The reductase NCB5OR is responsive to the redox status in β-cells and is not involved in the ER stress response

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The novel reductase NCB5OR (NADPH cytochrome b_5 oxidoreductase) resides in the ER (endoplasmic reticulum) and may protect cells against ER stress. Levels of BiP (immunoglobulin heavy-chain-binding protein), CHOP (CCAAT/enhancer-binding protein homologous protein) and XBP-1 (X-box-binding protein-1) did not differ in WT (wild-type) and KO (*Ncb5or*-null) tissues or MEFs (mouse embryonic fibroblasts), and XBP-1 remained unspliced. MEFs treated with inducers of ER stress demonstrated no change in *Ncb5or* expression and expression of ER-stress-induced genes was not enhanced. Induction of ER stress in β-cell lines did not change *Ncb5or* expression or promoter activity. Transfection with *Ncb5or*-specific siRNA (small interfering RNA) yielded similar results. Microarray analysis of mRNA from islets and liver of WT and KO animals revealed no significant changes in ER-stress-response genes. Induction of oxidative stress in βTC3 cells did not alter *Ncb5or* mRNA levels or promoter activity. However, KO islets were more sensitive to streptozotocin when compared with WT islets. MEFs incubated with nitric oxide donors showed no difference in cell viability or levels of nitrite produced. No significant differences in mRNA expression of antioxidant enzymes were observed when comparing WT and KO tissues; however, microarray analysis of islets indicated slightly enhanced expression of some antioxidant enzymes in the KO islets. Short-term tBHQ (t-butylhydroquinone) treatment increased *Ncb5or* promoter activity, although longer incubation times yielded a dose-dependent decrease in activity. This response appears to be due to a consensus ARE (antioxidant-response element) present in the *Ncb5or* promoter. In summary, NCB5OR does not appear to be involved in ER stress, although it may be involved in maintaining or regulating the redox status in β-cells.

Key words: antioxidant, β-cell, endoplasmic reticulum, NADPH cytochrome b_5 oxidoreductase (Ncb5or), oxidative stress, reductase.

INTRODUCTION

The novel reductase NCB5OR (NADPH cytochrome b_5 oxidoreductase; also known as b5 + b5R, Cyb5r4 [1]) is critical for β-cell survival. KO (*Ncb5or*-null) mice are viable and initially have normal blood glucose levels [2]. However, at around 4 weeks of age, these mice begin to lose β-cells from the pancreatic islets. At 6 weeks of age, these mice are hyperglycaemic, owing to a decrease in serum insulin. Insulin tolerance in these animals is normal [2]. NCB5OR resides in the ER (endoplasmic reticulum) [3], a site in the β-cell which is under considerable oxidative stress [4]. The β-cell displays exceptional biosynthetic capability with production of insulin, while retaining tight quality control to ensure it is folded properly with appropriate disulfide bond formation. Several mechanisms enable the β-cell to cope should the process be disrupted. When misfolded protein accumulates, a signal triggers transcription of ER chaperone proteins, including BiP (immunoglobulin heavy-chain-binding protein)/Grp78 (glucose-regulated protein of 78 kDa). Accumulation of misfolded and denatured protein beyond a certain threshold triggers apoptosis, through such mediators as the transcription factor CHOP (CCAAT/enhancer-binding protein homologous protein). Since NCB5OR is a reducing enzyme that resides in the ER, we hypothesized that it was involved in the ER-stress-response pathway, functioning to protect the cell from excess build-up of ROS (reactive oxygen species). Supporting this idea, KO mice display enhanced sensitivity to the β-cell toxin STZ (streptozotocin) [2]. The present study examined the role of NCB5OR and regulation of its gene expression following exposure to oxidant and ER stress in a variety of model cell systems. We have also examined the response of antioxidant enzymes in tissues and cells lacking NCB5OR.

EXPERIMENTAL

Northern blotting

Total RNA was isolated from tissues of WT (wild-type) and KO mice using a standard TRIzol® protocol and separated on 1.25 % agarose formaldehyde denaturing gels (15 μg per lane). The gels were blotted on to nylon membranes (GE Healthcare) and fixed with 0.05 M NaOH. Pre-hybridization of blots was carried out at 55 °C with Ultrahybe buffer (Ambion) in a hybridization oven for >30 min. Radiolabelled probe was synthesized from cDNA template using random priming (Megaprime, GE Healthcare) and [α-^{32}P]dCTP (GE Healthcare). Probe was boiled for 5 min, snap-cooled on ice, then added to the hybridization solution. Hybridization was conducted at 43 °C overnight (at least 16 h), followed by washing four times for 10 min with Northern blot wash solution [0.1× SSC (1× SSC is 0.15 M NaCl/0.015 M

Abbreviations used: ARE, antioxidant-response element; BiP, immunoglobulin heavy-chain-binding protein; CHOP, CCAAT/enhancer-binding protein homologous protein; CYGB, cytoglobin; DETA, diethylenetriamine; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; ER, endoplasmic reticulum; ERSE, ER-stress-response element; FBS, fetal bovine serum; GPX, glutathione peroxidase; Grp78, glucose-regulated protein of 78 kDa; MEF, mouse embryonic fibroblast; NAC, *N*-acetylcysteine; Ncb5or, NADPH cytochrome b_5 oxidoreductase; KO, *Ncb5or*-null; PAO, phenylarsine oxide; RNAi, RNA interference; ROS, reactive oxygen species; RT, reverse transcription; siRNA, small interfering RNA; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; STZ, streptozotocin; tBHQ, t-butylhydroquinone; WT, wild-type; XBP-1, X-box-binding protein-1.

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sodium citrate) and 0.1 % SDS] at 55 °C. Blots were exposed to a phosphoimager screen, and densitometric analysis was performed using Imagequant (GE Healthcare).

Semi-quantitative RT (reverse transcription)–PCR

Total RNA was isolated from livers of 4- and 6-week-old WT and KO mice using a standard TRIzol® protocol and resuspended in double-distilled water. RT was performed in a thin-walled PCR tube in a final volume of 20 μl, consisting of 1× RT buffer (Promega), oligo-dT primer (1 μM), dATP/dTTP/dGTP (0.5 mM each) and total RNA (1 μg). The reaction mixture was placed in a heating block and RT–PCR was initiated: 70 °C for 10 min, 62 °C for 2.5 min and 37 °C for 5 min. AMV (avian myeloblastosis virus) reverse transcriptase (25 units) was added to the tube to begin RT, and the reaction mixture was incubated at 42 °C for 1.5 h. The temperature of the heating block was changed to 94 °C for 10 min, followed by cooling of the reaction mixture on ice. The sample cDNA was diluted by adding double-distilled water (30 μl) and a 4 μl aliquot was placed into a new tube for PCR. PCR was performed in a final volume of 20 μl with gene-specific primers for each gene (1 μM), 1× PCR buffer, dNTPs (0.25 mM each), and Taq (0.5 μl), using a hot-start protocol (2 min at 94 °C, then 24–35 cycles as indicated of 94 °C for 20 s, 56–60 °C for 1 min and 72 °C for 1.5 min, then 5 min at 72 °C). Samples were separated on a 1.25 % agarose gel and visualized with ethidium bromide staining.

MEF (mouse embryonic fibroblast) preparation

MEFs were prepared from 13.5-day-old embryos of WT and KO Balb/c mice using trypsin digestion. Freshly digested cells were incubated in DMEM (Dulbecco's modified Eagle's medium) (high glucose, pyridoxine/HCl and sodium pyruvate; without glutamine) supplemented with FBS (fetal bovine serum; Gibco/Invitrogen) (10 %), L-glutamine (2 mM), and penicillin/streptomycin (100 μg/ml). Experiments were initiated from the first passage of MEFs (P1) and cells were passaged no more than three times for experiments examining ER stress, NO donors or antioxidant enzyme gene expression.

Cell culture, transfection and promoter activity measurements

Promega's luciferase assay system was used to analyse the *Ncb5or* promoter [5] according to the manufacturer's instructions. Mouse βTC3 insulinoma cells were grown in 1× DMEM (4.5 g/l glucose, L-glutamine and sodium pyruvate; Mediatech) supplemented with 10 % FBS and 1× penicillin/streptomycin antibiotics (Gibco/Invitrogen) at 37 °C under 5 % CO_2. Cells were seeded on to 24-well plates and incubated for 24 h before transfection of experimental plasmid (0.25 μg) and pSV-β-galactosidase (0.2 μg), included as an internal control to standardize transfection efficiency. For each transfection, 50 μl of OptiMEM (without serum; Invitrogen), 1–2 μl of plasmids and 1 μl of Lipofectamine™ (Invitrogen) were pre-incubated at room temperature (24 °C) for 15 min. The DNA/Lipofectamine™ mixture was added dropwise to the βTC3 cells, which contained 300 μl of OptiMEM medium/well. Chemical treatments were initiated at the appropriate time before cell harvesting to allow for the incubation times indicated: tunicamycin (1.5 μg/ml; 4 h), calcium ionophore A23187 (7 μM; 4 h), $NaAsO_4$ (100 μM; 1 h) and tBHQ (t-butylhydroquinone) (1–100 μM; 1–16 h). At 24 h post-transfection, cell lysates were prepared using 1× reporter lysis buffer (200 μl; Promega) and transferred to pre-chilled tubes on ice. All subsequent manipulation steps were performed on ice. Clarified samples (50 μl) were transferred into an opaque 96-well plate (Costar). Plates were scanned using a Fluostar luminometer set for a 0.2 s delay with light production measured for a period of 20 s. β-Galactosidase activity was measured from the same sample (5 μl) by means of a colorimetric assay and was used to normalize the luciferase values. At least four or more independent transfections were evaluated for each construct.

EMSAs (electrophoretic mobility-shift assays)

EMSAs were performed as described previously [5]. Briefly, complimentary oligonucleotides containing built-in 3′-overhangs were mixed together in a 1:1 molar ratio, incubated in a beaker of boiling water for 5 min and were allowed to slowly cool to room temperature. Annealed oligonucleotides (1 μg) were incubated with 1× Klenow buffer, Klenow enzyme (1 unit), dNTPs (dATP, dCTP, dGTP; 0.1 mM each) and [^{32}P]dCTP (15 μCi) in a final volume of 20 μl for 15 min at 30 °C. The reaction was terminated with addition of 1 μl of 0.5 M EDTA, and unincorporated nucleotides were removed using G50 spin columns (Roche). EMSA mixtures consisted of 7.5 μg of βTC3 nuclear extract in a binding buffer containing 20 mM Hepes pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 4 % glycerol, in a final reaction volume of 20 μl. Poly(dI-dC) • (dI-dC) (150 ng) and a non-specific oligonucleotide duplex (5′-CTCCGCATCCGATCCGATTC-3′) were included in each incubation to reduce non-specific interactions. Reactions were incubated on ice for 10 min, followed by addition of labelled probe with continued incubation for an additional 20 min at room temperature. Immediately following incubation, samples were loaded on to a native 5 % polyacrylamide gel (pre-run for 30 min) and electrophoresis was performed at 30 mA for 4–5 h in 0.25× TAE running buffer (6.7 mM Tris, pH 7.9, 3.3 mM sodium acetate and 1 mM EDTA). The gel was vacuum-dried for 1 h at 80 °C, placed on a phosphoscreen overnight, and analysed using a Typhoon 9410 phosphoimager.

RNAi (RNA interference) experiments and cell treatments

All chemicals were obtained from Sigma–Aldrich, unless indicated otherwise. Before transfection, βTC3 cells were grown to confluence and were incubated in fresh medium overnight at 37 °C. The following day, cells were trypsinized, counted and resuspended in an appropriate volume of 2× HEBS buffer (50 mM Hepes, pH 7.4, 280 mM NaCl and 1.5 mM Na_2HPO_4). The suspension was split evenly into cuvettes with *Ncb5or* specific (5′-AUGGCAAACAGGGACACAU-3′; 5′-GGGUCAAUUA-UGAAUCCAUGC-3′) or scrambled control (5′-CCGUAUU-GGAUGUAUCAGAAC-3′) siRNA (small interfering RNA) oligonucleotides (0.2 nmol/million cells; Dharmacon) and/or *Ncb5or* specific morpholino oligonucleotide (5′-CTGGGAA-GGGACGTTCAGCATG-3′; 0.2 nmol/million cells; Gene Tools) and electroporation was carried out at 280 V. Cells were seeded into six-well plates and were incubated for 72 h followed by standard TRIzol® RNA isolation. For chemical treatments (1 μg/ml tunicamycin or 2 μM A23187; 18 h) incubation was timed such that RNA would still be harvested at 72 h post-transfection.

For cell induction assays, βTC3 or MIN6 cells were seeded into six-well plates and grown to ~80 % confluence. Cells were washed once with PBS, followed by addition of serum-free DMEM to each well with subsequent addition of the compound being tested at the concentrations indicated. Compounds included alloxan, STZ, tunicamycin, A23187, thapsigargin, NAC (*N*-acetylcysteine), $NaAsO_4$, PAO (phenylarsine oxide), $ZnCl_2$, $CdCl_2$, $HgCl_2$ and tBHQ. Cells were incubated for the times outlined in each respective Figure, followed by total RNA isolation and Northern blotting. Expression levels of probes

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were normalized using 36B4 (which encodes acidic ribosomal phosphoprotein) as a constitutively expressed gene.

Oxidative stress and β-cells

Islets were isolated from WT or KO animals by perfusing the pancreas with liberase (via the bile duct) followed by standard digestion for 18 min at 37 °C and washing with PBS. Islets were separated by differential centrifugation through a Ficoll density gradient (900 ***g*** at 4 °C for 20 min, no brake), hand picked and washed in a Petri dish containing DMEM. Islets (>150 for each treatment) were dispersed into single β-cells using a syringe and needle. Dispersed cells were transferred into wells of a 96-well plate and treated with vehicle alone (PBS) or STZ (5 mM) for 16 h. Viability assays were performed by mixing collected cells with a 0.4 % (w/v) Trypan Blue solution (1:1). The counting chamber of a haemocytometer was loaded with each sample, and the number of stained and unstained cells was counted and the total number of cells was determined. The percentage of unstained cells was calculated for $n = 3$ samples (>50 cells counted per sample) giving the percentage of viable cells.

NO donor assays

MEFs isolated from WT or KO animals and βTC3 insulinoma cells were seeded into a 96-well plate (20 000 cells/well for MEFs and βTC3 cells; 9000 cells/well for dispersed β-cells) in a total volume of 100 μl and cultured overnight in complete DMEM medium (10 % FBS; MEFs and βTC3 cells) or in complete RPMI 1640 medium (10 % FBS; dispersed β-cells). Before NO treatment, medium was changed (100 μl). A stock solution was made of SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine) (0.1 M in ethanol) or DETA (diethylenetriamine) NONOate (0.1 M in 0.01 M NaOH). βTC3 cells were treated with varying SNAP concentrations (0–1.0 mM) for a time course of 1–6 h for Northern blotting and 6 h for MEF viability assays. MEFs were also treated with various DETA NONOate concentrations (0–5.0 mM) for an incubation time of 24 h. Viability assays were performed by trypsinizing cells and mixing (1:1) with a 0.4 % (w/v) Trypan Blue solution. The counting chamber of a haemocytometer was loaded with each sample and the number of stained and unstained cells was counted and a total number of cells was determined. The percentage of unstained cells was calculated for $n = 3$ samples (> 200 cells counted per sample) for MEFs and for $n = 1$ sample (25–100 cells counted per treatment) for β-cells, giving the percentage of viable cells. For the DETA NONOate incubation experiments with the MEFs, medium was removed from each of the wells and immediately frozen in liquid nitrogen. Samples were later used to determine total nitrite using a nitrate/nitrite colorimetric assay kit (Cayman Chemical). The kit provides a standard Griess reaction (after conversion of nitrate into nitrite).

Assay for CYGB (cytoglobin) reductase and nitrate reductase

Human full-length NCB5OR recombinant protein, prepared as described previously [3], was used (50 nM) to catalyse the NADH consumption (100 μM) in the presence of CYGB or nitrate *in vitro*. The rate of NADH consumption was used to quantify the electron transport between NCB5OR and CYGB or nitrate.

Microarrays

Samples were prepared from 4.5-week-old (islets; pre-diabetic) or 12-week-old (liver; diabetes corrected with islet transplant [6]) male litter-matched WT and KO mice. Islets were incubated in RNALater (Qiagen), and frozen at −80 °C. Total RNA was isolated from islets and liver using the Qiagen mini-RNA isolation kit, with quality and concentration determined via Bioanalyzer Quality Control analysis. Islet samples containing RNA of sufficient quality and concentration (>100 ng) were selected for further amplification using the RiboAmp HS RNA amplification kit (Arcturus). Hybridization of samples to Affymetrix mouse expression arrays (430A 2.0) was performed at the Harvard Partners Center for Genetics and Genomics (HPCGG).

RESULTS AND DISCUSSION

ER stress and the *Ncb5or*-null model

Since NCB5OR resides in the ER, we hypothesized that this enzyme may protect cells against ER stress. The mRNA expression of markers of ER stress was examined in selected tissues of our *Ncb5or*-null model system via Northern blotting. The ER chaperone protein, BiP (also known as Grp78) serves as a sensor and regulator of the ER stress response [7]. CHOP is a transcription factor that is normally expressed at very low levels, but is markedly up-regulated with ER stress [8]. BiP and CHOP expression did not differ when comparing mRNA levels in WT and KO tissue samples (Figure 1A). Semi-quantitative RT–PCR was performed on liver mRNA samples to confirm Northern blotting results showing that levels of ER stress markers did not change. Control reactions that varied initial concentration of cDNA and PCR cycle number were performed with each gene to determine optimal conditions for analysis (a sample reaction for BiP is shown in Figure 1B).

XBP-1 (X-box-binding protein-1) mRNA exists in the cell as a ‘precursor’ that, during ER stress, is cleaved by IRE-1 (insulin-response element 1) resulting in a transcript containing a frameshift. This ‘mature’ transcript encodes XBP-1, which is a transcription factor that regulates a subset of ER-resident chaperone genes that are essential for protein folding, maturation and degradation in the ER [9]. Expression of XBP-1 mRNA was examined in WT and KO liver samples. Using a size differential assay [10], we were able to detect the unspliced 254 bp band (Figure 1C). The smaller 229 bp band was not detected, suggesting absence of ER stress. The relative levels of XBP-1 also remained unchanged. BiP and CHOP expression did not differ in liver samples prepared from animals aged 4 (pre-diabetic) and 6 (onset of hyperglycaemia; loss of β-cells) weeks, confirming the Northern blotting result. Similar analysis was performed on WT and KO MEFs. MEFs were treated for 6 h (results not shown) and 18 h (Figure 1D) with known inducers of ER stress, including tunicamycin, A23187, and thapsigargin. No change was observed in *Ncb5or* expression in response to treatment, and the expression of ER-stress-induced genes was not enhanced under these conditions (Figure 1D). No change in viability was evident (results not shown).

Examination of ER stress and the role of NCB5OR in the β-cell

It is possible that ER stress may only occur in the β-cell where the primary diabetic phenotype is observed. βTC3 cells, an insulin-producing β-cell line, were treated with tunicamycin and A23187 to determine whether *Ncb5or* was differentially expressed in response to acute ER stress in insulinoma cells. As expected, BiP and CHOP were up-regulated by these inducers of ER stress. However, Ncb5or expression was unchanged (Figure 2A). To confirm this result, an *Ncb5or* promoter construct attached to luciferase was transfected into βTC3 cells and exposed to a similar induction treatment. Promoter activity, measured as luciferase activity, did not change with tunicamycin or A23187 treatment (Figure 2B). The promoter for *Ncb5or* was examined

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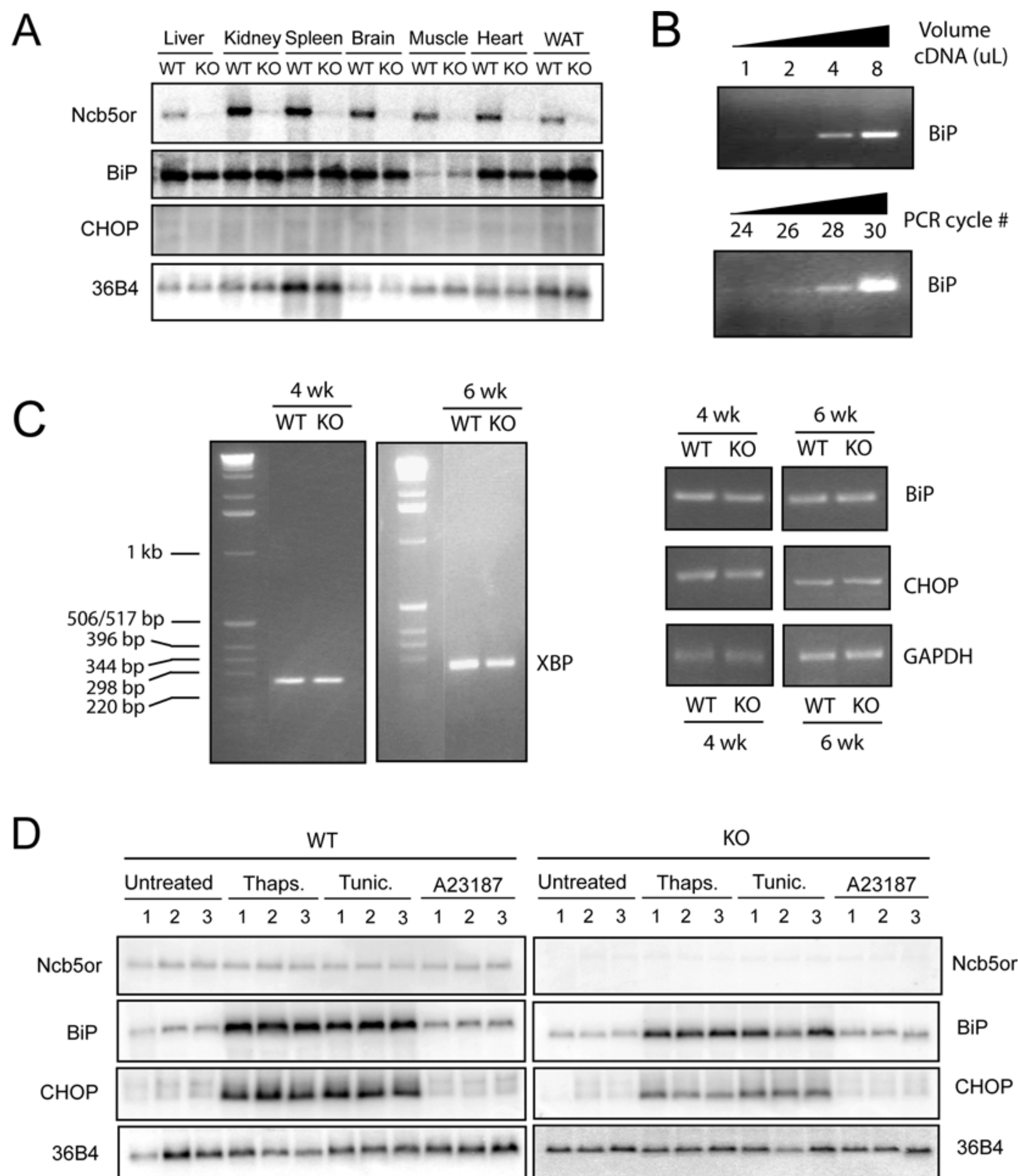

Figure 1 Examination of the ER stress response in the presence and absence of NCB5OR

(**A**) BiP and CHOP expression was determined in tissues of WT and KO mice with Northern blotting. WAT, white adipose tissue. Standardization and validation of semi-quantitative RT–PCR was completed using BiP (**B**) and then used to examine the expression of ER stress markers XBP-1, BiP and CHOP in RNA prepared from the liver of WT and KO animals (**C**). Samples were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and actin (not shown). (**D**) BiP and CHOP were examined in MEFs from Ncb5or WT (left-hand panel) and KO (right-hand panel) mice treated with the ER-stress-inducing agents thapsigargin (Thaps.; 2 μM; 18 h), tunicamycin (Tunic.; 1 μg/ml; 18 h) and A23187 (2 μM; 18 h).

for a potential ERSE (ER-stress-response element) containing the consensus sequence CCAATN$_9$CCACG [11]. No such element was detected, supporting the absence of a transcriptional response by *Ncb5or* to induced ER stress.

RNAi was used in combination with translation-blocking morpholinos to determine whether a lack of NCB5OR results in an increase in the ER stress response. Transfection of βTC3 cells with *Ncb5or*-specific siRNA sequences effectively knocked down *Ncb5or* expression to barely detectable levels (Figure 2C). Cells were then treated with the same inducers as above to determine if the ER stress response was altered when *Ncb5or* is knocked down. The induction of these markers was not affected by suppression of *Ncb5or* expression (Figure 2C), and cell viability was also unaffected (results not shown).

Since mRNA knockdown may not be 100 % and occurs within a short time frame, we examined islets and liver prepared from WT and KO animals using microarray technology. As expected, selected genes and pathways were differentially expressed. No significant changes occurred in genes known to be involved in the ER stress response in either islets or liver (Figure 2D), although a damage-specific DNA-binding protein was expressed at a higher level in the KO islets.

Although NCB5OR is located in the ER, the following evidence argues against involvement in the ER stress response. Analysis

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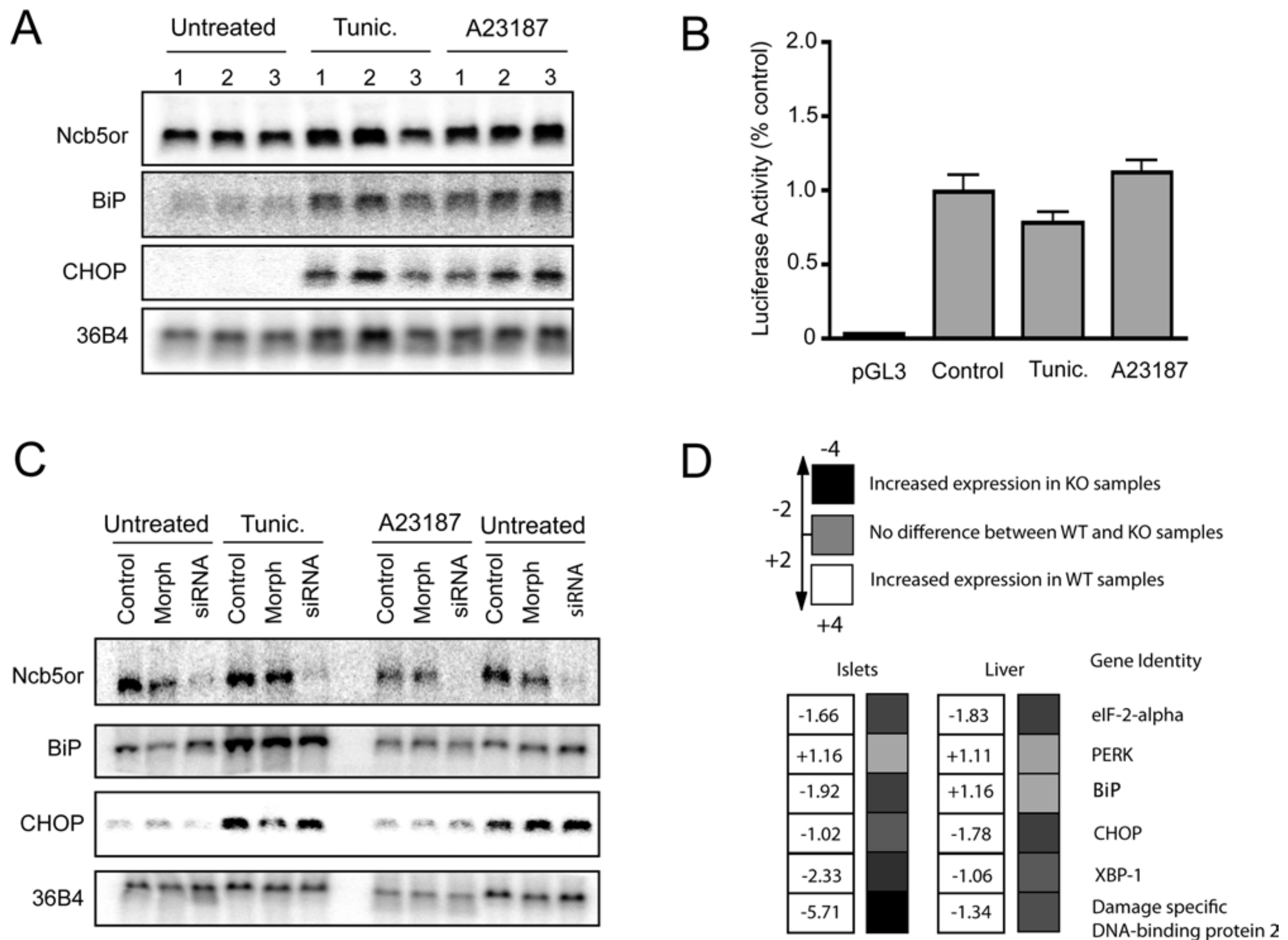

Figure 2 Examination of the ER stress response in insulinoma β-cells in the presence and absence of NCB5OR

(**A**) Effects of ER-stress-inducing agents on *Ncb5or* transcript expression in βTC3 cells. (**B**) βTC3 cells transfected with an *Ncb5or* promoter construct were incubated with vehicle (control) or ER-stress-inducing agents (tunicamycin/A23187), and luciferase activity was recorded. (**C**) *Ncb5or* transcript levels were knocked using siRNA oligonucleotides, in combination with *Ncb5or*-specific morpholino (Morph) and cells were exposed to the same ER-stress-inducing agents as in (**A**). The levels of ER stress marker genes were determined and normalized to those of 36B4. (**D**) Specific genes known to be involved in ER stress were examined in islets (left-hand panel) and liver (right-hand panel) prepared from WT and KO mice using Affymetrix microarrays. eIF2-alpha, eukaryotic initiation factor α; Tunic., tunicamycin.

of the *Ncb5or* promoter failed to identify an ERSE consensus sequence, and known inducers of ER stress did not affect *Ncb5or* expression in insulinoma cells lines. Additionally, a lack of NCB5OR in βTC3 cells, tissues of the KO mouse and KO MEFs did not appear to exacerbate the endogenous ER stress response or the response to ER-stress-inducing agents. Taken together, these results suggest that ER stress is not a likely mechanism for β-cell death in KO mice [2,3].

NCB5OR and the oxidative stress response

We next examined the effect of oxidative stress treatments on *Ncb5or* expression. Induction of oxidative stress in βTC3 cells with $NaAsO_4$ did not alter *Ncb5or* mRNA expression (Figure 3A) or promoter activity (Figure 3B). The same results were observed with other oxidative stress inducing compounds, including PAO, $ZnCl_2$, $CdCl_2$ and $HgCl_2$ (results not shown).

Alloxan and STZ are known β-cell toxins [12,13], and β-cells of KO mice display enhanced susceptibility to STZ [2]. Several lines of evidence indicate that free radicals play an essential role in the mechanism of DNA damage and cytotoxicity by STZ. It has been found that STZ enhances O_2 radical generation by the xanthine oxidase system of pancreatic β-cells [14] and stimulates H_2O_2 generation and causes DNA fragmentation in isolated rat pancreatic islets [15]. βTC3 (Figure 3B) and MIN6 cells (results not shown) were incubated with each of these compounds for 6–18 h at various concentrations. Neither compound induced a change in Ncb5or expression (Figure 3B). Islets prepared from WT and KO mice were dispersed into single β-cells and were incubated overnight (16 h) with 5 mM STZ. Viable cells were assessed following treatment and β-cells from KO islets were more sensitive to STZ when compared with β-cells from WT islets (Figure 3C).

NO formation is a source of reactive oxidant compounds and can lead to the development of insulin-dependent diabetes. We tested the hypothesis that NCB5OR can protect cells from NO accumulation, thus contributing to the cellular defence against oxidative stress. We examined the impact of NO on *Ncb5or* gene expression as well as the viability of KO cells. Incubation of βTC3 cells with the NO donor SNAP for a range of concentrations and time periods (Figure 4A) did not alter *Ncb5or* expression. When MEFs prepared from WT and KO animals were incubated

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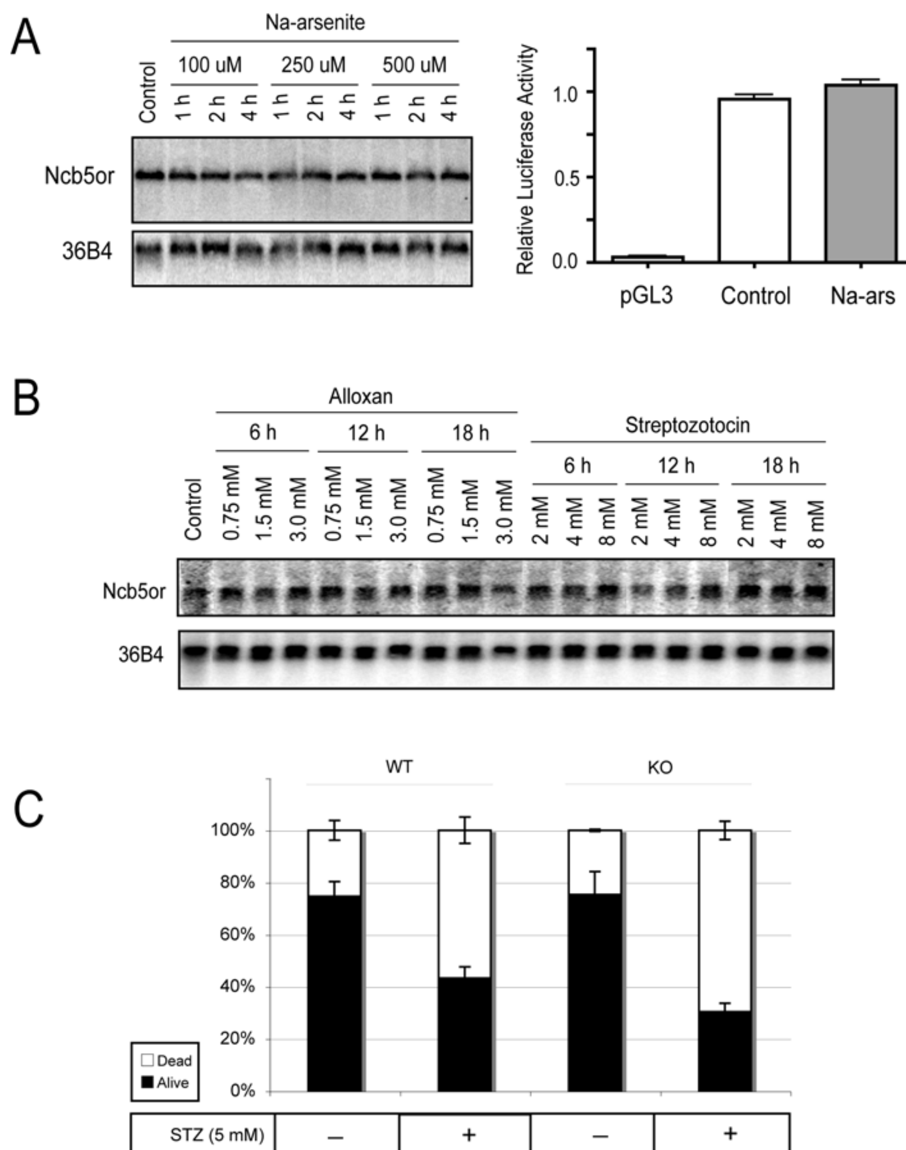

Figure 3 Effects of oxidant stress on *Ncb5or* transcript expression and promoter activity

(**A**) *Ncb5or* expression was determined following treatment of βTC3 cells with various concentrations of $NaAsO_4$ (Na-arsenite/Na-ars), for various incubation times (left-hand panel). *Ncb5or* promoter activity was determined in βTC3 cells following similar treatment (right-hand panel). (**B**) *Ncb5or* expression was determined following treatment of βTC3 cells with various concentrations of alloxan and STZ, for various incubation times. (**C**) Dispersed β-cells from WT and KO islets were incubated overnight with STZ (5 mM), and cell viability was determined.

with SNAP, we observed no difference in the number of viable cells (Figure 4B). Experiments were repeated with a longer-acting NO donor (NONOate). Similarly to SNAP treatment, no significant difference was observed between WT and KO MEF viability following 24 h of treatment (Figure 4C). Cells treated with 5.0 mM NONOate all died, regardless of genotype. Levels of nitrite produced during the incubation period were analysed, and no significant difference was observed (Figure 4D). This result argues against NCB5OR functioning as an NO dioxygenase.

NCB5OR bears sequence similarity to the single-domain cytochrome b_5 and its reductase, both of which have been shown to reduce methaemoglobin in red cells. The yeast flavohaemoglobin, a fusion protein containing both globin and b_5 reductase domains, has been shown to function as an NO dioxygenase. Therefore we examined whether NCB5OR can act as a reductase of met-CYGB and whether these two proteins together can function as an NO dioxygenase. Kinetic measurements for the NADH-consumption rate as a function of concentration of CYGB or NCB5OR showed K_m values of $>5\ \mu$M CYGB (with 100 nM NCB5OR) and $>0.2\ \mu$M NCB5OR (with 0.5 μM CYGB), and a k_{cat} of $<1\ s^{-1}$, suggesting that the electron-transport activity from NCB5OR to CYGB is too weak to be physiologically valid. In addition, *in vitro* pull-down of NCB5OR did not yield any detectable complex with CYGB, suggesting no physical interaction under our experimental conditions (results not shown).

Alternatively, NCB5OR may function as a nitrate reductase to remove the oxidized product of NO. This hypothesis arose from the fact that NCB5OR shares a similar structure with nitrate reductase in plants, both containing both cytochrome b_5 and cytochrome b_5 reductase domains. However, NCB5OR has a hinge ($\sim$90 residues), which is not present in nitrate reductase, while the latter has an additional domain at the N-terminus with

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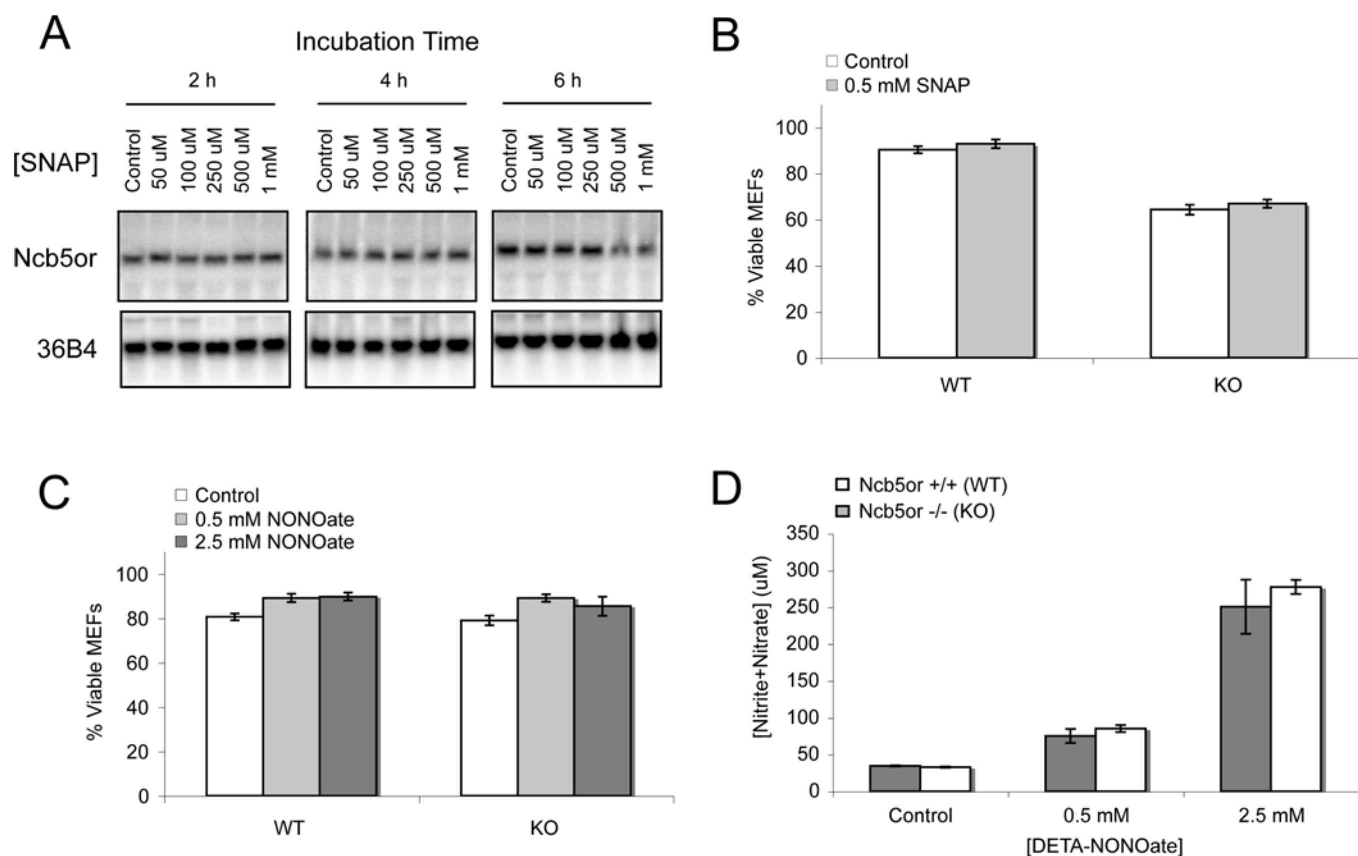

Figure 4 Examination of the response of NCB5OR to NO in β-cells and determination of MEF (WT and KO) viability following incubation with NO donors

(**A**) βTC3 insulinoma cells were treated with various SNAP concentrations (0–1.0 mM) for 1–6 h. The expression of *Ncb5or* was determined and normalized to control gene 36B4. WT and KO MEFs were treated with (**B**) SNAP (0.5 mM) or (**C**) various NONOate concentrations (0–5.0 mM) for incubation times of 6 and 24 h respectively. Viability assays (dye exclusion) were performed, and the number of viable cells was calculated. (**D** Medium was collected from the MEFs incubated with NONOate in (**C**), and total nitrite was determined using a standard Griess reaction (after conversion of nitrate into nitrite).

molybdenum as a cofactor. Kinetic measurements for the NADH-consumption rate as a function of nitrate concentration showed a K_m of >10 mM and a k_{cat} of <0.1 s^{-1} (with 50 nM NCB5OR), again an activity too weak to be physiological.

NCB5OR, antioxidants and cellular redox status

The KO mouse may have an impaired system for dealing with oxyradicals. Select tissues from WT and KO mice were examined for expression of known antioxidant enzymes, including Mn- and Cu/Zn-superoxide dismutase, GPX (glutathione peroxidase)-1 and catalase. No significant differences in mRNA expression were observed (Figure 5A). This result was confirmed using semi-quantitative RT–PCR analysis of RNA prepared from liver of 4- and 6-week-old WT and KO animals (Figure 5B). Microarray analysis indicated slightly enhanced expression of some antioxidant enzymes in the KO islets, GPX-2–4 in particular, with a slight reduction in the expression of catalase (Figure 5C). No change was observed in liver samples, with the exception of a slight increase in GPX-3 in the KO samples. We are currently breeding our KO allele into both ALR (alloxan-resistant) and ALS (alloxan-sensitive) mice [16] to see whether the onset of diabetes can be delayed.

It is possible that NCB5OR is involved in the antioxidant response in the cell. tBHQ is a phenolic antioxidant and electrophilic phase II enzyme inducer that has been reported to stimulate and/or repress transcription through binding at AREs (antioxidant-response elements) located in selected genes [17]. Treatment with tBHQ yielded an increase in promoter activity following a short incubation (1 h), although longer incubation times (2–4 h) yielded no change, and extended incubation times (6–16 h) demonstrated a decrease in activity (Figure 6A). This effect appeared to be dose-dependent (Figure 6B). However, when βTC3 and MIN6 cells were incubated with tBHQ for the same exposure times and tBHQ concentrations, *Ncb5or* mRNA expression did not change significantly (results not shown).

Since *Ncb5or* expression may be repressed following antioxidant exposure, we examined the mouse promoter for potential AREs. AREs are *cis*-acting sequences found in the 5′-regulatory region of a number of genes encoding enzymes involved in the phase II metabolism of xenobiotics [17]. AREs have been shown to be involved in the activation of a number of genes, many of which play a role in oxidation and reduction reactions [18] or are detoxifying enzymes [19]. The mouse *Ncb5or* sequence contains four putative ARE consensus sites designated ARE1–ARE4 (Figure 6C). When compared with the human *Ncb5or* promoter sequence, ARE2 is identical, ARE1 contains a single mismatch, and ARE3 and ARE4 are not present. Identity between the mouse and human sequence at ARE2 suggested this site may be important in the *Ncb5or* promoter. Probes representing each putative ARE (ARE1–ARE4) were used for EMSAs (Figure 6C). A specific complex was observed only with the ARE2 probe using

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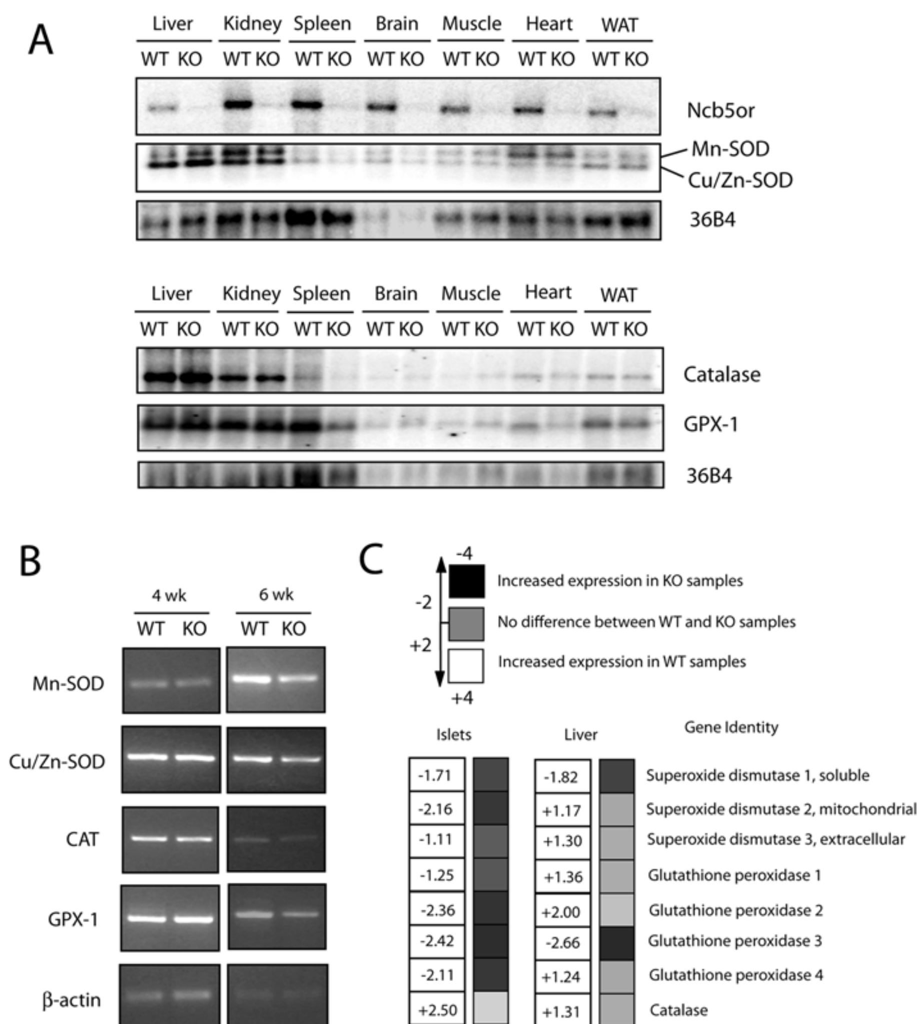

Figure 5 Examination of antioxidant enzyme expression in the presence and absence of NCB5OR

(**A**) Expression of superoxide dismutase (Mn-SOD and Cu/Zn-SOD), GPX-1 and catalase was determined in tissues of WT and KO mice using Northern blotting. WAT, white adipose tissue. (**B**) Semi-quantitative RT–PCR was used to examine the expression these genes in RNA prepared from the liver of WT and KO animals. Samples were normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and actin (not shown). (**C**) The antioxidant genes examined above, as well as other known isoforms, were examined in islets (left-hand panel) and liver (right-hand panel) prepared from WT and KO mice using Affymetrix microarrays.

nuclear extract from βTC3 cells (Figure 6C). The probes ARE1, ARE3, and ARE4 contained an acceptable consensus sequence for an ARE, but did not generate a band shift when incubated under similar conditions. These probes were used to gauge the specificity of complex binding to ARE2. The complex formed with ARE2 could not be displaced by these probes (Figure 6C). Bound complexes at ARE2 were displaced by unlabelled probe, whereas probes containing point mutations in the ARE consensus sequence did not displace the binding factors (Figure 6D).

The putative ARE in the *Ncb5or* promoter spans nucleotides −141 to −132 and conforms to the core consensus sequence 5′-RTGAYNNNGC-3′, as proposed by Nioi et al. [20]. The sequence of the ARE presented here is similar to that of the EpRE (electrophile-response element) (core sequence 5′-G/CTGAC/GNNNGCA/G-3′) and the T-MARE (PMA-responsive element-type Maf-recognition element) (5′-GCTGAG/CTCAGCA-3′), suggesting that transcription factors recognizing these sequences may bind to and regulate *Ncb5or* expression in this region. It will be interesting to determine the potential role Nrf2 and members of the Maf family may play in regulating transcription of *Ncb5or*. NCB5OR fits in the class of enzymes demonstrated previously to be regulated by such factors, although its gene does not appear to be inducible. It does, however, appear to be regulated when challenged with agents that affect cellular antioxidant status. It has been demonstrated that c-Maf acts as a transcriptional repressor of ARE-mediated transcription [21]. The authors demonstrated that c-Maf expression increased following short-term tBHQ treatment, which led to repression of transcription [21]. The same group demonstrated that Bach1 in association with small Maf proteins repressed ARE-mediated gene expression in response to tBHQ [22]. Additional factors, including MafK [23], c-Fos and Fra1 [24], and Nrf3 [25] negatively regulate ARE-mediated gene expression for genes that encode similar enzymes. The hypothesis put forth by de Macario and Macario [26] and Dhakshinamoorthy and Jaiswal [21] may explain the observed repression of *Ncb5or* expression. They suggested that small amounts of ROS are required to maintain active cellular defence. Activation of protective enzymes generally results in reduction in ROS levels. Since a certain threshold of ROS levels may be required within a cell, negative regulatory factors may act

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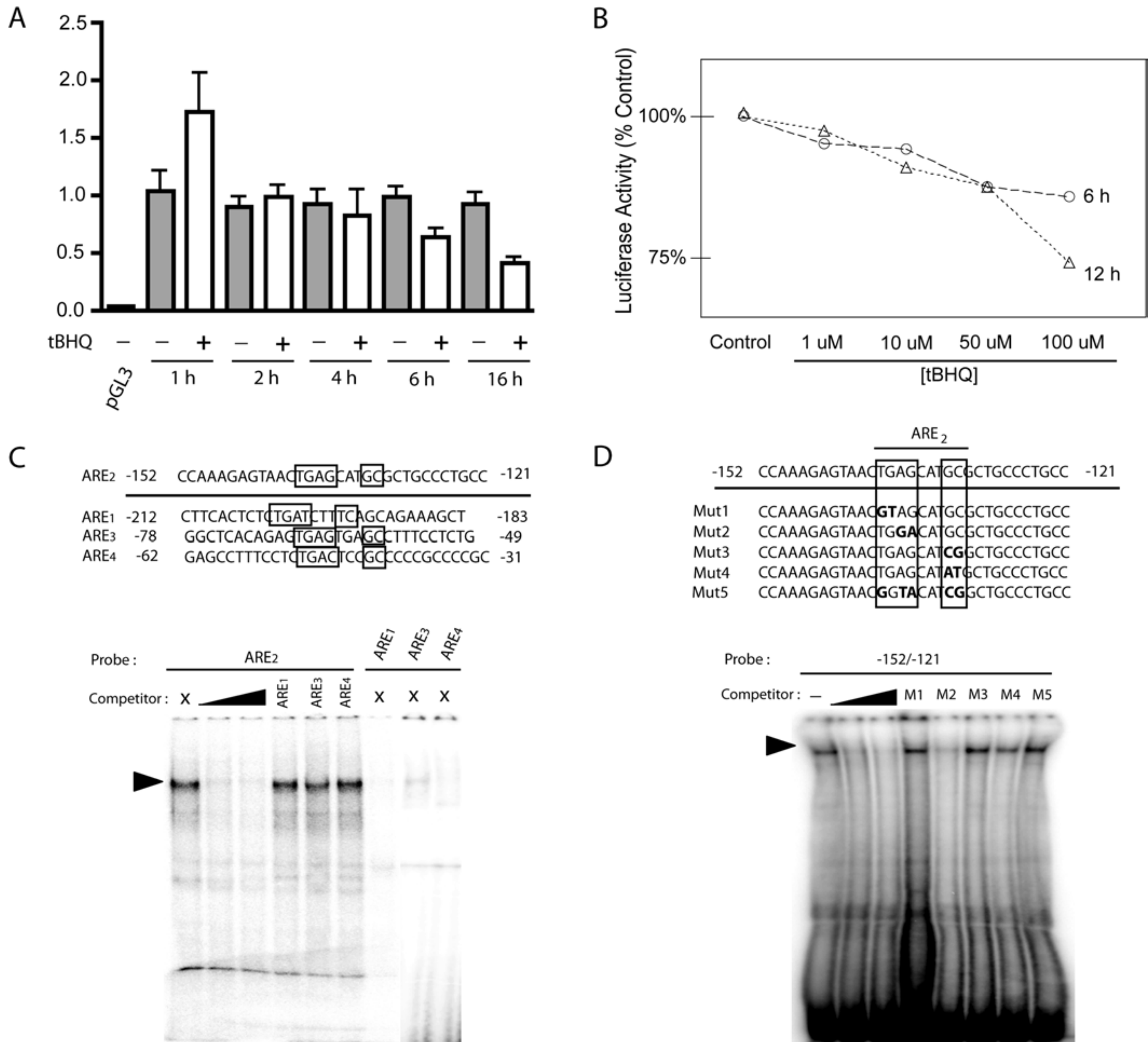

Figure 6 Effects of antioxidants on *Ncb5or* promoter activity and transcript expression

An *Ncb5or* promoter construct was used to monitor promoter activity in βTC3 cells following (**A**) incubation with tBHQ (100 μM) for 1–16 h and (**B**) incubation with a range of tBHQ concentrations (1–100 μM) for 6 and 12 h. (**C**) A putative ARE identified in the *Ncb5or* promoter, designated ARE_2 (−152/−121), demonstrated binding of a specific complex following incubation with nuclear extract from βTC3 cells. Complexes were competed out using unlabelled probe, but not by other potential ARE-binding sites from the *Ncb5or* proximal promoter, including ARE_1 (−212/−183), ARE_3 (−78/−49) or ARE_4 (−62/−31). (**D**) Mutated probes [Mut1 (M1)–Mut5 (M5)] identified critical nucleotides for complex formation.

to balance the basal expression of *Ncb5or* with respect to the antioxidant status in the cell. It is interesting to note that treatment of βTC3 cells with the antioxidant NAC also resulted in a decrease in *Ncb5or* expression (results not shown), supporting the idea that *Ncb5or* is involved in the antioxidant status in the β-cell. Since antioxidant treatment appears to have an effect on *Ncb5or* expression, it is likely that NCB5OR is involved in maintaining or regulating the redox status in β-cells.

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REFERENCES

1 Zhu, H., Qiu, H., Yoon, H. W., Huang, S. and Bunn, H. F. (1999) Identification of a cytochrome *b*-type NAD(P)H oxidoreductase ubiquitously expressed in human cells. Proc. Natl. Acad. Sci. U.S.A. **96**, 14742–14747
2 Xie, J., Zhu, H., Larade, K., Ladoux, A., Seguritan, A., Chu, M., Ito, S., Bronson, R. T., Leiter, E. H., Zhang, C. Y. et al. (2004) Absence of a reductase, NCB5OR, causes insulin-deficient diabetes. Proc. Natl. Acad. Sci. U.S.A. **101**, 10750–10755
3 Zhu, H., Larade, K., Jackson, T. A., Xie, J., Ladoux, A., Acker, H., Berchner-Pfannschmidt, U., Fandrey, J., Cross, A. R., Lukat-Rodgers, G. S. et al. (2004) NCB5OR is a novel soluble NAD(P)H reductase localized in the endoplasmic reticulum. J. Biol. Chem. **279**, 30316–30325

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4 Harding, H. P. and Ron, D. (2002) Endoplasmic reticulum stress and the development of diabetes: a review. Diabetes **51** (Suppl. 3), S455–S461
5 Larade, K. and Bunn, H. F. (2006) Promoter characterization and transcriptional regulation of Ncb5or, a novel reductase necessary for pancreatic β-cell maintenance. Biochim. Biophys. Acta **1759**, 257–262
6 Montana, E., Bonner-Weir, S. and Weir, G. C. (1993) β-Cell mass and growth after syngeneic islet cell transplantation in normal and streptozocin diabetic C57BL/6 mice. J. Clin. Invest. **91**, 780–787
7 Zhang, K. and Kaufman, R. J. (2004) Signaling the unfolded protein response from the endoplasmic reticulum. J. Biol. Chem. **279**, 25935–25938
8 Wang, X. Z., Lawson, B., Brewer, J. W., Zinszner, H., Sanjay, A., Mi, L. J., Boorstein, R., Kreibich, G., Hendershot, L. M. and Ron, D. (1996) Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol. Cell. Biol. **16**, 4273–4280
9 Lee, A. H., Iwakoshi, N. N. and Glimcher, L. H. (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol. Cell. Biol. **23**, 7448–7459
10 Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P. and Ron, D. (2004) CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. **18**, 3066–3077
11 Roy, B. and Lee, A. S. (1999) The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. Nucleic Acids Res. **27**, 1437–1443
12 Konrad, R. J. and Kudlow, J. E. (2002) The role of O-linked protein glycosylation in β-cell dysfunction. Int. J. Mol. Med. **10**, 535–539
13 Szkudelski, T. (2001) The mechanism of alloxan and streptozotocin action in β cells of the rat pancreas. Physiol. Res. **50**, 537–546
14 Nukatsuka, M., Sakurai, H., Yoshimura, Y., Nishida, M. and Kawada, J. (1988) Enhancement by streptozotocin of O_2^- radical generation by the xanthine oxidase system of pancreatic β-cells. FEBS Lett. **239**, 295–298
15 Takasu, N., Komiya, I., Asawa, T., Nagasawa, Y. and Yamada, T. (1991) Streptozocin- and alloxan-induced H_2O_2 generation and DNA fragmentation in pancreatic islets: H_2O_2 as mediator for DNA fragmentation. Diabetes **40**, 1141–1145
16 Mathews, C. E. and Leiter, E. H. (1999) Constitutive differences in antioxidant defense status distinguish alloxan-resistant and alloxan-susceptible mice. Free Radical Biol. Med. **27**, 449–455
17 Nguyen, T., Sherratt, P. J. and Pickett, C. B. (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu. Rev. Pharmacol. Toxicol. **43**, 233–260
18 Nguyen, T., Yang, C. S. and Pickett, C. B. (2004) The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radical Biol. Med. **37**, 433–441
19 Jaiswal, A. K. (2004) Regulation of antioxidant response element-dependent induction of detoxifying enzyme synthesis. Methods Enzymol. **378**, 221–238
20 Nioi, P., McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J. D. (2003) Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. Biochem. J. **374**, 337–348
21 Dhakshinamoorthy, S. and Jaiswal, A. K. (2002) c-Maf negatively regulates ARE-mediated detoxifying enzyme genes expression and anti-oxidant induction. Oncogene **21**, 5301–5312
22 Dhakshinamoorthy, S., Jain, A. K., Bloom, D. A. and Jaiswal, A. K. (2005) Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. J. Biol. Chem. **280**, 16891–16900
23 Nguyen, T., Huang, H. C. and Pickett, C. B. (2000) Transcriptional regulation of the antioxidant response element: activation by Nrf2 and repression by MafK. J. Biol. Chem. **275**, 15466–15473
24 Venugopal, R. and Jaiswal, A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc. Natl. Acad. Sci. U.S.A. **93**, 14960–14965
25 Sankaranarayanan, K. and Jaiswal, A. K. (2004) Nrf3 negatively regulates antioxidant-response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. J. Biol. Chem. **279**, 50810–50817
26 de Macario, E. C. and Macario, A. J. (2000) Stressors, stress and survival: overview. Front. Biosci. **5**, D780–D786

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