression experiments, total RNA was extracted using SV Total RNA Isolation kit (Promega). RNA was DNase treated and 1 μ g total RNA was reverse transcribed using a first-strand cDNA synthesis kit (PrimerDesign, Southampton, UK). Ninety-eight microlitres of the resulting cDNA were mixed with 1225 μ l of SYBR Green PCR master mix (PrimerDesign) and 1127 μ l of PCR water (Ambion, Warrington, UK) and were aliquoted out into a commercially available diabetes pathway-specific real-time PCR array (Tebu-Biosciences, Peterborough, UK). Samples were run as duplicates for 40 cycles using an iCycler thermal cycler (Bio-Rad). Cycle thresholds were measured, and the relative expression of genes was calculated by comparison of C_t values. The mean for all

samples was calculated and subsequently normalized to the housekeeping genes β -glucuronidase, hypoxanthine phosphoribosyltransferase 1, heat shock 90 kDa protein 1 β , β -actin and glyceraldehydes-3-phosphate dehydrogenase. Melt-curve analysis was used to confirm single amplicon production for each gene tested. Genomic contamination was assessed by non-reverse transcribed samples (RT-ve). Pre-validated SYBR Green PCR primers (PrimerDesign) were used to confirm PCR array results in a separate set of experiments (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Insulin secretion

Cells were treated with a dose range (0–200 ng/ml) of visfatin for 1 h. Supernatants were collected and centrifuged to remove any cells, and insulin content was measured using a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden).

Insulin receptor activation

To measure the effect of visfatin on insulin receptor activation, sandwich ELISA using phospho-specific anti-insulin receptor antibodies was employed (R&D, Abingdon, UK). Phospho-specific western blotting was used to qualitatively confirm the results. Cells were cultured as described above and were treated with visfatin in a concentration-dependent (0–200 ng/ml) and time-dependent (0, 2, 5, 15, 30 and 60 min) manner. Cell lysates were collected using RIPA buffer containing phosphatase and protease inhibitors for assay. Insulin treatment of 100 nmol/l was used as a positive control for insulin receptor activation.

ERK1/2 activation assay

To study the activation of intracellular p42–44 ERK signalling, a proprietary phospho-ERK1/2 ELISA was used (RayBiotech, Norcross, GA, USA). Cells were plated out in 96-well plates and treated over a time period (0, 2, 5, 15, 30 and 60 min) with 200 ng/ml visfatin before being fixed and assayed. Five-minute phorbol myristate acetate (PMA) treatment was used as a positive control for ERK1/2 activation, $n=5$.

Statistical analysis

Statistical analysis for PCR array was undertaken using a Mann–Whitney U test. For insulin secretion, ERK1/2 activation and cell viability experiments, ANOVA followed by Tukey's *post-hoc* test was used; $P \leq 0.05$ was taken as a point of significance.

Table 1 Visfatin regulation of β -cell mRNA expression

Gene	Fold change	<i>P</i> value
	Compared to control	
Angiotensin-converting enzyme	−3·732	0·019
ATP citrate lyase	2·701	0·471
Adrenergic receptor 1a	4·925	0·518
Adrenergic receptor 3b	1·231	0·334
Angiotensinogen	1·823	0·845
V-Akt murine thymoma viral oncogene homolog 2	24·251	0·002
Aquaporin 2	1·260	0·338
Chemokine ligand 5	7·294	0·052
Chemokine receptor 2	3·175	0·330
CD28	97·006	0·009
CeaCam1	−2·828	0·027
CCAAT/enhancer-binding protein (C/EBP), α	4·387	0·407
Cytotoxic T-lymphocyte-associated protein 4	8·574	0·167
Dipeptidylpeptidase 4	1·097	0·075
Dual specificity phosphatase 4	3·647	0·419
Ectonucleotide pyrophosphatase/phosphodiesterase 1	1·516	0·663
Fructose-1,6-bisphosphatase 1	−2·000	0·158
Forkhead box C2	1·741	0·831
Forkhead box G1	1·414	0·801
Forkhead box P3	−1·289	0·087
Glucose-6-phosphatase, catalytic subunit	4·925	0·075
Glucose-6-phosphate dehydrogenase 2	1·910	0·775
Glucagon	1·782	0·652
Glucagon receptor	2·297	0·873
Glucose transporter type 4	2·701	0·624
Glucagon-like peptide 1 receptor	1·072	0·284
Glycerol-3-phosphate dehydrogenase 1 (soluble)	2·962	0·172
Glycogen synthase kinase 3 β	−1·447	0·266
Heme oxygenase (decycling) 1	3·647	0·202
HNF β 1	32·000	0·011
HNF4 α	16·000	0·020
Intercellular adhesion molecule 1	−1·260	0·363
Insulin-degrading enzyme	1·097	0·396
Interferon γ	2·764	0·586
IGF-binding protein 5	2·764	0·844
Inhibitor of nuclear factor κ -B kinase β subunit	1·516	0·727
IL10	3·127	0·482
IL12b	4·812	0·664
IL4 receptor, α	12·409	0·103
IL6	2·351	0·692
Inositol polyphosphate phosphatase-like 1	2·462	0·371
Insulin	8·980	0·037
Insulin receptor substrate 1	4·287	0·320
Mitogen-activated protein kinase 14	2·639	0·771
Mitogen-activated protein kinase 8	−1·122	0·039
Neurogenic differentiation 1	1·122	0·118
Nuclear factor κ -B DNA binding subunit	40·317	0·019
Nitric oxide synthase 3	3·980	0·360
Nuclear respiratory factor 1	1·587	0·391
<i>N</i> -ethylmaleimide-sensitive factor	2·245	0·901
Pancreatic and duodenal homeobox 1	2·047	0·922
Poly (ADP-ribose) polymerase family member 1	1·176	0·169
Paired box gene 4	16·000	0·114
Phosphoenolpyruvate carboxykinase 1	3·482	0·108
6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase	3·482	0·451
Phosphatidylinositol-3-kinase catalytic δ polypeptide	2·095	0·967
Phosphoinositide-3-kinase, regulatory subunit 1 (α)	2·297	0·947
PPAR α	−1·260	0·043

(continued)

Table 1 Continued

Gene	Fold change	P value
	Compared to control	
PPAR γ	2.895	0.762
PPAR γ , coactivator 1 α	5.528	0.404
Protein tyrosine phosphatase, non-receptor type 1	2.297	0.904
Phosphorylase, glycogen, liver	2.000	0.777
RAB4A, member RAS oncogene family	1.176	0.284
Resistin	3.564	0.499
Selectin	15.277	0.119
Serine (or cysteine) peptidase inhibitor, clade E, member 1	1.289	0.553
Solute carrier family 14 (urea transporter), member 2	2.351	0.874
Synaptosome-associated protein, 23 kDa	−1.047	0.417
Synaptosome-associated protein, 25 kDa	−2.144	0.128
Superoxide dismutase 2	1.414	0.692
Sterol regulatory element binding transcription factor 1	1.721	0.813
Syntaxin 4	17.549	0.035
Syntaxin-binding protein 1	−11.055	0.319
Syntaxin-binding protein 4	2.962	0.405
Transforming growth factor, β 1	54.443	0.155
TNF	2.351	0.891
TNF receptor superfamily, member 1a	1.662	0.737
TNF receptor superfamily, member 1b	2.406	0.861
Tribbles homolog 3	2.351	0.953
Uncoupling protein 2	−1.289	0.005
VAMP 2	1.289	0.331
VAMP 3	−6.350	0.307
VAMP-associated protein A	−30.555	0.254
Vascular endothelial growth factor A	−4.702	0.390

SYBR Green real-time PCR array analysis of diabetes-related genes. Treatment with 50 ng/ml visfatin resulted in a significant up- or down-regulation of 12 genes compared to untreated cells. Data are representative of three independent experiments. HNF, hepatocyte nuclear factor; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; TNF, tumour necrosis factor; VAMP, vesicle-associated membrane protein. Figures in bold represent statistically significant finding.

Results

Visfatin regulates mRNA expression of key diabetes genes in BTC cells

Real-time SYBR Green PCR array analysis of a diabetes-specific gene array was performed. Data analysis demonstrated that 24-h treatment of BTC cells with 50 ng/ml recombinant visfatin induced significant changes in expression (ranging from $P=0.0031$ to $P=0.0434$) compared to untreated cells in 12 of the 84 genes tested (Table 1).

Visfatin but not NMN increases insulin secretion at low glucose

Visfatin has previously been suggested to have a role in glucose-induced insulin secretion. We therefore investigated the effect of a static 1-h incubation of BTC cells with a range of visfatin concentrations at low and high glucose levels. Incubation with 200 ng/ml visfatin for 1 h at 2.2 mmol/l (low) glucose caused a significant 46% increase in insulin secretion compared to control

($P<0.001$; Fig. 1). Lower concentrations of visfatin (0–100 ng/ml) caused no significant change in insulin secretion. Co-incubation of visfatin with the specific NAMPT inhibitor FK866 significantly blocked the insulinotropic effect of visfatin ($P<0.05$) compared to 200 ng/ml visfatin alone. No effect of visfatin on insulin secretion at high glucose was seen (data not shown). To investigate the involvement of ERK1/2 mitogen-activated protein kinase (MAPK) in visfatin-induced insulin secretion, cells were co-incubated with 200 ng/ml visfatin, and the ERK1/2 inhibitor U0126 and insulin secretion was measured. Co-incubation of cells with 200 ng/ml visfatin and 1 μ mol/l U0126 caused no significant difference in insulinotropic effect compared with the treatment with visfatin alone. Incubation of cells with NMN alone resulted in a small non-significant increase in the levels of insulin secretion in the BTC cells ($P=0.1$).

Incubation with visfatin induces insulin receptor phosphorylation

Visfatin has previously been described as having the ability to activate the insulin receptor (Chan *et al.* 2007).

Figure 1 (A) Insulin secretion measured over 1-h static incubation. Treatment of BTC with visfatin (200 ng/ml) caused a significant increase in insulin secretion at low glucose compared to untreated cells ($P < 0.01$). Co-incubation with FK866 significantly blocked this increase in insulin secretion ($P < 0.01$). U0126 co-incubation did not significantly alter the effects of 200 ng/ml visfatin on BTC insulin secretion compared to visfatin alone, $n = 6$. (B) Incubation with 10 and 100 μ M NMN caused a small but non-significant increase in insulin secretion at low glucose, $n = 5$.

We therefore investigated the activation of the insulin receptor in BTC by visfatin. Insulin receptor activation was assessed by phospho-specific insulin receptor sandwich ELISA. Using 50, 200 and 500 ng/ml visfatin, significant increases in the level of insulin receptor phosphorylation were seen after 15 min ($P < 0.001$), with increases being greater at 200 and 500 ng/ml than at 50 ng/ml. This phosphorylation decreased over the subsequent 45 min. NMN alone also caused a significant increase in insulin receptor phosphorylation at the same time point ($P < 0.05$). Co-incubation of cells with 200 ng/ml visfatin and the specific visfatin inhibitor FK866 caused a partial reduction in the level of insulin receptor phosphorylation compared to cells treated with 200 ng/ml visfatin alone (Fig. 2).

Visfatin and NMN cause activation of ERK1/2 but not of p38 MAPK

Visfatin has previously been shown to cause activation of the ERK1/2 and p38 MAPKs (Chen *et al.* 2006, Adya *et al.* 2008a,b). We therefore investigated the activation of these MAPKs by both visfatin and NMN. Treatment of cells with 200 ng/ml visfatin caused a significant 122%

up-regulation in ERK1/2 phosphorylation after 15 min ($P < 0.001$; Fig. 3). This result was mirrored by a similar 15-min increase in ERK1/2 phosphorylation of 105% by NMN ($P < 0.001$; Fig. 3). Co-incubation with the specific visfatin inhibitor FK866 significantly reduced ERK1/2 activation by visfatin compared to visfatin alone ($P < 0.05$; Fig. 3). Neither visfatin nor NMN caused any significant change in p38 activation compared to basal (Fig. 3). PMA positive control showed an expected significant increase in both p38 and ERK1/2 MAPK activation.

Discussion

Despite significant interest and controversy over the effects of visfatin and insulin receptor signalling, there have been relatively little data published which have suggested precisely what the biological functions of visfatin are, and more importantly, how these functions might fit into the models of metabolic disorder. Here, we can report for the first time that visfatin is able to regulate the mRNA expression of a variety of genes which are key to maintaining normal pancreatic β -cell function. We found that 12 of the 84 genes tested were significantly up- or down-regulated by treatment with 50 ng/ml visfatin (a concentration only slightly higher than that reported in the circulation of obese/diabetic subjects (Chen *et al.* 2006); Table 1), including insulin and syntaxin 4 (a protein known to facilitate glucose-stimulated insulin secretion (Spurlin & Thurmond 2005)). We have subsequently confirmed visfatin regulation of mRNA expression of several of these genes using a separate real-time PCR using pre-validated primers (Supplementary Figure). Previously published reports have highlighted the importance of these genes in β -cell function, particularly hepatocyte nuclear factor (HNF)4 α and HNF1 β (Maestro *et al.* 2007), two genes which form single gene defects in forms of maturity onset diabetes of the young (MODY1 and MODY5 respectively). The marked up-regulation of these important transcription factors fits well with the observation here that visfatin causes an increase in insulin secretion (Fig. 2), as they are both thought to be involved in insulin gene transcription (Bernardo *et al.* 2008).

Significant increases were also observed in some unexpected genes, including CD28 (a 97-fold increase). CD28 is more commonly associated with type 1 diabetes and immune responses, and any potential role in T2D is still unclear. The increase we report in nuclear factor- κ B (NF- κ B) fits well with previously published data regarding visfatin and NF- κ B activity in endothelial cells (Adya *et al.* 2008a,b). Visfatin has previously been suggested to have a role in the regulation of insulin secretion, where NMN was shown to be able to rescue

Figure 2 Phospho-specific analysis of insulin receptor activation. (A) Visfatin (50 and 500 ng/ml) caused significant 104% ($P < 0.001$) and 178% ($P < 0.001$) increases in insulin receptor phosphorylation respectively. This was confirmed by phospho-specific western blotting (data not shown). $I = 100$ nM insulin-positive control, $n = 4$. (B) Time-dependent analysis of insulin receptor phosphorylation (using 200 ng/ml visfatin) showed that a significant ($P < 0.001$) activation of the insulin receptor compared to basal occurred after 15 min, and remained significantly higher than basal for 60 min ($P < 0.05$). (C) Incubation with 100 μ M NMN also caused a significant increase in insulin receptor phosphorylation ($P < 0.01$) after 15 min in these cells, $n = 4$. (D) During co-incubation of visfatin (200 ng/ml) and 10 nM FK866, significantly less insulin receptor phosphorylation was seen than that seen with visfatin treatment alone ($P < 0.05$). (E) Western blot images for phospho-insulin receptor (p-IR) and total insulin receptor (t-IR) in cells treated with 200 ng/ml visfatin and 100 nM insulin as positive controls. Images are representative of four independent experiments.

insulin secretion in islets isolated from visfatin knock-out mice (Revollo *et al.* 2007). This report, however, focussed on the intracellular actions of visfatin, as opposed to administered visfatin. Our observations suggest that visfatin therefore has an important role as both an intra- and an extracellular regulator of pancreatic β -cell function.

Interestingly, the significant increase in insulin secretion from BTC cells seen here was blocked by co-incubation with the specific NAMPT inhibitor FK866 (Fig. 1). This might initially suggest that the mechanism by which visfatin is regulating insulin secretion is through its ability to synthesize NMN (as FK866 non-competitively binds the enzymatic region of visfatin

which normally binds nicotinamide; Kim *et al.* 2006). However, NMN itself did not significantly increase insulin secretion in the BTC cells, suggesting a more complex mechanism for this action of visfatin. It is possible that FK866 not only blocks the ability of visfatin to bind nicotinamide, but also blocks other as yet undescribed interactions, possibly those involving the insulin receptor. Clearly, further research is needed to fully assess the physiological relevance of these observations.

Curiously, we found that at a supraphysiological concentration (500 ng/ml) of visfatin caused a decrease in cell viability over 24 h (data not shown). Although most reports of the circulating levels of

Figure 3 Analysis of MAPK activation by visfatin. The effect of visfatin/NMN on MAPK activation was measured using a phospho-specific ERK1/2 cell-based assay. (A) Visfatin (200 ng/ml) treatment resulted in a significant 130% up-regulation of ERK1/2 phosphorylation after 15 min compared to basal ($P < 0.001$). (D) Co-incubation with 10 nM FK866 significantly inhibited this ERK1/2 activation ($P < 0.01$). (B) No significant effect of 200 ng/ml visfatin was seen on p38 activation compared to basal. (C) Incubation of BTC with 100 μ M NMN also caused a significant increase of 105% after 15 min ($P < 0.001$).

visfatin are well below this concentration, some have suggested far higher circulating concentrations of visfatin, potentially placing this within the physiological range (Chan *et al.* 2007), which suggests that visfatin may have a more complex role in the pancreas than our data suggest. One previous report has linked β -cell dysfunction to elevated serum visfatin levels (Lopez-Bermejo *et al.* 2006), and the potential exists that although a short-term increase in insulin secretion is seen, longer term effects of exposure to elevated visfatin might be more deleterious to the β -cell.

The role of visfatin in insulin receptor signalling is a controversial one. We report for the first time that in the pancreatic β -cell, visfatin has a definite role in insulin receptor signalling, with a significant up-regulation of insulin receptor phosphorylation occurring when BTC cells are exposed to a range of visfatin concentrations. This matches previous reports of an interaction of visfatin with the insulin signalling pathway in at least one other tissue (Xie *et al.* 2007). As insulin signalling has been shown to be essential for β -cell function (Otani *et al.* 2004), this confirms the importance of this role for visfatin in the pancreas, a role which exists potentially via multiple mechanisms (both intra- and extracellular) involving both insulin secretion and signalling.

In conclusion, these data suggest that visfatin has a role in multiple aspects of pancreatic β -cell biology, including a role in the regulation of insulin secretion and receptor signalling. These findings confirm the previously reported modulatory role of visfatin in the insulin signalling pathway, and suggest that these actions occur via its ability to synthesize NMN.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JME-09-0071>.

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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