Dysregulation of Hnf1b Gene Expression in Cultured Beta-Cells in Response to Cytotoxic Fatty Acid

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ABSTRACT

Context Increased levels of circulating fatty acids deriving from over-nutrition are thought to contribute to the progressive beta-cell failure associated with type 2 diabetes. Pancreatic beta-cells in culture are sensitive to exposure to long-chain saturated fatty acids (e.g. palmitate) which cause cytotoxicity, whereas the monounsaturated equivalents (e.g. palmitoleate) are cytoprotective.

Objectives In this study we sought to determine whether of members of the hepatocyte nuclear factor (HNF) family of transcription factors, which are mutated in familial, young-onset, monogenic beta-cell diabetes, could play a role in fatty acid-mediated cytotoxicity in cultured beta-cells. Design We used real-time PCR to determine whether hepatocyte nuclear factor gene expression was altered in response to palmitate exposure in the BRIN-BD11 beta-cell line. Results We found that the Hnf isoforms expressed in BRIN-BD11 cells are dysregulated by palmitate exposure. The expression of Hnf1b is specifically reduced by exposure to palmitate, and this response is prevented by co-incubation with palmitoleate. Conclusions Down-regulation of Hnf1b gene expression accompanies palmitate-mediated cytotoxicity in cultured beta-cells.

INTRODUCTION

The rising incidence of type 2 diabetes correlates with spiralling levels of obesity [1] and it is thought that increased circulating free fatty acids, associated with over-nutrition and adiposity, may contribute to the progression of type 2 diabetes [2, 3]. Type 2 diabetes occurs when pancreatic beta-cells are unable to secrete sufficient insulin to compensate for peripheral insulin resistance, most likely due to a combination of beta-cell dysfunction and decreased beta-cell mass [4]. After the onset of type 2 diabetes, the combined effects of increasing hyperglycaemia and hyperlipidaemia contribute to a further deterioration in beta-cell function and mass [5].

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Key words Hepatocyte Nuclear Factor 1-beta; Palmitic Acid; palmitoleic acid

Abbreviations HNF: hepatocyte nuclear factor

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in lipid metabolism [13] and has recently been shown to bind endogenous fatty acid in vivo, although the ligand does not appear to enhance transcriptional activity [14].

Haploinsufficiency for HNF4A (the gene which encodes the HNF4alpha protein) causes a rare form of monogenic beta-cell diabetes with autosomal dominant inheritance, early age of onset, impaired insulin secretion but detectable C-peptide (formerly known as maturity onset diabetes of the young or MODY) [15]. Familial, young-onset, monogenic beta-cell diabetes is characterised by progressive beta-cell dysfunction and is also caused by heterozygous mutations in two other HNF homeobox 1 transcription factors, HNF1A and HNF1B. These transcription factors interact in a pancreatic islet-specific network and thus changes in the expression of one can have an impact on other members of the network [16]. One of the key interactions is a cross-regulatory relationship between HNF1alpha and HNF4alpha, apparently unique to the pancreas and perhaps best reflected in the overlapping beta-cell phenotype of HNF1A and HNF4A monogenic beta-cell diabetes [16]. Pancreatic beta-cells appear to be particularly sensitive to dosage perturbations since monogenic beta-cell diabetes is the primary clinical phenotype associated with both HNF1A and HNF4A heterozygous mutations despite their expression in other tissues [17].

In this study we sought to investigate whether the regulation of any of these transcription factors was altered in response to exposure to cytotoxic fatty acids using a well-characterised rodent beta-cell system.

METHODS

Treatment of Cells with Fatty Acids

The rat pancreatic beta-cell lines BRIN-BD11 [18] and INS-1 [19] were cultured under standard conditions in RPMI-1640 medium containing 11 mM glucose [20]. Six-well plates were seeded with 10^5 cells/well 24 h prior to treatment. Palmitate (Sigma-Aldrich, Gillingham, United Kingdom) was dissolved in 50% ethanol at 70°C and palmitoleate (MP Biomedicals, Cambridge, United Kingdom) dissolved in 90% ethanol. Fatty acid was bound to 10% fatty acid-free bovine serum albumin (BSA) (MP Biomedicals, Cambridge, United Kingdom) by incubation for 1 h at 37°C prior to addition to cells. During treatment all cells were cultured in the absence of foetal bovine serum (FBS) in 1% BSA and 0.5% ethanol. BRIN-BD11 cells were treated with fatty acid/BSA complexes for 18 h and INS-1 cells for 48 h. For serum starvation, BRIN-BD11 cells were grown in supplemented RPMI-1640 lacking FBS, in 1% BSA and 0.5% ethanol for 48 h. Each condition had three replicates and BRIN-BD11 cells were treated with palmitate in five independent experiments. Cell death was estimated by microscopy and was consistent with previous results [11, 21]. BRIN-BD11 cells under control conditions underwent 10-20% cell death, 0.1 mM palmitate with 40-60% cell death and 0.25 mM palmitoleate with 70-90% cell death. Palmitoleate (0.25 mM) reduced cell death to below control levels and laurate (0.25 mM) did not influence cell viability.

RNA Extraction and Reverse Transcription

RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, United Kingdom). cDNA was synthesised from total RNA using random hexamers (Qiagen, Crawley, United Kingdom). cDNA was normalised to beta-2 microglobulin (B2m) [22]. The gene expression assay for the endogenous control gene beta-2 microglobulin (B2m) is commercially available (Rn00560865_m1, Assays-on-Demand, Applied Biosystems, Warrington, United Kingdom). Quantitative PCR reactions were performed in triplicate on the ABI Prism 7900HT platform using TaqMan Fast Universal PCR Master Mix (no AMPerase) (Applied Biosystems, Warrington, United Kingdom). The relative expression of each isoform under various conditions was determined by normalisation to B2m and comparison to the average of the three replicate controls within an experiment using the 2^(-ΔΔCt) method [23].

STATISTICS

Statistical significance was assumed for two-tailed P values less than 0.05 and was determined using the non-parametric Mann Whitney-U test in SPSS (v15.0, Chicago, IL, USA).

RESULTS

Expression Profile of HNF Transcription Factor Isoforms in the Rodent Beta-Cell Line BRIN-BD11

BRIN-BD11 cells were used in this study as their viability in response to saturated and monounsaturated fatty acids is similar to that seen in isolated human islets [10, 24]. Gene expression profiling using a series of TaqMan gene expression assays [22] designed to detect individual isoforms of Hnf1a (a, b, c), Hnf1b (a, b, c) and Hnf4a (7, 8, 9) was performed on BRIN-BD11 cells. Hnf1a(a) was abundant and Hnf1b(a) and Hnf1b(b) were also detected robustly (data not shown). The isoform expression profiles for Hnf1a and Hnf1b were consistent with data from rodent tissues [22]. All other isoforms were expressed at very low levels. Palmitate (0.25 mM) exposure was associated with high levels of cell death and the resulting yields of RNA were too low to generate reliable data for the remaining transcription factor isoforms.

Hnf1a Expression is Up-Regulated in Response to Saturated Fatty Acid Exposure

In BRIN-BD11 cells the expression of Hnf1a(a) increased significantly in response to palmitate, with a 39% increase in expression in the presence of 0.1 mM
Palmitate (P=0.001) and a 59% increase in the presence of 0.25 mM palmitate (P=0.013) (Figure 1). This increased expression was suppressed when cells were incubated with 0.25 mM palmitate in the presence of 0.25 mM palmitoleate (Figure 1). Incubation in the presence of 0.25 mM palmitoleate alone did not influence the expression of Hnf1a (Figure 1). The dose-dependent increase in expression of Hnf1a(a) in BRIN-BD11 cells exposed to palmitate had potentially important implications and we therefore investigated it further. First it was critical to establish whether this effect was a general response to cell death and, therefore, expression of Hnf1a(a) was measured in response to a different pro-apoptotic stimulus; serum starvation. Withdrawal of serum from the culture medium led to the loss of viability of BRIN-BD11 cells in the presence of 1% BSA and 0.5% ethanol and the magnitude of this response reached comparable levels within 48 h (50-60% cell death) to that seen during palmitate exposure. Under these conditions Hnf1a(a) expression was not increased compared to the 18 h control (Figure 1). This implies that the increase in Hnf1a(a) expression was a specific response to saturated fatty acid exposure. We therefore tested the response to a shorter chain saturated fatty acid, laurate (C12:0), which is not cytotoxic. In this case a small (non-significant) dose-dependent increase in Hnf1a(a) expression was observed compared to control conditions (Figure 1), and it did not differ significantly from the response to palmitate. Interestingly, we found that Hnf1a(a) expression was unchanged in a second rat pancreatic beta-cell line, INS-1, following palmitate exposure (Figure 1).

**Hnf1b Isoforms Show a Specific Response to Cytotoxic Saturated Fatty Acid**

We analysed the expression of Hnf1b isoforms in BRIN-BD11 cells in response to fatty acid exposure. Two gene expression assays were used to detect all three Hnf1b isoforms. Hnf1b(a) detects isoforms a and c but since isoform c accounts for less than 10% of the transcripts under all conditions tested (data not shown) it has been simplified to Hnf1b(a). Again, it was found that Hnf1b expression showed a dose-dependent change in response to palmitate exposure but, in this case, the direction of change was opposite to that seen for Hnf1a (Figure 2). Hnf1b(a) showed an 18% and Hnf1b(b) a 19% decrease in expression in response to 0.1 mM palmitate and the magnitude of these decreases was increased further when a higher concentration (0.25 mM) was employed. Under these conditions, Hnf1b(a) decreased by 33% and Hnf1b(b) by 28%. As was the case for Hnf1a(a), incubating cells with 0.25 mM palmitate in the presence of 0.25 mM palmitoleate prevented the response, while 0.25 mM palmitoleate alone did not have any effect on the expression of Hnf1b (Figure 2). Again we were able to show that the response was specific to fatty acid exposure and did not appear to represent a general pro-apoptotic response. Under conditions of serum starvation (48 h) the expression of all Hnf1b isoforms was comparable to that of the 18 h control and they differed significantly from the levels achieved with 0.25 mM palmitate (P<0.018). Exposure to laurate did not significantly alter Hnf1b expression. The INS-1 cell line used in this study did not express detectable levels of any of the

**Figure 1. Hnf1a expression is upregulated in response to saturated fatty acids.** The results of quantitative PCR (qPCR) analysis of Hnf1a(a) expression in cultured rat beta-cells exposed to a variety of stimuli. BRIN-BD11 cells were treated with palmitate or laurate for 18 h or were serum starved (-serum) for 48 h. INS-1 cells were exposed to palmitate for 48 h. The medians and interquartile ranges are plotted (n=6-15 times in each experiment). P values were derived using the Mann Whitney-U test. Values which differ significantly from the BRIN-BD11 palmitate control (BSA/ethanol only) are indicated. BSA: bovine serum albumin; FA: saturated fatty acid; PO: palmitoleate.

**Figure 2. Hnf1b expression is downregulated in response to cytotoxic fatty acid.** Hnf1b isoform expression was analysed in BRIN-BD11 cells exposed to fatty acids for 18 h or serum starved (-serum) for 48 h. The medians and interquartile ranges are plotted (n=6-15 times in each experiment). P values were derived using the Mann Whitney-U test. Values which differ significantly from the BRIN-BD11 palmitate control (BSA/ethanol only) are indicated. BSA: bovine serum albumin; FA: saturated fatty acid; PO: palmitoleate.
Hnf1b isoforms under the conditions employed. Since the changes in expression of Hnf1b(a) and Hnf1b(b) are reciprocal, this implies that they arise from a specific transcriptional response elicited by palmitate, rather than from a process controlling post-transcriptional processing.

DISCUSSION

We have shown that expression of the predominant Hnf isoforms present in BRIN-BD11 cells is altered in response to chronic exposure to the saturated fatty acid palmitate. As these transcription factors act within a complex cross-regulatory circuit [16], this has general implications for the network.

With respect to palmitate-mediated beta-cell toxicity, the most significant response detected was a decrease in expression of Hnf1b. Palmitate exposure resulted in a decrease in expression of all Hnf1b isoforms, a response which was not elicited by a non-cytotoxic saturated fatty acid (laurate) or by an alternative cytotoxic mechanism (serum starvation). In addition, and perhaps most significantly, Hnf1b expression was not down-regulated when palmitate was co-incubated with the cytoprotective monounsaturated fatty acid, palmitoleate. A similar suppression of the molecular response to palmitate by co-incubation with palmitoleate is also seen for components of the endoplasmic reticulum stress response [25], a pathway thought to be involved in palmitate-mediated cytotoxicity [26, 27]. HNF1B is highly dosage-sensitive. A decrease in gene dosage is clearly detrimental to beta-cell function as haploinsufficiency for HNF1B is sufficient to cause monogenic beta-cell diabetes [28] and, in a conditional mouse model, incomplete deletion is sufficient to impair glucose tolerance [29]. In addition, over-expression of HNF1beta is associated with reduced viability in INS-1 beta-cells [30]. The HNF1beta protein is not readily detected in mature pancreatic beta-cells [31], making it difficult to determine whether the reduction in gene expression observed here was reflected by a change in the protein level of HNF1beta. Perhaps the most meaningful way to determine the effects of reduced Hnf1b expression will be to examine the effect of acute exposure to palmitate on downstream targets of HNF1beta. Conditional deletion of Hnf1b in mouse beta-cells leads to a decrease in Hnf4a expression suggesting that Hnf1b may be required for Hnf4a expression in murine islets [29]. We were unable to determine whether the same effect was seen in BRIN-BD11 cells in response to palmitate as Hnf4a transcripts are expressed at very low levels. Hnf1a expression was increased during palmitate exposure. Unlike Hnf1b, this response does not appear to relate to cytotoxicity as there was also a trend towards increased expression in response to laurate. Hnf1a expression did not increase in response to palmitate in a second beta-cell line, INS-1. In a beta-cell-specific conditional gene knockout of Hnf1b, the expression levels of Hnf1a increased in response to loss of Hnf1b [29] and thus up-regulation of Hnf1a in BRIN-BD11 cells in response to palmitate could be a secondary effect of reduced Hnf1b expression. This could also partly account for the difference in the Hnf1a response between BRIN-BD11 and INS-1 cells, which express very low levels of Hnf1b. Palmitate is also toxic to INS-1 cells but toxicity does not develop until up to 48 h of exposure, compared to as little as 18 h for BRIN-BD11 cells. We conclude that induction of Hnf1a(a) expression is a specific response to exposure to palmitate in BRIN-BD11 cells but it does not relate to cytotoxicity.

In summary, we have shown that there are changes in the expression of the Hnf transcription factor isoforms expressed in BRIN-BD11 cells in response to palmitate exposure. Most significantly we observed a dose-dependent decrease in Hnf1b expression in response to palmitate exposure. This response is specific to palmitate-mediated cytotoxicity and is prevented by co-incubation with monounsaturated fatty acid. We also detected a dose-dependent increase in the expression of Hnf1a in response to saturated, but not monounsaturated, fatty acid exposure. We conclude that reduced Hnf1b gene expression could represent one contributory factor in palmitate-mediated cytotoxicity in cultured beta-cells.

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