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Impaired expression of transcription factor IUF1 in a pancreatic β-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis)

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Abstract Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), or nesidioblastosis, is a rare disorder which may be familial or sporadic, and which is characterized by unregulated secretion of insulin and profound hypoglycaemia in the neonate. The defect has been linked in some patients to mutations in the sulphonyl urea receptor gene (SUR). The present study investigated potential defects in the regulation of the insulin gene by glucose in a β-cell line (NES 2Y) derived from a patient with PHHI. The results show that the insulin promoter is unresponsive to glucose in PHHI, and that this defect can be attributed to impaired expression of the transcription factor IUF1. Because IUF1 is involved not only in linking glucose metabolism to the control of the insulin, but is also a major regulator of β-cell differentiation during embryogenesis, we propose that impaired expression of IUF1 contributes to β-cell dysfunction in PHHI by leading to abnormal β-cell differentiation.

Key words: Insulin gene; Transcription; Islet of Langerhans; Nesidioblastosis; Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI)

1. Introduction

Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), or nesidioblastosis, although a rare disorder characterized by unregulated secretion of insulin and profound hypoglycaemia in the neonatal period [1], is the most common cause of persistent hypoglycaemia in childhood. Newly born children with the disease can suffer severe brain damage if not diagnosed and treated immediately. Treatment in severe cases usually involves partial or even total pancreatectomy.

There is controversy over the aetiology of the disease [2]. The term nesidioblastosis was coined in recognition of the histological appearance of endocrine cells lying in duct epithelium with an apparent failure to aggregate into discrete islets of Langerhans [3]. However, severe hyperinsulinaemia can also occur in the presence of apparently normal islets, and doubt has been expressed over the concept that the condition is entirely due to a defect in β-cell differentiation [1]. The disease occurs either in families, particularly in the Middle East, or sporadically. In some patients the disease has been mapped recently to mutations in the sulphonyl urea receptor (SUR) gene [4]. The sulphonyl urea receptor is a component of the K_{ATP} channel that plays an important role in the mechanism whereby glucose metabolism in β cells is linked to insulin secretion [5]. Evidence linking the disease to abnormal K_{ATP} channel activity comes from the observation that cell lines derived from five patients with PHHI lacked such activity with a loss of glucose-insulin secretion coupling [6].

However, in addition to stimulating insulin secretion, glucose metabolism also regulates transcription of the insulin gene through modulation of the phosphorylation state and DNA binding activity of the homeodomain transcription factor IUF1 [7], which is also known as IPF1, IDX1, STF1 or PDX1 [8–10], and possibly other factors [11,12]. In the adult, expression of IUF1 is restricted to pancreatic β cells and somatostatin secreting cells of the duodenum [9]. It binds to the consensus sequence C(T/C)TAAG located at four sites (the A boxes at −77, −129, −210 and −313) in the human insulin gene promoter [13,14]. IUF1 (PDX1) also plays an important role in lineage determination in the developing pancreas [15,16].

Because PHHI may be associated with β-cell hyperplasia during fetal development [17], and since IUF1 is involved not only in linking glucose metabolism to the insulin gene but is also a major regulator of β-cell differentiation during embryogenesis, we hypothesised that defects in both these processes would explain some of the pathologies associated with PHHI. This study was undertaken to explore this hypothesis in a β-cell line derived from a patient with PHHI.

2. Materials and methods

2.1. Cell culture

The patient's pancreas had the appearance of diffuse nesidioblastosis and islets of Langerhans were isolated by a standard method using collagenase digestion, and plated out in non-tissue culture Petri dishes (Sterilin). Seven days later the islets were transferred to T25 culture flasks and allowed to acquire confluency. The cells were then harvested with trypsin/EDTA and plated out in Sterilin dishes again. After 7 days the cells were replated in T25 culture flasks and the cell line derived by continual subculture. Cultures were monitored for human insulin production to help derive an active population. The islets were plated out into 10 cm Nunc Petri dishes using Gibco RPMI 1640 medium containing 11 mM glucose, supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine. MIN6, a β-cell line derived from transgenic mice expressing the SV40 large T antigen under the control of the rat insulin promoter [18] were grown in DMEM containing 5mM glucose, supplemented with 15% heat inac-

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Abbreviations: PHHI, persistent hyperinsulinaemic hypoglycaemia of infancy; EMSA, electrophoretic mobility shift assay; TKp, thymidine kinase promoter

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tivated myoclone foetal calf serum (Sigma) and 2mM L-glutamine. MIN6 cells were used between passage 26 and 30 for all experiments.

2.2. Plasmids

The control construct pGL-LUC was based on the plasmid pGL2 (Promega), with the thymidine kinase promoter (TKp) from the herpes simplex virus cloned 5′ to the firefly luciferase gene. The construct pGL-LUC200 varies from this in that it contains a −50 to −250 base-pair fragment of the human insulin gene promoter cloned upstream of the TKp [19]. The IUF1 cDNA [19] was cloned into the pCR3 vector (Invitrogen) (to generate pCR3-IUF1) by virtue of the overhanging A's which are the natural result of PCR amplification with Taq DNA polymerase (Promega).

2.3. Transfections and luciferase assays

Transfection conditions and luciferase assays were as previously described [19].

2.4. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared and EMSAs performed as previously described [13].

2.5. Western blotting

For Western blot analysis, 5 μg samples of nuclear extract were fractionated by SDS-PAGE and blotted on to ECL-nitrocellulose membrane (Amersham) and incubated for 60 min in buffer containing 10 mM Tris-HCl, 0.05% (v/v) Tween 20, 0.5 M NaCl, and a 1:1000 dilution of anti-IUF1 antibody [19]. The antibody-antigen complex was then detected by incubating the membrane for a further 60 min in buffer containing a 1:5000 dilution of horse-radish peroxidase-conjugated anti-rabbit IgG secondary antibody (ECL, Amersham).

2.6. RT-PCR

Total RNA was isolated following lysis of cells in 4 M guanidinium isothiocyanate, 0.25 M sodium citrate, 5% (wt/vol) sodium sarcosyl, 0.2 M sodium acetate, pH 4.0. For cDNA synthesis, the RNA sample was heated at 65°C for 10 min, and the transcription reaction performed at 37°C for 60 min in a mixture containing the following: 1× reverse transcriptase reaction buffer (Promega, Southampton, UK), 20 units RNasin (Promega), 40 pmoles driving primer, 0.25 mM dATP, dCTP, dTTP, and dGTP, 25 mM dithiothreitol, 0.5 μg RNA, and 50 units AMV reverse transcriptase (Promega). For quantitative RT-PCR, 10%, 5%, 2.5%, 1%, 0.1% and 0.01% of the total cDNA was amplified with the primers described below, using a 35-cycle PCR with an annealing temperature of 60°C. Twenty percent of the PCR product was then run on an ethidium bromide-stained 1% agarose gel.

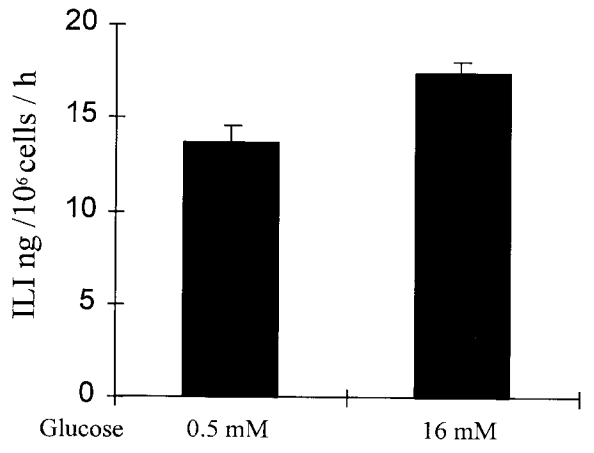

Fig. 1. Effect of glucose on insulin secretion from NES 2Y cells. NES 2Y cells were preincubated for 180 min in media containing 0.5 mM glucose. The media was then replaced with that containing the indicated amount of glucose and media samples assayed for insulin by radioimmunoassay after a further 60 min incubation. The data are representative of two experiments and show the mean ± standard deviation of six separate determinations.

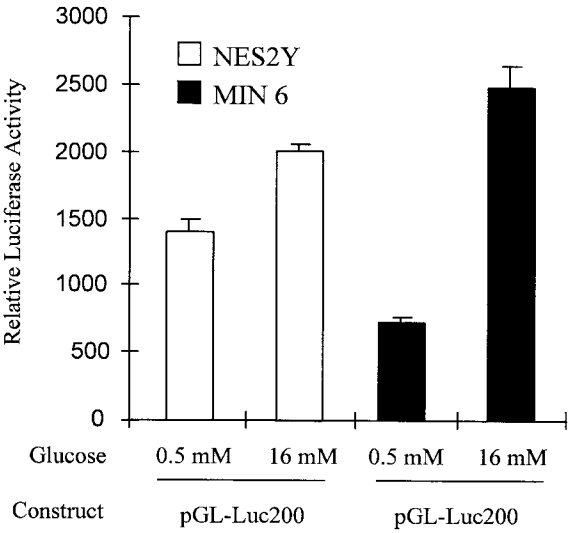

Fig. 2. Effect of glucose on pGL-LUC200 constructs in transfected MIN6 and NES 2Y cells. NES 2Y cells or Min6 cells were transfected with pGL-LUC200 constructs. After 24 h, cells were preincubated for 3 h in 0.5 mM glucose and then incubated in low (0.5 mM) or high (16 mM) glucose for 24 h. Values are shown as relative luciferase activity standardised against protein content. The data are representative of two separate experiments and show the mean ± standard deviation for four separate determinations.

2.7. Oligonucleotides

Oligonucleotides used in EMSAs for IUF1, IEF1 and USF have been described elsewhere [13,20]. The PCR primers used to quantify IUF1 mRNA corresponded to the mouse IPF1 sequences [8] 5′-ACCATGAATAGTGAGGAGCA-3′ and 5′-TCACCGGGGTTCCTGCGGTCGCAGTGGGATCGC-3′, respectively. Primers Ins1 and Ins2 used in quantification of insulin mRNA corresponded to the human insulin sequences 5′ GCGGGCTGCGTCTAGTTGCAGTAG-3′ and 5′ ATGGCCCTGTGGATGCGCCTCCTG-3′, respectively, and primers USF1 and USF2, used in quantification of USF mRNA corresponded to USF sequences 5′ ATGAAGGGGCAGCAGAAAACAGC-3′ and 5′-TTAGTTGCTGTCATTCTTGATGAC-3′, respectively.

2.8. Insulin measurements

Insulin was measured by radioimmunoassay using an anti-guinea pig insulin antibody and ^{125}I human insulin (Amersham).

3. Results

The NES2Y cells were derived from a patient (patient number 2 in [6]) who exhibited sporadic PHHI, with no parental consanguinity and no other affected siblings. The cell line was derived by continual subculture. In keeping with the characteristics of insulin secretion from PHHI islets in culture [1,21], secretion of insulin from the NES2Y cells was unresponsive to glucose stimulation (Fig. 1). Glucose-stimulated insulin transcription was also defective in the NES 2Y cells (Fig. 2). This was demonstrated by transfecting the plasmid pGL-LUC200 which contains a −50 to −250 fragment of the human insulin gene promoter into NES 2Y or MIN6 cells. In MIN6 cells the pGL-LUC200 construct gave a five-fold increase in activity in 16 mM glucose compared with 0.5 mM glucose. In NES 2Y cells there was no effect of glucose on the pGL-LUC200 construct. A control vector pGL-LUC which lacked the insulin reporter sequences was unaffected by glucose in both cell types (data not shown).

Recent studies have demonstrated that glucose activates

insulin gene transcription by modulating the phosphorylation state of the transcription factor IUF1 [7,19]. IUF1 binding was therefore examined by EMSA in NES 2Y and MIN6 cells. There was no detectable IUF1 binding activity in the NES 2Y cells (Fig. 3). Under similar conditions IUF1 binding activity could be detected in MIN6 cells. This was not due to differences in the quality of the nuclear extracts, since the activity of other transcription factors known to bind to the insulin promoter, i.e. IEF1 and USF, could be readily detected in both MIN6 and NES 2Y cells (Fig. 3). IUF1 protein could not be detected by Western blot analysis in NES 2Y cells (Fig. 4). The 46 kDa form of IUF1 was present in MIN6 cells, but as previously reported [7], it was absent from the non-insulin expressing cell lines α-TC and BHK. The high molecular weight protein (55 kDa) seen in tracks 2 and 3 is always observed in β-cell lines, but does not represent IUF1.

The NES 2Y cells were shown by reverse transcriptase polymerase chain reaction (RT/PCR) to have slightly elevated

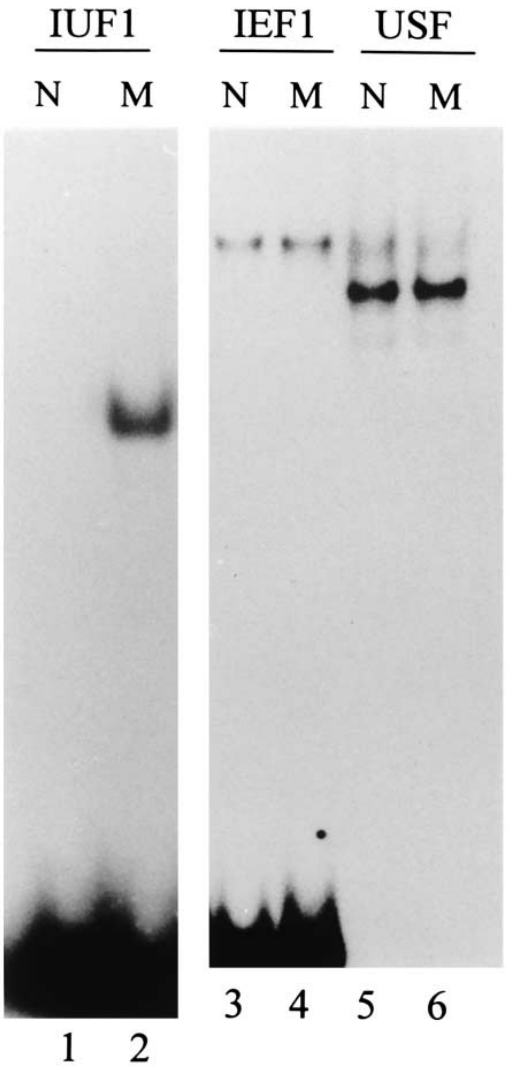

Fig. 3. EMSA analysis of transcription factor binding activity in NES 2Y (N) and MIN6(M) cells. NES 2Y and MIN6 cells were incubated for 1 h in 16 mM glucose and analysed by EMSA for the binding activities of IUF1 using (oligonucleotide B [13], lanes 1 and 2), USF (oligonucleotide USF [20] lanes 5 and 6), and IEF1 using oligonucleotide Jr1 [20], lanes 3 and 4.

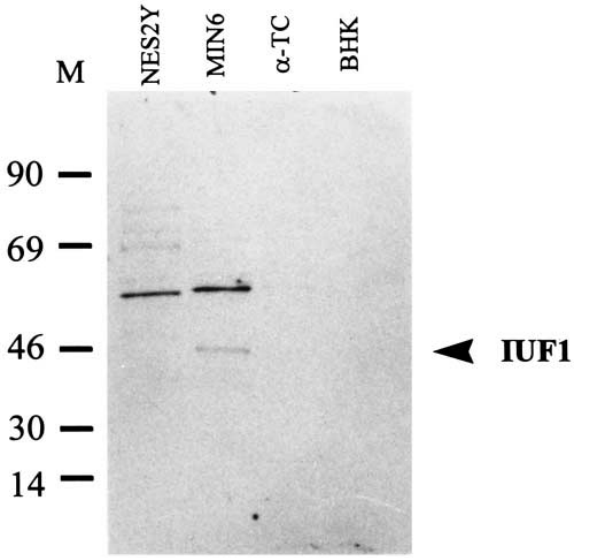

Fig. 4. Western blot analysis of IUF1 in NES 2Y, MIN6, αTC and BHK cells. The indicated cell lines were analysed for the IUF1 protein using a specific IUF1 antibody. The arrow indicates IUF1, which has a molecular weight of 46 kDa. The higher molecular weight band in tracks 2 and 3 is observed in all β cells, and does not represent IUF1. Lane 1 contains rainbow molecular size markers.

insulin mRNA levels compared to MIN6 cells, while USF mRNA levels were similar in both cell types (Fig. 5). However, in NES 2Y cells the IUF1 mRNA levels were more than 10-fold less than USF mRNA, and about 10^3-fold less than insulin mRNA. In MIN6 cells IUF1 and USF mRNA levels were similar, both being about 10^2-fold less than insulin mRNA levels. These results confirmed that the IUF1 gene was expressed in NES 2Y cells but at very low levels. The sequence of the IUF1 cDNA synthesised from NES 2Y mRNA was identical to the known sequence of human IUF1 [19] (data not shown).

To investigate whether the defect in glucose regulation of the insulin promoter in NES 2Y cells could be corrected by IUF1, a mammalian expression vector harbouring an IUF1 cDNA was co-transfected into NES 2Y cells along with the pGL-LUC and pGL-LUC200 constructs. IUF1 had no effect on the control vector (data not shown). In the absence of IUF1 glucose had no specific effect on the pGL-LUC200 vector, but in the presence of IUF1 glucose stimulated the pGL-LUC200 activity 5-fold (Fig. 6). Thus a full transcriptional response to glucose could be restored by transfecting the NES 2Y cells with IUF1.

4. Discussion

PHHI, or nesidioblastosis, is a complex condition, manifested in erratic secretion of insulin, and accompanied by a variety of histological appearances of the endocrine pancreas ranging from apparently normal islets of Langerhans, to one in which no recognisable islets are seen, the endocrine cells lying in duct epithelium. Hyperplasia of insulin cells with 'giant' nuclei has been considered pathogenic [1–3,22]. The observed effects on glucose-regulated insulin secretion may be attributed to loss-of-function mutations in the NBF-2 region of the sulphonyl urea receptor gene (SUR) [6]. However,

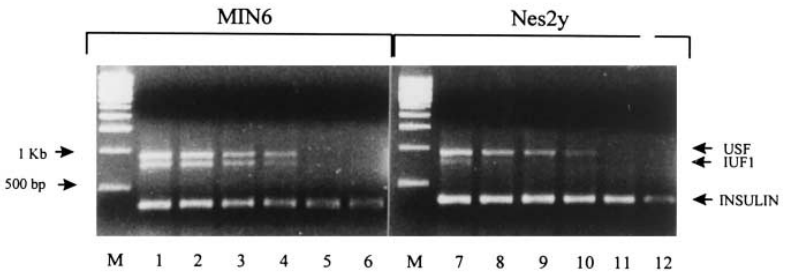

Fig. 5. Quantitative RT-PCR of IUF1, insulin and USF mRNA levels. Comparison of quantitative PCR with primers specific for IUF1 (NES 2Y, lanes 1–6; MIN6, lanes 7–12) insulin, and USF. Lanes 1–6 and 7–12 represent 10%, 5%, 2.5%, 1%, 0.1% and 0.01% of total cDNA as template, respectively. M indicates kilobase size markers. The amplified IUF1 fragment has an expected size of 850 base pairs, USF 900 base pairs, and insulin 350 base pairs.

these genetic defects may not account for all cases of PHHI, or for the full range of observed pancreatic abnormalities, and further candidate genes for this disorder must include those involved in islet cell differentiation, as well as those controlling insulin secretion. The present study has established that the insulin promoter is unresponsive to glucose in PHHI cells (NES 2Y) and that this can be attributed to reduced expression of the transcription factor IUF1. Overexpression of IUF1 fully restored glucose control of the promoter in these cells.

Recent progress has been made in understanding how glucose metabolism regulates IUF1 activity in adult human islets and MIN6 cells. Glucose, and possibly other nutrients, activate a novel stress-activated pathway involving the MAP kinase homologue, SAPK2 [23], leading to phosphorylation of IUF1 by an unknown IUF1 kinase [19]. Because of the low levels of IUF1 mRNA and undetectable levels of the protein, it is unlikely that the defect in the NES2Y cells is in the post-translational modification of IUF1. Rather, the data suggest impaired regulation of the IUF1 gene as a result, either of a functional mutation within the IUF1 enhancer/promoter, or a defect in a transcription factor(s) controlling the gene. Little is known about the regulation of the IUF1 gene [24,25]. There is, however, evidence that IUF1 gene expression can be modulated by glucose. Thus, when HIT T15 β cells were incubated in media containing elevated glucose levels for prolonged periods of time there was a concomitant decrease in IUF1 and insulin mRNA levels [26,27]. In the present study the decreased IUF1 mRNA levels were not associated with decreased insulin mRNA levels.

There are no available data on the regulation of IUF1 in normal human fetal islets. However, the role of IUF1 in islet ontogeny has been studied in mice. In this species the endocrine pancreas originates from endodermal cells in the gut of embryos of about 20 somites [28,29]. All four islet cell types (β, α, δ and PP) arise from a common pluripotent precursor. IUF1 is expressed at the earliest stages and is essential for pancreatic development [30]. Co-localisation studies show that the endocrine cells pass through various stages where IUF1 is co-expressed with combinations of other hormones. IUF1 is subsequently extinguished in the developing fetus in α, δ, and PP cells, remaining co-expressed with insulin in β cells in the adult [16]. It has been known for some time that the β-cell hypertrophy and islet hyperplasia associated with PHHI are often accompanied by abnormalities in the distribution and number of glucagon (α), somatostatin (δ) and pancreatic polypeptide (PP) producing cells. This led to the suggestion that the histological resemblance of nesidioblastotic pancreata to those of immature foetuses may have arisen as the result of inappropriate control during the earliest phases of endocrine pancreatic development [17]. Further studies supported this conclusion, adding that in PHHI, the β cells resemble cells in the early fetal pancreas not only anatomically, but by their defective recognition of nutrient secretagogues, and lack of glucose dependency of their c-AMP mediated insulin release [21]. The results of the present study are therefore consistent with a view that a defect in the expression of IUF1, results in aberrant development of the β cells in PHHI. It remains to be established how this defect relates to the K_{ATP} channel abnormality in this patient. PHHI may be a manifestation of poor nutrient sensing in the developing endocrine pancreas, raising the intriguing possibility that a similar defect in IUF1 expression may also contribute

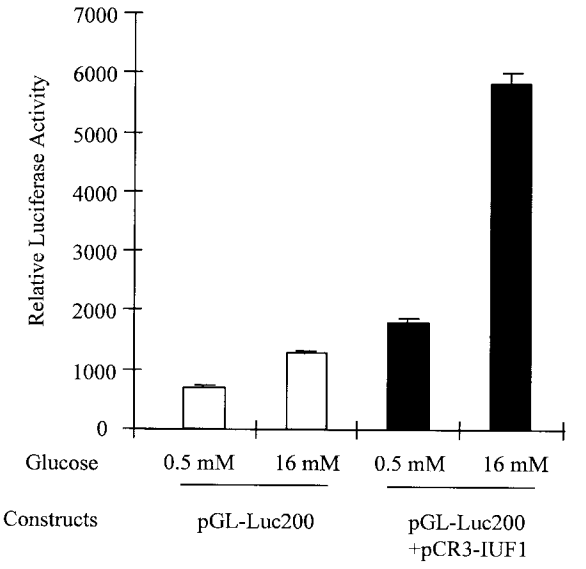

Fig. 6. IUF1 restores glucose responsive regulation of the insulin promoter in transfected NES 2Y cells. NES 2Y cells were transfected with pGL-LUC200, or co-transfected with pGL-LUC200 and pCR3-IUF1, as indicated. Cells were then incubated in 0.5 mM glucose or 16 mM glucose for 24 h. Values are shown as relative luciferase activity standardised against protein content. Data are representative of two experiments and show the mean ± S.D. of four separate determinations.

to the β-cell dysfunction associated with NIDDM. The interrelations of fetal nutrient supply, IUF1 expression and the functional development of postnatal insulin secretion is clearly an area demanding further study.

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