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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.

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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

performed using a *pcDNA3.1* plasmid coding for the full length human *Nupr1* gene as previously reported [18,24].

2.9. MIN6 beta cell transfection

For gene reporter assay studies, MIN6 beta cells were transiently transfected using a Nucleofector II electroporation device as previously described [23] and maintained in culture overnight at 37 °C in DMEM (25 mM glucose) supplemented with 2 mM glutamine, 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Promoter activity levels were then determined by bioluminescence using luciferase assay kits and normalised to β-galactosidase expression which was co-transfected using *pSV-β-Gal* as described [18].

2.10. Data analysis

Numerical data are expressed as means ± SEM. The differences between groups were assessed using Student's unpaired t-tests for two group analyses, and one- or two-ways ANOVA followed by the Tukey's HSD test for more than 3 groups or for multiple variable analysis (genotype and diet), respectively. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Nupr1 haplodeficiency does not alter islet area or number

Quantitative RT-PCR analyses indicated that pancreatic *Nupr1* mRNA levels were reduced by approximately 50% in *Nupr1* haplodeficient mice (Fig. 1A), confirming the gene dosage reduction in expression with deletion of only one *Nupr1* allele. Morphometric analyses of the endocrine pancreases of 3-month-old wild-type and *Nupr1* haplodeficient mice indicated that the absence of one copy of the *Nupr1* gene did not significantly affect islet area or number, nor were there any alterations in the endocrine cell ratios (Table 1). Consistent with these results, no significant differences in the number of proliferating BrdU-positive beta cells were observed between pancreases obtained from wild-type and *Nupr1* haplodeficient mice that had been exposed to 1 mg/ml BrdU in drinking water for 7 days (Fig. 1B–D).

3.2. Improved insulin sensitivity in Nupr1 haplodeficient mice

We have previously reported that complete deletion of *Nupr1* resulted in impaired insulin sensitivity without any significant differences in glucose tolerance, weight gain, or in gastrocnemius muscle and perigonadal fat masses [18]. These metabolic and weight parameters were therefore monitored in mice heterozygous for the *Nupr1* gene and compared to those obtained from their age-matched wild-type littermates. No significant differences in weight gain over 13 weeks were observed between wild-type and *Nupr1* haplodeficient mice (Fig. 2A and B). Similarly, the pancreas and gastrocnemius masses were also very similar in 3-month-old wild-type mice and those lacking one *Nupr1* allele (Fig. 2C and D). We consistently observed that perigonadal fat accumulation was reduced by 10–15% in *Nupr1* haplodeficient mice (Fig. 2E), although this reduction was not statistically significant ($p < 0.09$).

To characterise the metabolic phenotype of *Nupr1* haplodeficient mice, intraperitoneal glucose tolerance and insulin sensitivity tests were performed, and serum insulin levels in response to glucose administration were also measured in 3-month-old animals after 16 h of

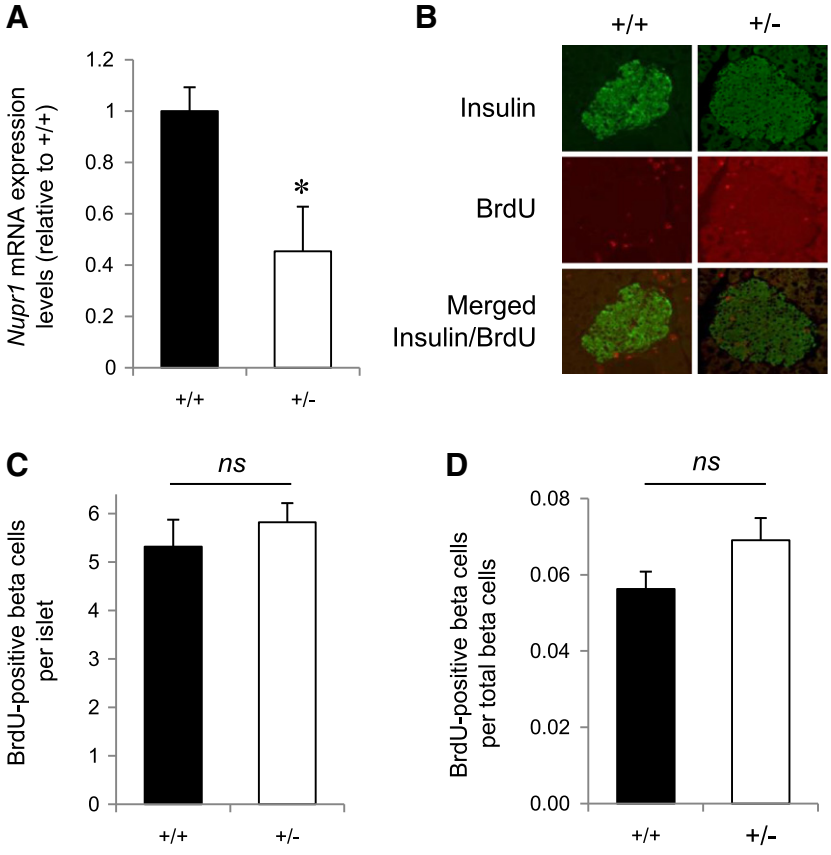

Fig. 1. *Nupr1* gene expression and beta cell proliferation. (A) *Nupr1* gene expression levels in the pancreas of wild-type (+/+, black bars) and *Nupr1* haplodeficient (+/−, white bars) mice. (B) Representative immunographs of pancreas sections obtained from 3-month-old wild-type (+/+) and *Nupr1* haplodeficient (+/−) mice showing proliferative BrdU-positive (red) beta cells (green). (C) Number of BrdU-positive beta cells per islet and (D) number of BrdU positive beta cells as a proportion of the total beta cell number in wild-type (+/+, black bars) and *Nupr1* haplodeficient (+/−, white bars) mice. Data are mean ± sem, n = 4 for both genotypes, $^{*}p < 0.05$.

Table 1
Islet characteristics in wild-type and *Nupr1* haplodeficient mice. Alpha, beta and delta cell areas and the beta/alpha, alpha/delta and beta/delta cell ratios were measured by point-counting morphometry from pancreas sections immunolabelled for insulin, glucagon and somatostatin in wild-type ($Nupr1^{+/+}$) and *Nupr1* haplodeficient ($Nupr1^{+/-}$) mice. Data are mean ± sem, n = 4 for each genotype.

	Islet area/pancreas (% of total)	Islet number per mm^2 pancreas	Mean cell areas per islet (μm^2)			Cell ratios per mm^2 pancreas		
			Alpha cell	Beta cell	Delta cell	Beta/alpha cell ratio	Alpha/delta cell ratio	Beta/delta cell ratio
$Nupr1^{+/+}$	0.54 ± 0.09	0.47 ± 0.05	340 ± 71	10190 ± 1095	77 ± 19	24.4 ± 2.7	6.3 ± 1.3	138.7 ± 31.6
$Nupr1^{+/-}$	0.72 ± 0.15	0.51 ± 0. 09	276 ± 51	8072 ± 1031	110 ± 40	28.4 ± 2.4	4.3 ± 0.8	125.8 ± 15.8
p-Value	0.08	0.63	0.56	0.21	0.49	0.3	0.24	0.7

fasting. The tolerance to glucose (Fig. 3A) and the *in vivo* insulin secretion profiles (Fig. 3B) were not significantly different in *Nupr1* haplodeficient mice, but these animals showed a significant improvement in insulin sensitivity 30 and 45 min after insulin (0.75 U/kg) administration (Fig. 3C). Statistical comparisons of the areas under the curves (insulin sensitivity curves (AUC): 1367.4 ± 63.2 ($Nupr1^{+/+}$) *vs.* 1119.3 ± 80.8 ($Nupr1^{+/-}$), $p < 0.05$) confirmed these results.

In *in vitro* experiments in which isolated islets were perifused using buffers supplemented with 4–20 mM glucose, 100 nM PMA or 100 μM tolbutamide, although the first phase of glucose-stimulated insulin release was slightly reduced in *Nupr1* haplodeficient islets, there were no significant differences between the insulin secretion profiles of wild-type and *Nupr1* haplodeficient islets (Fig. 3D). Taken together, our results indicate that the reduced *Nupr1* expression in *Nupr1* haplodeficient mice improves insulin sensitivity without altering beta cell mass or beta cell function and this enhanced insulin sensitivity is not associated with improved glucose tolerance under standard diet and environmental conditions of maintenance.

3.3. Reduced levels of Nupr1 protect against HFD-induced obesity and obesity-associated metabolic defects

Because insulin sensitivity was significantly improved and there was a trend towards reduced perigonadal fat deposition in *Nupr1* haplodeficient mice fed normal chow, we further assessed the *in vivo* effect of reduced *Nupr1* expression on the weight and metabolic parameters following the administration of a high- (55%) fat diet (HFD) for 16 weeks. As expected, prolonged maintenance of wild-type mice on the HFD resulted in impaired glucose tolerance that was characterised by significant elevation in fasting hyperglycaemia and serum insulin compared to wild-type mice fed standard chow containing 4% fat (Table 2). In contrast, HFD-fed *Nupr1* haplodeficient animals did not show significant increases in circulating glucose or insulin concentrations, and *Nupr1* reduction also partially protected against HFD-induced body weight gain (Table 2). In a similar manner, there was a 50% lower perigonadal fat deposition in $Nupr1^{+/-}$ mice over 16 weeks than in age-matched HFD-fed wild-type mice (Table 2). Moreover,

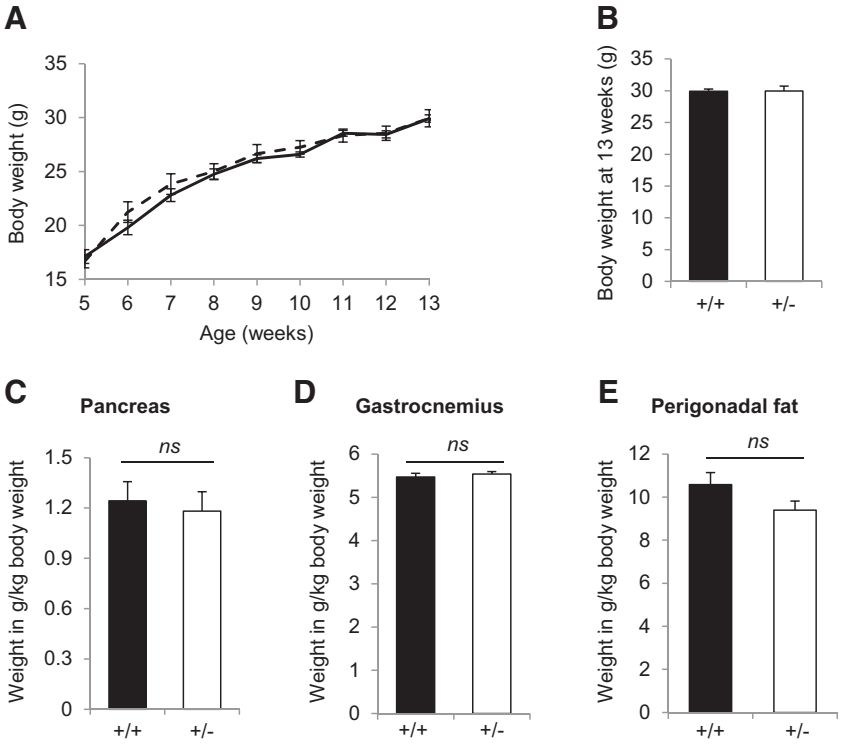

Fig. 2. Weight variables in wild-type and *Nupr1* haplodeficient mice. (A–B) Total body weights of wild-type (solid line) and *Nupr1* haplodeficient (dashed line) mice measured from week 5 to week 13. (C–E) Mean wild-type (+/+, black bars) and *Nupr1* haplodeficient (+/−, white bars) pancreas (C), gastrocnemius muscle (D) and perigonadal fat pad (E) weight indices expressed as gram per kg of body weight at week 13. Total body weights: n = 66 (+/+), n = 69 (+/−) in total; pancreas weight indices: n = 12 (+/+ and +/−); gastrocnemius muscle and perigonadal fat pad indices: n = 15 (+/+), n = 20 (+/−).

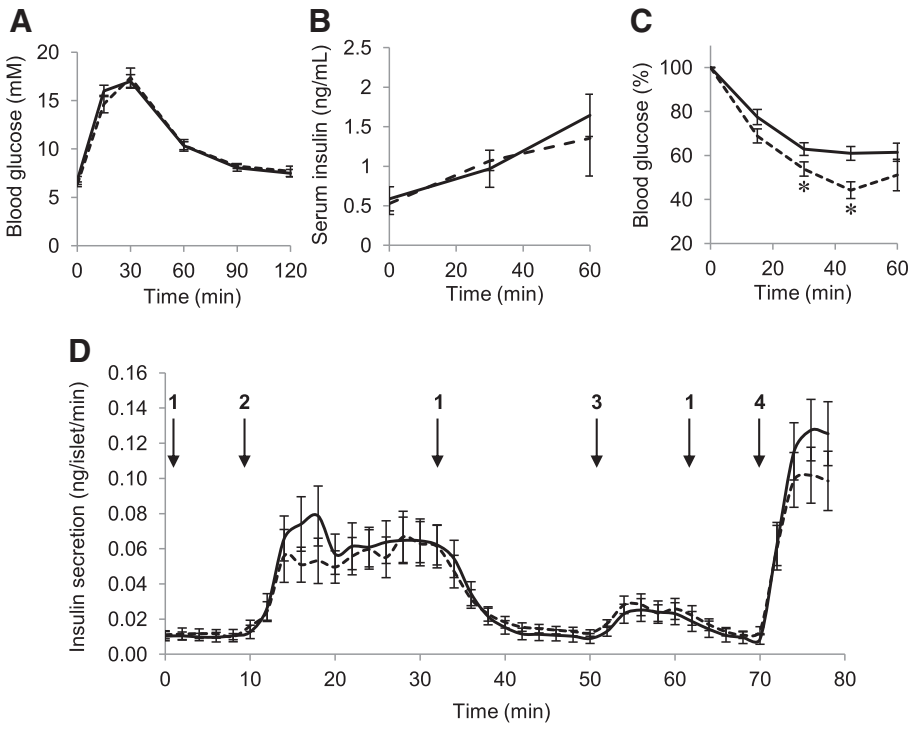

Fig. 3. Metabolic variables. (A) Glucose tolerance tests, (B) serum insulin measurements, and (C) insulin sensitivity tests performed on 3-month-old wild-type (solid line) and *Nupr1* haplodeficient (dashed line) mice. (D) Determination of the insulin secretion profiles of wild-type (solid line) and *Nupr1* haplodeficient (dashed line) islets perifused in buffers containing 4 mM (1) or 20 mM glucose (2), 100 μM tolbutamide (3) or 100 nM phorbol myristate acetate (PMA) in the presence of 20 mM glucose (4). Data are means ± sem. *$p < 0.05$. Glucose tolerance tests: n = 12 (+/+ and +/−); serum insulin measurements: n = 5 (+/+) and n = 6 (+/−); insulin sensitivity tests: n = 15 (+/+ and +/−); insulin secretion profiles: n = 16 (+/+) and n = 15 (+/−).

although insulin sensitivity was significantly impaired in HFD-fed wild-type mice 15, 30 and 45 min-post insulin administration, HFD-fed *Nupr1* haplodeficient animals showed similar insulin-induced glucose uptake curves to those fed a NFD (Fig. 4A), suggesting that reduced levels of *Nupr1* also confer a protection against obesity-induced insulin resistance. Improved insulin sensitivity of *Nupr1* haplodeficient mice was confirmed by phospho-Akt immunoblot analysis, which indicated that *Nupr1*$^{+/-}$ mice did not show reduced insulin-induced Akt phosphorylation in gastrocnemius muscle (Fig. 4D) and perigonadal fat (Fig. 4E) after maintenance on a HFD for 16 weeks, whereas in HFD-fed wild-type mice Akt phosphorylation was reduced by approximately 50%. Thus, not surprisingly, serum insulin levels were increased in wild-type animals fed a HFD, but this did not occur in mice heterozygous for *Nupr1* (Table 2 and Fig. 4B). As a result, glucose tolerance was significantly impaired in HFD-fed wild-type mice after 16 weeks of treatment, but it was not altered in mice heterozygous for *Nupr1* (Fig. 4C), and these observations were confirmed by quantification of area under the curve measurements (Fig. 4F), which indicated that glucose tolerance was significantly improved in the *Nupr1*$^{+/-}$ mice after 16 weeks on a HFD compared to age-matched wild-type mice on the HFD. Insulin resistance was assessed using the homeostasis model assessment, HOMA-IR, and this further confirmed that reduced *Nupr1* expression improved insulin sensitivity in HFD-fed animals while it was reduced by more than 3-fold in obese wild-type mice (Table 2). The improved glucose handling was not increased related to beta cell mass expansion since immunohistochemical analyses of pancreases retrieved from HFD-fed wild-type and *Nupr1* haplodeficient mice that had been administered 1 mg/ml BrdU for 7 days prior to sacrifice indicated that there were no significant differences in beta cell number between genotypes (BrdU-positive beta cells per islet, *Nupr1*$^{+/-}$ mice: 78.5 ± 36.6% of wild-type; BrdU-positive cells per total beta cells, *Nupr*$^{+/-}$ mice: 81.8 ± 48.5% of wild-type, $p > 0.2$, n = 4 for each genotype).

Table 2
Metabolic variables from NFD- and HFD-fed wild-type and Nupr1 haplodeficient mice. Body and perigonadal fatweight gain, fasting blood glucose and seruminsulin levels were measured in NFD- and HFD-fed wild-type (*Nupr1*$^{+/-}$) and Nupr1 haplodeficient (*Nupr1*$^{+/-}$) mice after 16 weeks. Insulin resistance was assessed using the homeostatic model assessment HOMA-IR. Data are mean ± sem, n = 3 for each genotype and diet.

	Nupr1$^{+/+}$		*Nupr1*$^{+/-}$	
	NFD	HFD	NFD	HFD
Body weight gain (g)	2.5 ± 0.95	119.5 ± 1.7*	9.3 ± 1.3	14.5 ± 3.1
Perigonadal fat (g/kg body weight)	9.8 ± 0.7	33.4 ± 5.3*	8.9 ± 0.9	20.6 ± 2.5
Blood glucose (mM)	5.9 ± 0.53	1 ± 0.44*	6.0 ± 0.49	6.9 ± 0.19
Serum insulin (ng/mL)	0.6 ± 0.03	1.2 ± 0.16*	0.5 ± 0.09	0.4 ± 0.02**
HOMA-IR	3.5 ± 0.06	11.2 ± 1.53*	3.4 ± 0.36	2.7 ± 0.46**

* $p < 0.05$ relative to NFD wild-type mice.
** $p < 0.05$ relative to HFD-fed wild-type mice.

3.4. Nupr1 controls Hsp70 promoter activity and mRNA expression

It is well-established that reductions in body mass index are associated with improved insulin sensitivity, and increased levels of visceral fat-derived pro-inflammatory adipokines involved in insulin resistance occur in obesity [25]. It is therefore likely that the protection against excessive weight gain and perigonadal fat deposition shown by HFD-fed *Nupr1*$^{+/-}$ mice protected them against metabolic derangement. We sought to identify a Nupr1-dependent mechanism of improved glucose handling in these mice, and focused on heat shock protein 70 (Hsp70) since this protein is known to reduce visceral adiposity and insulin resistance in mice and humans [9,26], and our microarray analysis indicated that complete ablation of *Nupr1* affects expression of *Hspa1a* (also known as *Hsp70-1* or *Hsp72*), which encodes Hsp70 [18]. Thus, given our demonstration that insulin sensitivity is significantly improved in *Nupr1* haplodeficient mice, we monitored the expression of *Hspa1a* mRNA in insulin-sensitive tissues and islets when *Nupr1* expression was reduced. Quantitative RT-PCR indicated that *Hspa1a*

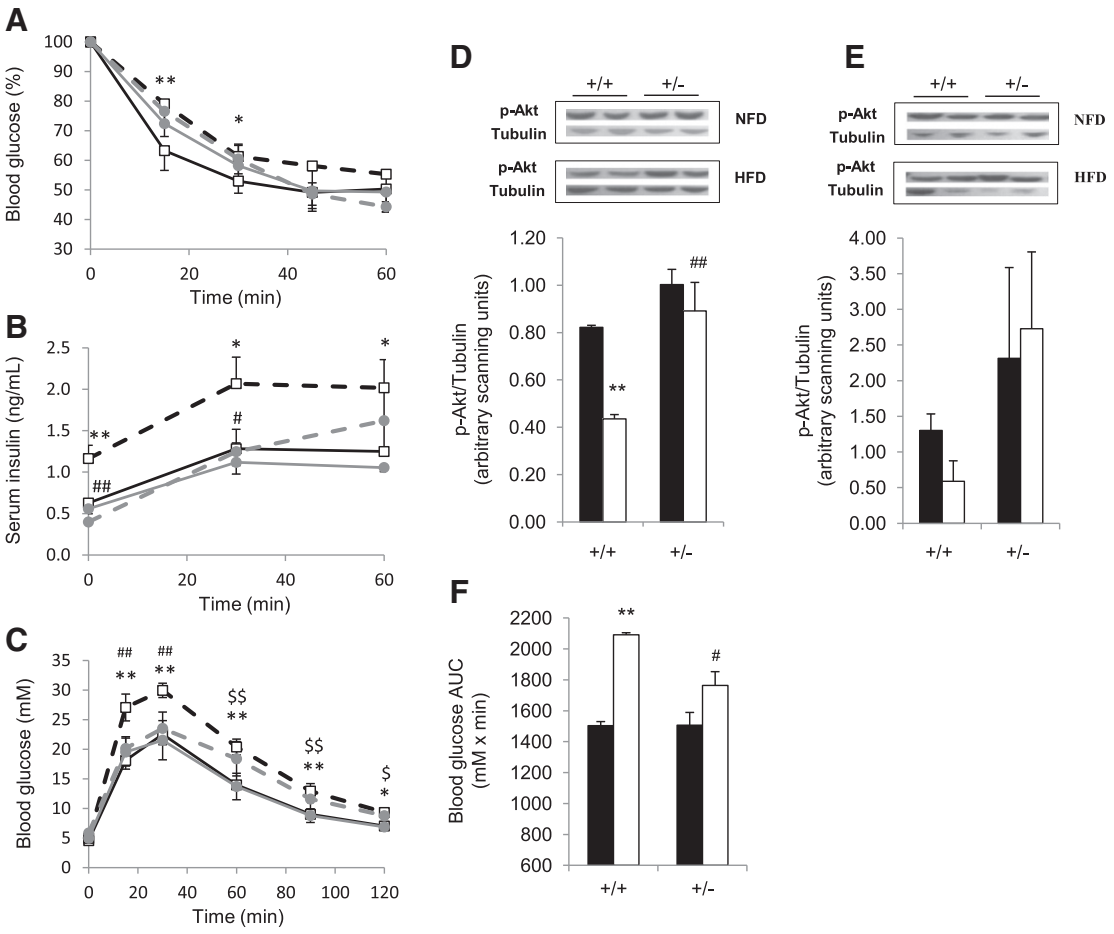

Fig. 4. Metabolic variables during a high fat diet challenge. Determination of NFD- (solid lines and black bars) and HFD-fed (dashed lines and white bars) wild-type (+/+) and *Nupr1* haplodeficient mice (+/−) glucose (A, C) and serum insulin (B) levels after 16 weeks of treatment. (A) Insulin sensitivity tests were performed after administration of 0.75 U/kg insulin. (B) Serum insulin levels were determined at fast and after an i.p glucose challenge. (C) Glucose tolerance tests were carried out following i.p. administration of glucose. (D, E) Akt phosphorylation was determined in gastrocnemius muscle (D) or perigonadal fat (E) 10 min after i.p. insulin injection and compared with tubulin expression in the same samples (upper panels). Densitometric data of immunoreactive protein densities are shown in the lower panels. (F) Areas under the curves (AUC) were calculated from the glucose tolerance curves in panel C. Data are mean ± sem of repeated measures, n = 3 for each genotype and diet condition, (*, $^{\$}$, $^{\#}$) $p < 0.05$ and (**, $^{\$\$}$, $^{\#\#}$) $p < 0.01$ relative to NFD-fed wild-type mice (*, **), NFD-fed heterozygous mice ($^{\$, \$\$}$) and HFD-fed wild-type mice ($^{\#, \#\#}$), respectively.

mRNA levels were up-regulated in perigonadal fat, liver and islets of *Nupr1*$^{+/-}$ mice fed on standard chow (Fig. 5, black bars), consistent with the improved insulin sensitivity of these mice. Moreover, while both wild-type and *Nupr1*$^{+/-}$ mice maintained on a HFD showed significant reductions in *Hspa1a* mRNA in fat and liver, the levels in *Nupr1* haplodeficient HFD mice were not significantly different from those observed in wild-type mice on the NFD diet (Fig. 5). Furthermore, islets retrieved from *Nupr1*$^{+/-}$ mice did not show the HFD-induced reduction in *Hspa1a* mRNA levels that occurred in wild-type mice on the HFD (Fig. 5). The reasons for this are not entirely clear, but the data are consistent with improved *Hspa1a* mRNA levels under conditions of metabolic stress. Overall, *Nupr1* haplodeficiency was associated with significantly increased *Hspa1a* mRNA expression in all tissues examined in both NFD and HFD conditions.

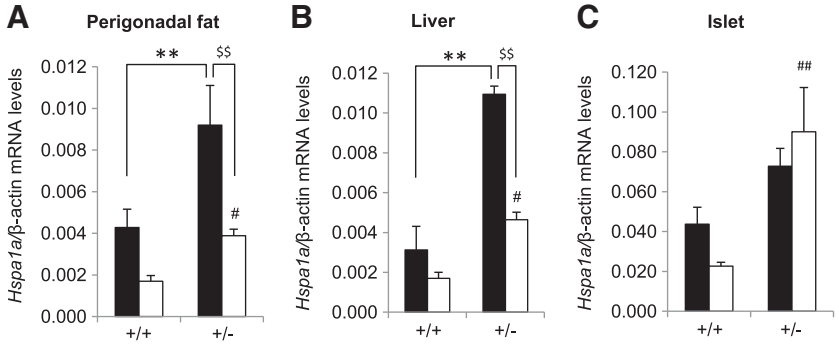

Fig. 5. Increased *Hspa1a* expression in *Nupr1* haplodeficient tissues. Quantitative RT-PCR amplification data showing gene expression levels of *Hspa1a* in (A) perigonadal fat, (B) liver, and (C) islets retrieved from NFD- (black bars) and HFD-fed (white bars) wild-type (+/+) and *Nupr1* haplodeficient (+/−) mice. Values are mean ± sem of repeated measures, n = 3 for each genotype and diet, ($^{\#}$) $p < 0.05$ and (**, $^{\$\$, \#\#}$) $p < 0.01$ relative to NFD-fed wild-type mice (**), NFD-fed heterozygous mice ($^{\$\$}$) and HFD-fed wild-type mice ($^{\#, \#\#}$), respectively.

To determine whether Nupr1 plays a role in regulating *Hspa1a* expression mouse MIN6 beta cells were transiently co-transfected either with the promoterless *pGL2/Luc* plasmid (*pGL2*) or *Hspa1a* promoter-driven *pGL2/Luc* vectors (*Hspa1a/pr*), and *pcDNA3.1/NUPR1* and/or *pcDNA3.1/Hsf-1* coding for the full-length forms of the human NUPR1 and mouse Hsf-1 proteins, respectively. As expected, the basal *Hspa1a* promoter activity was low under standard culture conditions and a 4-fold stimulation was observed following Hsf-1 overproduction (Fig. 6A). Overexpression of NUPR1 did not significantly alter the *Hspa1a* promoter activity in the absence of Hsf-1-induced stimulation, but it significantly repressed *Hspa1a trans*activation by 38 ± 6% following Hsf-1 overproduction (Fig. 6A). Similar results were obtained in gene reporter assays in which the luciferase activity was driven by the −1491/-1 bp mouse *Hspa1b* promoter (Fig. 6B) which drives the expression of a second Hsp70 isoform known as Hsp70-2. In these experiments NUPR1 overexpression did not alter basal *Hspa1b* promoter activity, but it significantly reduced (by 47 ± 5%) activity induced by Hsf-1 (Fig. 6B).

4. Discussion

We previously reported that the stress-activated protein NUPR1 acts as a tonic endogenous inhibitor of beta cell proliferation and expansion *via* molecular mechanisms involving repression of cell-cycle regulatory genes such as *TCF19* and *CCNA2* [18]. An increased beta cell mass in *Nupr1* null mice conferred protection against high fat diet (HFD)-induced obesity, but on normal diets deletion of *Nupr1* was associated with insulin resistance.

This study used *Nupr1* haplodeficient mice to test whether reducing rather than ablating *Nupr1* expression could be beneficial for maintenance of glucose homeostasis and we now confirm that *Nupr1* is indeed a potential molecular target for future drug-based therapies to treat obesity and obesity-associated metabolic defects. Thus, as we previously reported for *Nupr1* knockout mice, halving *Nupr1* expression levels was sufficient to protect mice from the metabolic stresses induced by maintenance on a 55% fat diet for 16 weeks. We have found that high fat fed *Nupr1* haplodeficient mice were normoglycaemic, glucose tolerant and sensitive to insulin, while wild type mice showed the typical metabolic derangements associated with prolonged high fat feeding. The improvements in glucose handling in *Nupr1*$^{+/-}$ mice were not secondary to increased beta cell mass, but to direct improvements in insulin sensitivity that was associated with lower body weight gain and reduced visceral fat accumulation. In addition, perigonadal fat mass accumulation was also reduced and insulin sensitivity increased in *Nupr1* haplodeficient animals fed a normal diet, in contrast to the impaired insulin sensitivity that we had previously seen in *Nupr1* knockout mice maintained on a normal diet [18].

One of the key signal transduction cascades downstream of insulin binding to its receptor is through phosphorylation and activation of the serine/threonine kinase Akt, which is responsible for GLUT4 translocation to the plasma membrane to allow glucose uptake into adipocytes and myocytes [27]. It has been reported that Hsps induction *via* heat shock and mild electrical stimulation can ameliorate insulin resistance by enhancing insulin signalling in the liver, where increased Akt phosphorylation was observed [12,26]. Here, we demonstrate that *Nupr1* haplodeficient mice show insulin-induced Akt phosphorylation in muscle and perigonadal fat even when fed a high fat diet, while wild-type mice exhibited insulin resistance, as expected, on this diet. Our observations of increased expression of *Hspa1a*, a mRNA encoding a heat shock protein 70 (HSP70) family member, in perigonadal fat, liver and islets of insulin-sensitive *Nupr1* haplodeficient mice, are consistent with Hsp70 playing a role in the increased insulin sensitivity of these mice. Although Hsp70 protein levels were not quantified in the current study, there is a close correlation between *Hspa1a* mRNA levels and Hsp70 expression [28–30]. In addition, when Hsp70 has completed its functional effects it undergoes ubiquitin-dependent degradation to decrease its levels [30] or it may show increased stability when further cellular protection is required [31].

Heat shock proteins function as chaperone molecules and can provide protection against obesity-associated metabolic dysfunction [9]. For example, *HSPA1A* expression is reduced in muscle biopsies of diabetic patients [10,11] and impaired induction of its *trans*activating factor HSF-1 in hyperglycaemic diabetic monkeys has been reported [32]. Consistent with these observations, oral administration of chemical chaperones to obese and type 2 diabetic animals can restore glucose homeostasis and insulin sensitivity [33] and Hsp70 overexpression in high fat fed mice is reported to prevent the development of insulin resistance by inhibiting JNK-mediated inflammation [9]. Strategies directed at increasing heat shock protein expression have been proposed as a means of improving insulin sensitivity [26,34–36] and it has been reported that mean body weight, fasting plasma glucose and glycated haemoglobin levels of individuals with T2D were significantly decreased following a three-week heat shock treatment [34]. Further support for a role for heat shock proteins in counteracting insulin

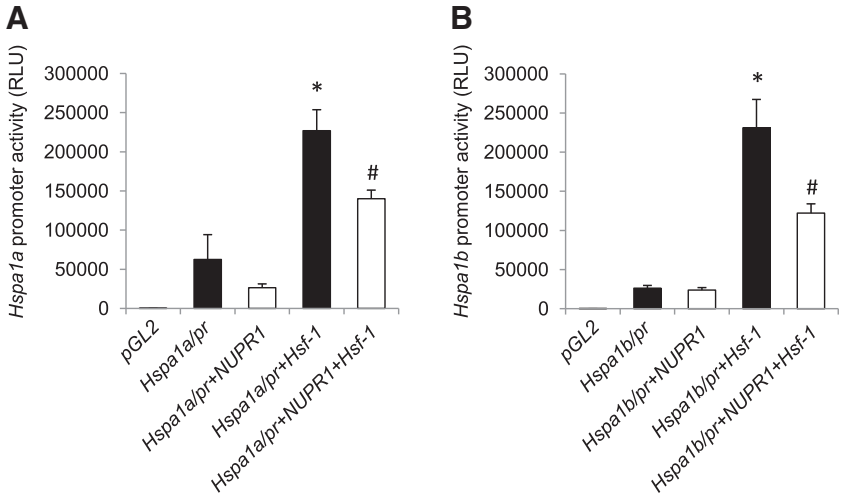

Fig. 6. Nupr1 down-regulates *Hspa1a* and *Hspa1b* promoter activities. (A) *Hspa1a* and (B) *Hspa1b* promoter activities were determined in MIN6 beta cells by gene reporter assays following the (co)transfection of the promoterless *pGL2/luciferase* plasmid (*pGL2*), the *−1512/0 Hspa1a* (*Hspa1a/pr*) or *−1491/-1 Hspa1b* (*Hspa1B/pr*) promoter-driven luciferase expression vectors, and pcDNA3.1 coding for the full length human NUPR1 or mouse Hsf-1 proteins. Data are mean ± sem, n = 3, $^{*}p < 0.05$ relative to *Hspa1a/pr* (A) *or Hspa1b/pr* (B), $^{\#}p < 0.05$ relative to *Hsf-1*-stimulated *Hspa1a* or *Hspa1b* promoter activities.

resistance has been demonstrated by improvements in insulin sensitivity and glucose utilisation following the administration of BGP-15, a co-inducer of heat shock proteins, to non-diabetic patients with impaired glucose tolerance [35].

The improved insulin sensitivity and increased *Hspa1a* mRNA expression that we observed in *Nupr1*$^{+/-}$ mice suggest that deletion of one *Nupr1* allele permitted up-regulation of Hsp70 in insulin-sensitive tissues as a mechanism of protecting against diet-induced insulin resistance. We therefore investigated whether Nupr1 acts as a tonic inhibitor of Hsp70 expression using gene reporter assays and found that *Nupr1* overexpression significantly repressed Hsf-1-induced *Hspa1a* promoter *trans*activation, and similar data were obtained when we measured promoter activity of the other Hsp70 family member, *Hspa1b*. It has previously been reported that Nupr1 can regulate *trans*activation of transcription factors through binding to protein partners including p300 [37]. It is possible that Nupr1 inhibits Hsf-1-mediated induction of *Hspa1a*/*b* through interaction with p300 since it is known that this cofactor plays a role in Hsp70 transcription [38,39]. Thus, our observations are consistent with a molecular model in which Nupr1 negatively regulates HSF-1 DNA binding activity and Hsp70 gene *trans*activation, and they are likely to be important in explaining the improved metabolic status of *Nupr1*$^{+/-}$ mice fed on a high fat diet.

In summary, our study demonstrates for the first time that the stress-regulated Nupr1 protein plays a central role in the regulation of mRNA encoding Hsp70 expression in tissues involved in the control of glucose homeostasis, and that reduction in Nupr1 expression and/or activity could represent a novel therapeutic approach to reduce obesity-associated adipose tissue accumulation and improve insulin sensitivity and glucose tolerance.

6. Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

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