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# Reduced nuclear protein 1 expression improves insulin sensitivity and protects against diet-induced glucose intolerance through up-regulation of heat shock protein 70

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ABSTRACT

We recently reported that deletion of the stress-regulated nuclear protein 1 (Nupr1) protected against obesity-associated metabolic alterations due to increased beta cell mass, but complete Nupr1 ablation was not advantageous since it led to insulin resistance on a normal diet. The current study used *Nupr1* haplodeficient mice to investigate whether a partial reduction in *Nupr1* expression conferred beneficial effects on glucose homeostasis. Islet number, morphology and area, assessed by immunofluorescence and morphometric analyses, were not altered in *Nupr1* haplodeficient mice under normal diet conditions and nor was beta cell BrdU incorporation. Glucose and insulin tolerance tests indicated that there were no significant changes in *in vivo* insulin secretion and glucose clearance in *Nupr1* haplodeficient mice, and beta cell function *in vitro* was normal. However, reduced *Nupr1* expression decreased visceral fat deposition and significantly increased insulin sensitivity *in vivo*. In contrast to wild type animals, high fat diet-fed *Nupr1* haplodeficient mice were not hyperinsulinaemic or glucose intolerant, and their sustained insulin sensitivity was demonstrated by appropriate insulin-induced Akt phosphorylation, as determined by Western blotting. At the molecular level, measurements of gene expression levels and promoter activities identified *Nupr1*-dependent inhibition of heat shock factor-1-induced heat shock protein 70 (Hsp70) expression as a mechanism through which Nupr1 regulates insulin sensitivity. We have shown for the first time that Nupr1 plays a central role in inhibiting Hsp70 expression in tissues regulating glucose homeostasis, and reductions in Nupr1 expression could be used to protect against the metabolic defects associated with obesity-induced insulin resistance.



## 1. Introduction

Diabetes is one of the major causes of mortality worldwide with a predicted incidence of 592 million by 2035 according to the Sixth International Diabetes Federation (IDF) Diabetes Atlas [1]. Hyperglycaemia is the main feature of type 2 diabetes (T2D), which occurs as a consequence of defective insulin action in peripheral tissues in combination with impaired insulin secretion from islet beta cells [2,3]. It has become evident that T2D development and obesity are linked to chronic inflammation and insulin resistance [4], resulting in decreased insulin-stimulated Akt phosphorylation and consequent impaired GLUT4 glucose transporter translocation to the plasma membrane in the muscle and fat [5,6]. There is increasing evidence that the induction of heat shock protein 70 (Hsp70), the major inducible molecular chaperone [7,8], is linked to improved sensitivity in high-fat diet-fed mice [9]. In contrast, Hsp70 is decreased in skeletal muscle of T2D patients, and its reduced expression is correlated with the degree of insulin resistance [10,11]. Furthermore, increased insulin-stimulated Akt phosphorylation in liver cells has been observed *in vitro* following Hsp70 up-regulation [12]. Thus, increasing Hsp70 expression might be helpful in treating patients with insulin resistance to improve glucose homeostasis.

Heat shock proteins (Hsps) are a large family of proteins, many of which are ubiquitously expressed in eukaryotic cells, whose expression levels increase under stress conditions such as elevated temperature and hypoxia [13–15]. When Hsps are up-regulated in cells they confer protection against the stressful stimuli and allow maintenance of cellular function [14,15]. Transcription of Hsps is regulated by the heat shock transcription factors (Hsfs), and Hsf-1 is the main regulator of

*Abbreviations:* HFD, high fat diet; HOMA-IR, homeostasis model assessment-insulin resistance; Hsf-1, heat shock transcription factor 1; Hsp70, heat shock protein 70; NFD, normal fat diet; Nupr1, nuclear protein, transcriptional regulator, 1; T2D, type 2 diabetes

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short-term induction of Hsps [16]. Thus, under stress conditions Hsf-1 monomers dissociate from Hsps in the cytosol, are transported to the nucleus where they form phosphorylated homotrimers and bind to the *hsp* gene promoter, stimulating transcription. Direct binding of Hsps to the Hsf-1 *trans*activation domain results in its inactivation and subsequent reduction in Hsps expression [7,17].

We recently demonstrated that global deletion of *Nupr1*, a stress-regulated protein, in mice promoted basal and glucose-stimulated insulin secretion *in vivo* due to increased beta cell mass [18]. In addition, deletion of *Nupr1* protected mice against obesity-associated perigonadal fat mass accumulation, glucose intolerance and insulin resistance associated with prolonged maintenance on a high fat diet. However, *Nupr1* knockout mice fed a normal diet were insulin resistant, so complete ablation of Nupr1 is not an ideal scenario for appropriate control of glucose homeostasis. Our microarray analysis indicated that *Nupr1* regulated expression of *Hspa1a*, which codes for Hsp70 expression [18], so we hypothesised that partial loss of *Nupr1* could circumvent the potentially deleterious effects seen with complete Nupr1 knockout on a normal diet, while conferring beneficial effects through increased expression of Hsp70 and consequent improved insulin sensitivity. In the current study we therefore characterised the metabolic phenotype of *Nupr1* haplodeficient C57BL/6 mice under normal conditions and when fed a high fat diet to induce insulin resistance and glucose intolerance.

## 2. Materials and methods

### 2.1. Materials

Cell culture reagents were obtained from Sigma Aldrich (Poole, UK). Molecular biology reagents were purchased from Invitrogen (Paisley, UK) and Promega (Southampton, UK). SYBR Green and BrdU proliferation kits were from Roche (Burgess Hill, UK). Antibodies against insulin, glucagon and somatostatin were from Dako UK (Ely, UK), and the anti-phospho Akt(Ser473) antibody was supplied by New England Biolabs (Hertfordshire, UK). Fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-labelled secondary antibodies were from Jackson Immunoresearch (Newmarket, UK). Metafectene Pro was from Biontex Laboratories (Martinsried/Planegg, Germany). Ultrasensitive mouse insulin ELISA kits were obtained from Mercodia (Uppsala, Sweden).

### 2.2. Animals

The *Nupr1* transgenic C57BL/6 mouse model was generated by homologous recombination to delete exon 2 which encodes 60% of the murine Nupr1 protein [19]. All procedures performed in this study were approved by the local ethical committee and were in accordance with the United Kingdom Home Office standards. Mice were kept under a light–dark cycle of 12 h and bred to generate wild-type and *Nupr1*-haplodeficient littermates. Mice were fed *ad libitum* with a standard normal-fat diet (NFD; 4% fat), and for some experiments they were fed a high-fat diet (HFD; 55% fat) for 16 weeks, starting at 5 weeks of age. All experiments were performed using age-matched animals.

### 2.3. Metabolic studies

After i.p. administration of 2 g/kg glucose, glucose tolerance tests were performed by quantification of blood glucose concentrations with a glucose meter. Serum insulin levels were determined using ultrasensitive ELISA kits. Insulin tolerance tests were performed following i.p. administration of 0.75 U insulin per kg body weight. Weight gain was recorded weekly until the day the mice were killed. Pancreases, perigonadal fat pads and gastrocnemius muscles were retrieved on the day the mice were killed, and were weighed.

### 2.4. Immunohistochemistry and morphometric analysis

Pancreases were retrieved from wild-type and *Nupr1*-haplodeficient mice, fixed in 4% paraformaldehyde, cut into 5 μm sections and morphometric analyses were performed on every tenth section throughout each pancreas. For quantification of islet number and alpha, beta and delta cell areas, sections were incubated with antibodies against glucagon, insulin and somatostatin, and then with FITC- and TRITC-conjugated secondary antibodies. Islet size and the area of glucagon-, insulin- and somatostatin-labelled cells on each section were quantified using ImageJ software (ImageJ 1.45: http://rsbweb.nih.gov/ij/download.html).

Beta cell proliferation was estimated following the administration of 1 mg/ml BrdU in drinking water to the mice for 7 days. Then, as described above, fixed pancreas sections were immunostained to detect BrdU positive beta cells using anti-BrdU and anti-insulin antibodies.

### 2.5. Mouse islet isolation and dynamic insulin secretion

Islets were isolated by collagenase digestion of pancreases retrieved from 3-month-old mice as described previously [20], and maintained in culture for 24–48 h in RPMI-1640 medium (11 mM glucose) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The insulin secretory responses to 4 mM and 20 mM glucose, 100 nM 4β-phorbol myristate acetate (PMA) and 100 μM tolbutamide were then assessed using a temperature-controlled perifusion device for sample collection [21] and quantified by radioimmunoassay as previously described [22].

### 2.6. Insulin-stimulated Akt phosphorylation

Insulin (5 U per kg body weight) was administered i.p. to 14 h fasted wild-type and *Nupr1* haplodeficient mice, which were culled 10 min later. Samples of gastrocnemius muscle and perigonadal fat were rapidly retrieved, frozen in liquid nitrogen and stored at −85 °C. Protein immunoblot analysis [23] was performed using an anti-phospho Akt antibody (1:1000 dilution) and normalised against tubulin expression (1:2000 dilution of antibody) in the same samples.

### 2.7. Gene expression analyses

Messenger RNAs were extracted from pancreas, islets, MIN6 beta cells, perigonadal fat and liver, purified and concentrated using Invitrogen RNeasy minikits before being reverse-transcribed into cDNAs [18]. Quantitative PCR amplifications of *Nupr1*, *Hsp1a* and β-actin were performed using the following conditions: denaturation at 95 °C for 10 min, DNA amplification using 35–40 cycles at 95 °C for 1 s, 60 °C for 10 s and 72 °C for 15 s. The primers used were: *Nupr1* (F) *5′-gaagctgctgccaataccaacc-3′* and (R) *5′-tagctctgcccgtctaccctc-3′*; *Hspa1a* (F) *5′-gcactgccccgctgatgtga-3′* and (R) *5′-gtggcccaggggagagtcca-3′*; β-actin (F) *5′-atgaagtgtgacgttgacatccgt-3′* and (R) *5′-cctagaagcatttgcggtgcacgatg-3′*.

### 2.8. Plasmids

*Hsp70* promoter activities were assessed using −1512/0 mouse *Hspa1a* and −1491/-1 mouse *Hspa1b* promoter-driven luciferase expression cloned into *pGL2*. The −1512/0 *Hspa1a* and −1491/-1 *Hspa1b* promoter sequences were produced by PCR amplification using a C57BL/6 mouse islet DNA library. The following primers were used to introduce restriction sites for *HindIII* and *NheI* into *Hspa1a*: (F) 5′-*cacacgctagcgagtcagggtccaactatgt*agctcaggc-3′, (R) *5′-cacacaagc ttggcgccgcgctctgcttctggaaggctg-3′* and for *SacI* and *HindIII* into *Hspa1b*: (F) *5′-cacacgagctcggggcagagaaggagaaaaggggaca-3′* and (R) *5′-cacacaa gcttggcgccgcgctctgcttctggaaggct-3′*. The PCR products were purified, digested, inserted into the *pGL2* luciferase reporter vector and sequenced. Experiments in which *Nupr1* was overexpressed were