ARTICLE

Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice

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Abstract

Aims/hypothesis We previously observed hyperglycaemia, hyperinsulinaemia, insulin resistance and obesity in *Gpx1*-overexpressing mice (OE). Here we determined whether these phenotypes were eliminated by diet restriction, subsequently testing whether hyperinsulinaemia was a primary effect of *Gpx1* overexpression and caused by dysregulation of pancreatic duodenal homeobox 1 (PDX1) and uncoupling protein-2 (UCP2) in islets.

Methods First, 24 male OE and wild-type (WT) mice (2 months old) were given 3 g (diet-restricted) or 5 g (full-fed) feed per day for 4 months to compare their glucose metabolism. Thereafter, several mechanistic experiments were conducted with pancreas and islets of the two genotypes (2 or 6 months old) to assay for beta cell mass, reactive oxygen species (ROS) levels, mitochondrial membrane potential ($\Delta\psi_m$) and expression profiles of regulatory proteins. A functional assay of islets was also performed.

Results Diet restriction eliminated obesity but not hyperinsulinaemia in OE mice. These mice had greater pancreatic beta cell mass (more than twofold) and pancreatic insulin content (40%) than the WT, along with an enhanced $\Delta\psi_m$

and glucose-stimulated insulin secretion in islets. With diminished ROS production, the OE islets displayed hyperacetylation of H3 and H4 histone in the *Pdx1* promoter, elevated PDX1 and decreased UCP2.

Conclusions/interpretation Overproduction of the major antioxidant enzyme, glutathione peroxidase 1, caused seemingly beneficial changes in pancreatic PDX1 and UCP2, but eventually led to chronic hyperinsulinaemia by dysregulating islet insulin production and secretion.

Keywords Glutathione peroxidase · Histone acetylation · Hyperinsulinaemia · Insulin resistance · Insulin secretion · Islets · Oxidative stress · Selenium

Abbreviations

AKT	protein kinase B
ChIP	chromatin immunoprecipitation
GPX1	glutathione peroxidase 1
GSIS	glucose-stimulated insulin secretion
JNK	c-jun terminal kinase
OE	*Gpx1*-overexpressing mice
$\Delta\psi_m$	mitochondrial membrane potential
PDX1	pancreatic and duodenal homeobox 1
PTP1B	protein tyrosine phosphatase 1B
ROS	reactive oxygen species
UCP2	uncoupling protein 2
WT	wild-type

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Introduction

Pancreatic beta cells are considered to be low in antioxidant capacity and susceptible to oxidative stress [1]. Consequently, oxidative injury of beta cells has become a new aetiological focus of diabetes and insulin resistance [2].

Indeed, insulin synthesis or secretion in insulin-producing or -secreting cells is impaired by elevated intracellular reactive oxygen species (ROS), this impairment being partially alleviated by overproduction of antioxidant enzymes [3, 4]. However, overproduction of antioxidant proteins in animals, either specifically in pancreatic beta cells [5, 6] or ubiquitously in various tissues [7, 8], has generated highly variable or even negative phenotypes [9]. Most strikingly, we have observed spontaneous development of hyperglycaemia, hyperinsulinaemia, insulin resistance and obesity in mice overproducing Se-dependent cellular glutathione peroxidase-1 (GPX1), a major intracellular antioxidant enzyme [10].

While the physiological relevance of our paradoxical finding was shown by a strong correlation between increased erythrocyte GPX1 activity and insulin resistance in gestational diabetic women [11], our initial study [10] did not sort out the metabolic sequence of the phenotype in the *Gpx1*-overexpressing (OE) mice. It remained unclear whether hyperinsulinaemia and insulin resistance in OE mice were caused by or largely confounded by obesity. Because obesity may be eliminated or controlled by diet restriction [12], it is logical to examine whether obesity in OE mice can be prevented by restricted feeding, and whether their altered insulin status is independent of obesity.

Hyperinsulinaemia may be induced by elevated beta cell mass and/or excessive insulin synthesis and secretion [13, 14]. The transcriptional factor pancreatic duodenal homeobox 1 (PDX1) plays a pivotal role in pancreatic beta cell differentiation as well as insulin gene expression and synthesis [14–17]. Uncoupling protein 2 (UCP2) serves as a negative regulator of mitochondrial membrane potential ($\Delta\psi_m$) [18], which positively correlates with glucose-stimulated insulin secretion (GSIS) [19]. Levels and function of PDX1 and UCP2 are affected by intracellular ROS status [20, 21], glucotoxicity [22] and antioxidants [6, 23, 24]. However, it is not known whether in vivo global overexpression of *Gpx1* could overly diminish intracellular production of hydroperoxides and subsequently dysregulate production of PDX1 and UCP2 and their role in insulin synthesis and secretion.

Although post-transcriptional regulation of PDX1 by ROS or antioxidants has been postulated [20, 23–25], new mechanisms such as epigenetic modification [26] of *Pdx1* have not been studied. The proximal region of *Pdx1* promoter consists of an islet-specific expression consensus E-box motif, which predominantly binds the upstream transcription factor [27]. Site-specific acetylation or deacetylation of nucleosomal histones H3 and H4 is central to the switch between permissive and repressive chromatin structure and thus activation or repression of transcription [28]. With a high affinity for upstream transcription factor binding [29], H3 and H4 are the core histones with high levels of acetylation at the active transcriptional loci [30]. It would be of great interest to determine whether the presumed diminished intracellular ROS production in OE islets enhances *Pdx1* transcription via hyper-acetylating H3 and H4 in its proximal promoter region, causing hypertrophy of beta cells and overproduction of insulin. In addition, *Pdx1* transcription and PDX1 stability are affected by three key insulin signal proteins: c-jun terminal kinase (JNK), protein kinase B (AKT) and protein tyrosine phosphatase 1B (PTP1B) [20, 31, 32]. Because these proteins are highly sensitive to ROS [9, 33, 34], elevated GPX1 activity may impact on PDX1 via alteration of their production or phosphorylation.

In the present study, we first demonstrated that hyperinsulinaemia was a primary effect of *Gpx1* overexpression and was not prevented by diet restriction. Thereafter, we conducted a series of experiments to test whether: (1) hyperinsulinaemia in OE mice was attributable to increased beta cell mass, insulin synthesis, mitochondrial potential and GSIS in islets; (2) OE islets phenotype was due to altered PDX1 and UCP2 levels; and (3) the dysregulation of PDX1 and UCP2 was mediated by overly scavenged ROS level, hyperacetylation of H3 and H4 histone of *Pdx1* promoter and alterations of JNK, AKT and PTP1B. Our data indicate that hyperinsulinaemia in OE mice was associated with upregulated PDX1 and a downregulated UCP2 protein in islets, and that hyperacetylation of H3 and H4 histone of *Pdx1* promoter in response to *Gpx1* overexpression was a novel regulatory mechanism for this key transcriptional factor in vivo.

Methods

Transgenic mice and animal care All mouse experiments were approved by the Institutional Animal Care and Use Committee at Cornell University. The OE mice were derived from a B6C3 (C57B1×C3H) hybrid line (Taconic, Germantown, NY, USA) [35] and carried three additional copies of the *Gpx1* gene. The wild-type (WT) was derived from the non-transgenic littermates. Elevated GPX1 activity in the OE mice was confirmed in a number of tissues and showed no significant effect on other selenoproteins and antioxidant enzymes [35]. All mice were weaned at 3 weeks of age, given free access to a Torula yeast and sucrose-based diet (0.4 mg Se/kg) [35] and distilled water. Mice were individually reared in plastic cages in an animal room with constant temperature (22°C) and a 12 h light–dark cycle. All assays were conducted with male mice at 6 months of age unless otherwise indicated.

Diet restriction experiment A total of 12 WT and 12 OE male mice (2 months of age) were fed 5 g (full-fed) or 3 g

(diet-restricted) of the diet per day [36]. During the 4 month trial, body weights of mice were recorded monthly. Fasting (8 h, overnight) blood glucose concentrations of mice were measured monthly from tail blood using a glucometer (Bayer, Elkhart, IN, USA). At the end of the trial period, all mice were killed to collect blood (plasma), liver, pancreas and gastrocnemius muscle to assay for activities of GPX1, GPX3, thioredoxin reductase and total superoxide dismutase [10]. At 1 week prior to the end of the study, fasted mice were tested for insulin tolerance (0.5 units per kg body weight; Humulin R; Eli Lilly, Indianapolis, IN, USA), glucose tolerance (1 g/kg, D-glucose) and GSIS (1 g/kg) (tests conducted at 2 day intervals). Plasma insulin concentration was determined using a rat/mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) [10].

In vitro experiments and assays Detailed experimental protocols and complete information on reagents, materials and instruments are described in Electronic supplementary material (ESM). Briefly, total pancreatic insulin concentration was determined ($n=4$ mice per genotype) using the above-described kit after acid-ethanol extraction [37]. Pancreatic beta cell mass was determined by immunostaining of paraffin-embedded pancreatic sections ($n=3$ mice × three slides per genotype) [38] with a guinea pig polyclonal antibody against mouse insulin (Zymed, San Francisco, CA, USA). Immunostaining of pancreatic GPX1 protein ($n=3$ mice × three slides per genotype) was conducted using a rabbit polyclonal antibody against bovine GPX1 (Lab Frontier, Seoul, Korea). Islets were isolated from mice using a standard procedure [39] with minor modifications. Total GPX1 activity was assayed in homogenised islets isolated from the OE and WT mice ($n=3$ per genotype) [10]. For insulin secretion assays, islets (30 per sample, $n=3$ per genotype by treatment) were incubated for 1 h at 37°C in 1 ml Hanks' balanced salt solution containing 2.8 or 16.7 mmol/l glucose with or without 50 μmol/l H_2O_2. The released insulin (after 60 min incubation) in the medium supernatant fraction was quantified as described above. Intracellular ROS levels were detected using dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR, USA) [6]. Islet $\Delta\psi_m$ was determined using a mitochondrial membrane sensor kit (ApoAlert; Clontech Laboratories, Mountain View, CA, USA).

The mRNA levels of four genes in islets were determined by Q-PCR (7900HT; Applied Biosystems, Foster City, CA, USA). Total RNA was prepared from freshly isolated islets (200 per sample, $n=6$ mice per genotype) using Trizol (Invitrogen, Carlsbad, CA, USA). Protein concentrations of eight insulin-related signal molecules in homogenates of pancreas ($n=4$ per genotype) and islets (400 per sample, $n=4$ mice per genotype) were determined as previously described [10].

Two experiments were conducted to determine how elevated GPX1 activity in the OE islets affected acetylation of the H3 and H4 core histones in the *Pdx1* proximal promoter region [40] and also to determine their response to the H_2O_2-induced hypoacetylation. In both experiments, islets ($n=500$ per sample) were isolated from 16 mice ($n=4$ per genotype by age) and cultured for 48 h (RPMI 1640 medium) before the assay. In the second experiment, islets were further incubated with or without 50 μmol/l of H_2O_2 for 24 h. The histone cross-linking and chromatin immunoprecipitation (ChIP) were carried out by using a ChIP assay kit (Upstate/ Millipore, Temecula, CA, USA) following the manufacturer's instructions.

Ebselen, a seleno-organic compound that mimics the enzymatic function of GPX1 [41], was used to determine whether the decreased UCP2 protein levels in the OE islets were related to the enhanced GPX1 activity. Islets isolated from three WT mice were pooled and divided into three groups (200 islets per sample, $n=3$ per treatment): medium only (RPMI 1640, 10 mmol/l glucose and 10% [vol./vol.] fetal bovine serum), solvent vehicle control (0.25% [vol./vol.] DMSO) and 50 μmol/l ebselen. After 12 h incubation, islets were collected for western blot analysis of UCP2 as described above.

Statistical analyses Quantitative data were analysed using SAS (release 6.11; SAS Institute, Cary, NC, USA). Genotype or treatment effects were tested by one or two-way ANOVA and Student's *t* test. Data are presented as mean±SE and significance was set at $p<0.05$.

Results

Dietary restriction eliminated obesity, but not hyperinsulinaemia in OE mice Compared with the WT, the full-fed OE mice became heavier ($p<0.05$) (Fig. 1a) and exhibited hyperglycaemia ($p<0.05$) (Fig. 1c), insulin resistance (Fig. 1e) and hyperinsulinaemia with elevated GSIS (Fig. 1i) at various time-points. Diet restriction in the OE mice prevented all of these phenotypes (Fig. 1b,d,f) except for hyperinsulinaemia. The diet-restricted OE mice still had higher ($p<0.05$) plasma insulin concentrations at 0 (baseline, 66%) and 15 min (222%) after the glucose challenge (Fig. 1j) than did the WT mice. Despite slightly higher ($p<0.05$) blood glucose concentrations than WT at several time-points during the glucose tolerance test (Fig. 1g,h), the OE mice actually exhibited greater ($p<0.05$) glucose tolerance relative to their initial concentration (data not shown). Compared with the WT, the elevation of GPX1 activity in the OE mice (Table 1) was relatively greater in pancreas (23-fold) than in liver (24%) or muscle (4.2-fold), while there was no difference in activities of other enzymes.
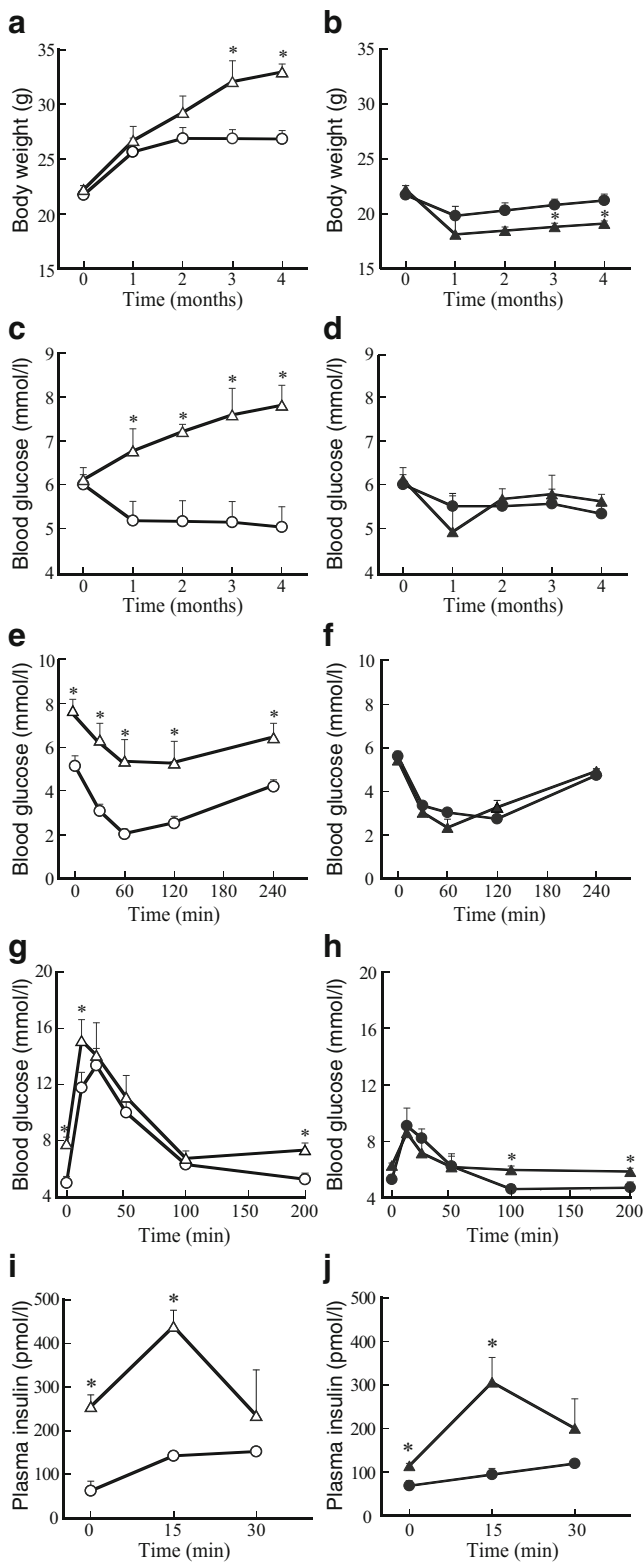
The OE mice had elevated beta cell mass, insulin synthesis and GSIS Pancreatic insulin content was 40% higher (*p*< 0.05) in the OE than the WT mice (Fig. 2a). After incubation with 2.8 and 16.7 mmol/l glucose, OE islets released 67 and 85% more (*p*<0.05) insulin into the media, respectively than those of WT (Fig. 2b). The H_2O_2 treatment decreased (*p*<0.05, 37%) GSIS in the WT islets treated with 16.7 mmol/l glucose, but not in the OE islets. Elevated GSIS was also seen in OE islets at 15 min incubation and different ages of mice (2 to 10 months old; data not shown). Pancreatic insulin staining showed a stronger colour and a larger area in the OE than in the WT mice (Fig. 2c). The beta cell mass represented only 0.48% of the total pancreas in the WT, but was elevated (*p*<0.05) to 1.27% in the OE mice.

Overproduction of GPX1 in the OE islets was accompanied by an attenuated ROS production and an elevated mitochondrial membrane potential The immunostaining of GPX1 protein in pancreatic sections (Fig. 3a) was stronger in the OE than in the WT mice. Islet GPX1 activity was 22-fold greater (*p*<0.05) in the OE than in the WT (Fig. 3b). As shown by the intensity of green fluorescence (Fig. 3c,), intracellular ROS production in the OE islets treated with 5 mmol/l glucose was lower than that of the WT islets. The green fluorescence in the WT islets was increased by exposure to 25 mmol/l glucose for 12 h and further intensified by treatment with 50 μmol/l H_2O_2 for 5 h. In contrast, these treatments in the OE islets induced no apparent increase in green fluorescence over baseline. At 5 mmol/l glucose, the OE islet image showed yellow instead of green fluorescence as in the WT, implying a greater $\Delta\psi_m$ in the OE than in the WT (Fig. 3d). At 25 mmol/l glucose, islet mitochondria from both genotypes were highly energised (red fluorescence).

The OE mice showed opposite changes in islet PDX1 and UCP2 The relative mRNA level of *Pdx1* in OE islets was approximately threefold (*p*<0.05) that in WT islets (Fig. 4). The mRNA levels of preproinsulin 1 and preproinsulin 2 in the OE islets were also double (*p*<0.05 to 0.07) those of the WT. In contrast, the mRNA level of *Ucp2* in the OE islets was 23% lower (*p*<0.05) than that in the WT islets. Western blot analyses of the islet homogenates indicated a 67% (*p*<0.05) increase in PDX1 and 31 to 57% decreases

Table 1 Effects of diet restriction and full feeding on activities of GPX1 and other enzymes in liver, pancreas, muscle and plasma of OE and WT mice

Parameter	Diet restriction		Full feeding	
	WT	OE	WT	OE
GPX1[a]				
Liver	798.4±36.2	991.2±147.1	807.3±42.6	993.5±40.4[†]
Pancreas	40.4±4.1[*]	762.1±133.6[*†]	50.3±1.4	1,366.5±69.1[†]
Muscle	69.6±1.6	289.1±14.0[†]	67.4±1.6	285.7±6.5[†]
Plasma GPX3[b]	45.5±2.3[*]	49.0±1.1[*]	39.0±1.9	36.8±2.2
Thioredoxin reductase[c]				
Liver	25.0±1.4	25.8±1.3	26.3±1.4	25.9±1.6
Pancreas	5.9±0.6[*]	7.4±1.0	9.5±0.8	7.5±0.5
Muscle	9.4±0.3[*]	9.7±0.6	7.9±0.5	8.2±0.4
Total SOD[d]				
Liver	913±23[*]	873±36[*]	1,038±23	1,115±44
Pancreas	405±36	434±22	502±24	470±24
Muscle	321±12[*]	323±4.2[*]	399±9.5	367±9.1

Values are mean±SE ($n=6$)

[a] Glutathione oxidised in nmol min^{-1} (mg protein)$^{-1}$

[b] Glutathione peroxidase 3 (GPX3), measured as glutathione oxidised in nmol min^{-1} (mg protein)$^{-1}$

[c] Measured as formation of 5′-thionitrobenoic acid in nmol min^{-1} (mg protein)$^{-1}$

[d] Superoxide dismutase (SOD), measured as 50% formazan dye formation rate inhibition per mg protein

[*] $p<0.05$ for diet-restriction effect within genotype; [†] $p<0.05$ for genotype effect vs WT within dietary treatment
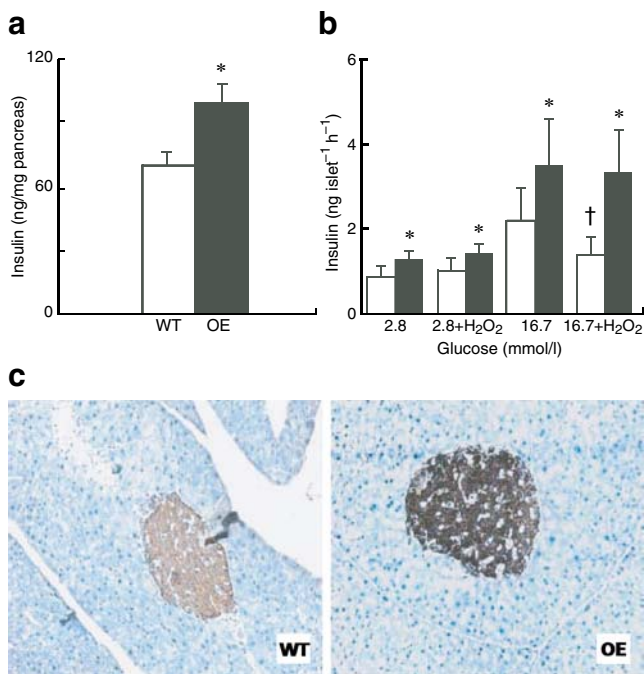

Fig. 2 Effects of *Gpx1* overexpression on pancreatic total insulin concentration (**a**), islet insulin secretion (**b**) and pancreatic beta cell mass (**c**) with representative image of insulin staining of pancreas sections and quantification of beta cell mass as indicated (WT, 0.48± 0.08%; OE, 1.27±0.36% of total pancreas). Data are mean±SE. [*]$p<0.05$ for difference between genotype (within treatment); [†]$p<0.05$ for H$_2$O$_2$ treatment effect in WT islets at the high glucose incubation. White bars, WT; black bars, OE

($p<0.05$) in UCP2, phosphorylated JNK, phosphorylated AKT on Thr-308, and PTP1B in the OE than the WT mice (Fig. 5a). Similar genotype differences in PDX1 and UCP2 proteins were detected in islets of 2-month-old mice (ESM Fig. 1). These assayed proteins were chosen to verify their genotype differences initially detected in the pancreas homogenates (ESM Fig. 2). In addition, the OE showed a 24% ($p<0.05$) decrease in total phosphorylated PDX1 protein in pancreas compared with WT, but the two genotypes had similar levels of pancreatic total JNK, total AKT and phosphorylated AKT on Ser-473. Compared with islets treated with medium only or the solvent vehicle-controls, the WT islets treated with 50 μmol/l ebselen had their UCP2 protein reduced to a minimal level (Fig. 5b). This implied that the decreased UCP2 protein in the OE islets was associated with elevated GPX1 activity.

The OE islets exhibited hyperacetylation of H3 and H4 histone at the proximal promoter of Pdx1 Both H3 and H4 acetylation were increased ($p<0.05$) in OE islets compared with WT islets (Fig. 6a,b). The genotype difference was numerically greater at 6 than at 2 months of age. Treating the islets with H$_2$O$_2$ decreased H3 and H4 acetylation by approximately 50% ($p<0.05$) in the WT, but not in the OE group (Fig. 6c,d). Apparently, hyperacetylation of H3 and H4 histone in the OE islets was associated with their enhanced capacity of H$_2$O$_2$ degradation resulting from *Gpx1* overexpression.

Discussion

Our most important finding is that hyperinsulinaemia in OE mice was not directly linked to obesity. Diet restriction eliminated obesity, hyperglycaemia and insulin resistance in the OE mice, but could not prevent fasting hyperinsulinaemia or elevated GSIS. In fact, similar outcomes were also produced by diet restriction of 6-month-old OE mice for 4 months (data not shown). Because the OE and WT mice shared similar activities in tissues for all the assayed antioxidant enzymes other than GPX1, hyperinsulinaemia

in the OE mice was mainly, if not solely, related to the elevated GPX1 activity. Without insulin resistance, hyperinsulinaemia in the diet-restricted OE mice was probably due to a non-compensatory overproduction and/or hypersecretion of insulin.

Indeed, the OE mice had a higher pancreatic beta cell mass and insulin content, and thus a greater capacity or potential for insulin production than the WT. The elevated islet mRNA levels of preproinsulin 1 and preproinsulin 2 were consistent with the increased pancreatic insulin content in the OE mice. Comparatively, neither baseline

Fig. 3 Effects of *Gpx1* over-expression on pancreatic GPX1 level (**a**), islet GPX1 activity (**b**), islet intracellular ROS production (**c**) and islet mitochondrial membrane potential (**d**). **a** Representative images of immunostaining of GPX1 protein in pancreatic sections of WT and OE mice as indicated. The dark brown staining in OE mice indicates a high level of GPX1 protein. Islet GPX1 activity (**b**) was enhanced 20-fold (*$p<0.05$) in the OE (black column) versus WT (white column) mice. **c** Decreased intracellular ROS production in islets of the OE compared with those of WT mice under various treatments as indicated. **d** OE islets had higher mitochondrial membrane potential than the WT islets under different treatments. In all panels, experiments were conducted on islets isolated from 6-month-old mice ($n=3$ to 5 per genotype). Scale bar, 10 μm. Magnification, 40×
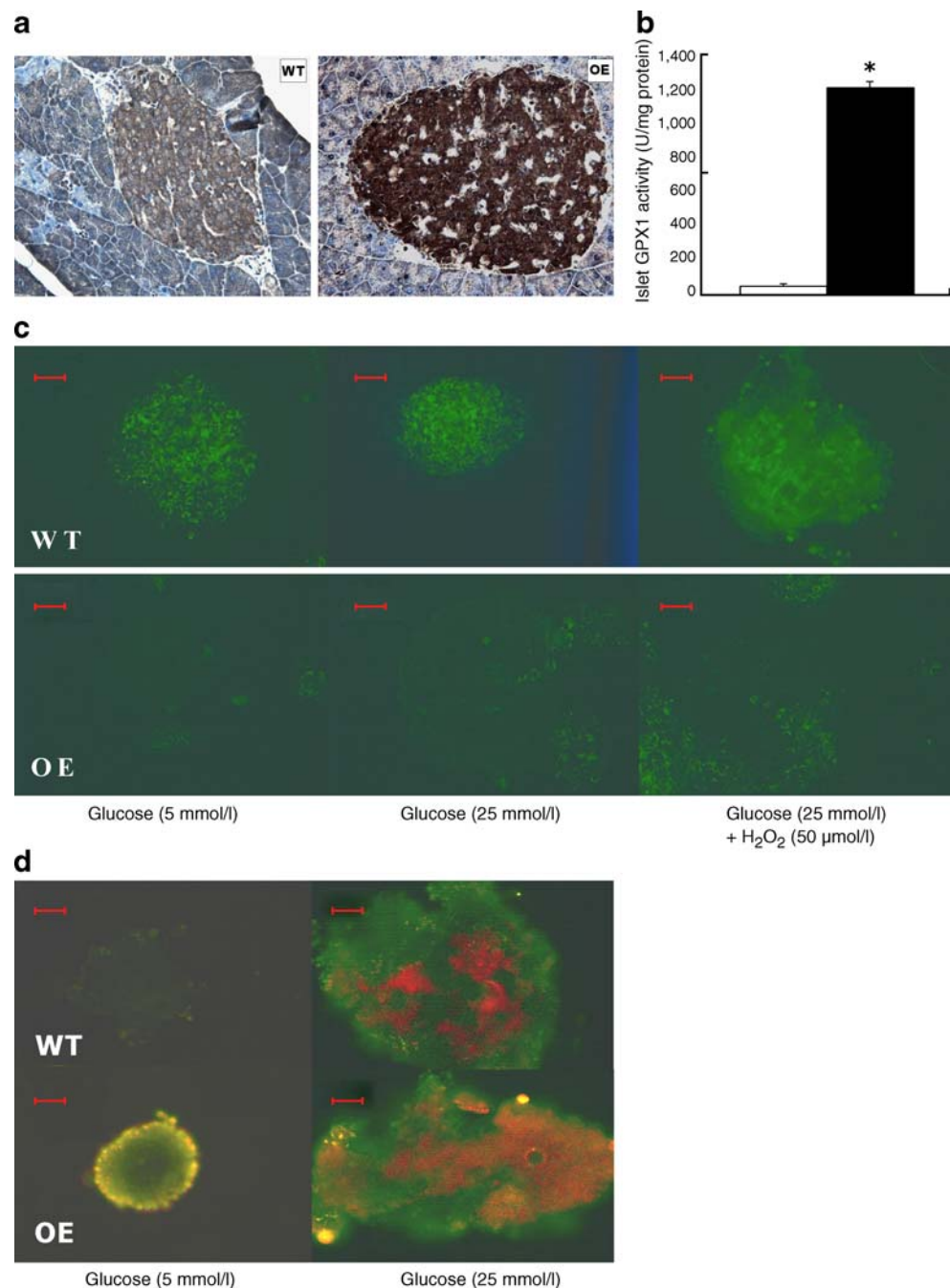
insulin concentration nor insulin gene expression in islets was altered by overexpressing catalase by up to 50-fold [9], two forms of metallothionein by up to 30-fold [9, 42] or three forms of superoxide dismutase enzymes by up to 10-fold [5, 7]. Furthermore, overproduction of metallothionein and catalase in beta cells actually accelerated cytokine-induced beta cell death [9]. Thus, the hypertrophic effect of GPX1 on beta cell mass and insulin synthesis is rather unique. Infection of rat islets with adenovirus encoding human *GPX1* gene protected against the ribose-induced loss of insulin mRNA, content and secretion. However, a sixfold increase in GPX1 activity and 72 h infection seemed to be insufficient to alter baseline levels of these three parameters [6, 24].

An enhanced functional PDX1 protein in the OE islets helps explain the hypertrophic effect of *Gpx1* overexpression on beta cell mass and insulin synthesis. Because in previous research only post-transcriptional regulation of *Pdx1* expression and function by ROS or antioxidant has been suggested [6, 20, 23, 25], our finding on the hyperacetylation of H3 and H4 histone at the proximal promoter of *Pdx1* in the OE mice reveals not only a novel, in vivo epigenetic, but also a potential transcriptional regulation for this key factor by antioxidant enzymes. Preceding transcriptional activation [43], hyperacetylation of H3 and H4 helps remodel the chromatin at *Pdx1* promoter to form a more accessible structure for transcription [44]. This modification helps explain the increased *Pdx1* mRNA levels in the OE islet and is strongly associated with the GPX1 function of H_2O_2 degradation. The latter statement is based on the negative correlation between histone acetylation and islet intracellular ROS production at 2 and 6 months of age, and on the fact that H_2O_2-induced histone hypoacetylation was prevented in the OE islets. Along with a modest decrease (24%) in pancreatic PDX1 phosphorylation (degradation) [25], the
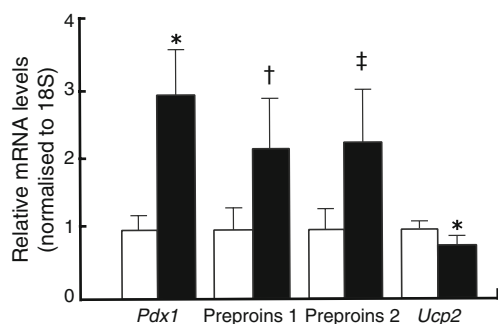
upregulation of *Pdx1* gene expression enabled the OE mice to maintain a higher level of functional PDX1 protein than the WT mice to promote beta cell differentiation and insulin synthesis. While similar positive effects on PDX1 protein were produced by three dietary antioxidant supplements in C57BL/KsJ-*db/db* mice [23], overproduction of catalase in beta cells accelerated cytokine-induced PDX1 protein disappearance [9]. It is intriguing to see such different impacts on PDX1 protein by GPX1 and catalase, given the so similar catalytic functions.

Decreased protein levels of phosphorylated JNK, phosphorylated AKT on Thr-308 and PTP1B in the OE islets were consistent with the attenuated ROS production, but presented mixed impacts on PDX1 abundance and stability. Suppressed phosphorylation of JNK was likely to promote PDX1 function by inhibiting ROS-induced nucleocytoplasmic translocation of PDX1 [20, 45] and to enhance *Pdx1*
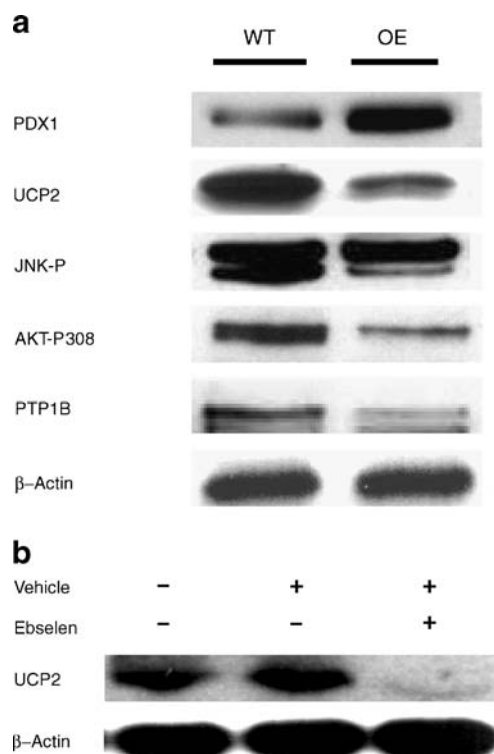

Fig. 5 Effects of *Gpx1* overexpression on islet protein levels of insulin-related signal molecules (**a**) and responses of islet UCP2 protein to GPX1 mimic ebselen (**b**). **a** Representative (*n*=4) immunoblots showing the protein levels (mean±SE, *n*=4) in OE islets relative to the WT islets (as 100%): PDX1 (167±17), UCP2 (43±16), phosphorylated JNK (JNK-P, 69±22), phosphorylated AKT on Thr-308 (AKT-P308, 49±27) and PTP1B (59±28) (all *p*<0.05 for difference between genotype). The relative protein level was normalised to that of ß-actin on the same membrane. **b** Representative (*n*=3) immunoblot showing responses of islet UCP2 protein to ebselen. The relative UCP2 protein level (mean±SE, *n*=3) was 100±5.4 for the blank control, 111.9±7.6 for the vehicle control, and 5.3±2.8 for the ebselen treatment, respectively (*p*<0.05 for effect of ebselen compared with vehicle and blank controls)

Fig. 4 Effects of *Gpx1* overexpression on islet mRNA levels of *Pdx1*, preproinsulin 1 (preproins 1), preproinsulin 2 (preproins 2) and *Ucp2* in 6-month-old mice. For each gene, the values were normalised to that of 18S rRNA in the same reaction. The gene expression level in the OE islets was presented as relative to the mean (set at 1) of WT. Data are means±SE (*n*=6). **p*<0.05; †*p*≤0.06; ‡*p*≤0.07 for difference between genotypes
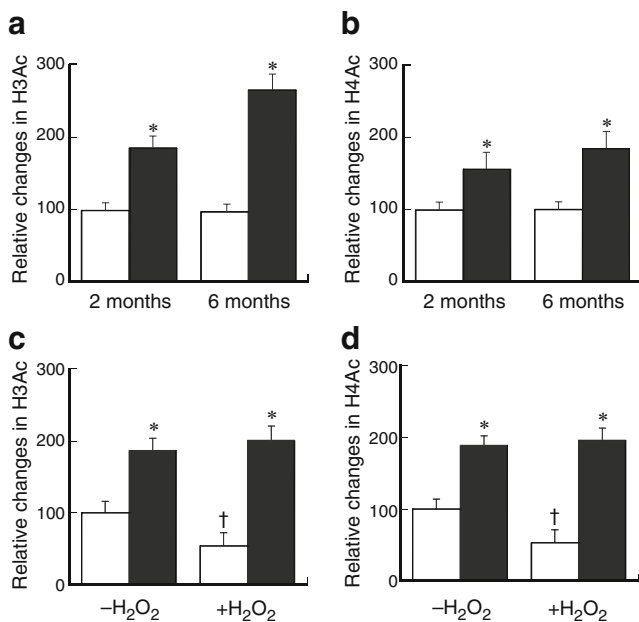
Fig. 6 Effects of *Gpx1* overexpression on islet H3 (**a**) and H4 (**b**) histone acetylation (Ac) in the proximal region of Pdx1 promoter in mice at 2 and 6 months of age ($n=4$ per genotype by age) and on the H_2O_2-induced hypoacetylation of islet H3 (**c**) and H4 (**d**) histone in 6-month-old mice ($n=4$ per genotype by treatment). Values are mean± SE ($n=4$) and expressed as per cent of WT. *$p<0.05$ for difference between genotypes within a given age or treatment; †$p<0.05$ for H_2O_2 treatment effect within the WT. White bars, WT; black bars, OE mice

transcription by protecting ROS-induced nuclear localisation of forkhead box O1 (an inhibitor of PDX1 transcription) [46]. The decreased levels of phosphorylated AKT on Thr-308 might attenuate H_2O_2-mediated phosphorylation (degradation) of PDX1 [25], but could also inhibit *Pdx1* transcription via reduced phosphorylation of forkhead box O1 [9, 47]. While the decrease of PTP1B protein with concomitant increases of PDX1 protein, beta cell mass and insulin synthesis in the OE islets resembled the phenotype of PTP1B-null mice [32], it does not support a possible increase of ROS inhibition of PTP1B as the ultimate mechanism for suppressing the AKT/PDX1 pathway by catalase overexpression [9].

The decreased expression of *Ucp2* mRNA and UCP2 protein in the OE islets may explain the enhanced $\Delta\psi_m$ [18] and the elevated GSIS [48]. A strong link between decreased levels of UCP2 and *Gpx1* overexpression is supported by the response of the WT islets to GPX1 mimic, ebselen, and the resistance of OE islets to H_2O_2-suppressed GSIS. However, the UCP2 decrease may not be simply attributable to the diminished intracellular ROS because of a possible two-way feedback mechanism for the interaction of UCP2 and H_2O_2 [49]. While expression and function of UCP2 are activated by ROS [21], mitochondrial H_2O_2 generation can be decreased by UCP2 [49]. Therefore, the concurrent decreases in both intracellular ROS and UCP2

in OE mice may reflect a disruption of the feedback mechanism. Probably, the overly scavenging intracellular H_2O_2 in islets of the OE mice allowed co-existence of highly coupled or energised mitochondria with lower levels of UCP2, nudging up the well-controlled GSIS and contributing to hyperinsulinaemia [18]. *Ucp2*, coincidentally, is located in regions of human chromosome 11 and mouse chromosome 7, which have been linked to hyperinsulinaemia and obesity [18].

In summary, chronic hyperinsulinaemia in OE mice was associated with a dysregulated functional production of PDX1 and UCP2. It is striking that seemingly beneficial effects of GPX1 on these two key factors, beta cell mass, and insulin synthesis and secretion eventually led to chronic hyperinsulinaemia. Apparently, our in vivo results are in stark contrast to the transient benefits of upregulating antioxidant capacity as shown in vitro [6, 7] or during short-term situations [23]. Therefore, this study reveals a long-term metabolic risk of disturbing the balance between intracellular antioxidant defence and ROS formation [9] and cautions against antioxidant strategies to prevent or treat diabetes [9, 50]. We have illustrated a decreased UCP2 protein concomitant with an increased mitochondrial potential in the OE islets, suggesting a new function of GPX1 in mitochondria. In contrast to the postulated post-transcriptional regulation of PDX1 [6, 20, 23, 25], we have revealed that histone hyperacetylation occurs at the *Pdx1* promoter and is a novel epigenetic regulation mechanism of the gene in vivo. This will offer a new view for interpreting outcomes associated with long-term human interventions of antioxidant supplementation to reduce insulin resistance and diabetes. Separating hyperinsulinaemia from obesity and insulin resistance in diet-restricted OE mice may help develop a specific model for the study of hyperinsulinaemia.

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