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Insulin secretion from beta cells is affected by deletion of Nicotinamide Nucleotide Transhydrogenase

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Abstract

NNT is an inner mitochondrial membrane transmembrane protein involved in regenerating NADPH, coupled with proton translocation across the inner membrane. We have shown that a defect in NNT function in the mouse, and specifically within the beta cell, leads to a reduction in insulin secretion. This chapter describes methods for examining NNT function in the mouse. This includes generating *in vivo* models with point mutations and expression of NNT by transgenesis, and making *in vitro* models, by silencing of gene expression. In addition techniques are described to measure insulin secretion, calcium and hydrogen peroxide concentrations, membrane potential and NNT activity. These approaches and techniques can also be applied to other genes of interest.

1 Introduction

We identified the nicotinamide nucleotide transhydrogenase (*Nnt*) gene as a possible candidate for the insulin secretory defect in C57BL/6J mice by quantitative trait loci mapping in an F2 mouse intercross between C57BL/6J and C3H mice (Toye *et al.*, 2005). In order to validate this gene we silenced it in an insulin secretory cell line and showed impairment of insulin secretion. We also identified two ENU alleles from the Harwell ENU DNA and sperm archive and demonstrated a glucose intolerant phenotype with an insulin secretory defect (Freeman *et al.*, 2006a). We hypothesised that NNT is required to generate NADPH needed to produce reduced glutathione in the mitochondria of the beta cell and that a lack of reduced glutathione for use by glutathione peroxidase (GPX) leads to an increase in reactive oxygen species (ROS)/Hydrogen peroxide. We proposed that this causes uncoupling via activation of uncoupling protein 2 (UCP2) and a reduction in membrane potential and a loss of ATP synthesis and thus a reduced ability of glucose metabolism to increase ATP/ADP ratios required for K_{ATP} channel closure and insulin secretion (reviewed Freeman

et al. 2006b and Freeman and Cox, 2006). Subsequent work in insulinoma cells using siRNAs has demonstrated the requirement for UCP2 for the effect on insulin secretion and membrane potential (manuscript in preparation). In C57BL/6J mice we demonstrated that replacement of the *Nnt* gene using a BAC transgene was able to complement the defect in glucose tolerance and insulin secretion (Freeman *et al.*, 2006c). This chapter describes the assays and techniques that we used to achieve this work.

2 Gene-driven ENU Screens

Gene-driven screens of mutagenised mice represent a powerful tool in the identification of allelic variants (Coghill *et al.*, 2002). *N*-ethyl-*N*-nitrosourea (ENU) is a germline mutagen that induces point mutations randomly and can be used to provide a mutant mouse resource (reviewed by Justice *et al.*, 1999). Male mice, exposed to ENU were used to create two parallel archives of frozen sperm and DNA. An archive was prepared from over 7,000 inbred mice that were F1 hybrids between BALB/c mutagenised males crossed with C3H females (Quwailid *et al.*, 2004, note that later portions of the archive are C57BL/6J x C3H F1s). Screening of the DNA archive can be used to identify an allelic series for any given gene of interest.

At present the archive is screened using high throughput mutation scanning methods (see section 2.1 and 2.2). Inevitably these miss a small proportion of mutations, but have the advantage of speed and cost efficiencies. When the cost of DNA sequencing becomes low enough we hope to sequence the DNA archive for all coding regions and thus generate a catalogue of mutations. Once a mutation is identified through DNA screening the mutant mice can be rederived easily by *in vitro* fertilisation (IVF) for subsequent phenotyping of live mice (see for example <http://www.har.mrc.ac.uk/services/fesa/> for a service or training). As ENU induces point mutations it can be expected that the full range of functional changes in any gene might be uncovered, including amorphs (null), hypomorphs, hypermorphs and neomorphs. The DNA archive is available to academics as a community resource; alternatively, MRC Harwell is now offering full training to visiting scientists to perform gene-driven screens within our mutation detection facility. See: http://www.har.mrc.ac.uk/services/dna_archive/

2.1 Denaturing High Performance Liquid Chromatography (DHPLC)

Initial screening of the Harwell ENU DNA archive was performed using DHPLC on the Transgenomic WAVE System (Coghill *et al.*, 2002; Quwailid *et al.*, 2004). Primers for polymerase chain reaction (PCR) amplification are designed to flank exons with at least a 20bp gap to allow complete sequencing of the region of interest including splice sites. Optimal fragment size is 150-450bp. To increase throughput, 12.5ng of archive DNA from each of 4 individual mice is pooled together. Conditions for PCR are as follows: 200uM dNTPs, 2.5mM MgCl₂, 0.2uM forward and reverse primers, 0.45U Applied Biosystems AmpliTaq Gold, 0.025U Stratagene Pfu Turbo polymerase and 1x AmpliTaq Gold buffer. A master mix of all PCR reagents is made and transferred to individual 96-well microtitre plates containing 50ng of DNA pools (final PCR volume 25-50ul). Touchdown PCR is performed on an MJ Research PTC 225 thermocycler using the following cycling

conditions: 95°C for 10 minutes, then 13 cycles of 94°C for 20 seconds, primer annealing temperature plus 7°C for 1 minute with a decrease of 0.5°C per cycle, and 72°C for 1 minute, followed by 19 cycles of 94°C for 20 seconds, primer annealing temperature for 1 minute, 72°C for 1 minute, and finally 72°C for 5 minutes and a 15°C hold. Following amplification, heteroduplexes are produced using the following conditions: 95°C for 4 minutes, followed by 45 cycles of 93.5°C for 1 minute with a reduction of 1.5°C per cycle, finally followed by holding at 25°C. When partially denatured, PCR products containing heteroduplexes elute quicker from the WAVE column and are detected by a UV monitor as additional peaks appearing before the homoduplex wildtype peak (Figure 1). Following identification of an interesting pool containing potential heteroduplexes, the individual DNAs making the pool are tested. The PCR amplification products containing heteroduplexes are purified using the QIAquick PCR purification kit (Qiagen) and then sent for sequence confirmation.

As DHPLC requires samples to be partially denatured to spot heteroduplexes, for some exons multiple temperatures are required to detect mutations across the entire PCR product. On a two-plate machine 2 plates with two methods can be run in 24 hours. Although a reliable method with approximately 95% detection efficiency (Dobson-Stone *et al.* 2000) there are potentially higher throughput methods now available (see section 2.2).

2.2 High-Resolution DNA Melting Analysis

The primary mode of screening the Harwell DNA archive is high-resolution DNA melting analysis performed on the Idaho Technology LightScanner. High-resolution DNA melting analysis has been successfully used for several years to perform mutation scans and genotyping (Wittwer *et al.* 2003). PCR is performed in the presence of the double-stranded DNA binding dye LCGreen. The LCGreen saturates heteroduplexes and they can be distinguished from the normal melt curve by an early decrease in fluorescence (Figure 2).

Primer pairs for PCR amplification are designed using Idaho Technology's LightScanner Primer Design Software. Fragment size ranges between 150-550bp with primers flanking the region of interest by 20bp to allow successful sequencing of the entire region. To increase throughput, 2.5ng of archive DNA from 4 individual mice is pooled together. Conditions for PCR are as follows: 1xLCGreen plus dye and 1xHotShot Mastermix, 0.2uM forward and reverse primers. Master mix of all PCR reagents are made, transferred to individual 96-well Framestar plates containing 10ng of DNA pools (final PCR volume 10ul) and overlaid with 20ul of mineral oil. PCR is performed using a GRI G-Storm GS4 Thermal Cycler using the following cycling conditions: 95°C for 2 minutes, then 44 cycles of 95°C for 30 seconds, primer annealing temperature for 30 seconds, 72°C for 30 seconds. Following initial PCR, heteroduplexes are produced by heating samples to 95°C and then rapidly cooling to 15°C. Samples are then centrifuged at 3000rpm for 3 minutes before running on the LightScanner. Following identification of an interesting pool, the individual DNAs making the pool are then tested alongside at least 12 wildtype DNA controls. The PCR amplification products containing heteroduplexes, are purified using QIAquick PCR purification kit (Qiagen) and sent for sequence confirmation.

This method is very rapid as once the PCR reactions are complete plates can be analysed at the rate of 1 plate every 8 minutes per machine. Unfortunately plate loading is not automated yet. We estimate that this method detects >90% of mutations.

2.3 Rederivation of live mice

Fifty nine new mutant lines have to date been successfully generated from the Harwell ENU archive. Embryos are either shipped at the 2 cell stage to the user's facilities or can be rederived in-house for phenotyping. F1 archived mice carry approximately 30 potentially *functional* mutations (Keays *et al.* 2006). The likelihood of more than one mutation confounding a given phenotype is very low however backcrossing of mice to C3H rapidly removes the unlinked mutations. After backcrossing for several generations homozygotes can be bred and if desired coding region surrounding the selected gene should be sequenced to confirm there are no mutations in closely linked genes although this is unnecessary particularly when several alleles are available to validate the phenotype (Keays *et al.* 2007).

3 Generating BAC transgenics

We originally identified *Nnt* as a candidate gene in a Quantitative Trait Locus (QTL) mapping experiment (Toye *et al.*, 2005). Having identified it as a strong functional candidate it was necessary to prove that it accounted for at least some of the glucose intolerance and insulin secretory phenotype in C57BL/6J mice. The effect was originally measured in an F2 C57BL/6J x C3H/HeH genetic environment. There are several ways to approach this problem and we decided to supplement the deleted copy of *Nnt* in C57BL/6J mice with a complete functional copy from the 129 inbred mouse. We achieved this by identifying a bacterial artificial chromosome (BAC) containing the entire *Nnt* gene and injecting this into the pronucleus of a fertilised C57BL/6J egg (Freeman *et al.* 2006c). The resulting offspring that carried transgenic integration events were examined for NNT expression and phenotyped.

3.1 Identification of a BAC clone

To identify a BAC containing all 21 exons of *Nnt*, the RPCI - 22 (129S6/SvEvTac) Mouse BAC Library (Children's Hospital Oakland Research Institute) was screened.

3.1.1 Generation of Probe—Exons 4 and 17 of NNT were PCR amplified to produce products of 443 and 462bp respectively. To make a probe, PCR products are purified using the QIAquick PCR purification kit (Qiagen). The purified PCR products are then radioactively labelled with [α -³²P]dCTP at 3000Ci/mmol using a Prime-It II Random primer labelling kit (Stratagene). Illustra NICK columns (GE Healthcare) are then used, to remove any unincorporated radiolabelled nucleotides from the labelling reaction. All kits are used according to manufacturer's instructions.

3.1.2 Hybridisation—Library membranes (filters) are wet in a minimum volume of Church Buffer (10g BSA, 2ml 0.5M EDTA, 500ml 1M NaHPO₄ (pH 7.2), 350ml 20% SDS, up to 1L with H₂O). Nylon mesh is placed between filters and filters placed in hybridisation bottles with 25ml Church Buffer. Filters are then pre-hybridised for one hour at 65°C after

which one of the labelled probes is denatured at 100°C for 5 minutes and then added. Filters are then left to hybridise overnight (16-18 hours) at 65°C.

After hybridisation, filters are washed 4 times with wash solution I (5g BSA, 1ml 0.5M EDTA, 40ml 1M NaHPO₄ (pH 7.2), 250ml 20% SDS, up to 1L with H₂O) at 65°C for 20 minutes each wash. Filters are then washed with wash solution II (2ml 0.5M EDTA, 40ml 1M NaHPO₄ (pH 7.2), 50ml 20% SDS, up to 1L H₂O) at 65°C for 20 minutes until the level of radioactive counts per minute (cpm) removed remained constant.

Filters are then rinsed in water and wrapped individually in cling film and exposed to film for 2-72 hours at -80°C. Positive clones are then identified. Filters are then stripped by incubating them at 65°C in a shaking water bath prior to repeating the hybridisation protocol again with the second labelled probe.

3.1.3 Identification of full length NNT BAC clone—Twenty two clones positive for both exons 4 and 17 were identified. To validate clones, each is streaked onto LB chloramphenicol (20µg/ml) agar plates and incubated overnight at 37°C. Individual colonies are then picked from each of these plates and PCR amplified directly for exons 1 and 21 of NNT. One of the 22 clones identified, BAC RP22-455H18, contained both exons 1 and 21.

3.1.4 DNA extraction—A positive colony is inoculated into 10ml of LB chloramphenicol (20µg/ml) broth and left shaking overnight at 37°C. The culture is then spun for 10 minutes at 2000 rpm and the supernatant poured off. The pellet is resuspended in 300µl of 50mM glucose, 25mM Tris, 10mM EDTA. Then 600µl of 1% SDS, 0.2M NaOH is added and the sample inverted several times (lysis for no longer than 5 minutes). Following this, 500µl 7.5M Ammonium acetate is added and the sample inverted several times and then left on ice for 10 minutes, inverting at intervals. The sample is then spun at 13,000 rpm for 20 minutes and the supernatant poured into a fresh tube and spun for a further 10 minutes at 13000 rpm. The supernatant is then poured into another fresh tube and 700ul isopropanol added. The sample is inverted several times and spun for 20 minutes at 13000 rpm. The supernatant is discarded and the pellet washed with 500µl 70% ethanol and then spun for 5 minutes at 13000 rpm. The supernatant is discarded and the pellet left to air dry for 5-10 minutes after which it is resuspended in 100ul 1X TE. RNase is added to a final concentration of 100ug/ml.

All 21 exons of *Nnt* were then PCR amplified. BAC RP22-455H18 was shown to contain all 21 exons of the *Nnt* gene, as well as considerable 5' and 3' flanking intergenic DNA.

3.2 BAC Transgenesis

A mouse transgenic model overexpressing NNT was created to perform a complementation test of the defect in insulin secretion and glucose intolerance phenotypes observed in male C57BL/6J mice. The chosen strategy for transgenesis is the pronuclear injection of a BAC. This is because the large cargo capacity of such vectors is likely to include the sequences that support the regulation of the expression of the endogenous *Nnt* gene, as well as coding information.

BAC DNA is prepared either as described by these workers (Freeman *et al.*, 2006c) or currently with a method based on an earlier publication (Carvajal *et al.*, 2001). Great care is taken to optimise every step of the preparation: bacteria are isolated on freshly prepared plates. A colony is transferred into a 5ml pre-culture, which is subsequently diluted into a 250ml culture. The BAC DNA is extracted employing the HiSpeed Maxiprep kit (Qiagen) with the following modifications. The bacterial pellet is resuspended in 50ml of ice-cold P1 buffer and incubated 10 minutes on ice. Then 50ml of freshly prepared P2 buffer is added. The preparation is mixed by inversion and incubated 5-10 minutes at room temperature. This is followed by 50ml of ice-cold N3 buffer and mixed by inversion. The tube is laid flat in a bed of ice with very gentle tilting for 20 minutes. After centrifugation, the supernatant is filtered on several layers of medical gauze and loaded on a column and washed as *per* manufacturer instructions. The DNA is eluted employing Elution Buffer pre-warmed to 55°C and precipitated as per manufacturer instructions. BAC DNA is resuspended overnight with very gentle agitation in BAC microinjection buffer (BAC MIB, 10mM Tris-HCl at pH 7.5, 0.1mM EDTA at pH 8.0, 100mM NaCl, filtered through 0.2µm). The DNA solution is dialysed overnight against BAC MIB employing a Slide-A-Lyzer MINI dialysis unit (Pierce) and quantified by spectrophotometry or Nanodrop. Preparations yielding less than a total of 30µg before dialysis are discarded.

Circular BAC DNA solution, no older than a month, is diluted with BAC MIB to a concentration of 1-2ng/µl on the day of injection. Pronuclear injection of C57BL/6J embryos and rearing of potential founders are performed according to standards protocols (Nagy *et al.*, 2003).

Tail biopsies are taken from potential founders and processed employing a DNeasy extraction kit (Qiagen), according to the manufacturer. As the C57BL/6J strain was shown to lack exons 7 to 11 of *Nnt*, PCR assays amplifying each of these exons were used to screen for potential founders obtained by pronuclear microinjection. It is noteworthy that a great proportion of the transgenic insertions obtained with the microinjection of the *Nnt* BAC showed at least some degree of rearrangement so one or several of exons 7 to 11 of NNT were missing from the transgene. In order to ascertain the integrity and functionality of the transgene in the positive founders, the expression of NNT transcripts and protein was checked by RT-PCR and Western Blot analysis, respectively (Freeman *et al.*, 2006c).

Once a transgenic line is established and validated, sperm or embryos are cryopreserved at an early generation. This is essential as loss of transgene activity through silencing or sequence rearrangements may occur in later generations. Figure 3 illustrates a NNT BAC line rederived from frozen embryos after loss of expression was observed in the stock being maintained on the shelf over several years.

4 Isolation of islets of Langerhans from mouse pancreas

To determine the phenotype of a mouse model, one can measure plasma glucose or insulin levels in response to a glucose challenge *in vivo* (for protocols see EMPRESS: <http://empress.har.mrc.ac.uk/browser/>). However a more sensitive description of insulin secretion can be obtained *in vitro* using isolated islets.

The cells of the endocrine pancreas are grouped into clusters known as islets of Langerhans. There are considered to be more than 10,000 islets per pancreas in rodents (Bonner-Weir and Orci, 1982). The size of an islet is 40-400 μm in diameter. Although islets are scattered throughout the pancreas, the majority are found in the body and tail of the organ, with only one third being found in the head of the pancreas (Orci, 1982). There are four major endocrine cells in the islet: insulin-producing β -cells, glucagon-producing α -cells, somatostatin-producing δ -cells, and pancreatic polypeptide-producing PP-cells. In rodents, the insulin-producing β -cells constitute about 60-70% of islets cells. They form the central core of the islet with the other non- β -cells forming a mantle around them (Orci et al., 1975; Erlandet et al., 1976).

In pancreatic β -cells, insulin secretion is regulated by K_{ATP} channels. At sub-stimulatory blood glucose levels, K_{ATP} channels in the β -cell membrane are open and efflux of K^+ ions through the channel generates a negative membrane potential. As a consequence, voltage-dependent Ca^{2+} channels (VDCC) remain closed (Ashcroft and Rorsmann 1989). However, when the blood glucose concentration is increased by food intake, β -cell glucose uptake and metabolism is stimulated, producing changes in the cytosolic concentration of adenine nucleotides that lead to closure of the K_{ATP} channel. This induces a membrane depolarization which opens the VDCC, allowing Ca^{2+} influx into the cell. The resulting increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) induces exocytosis of insulin granules (Henquin 2000).

Although single β -cells (or cultured β -cells such as HIT-T15 and β HC-9) are capable of secreting insulin in response to elevated level of glucose, it is known that the insulin release is enhanced when β -cells have contact with other cells in the islet (Bosco *et al.*, 1989; Soria *et al.*, 1991; Shimomura *et al.*, 2004; Saito *et al.*, 2003). Both electrical coupling between β -cells and the paracrine effects of hormones released from non- β -cells may contribute to the enhancement of insulin secretion (Meda *et al.*, 1983; Kantengwa *et al.*, 1997).

4.1 Isolation methods for obtaining islets

There are several ways to isolate islets from the pancreas. The simplest is the microdissection technique which involves physically cutting out islets with a sharp needle (30-31 gauge needle) under a binocular microscope (Henquin and Meissner 1984). Although the microdissection technique may avoid damage caused by the use of enzymes, it only isolates a very limited number of islets (10-50) as well as the risk of contamination with surrounding tissues.

A better technique is to digest the pancreas using enzymes such as liberase. Liberase is a blend of highly purified type I and type II collagenases and proteases that is specifically designed for rodent islet isolation. Although enzymes can damage the islet, this can be minimised by using the proper enzyme concentration and by prompt and swift washing of digested tissues. This technique can provide larger numbers (150-300) of high quality islets. In the liberase digestion technique, Hank's buffer with 0.2% BSA (Hank's-BSA) is used as the media throughout the procedure.

In order to release the maximum number of high quality islets, it is necessary to disperse the liberase solution (at 0.5mg/ml) throughout the pancreas. This can be achieved by injecting 2ml of solution, using a syringe fitted with a 30½ gauge needle, into the bile duct after obstructing the exit to the duodenum by ligating the bile duct-duodenum junction with surgical string or by clamping the Vater papilla in the duodenum using a surgical clamp (McDaniel et al., 1983; Wollheim et al., 1990). The needle is inserted where the common hepatic duct and cystic duct join as illustrated in Figure 4. When the injection is successful, the pancreas will inflate from head to tail. The inflation of the pancreas must be complete or the islet yield will be poor.

Once the injection is complete, the inflated pancreas should be carefully dissected away from other tissues, placed in 2ml of liberase solution and kept at 37°C for 20 minutes. After 20 minutes, the digested pancreas should be washed twice by adding 20ml of ice cold Hank's solution and centrifuging at 2,000 rpm for 2 minutes. After washing, the islets can be hand picked under the microscope. The purpose of hand picking is to exclude non-islet tissues from the digested pancreas and obtain pure islets. It can be done in two ways.

One way is to suck the islets up into a sterile pipette tip while visualizing them under a microscope. However, as this method often aspirates other tissues together with islets, the procedure needs to be repeated several times to obtain pure islets. Another way is to use a wire islet picker. This can be constructed by making a circle (with a diameter of approximately 1mm) at one end of a length of wire and gluing the other end to a rod so that it can be easily manipulated. By using a wire islet picker like a spoon, it is possible to physically pick up one islet at a time.

4.2 Insulin secretion experiment from static incubation

The simplest and most effective way to measure glucose-stimulated insulin secretion (GSIS) from islets is by the static incubation technique (Tanaka *et al.*, 2002; Freeman *et al.*, 2006a). Various concentrations of glucose in Krebs-Ringer buffer (KRB) solution are used to stimulate insulin secretion. All solutions should contain 0.1% BSA (to which insulin will complex) to prevent insulin sticking to the incubation tubes. The islets should be incubated in a low glucose solution (2mM) for 30 minutes prior to the experiment. This will ensure that insulin secretion is not dictated by the glucose concentration to which the islets were previously exposed (either in the animal or the culture solution)

A 24-well plate can be used for the static incubation and each well should contain 2ml of experimental solution containing either glucose at 2mM, 10mM, 20mM, or (if desired) a sulphonylurea such as tolbutamide. Five islets are then placed in each well and incubated for 1 hour at 37°C.

Immediately after incubation is completed, the supernatant (containing the secreted insulin) should be aspirated and centrifuged at 3,000rpm for 1 minute to remove any cells. Insulin in the solution can then be measured immediately or the solution can be stored at -20°C until desired.

The islets from each incubation tube are also collected under a microscope, transferred to an acidified-ethanol solution (95% ethanol and 5% acetic acid) and left overnight at -20°C. This lyses the islets (and β -cells) completely and releases insulin into the solution, enabling the insulin content to be measured. Since the insulin content level is 20-200 times higher than the secreted insulin level, it is necessary to dilute these samples before measurement.

Insulin in the samples can be measured by an insulin Enzyme Linked Immuno-assay (ELISA assay) (Linco, Rat/mouse ELISA kit) or an RIA assay using the 5ul protocol according to manufacturer's instructions. However, because the size of each islet varies, it is necessary to normalise the secreted insulin to insulin content in the same islets. Prior to the ELISA assay, supernatants are usually diluted 1 in 10-20, and content supernatants are diluted 1 in 200-400.

5 RNAi methodologies on insulinoma cell lines

RNA interference (RNAi), a gene silencing technology extensively used in *C.elegans*, was utilised to study loss of function of NNT in the insulin secreting cell line MIN6. Gene specific siRNA oligo duplexes are designed for the gene of interest and transfected into cells where they are incorporated into a ribonucleoprotein complex called RISC (RNA induced silencing complex). RISC mediates the unwinding of the siRNA duplex, by ATP-dependent RNA-helicase activity into a single stranded siRNA. The antisense strand of the siRNA duplex guides RISC to the homologous mRNA allowing it to bind in a sequence-specific manner, this mediates target mRNA endonucleolytic cleavage. This cleaved mRNA is then recognised by the cell as aberrant and destroyed, preventing translation resulting in the specific silencing of the target mRNA.

5.1 Insulinoma cell lines

We have used two insulin secreting cell lines (MIN6 and INS-1) for gene silencing in various projects. MIN6 is a mouse insulinoma cell line derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic beta cells. Glucose stimulated insulin secretion occurs progressively in this cell line with the amount of insulin secreted at 25mM glucose being 6-7 fold higher than that obtained at 5mM glucose. This cell line was obtained at passage 40 and is maintained at high confluence passaged every 3-4 days in DMEM containing 25mM glucose, supplemented with 15% heat inactivated fetal bovine serum in humidified 5% CO₂, 95% air at 37°C. INS-1 is a rat cell line derived from an x-ray induced rat transplantable insulinoma (Asfari *et al* 1992), which retains partial glucose responsiveness, with an optimal secretion observed at 11.2mM glucose which decreases slightly at higher levels (16.7mM). We have used this cell line to silence *Sox4* gene expression using siRNA (Goldsworthy *et al.* 2008). This cell line was obtained at passage 32 and is maintained in RPMI (11.11mM glucose concentration) supplemented with 10mM HEPES, 2mM L-Glutamine, 1mM sodium pyruvate and 50uM β -mercaptoethanol in humidified 5% CO₂, 95% air at 37°C.

5.2 Gene silencing using siRNA

siRNA oligos were designed and synthesised by Eurogentec on their exclusive siRNA design software, with a total of 4 siRNA duplexes used. Each siRNA was 19 nucleotides long with 2 DNA overhangs at the 3' end and a Cy3 tag on the 5' end of the sense strand. The presence of a Cy3 tag enabled transfected cells to be easily identified and transfection efficiency to be quantified. We used two independent *Nnt* siRNAs targeted to non-overlapping regions of the gene, an siRNA duplex specific to glucokinase (*Gck*) as a positive control (*Gck* is a key glycolytic enzyme which when absent abolishes glucose stimulated insulin secretion) and a scrambled (nonsense) siRNA as a negative control. A number of companies now provide pre-designed siRNAs that are ready to use.

Prior to transfection, cells are plated at a density of 1×10^5 and cultured on 24-well flat bottomed plates (Scientific Laboratory Supplies, Nottingham, UK) in their cell specific media and grown for 24-48 hours in humidified 5% CO₂, 95% air at 37°C. A cationic lipid mediated transfection (lipofectamine) is used to deliver the synthetic siRNA oligo duplex into the target cells. Incubation of lipofectamine and siRNA duplexes in serum free-media results in formation of unilamellar and multilamellar liposomes. The positively charged cationic lipids spontaneously interact with the negatively charged backbone of the siRNA to form complexes. These complexes then interact with the negatively charged cell membrane resulting in the delivery of the siRNAs into the cell. For each siRNA duplex a lipofectamine complex is set up by the addition of 20ul of lipofectamine to 2ml of serum free media, which is mixed gently by inversion and incubated at room temperature for 5 minutes to allow the liposomes to begin to form. After this incubation period siRNA duplexes are added at a final concentration of 50nM, mixed gently by inversion and incubated at room temperature for a further 20 minutes. Figure 5 illustrates the experiment schematically.

The 24-well plates of cells are prepared for transfection by aspiration of media and washing twice with PBS. Then, 200ul of the previously prepared lipofectamine/siRNA complex is added to the appropriate wells. To avoid shearing the complexes the solution should be pipetted slowly and carefully down the side of each well with a 1ml pipette and tip. Eighteen wells per siRNA should be transfected in order to perform an insulin secretion assay (in triplicate), extract RNA (to confirm silencing by real-time quantitative reverse transcription PCR) and protein (to confirm silencing by Western Blotting). Transfected plates are incubated at 37°C 5% CO₂, 95% air for 7-8 hours after which time the wells should be topped up with an additional 200ul of serum containing media and incubated for a total of 24 hours. After 24 hours the cells from 6 wells for each siRNA duplex are trypsinised and the resulting cell pellet divided into 2 aliquots for RNA and Protein extraction and stored at -70°C until use (Figure 5).

5.3 Confirmation of siRNA specific knockdown

In order to confirm the observed loss of insulin secretion was due to loss of silencing of *Nnt* specifically both mRNA and protein levels were measured utilising quantitative rtPCR and Western blotting. Total RNA is extracted from silenced cells using a Qiagen RNeasy mini-kit (Qiagen, Crawley UK) according to the manufacturers instructions, the optional on column DNase digestion is also performed. Concentration and quality of total RNA is

measured on a spectrophotometer (Nanodrop) and cDNA is then generated by Superscript II enzyme (Invitrogen) and analysed by quantitative RT-PCR using the taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer). Gene expression is normalised relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Inventoried Taqman probes with FAM tags can be purchased from Applied Biosystems. A student's *t* test was used to measure significance between nonsense siRNA transfected cells, *Gck* siRNA transfected cells, and *Nnt* siRNA transfected cells when amplifying for either *Gck* or *Nnt* gene expression.

Prior to western blotting protein is extracted from stored siRNA transfected cells by the addition of 50µl CelLytic™ M Cell Lysis Reagent (Sigma) containing complete mini proteinase inhibitor cocktail (Roche), cells are incubated on ice for 60 minutes prior to centrifugation at 13,000rpm for 15 minutes at 4°C. The supernatant is removed and protein quantified and stored at -20°C until use. For each well, 50µg of total protein is heated in sample buffer (Laemmli Sample Buffer, BioRad) for 3 minutes at 100°C prior to loading on 4-15% Tris-HCL Ready Gels (BioRad) and size separated by electrophoresis (100V for 1 hour). A SeeBlue (Invitrogen) protein ladder is included to allow molecular weights to be estimated. Proteins are transferred to Hybond-P membrane (Amersham, GE Healthcare) using a transblot system (Biorad) and hybridised with antibodies to GCK (Santa Cruz) and NNT (custom made). Bands are visualised on ECL film using ECL plus western Blotting detection system (Amersham, GE Healthcare) according to the manufacturers instructions. As expected NNT protein is present in nonsense and *Gck* transfected min6 cells but not *Nnt* siRNA transfected cells. GCK protein was detected in nonsense and *Nnt* siRNA treated cells but not *Gck* siRNA transfected cells. Membranes were re-probed with a mouse actin monoclonal antibody (Chemicon International) as a protein loading control.

Once the knockdown of the target gene is confirmed (usually by qRT-PCR alone), cells may be tested for insulin secretion as described in Section 4.2. Cells used for confirming siRNA knockdown and cells for testing insulin secretion should be harvested at the same time to ensure that the knockdown reflects the change in insulin secretion.

6 Measuring Intracellular Calcium

To measure intracellular calcium concentration in pancreatic islets the ratiometric Ca^{2+} probe fura-2 is used. The dye is introduced into the islets at room temperature by incubation in 3 µM of the lipophilic, cell-permeant acetoxymethyl ester (fura-2 AM), dissolved in EC Buffer (138mM NaCl, 5.6 mM KCl, 1mM MgCl_2 , 2.6mM CaCl_2 , 5mM HEPES, pH 7.4 with NaOH). The non-ionic detergent pluronic acid is used at a dilution of 0.01% to facilitate uptake of fura 2 AM. After 20 minutes, an islet is transferred into a perfusion chamber mounted on the stage of an *Axiinvert 200* microscope (Carl Zeiss Jena, Germany) where it is held in place by a suction glass pipette. To allow for complete de-esterification of intracellular AM ester and to remove external dye, the islet is perfused with warm EC buffer (33-35°C) for 15 minutes. During imaging, the islet is continuously perfused with warm EC buffer (33-35°C), containing different glucose concentrations or tolbutamide.

We used a fluorescence system (Photon Technology International) for ratiometric measurements with dual excitation at 340nm and 380nm. Emission is recorded at 510nm. Pancreatic islets are imaged at 2 frames per second and fluorescence intensity (F) is detected with a photomultiplier tube. Ca^{2+} signals are presented as the ratio of F at different excitation wavelengths (F340/F380). Data are analyzed using *FeliX32 Analysis* software. Examples of calcium measurement on wild type cells are shown in Figure 6.

7 Measuring Hydrogen Peroxide in mitochondria

Dichlorodihydrofluorescein (DCF) and its derivatives have been used to study free radical production in a variety of systems. However, DCF has several significant disadvantages: Firstly, the dyes are not specific, i.e. they are sensitive to multiple types of reactive oxygen species (ROS). Secondly, they cannot be targeted to specific intracellular compartments. Thirdly, and most importantly, they can produce ROS themselves on photoexcitation. An alternative to DCF is HyPer (Evrogen), which does not suffer from these disadvantages.

HyPer is a genetically encoded fluorescent protein capable of specifically detecting intracellular hydrogen peroxide (H_2O_2) (Belousov *et al.*, 2006). It consists of a yellow fluorescent protein (YFP) that has been inserted into the regulatory domain of the H_2O_2 -sensing transcription factor OxyR, expressed by *E. coli*. HyPer does not cause artificial ROS generation and can be used for detection of rapid changes of H_2O_2 concentrations within the physiological range. When the appropriate signal peptide is attached, HyPer can be expressed in different cell compartments. For example, addition of a mitochondrial targeting sequence (pHyPer-dMito) enables HyPer to be specifically expressed in mitochondria.

HyPer displays two excitation peaks (420, 500nm) and one emission peak (516nm). Upon exposure to H_2O_2 , the excitation peak at 420nm decreases in proportion to the increase in the excitation peak at 500nm. These spectral characteristics render it suitable for ratiometric measurements, thereby eliminating false results caused by photobleaching or differences in HyPer expression.

For detection of H_2O_2 in mitochondria, the insulinoma cell line MIN6 is transfected with a mammalian expression vector encoding mitochondria-targeted HyPer (Evrogen) using Lipofectamine (Invitrogen). The transfection protocol is carried out according to the manufacturer's instruction. HyPer fluorescence can be observed within 12 hours of transfection and shows no significant decline for about 7 days.

Before imaging, cells are incubated for 1 hour in EC buffer with 7% CO_2 and 93% air at 37°C. During the experiment, cells are continuously perfused with EC Buffer at 37°C and imaged on 35mm Fluoro Dishes (World Precision Instruments) using an epifluorescence microscope (Nikon, Eclipse, TE 2000-U) with a CFI Super Fluor 40x/1.3 oil objective (Nikon). An IonOptix fluorescence system (Boston, Massachusetts) for ratiometric measurements with a dual excitation at 480/20nm and 405/40nm and emission at 530/40nm filter (Chroma Technology) is used. Data are collected with a CCD camera (IonOptix). Cells are excited at one frame per three seconds or one frame per 10 seconds for experiments lasting for 35 minutes.

Using the technique described above, H_2O_2 levels in MIN6 cells can be measured in response to glucose stimulation. Within minutes of the addition of 20 mM glucose a steady increase in H_2O_2 is observed as a result of glucose metabolism (Figure 7).

HyPer can also be used to measure H_2O_2 levels in response to glucose in MIN6 cells with altered levels of NNT expression. Previous results suggest that NNT is involved in detoxification of ROS (Freeman *et al.* 2006a). MIN6 cells are co-transfected with pHyPer-dMito, nonsense and anti-*Nnt* siRNA duplexes (section 5) in 24-well cell culture plates. Then, 24 hours later ROS levels in response to glucose (0, 2, 20mM) and menadione (50 μM) are measured. Fluorescence is measured for 30 minutes on a Fluostar OPTIMA (BMG Labtech), 485nm excitation and 520nm emission. Reduced expression of NNT results in an increase in ROS detection in response to glucose but not with menadione (Figure 8). Menadione preferentially generates free radicals inside the mitochondrion and the effects are too rapid for NNT to assist in the detoxification of ROS accumulation.

As reported for most sensors based on YFP, HyPer fluorescence depends on pH (Belousov *et al.*, 2006). The titration curve of HyPer shows that with increasing pH the fluorescence ratio of 480nm to 405nm increases concomitantly. Thus in order to ascribe changes in HyPer fluorescence to a change in H_2O_2 concentration it is necessary to ascertain whether the pH stays unaltered throughout the experiment. The mitochondrial pH may be monitored by using a pH-sensitive probe such as mtAlpHi (Abad *et al.*, 2004) which is highly selective and can be targeted to the mitochondria. Using mtAlpHi, Wiederkehr and colleagues (2007) showed that glucose stimulation in both INS-1E cells and rat islets results in a gradual increase in matrix pH which reaches a steady state after 10-15minutes.

8 Imaging of mitochondrial membrane potential changes by confocal microscopy

A negative potential difference Ψ_m of ~ -200 mV exists between the cell cytosol and the mitochondrial matrix. This potential difference falls across the inner membrane of the mitochondrion and is the major driving force for proton influx into the mitochondrion. Because proton influx is essential for ATP synthesis, any damage to proteins that ensure the presence of potential difference across the inner membrane will lead to decreased ATP levels in the cell cytosol. Therefore, monitoring of the mitochondrial potential provides means to estimate the efficiency of ATP production in cells, as well as to detect malfunctioning of proteins involved.

Direct measurements of mitochondrial membrane potential have been performed on individual mitochondria with the help of the patch clamp technique that is widely used to study electrical properties of cell membranes (Jonas *et al.*, 1999). This method, though being reliable and providing a detailed information about voltages across the inner mitochondrial membrane and conductances present in it, is technically difficult to perform as well as time and work consuming. Additionally, it only allows one to investigate the properties of a small sample of mitochondria present in cell cytoplasm.

Methods traditionally used to measure the mitochondrial membrane potential employ lipophilic cations, such as tetraphenylphosphonium, that can penetrate the lipid bilayer and accumulate into different cellular compartments according to their electrical potential. The redistribution of lipophilic ions between the bulk solution containing a preparation of mitochondria and the mitochondrial matrix is usually monitored electrically with the help of a specific ion-sensitive electrode.

Another method is to use of fluorescent lipophilic ions. These substances, besides being capable of penetrating lipid bilayers, fluoresce under irradiation with light of specific wavelengths. Examples of such fluorescent lipophilic cations or fluorescent probes are rhodamine 123, tetramethylrhodamine ethyl and methyl esters (usually referred to as TMRE and TMRM, respectively), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidozolocarbocyanine (known as JC-1) and others. These fluorescent substances, because of the electrical charge present on their molecules, are sensitive to the electrical potential and redistribute between various compartments of the system if electrical potentials within it have changed. Consequently, the application of these substances to cells and tissues allows one to visualize the changes in the electrical potential across the membranes separating cellular compartments. The physical visualization or imaging of these changes can be performed on any of the variety of fluorescence microscopes that are in use today. The confocal laser scanning microscopy with its highly and easily controlled conditions for sample irradiation and the collection of emitted light, the possibility to select the position and width of an optical slice within the sample from which the fluorescent light is collected, is a very convenient tool to follow and make visible the changes of mitochondrial membrane potential in various preparations such as isolated mitochondria, live cells and tissues such as islets of Langerhans.

8.1 Confocal imaging of mitochondrial potential in pancreatic β -cells

Freshly prepared beta-cells are allowed to attach to glass coverslips and then loaded with TMRM (50 nM) for 30 min at 37 °C in standard extracellular solution of the following composition: 138mM NaCl, 5.6mM KCl, 2.6mM CaCl₂, 1.2mM MgCl₂, 5mM HEPES; pH is adjusted to 7.4 with NaOH.

Coverslips supporting TMRM-loaded cells are mounted on a custom-built microscope sample stage with an in-built perfusion bath. During the mounting procedure special care is taken to avoid the drying of cells. The sample stage is placed on a Zeiss Axiovert 200M microscope and cells are allowed to equilibrate for ~10 min while being perfused with the standard extracellular solution containing 50 nM TMRM at room temperature of ~22 °C. All subsequent experiments were also performed at this temperature.

The fluorescence of TMRM is monitored by Zeiss LSM 510 META confocal system attached to the Axiovert 200M microscope. The fluorescence of the dye is excited with the 543 nm line of the 15 mW HeNe laser at 1.8% delivered beam energy. The emitted light is collected within the range of wavelengths of 554 to 597 nm. The peak of TMRM fluorescence is well within this spectral band, and the exclusion of longer wavelengths diminishes the background noise present in the signal. Time series of images are obtained for individual cells or clusters of cells with a sampling rate of 10 s.

Examples of confocal images of TMRM loaded β -cells are seen in Figure 9. It is important to realize that in mitochondrial potential measurements fluorescent voltage sensitive dyes can be used, broadly speaking, in two different ways which are sometimes called the redistribution and quench/dequench methods (Duchen et al., 2003). If the quench/dequench method is used, cells are loaded with above mentioned dyes at comparatively high concentrations (1 to 20 μ M) for time periods not longer than 15 min. In these conditions, dyes accumulate in mitochondria to levels that cause autoquenching of the dye. If mitochondria are subsequently transferred into a medium that causes their depolarization, the dye redistributes and partly leaves the mitochondrial matrix. This reduces autoquenching and the depolarization of mitochondria is optically detected as an increase in fluorescent signal. In investigations described here the redistribution method was used.

For cells that are loaded with TMRM at very low concentrations (50 nM), the possibility of autoquenching within the mitochondrial matrix is excluded. The changes in mitochondrial potential then lead to the redistribution of fluorescent signal between the matrix and cell cytosol. A specific analysis of images has to be applied in order to extract information changes in mitochondrial potential. In this type of experiment it is important to keep the energy delivered by the laser beam to cells low for two main reasons. One is the photobleaching of the dye which may become a major problem in prolonged dynamic measurements of the mitochondrial potential changes. Besides keeping the energy of the laser beam low, bleaching can be reduced by increasing the interval between sampling time. The possibility of increasing the concentration of the dye in cells in order to avoid effects of bleaching is severely limited by another reason why the beam energy has to be low. This is the phototoxicity of fluorescent dyes in general and TMRM in particular. Irradiation of fluorescent molecules leads to the increased production of free radicals within cells, and these may have adverse effects on various cellular processes. It is not so damaging in case of dyes that are evenly distributed within the whole cytosol. However, if dye is concentrated in the mitochondrial matrix, the effect of increased production of free radicals may be problematic. It was observed that mitochondria in MIN6 cells bathed in solution containing 50 nM TMRM depolarized quickly on irradiation with a 543 nm beam from 15 mW HeNe laser at 5% level. The effect was identical to the depolarization observed after application of a high concentration of FCCP. It was necessary to keep the beam energy below 2% to avoid phototoxic effects even at concentrations of TMRM as low as 50 nM.

8.2 Analysis of confocal images representing the mitochondrial membrane potential

Images seen in Figure 9 contain information about the fluorescence changes caused by altered potential differences after application of different agents (high glucose, FCCP, menadione etc.) on both the cell membrane and the inner membrane of mitochondria. If conclusions about the mitochondrial potential are to be made, the analysis of these images has to be performed in a way that targets properties of fluorescent signal characteristic or linked to the mitochondrial potential.

This can be achieved by measuring the standard deviation of the fluorescent signal intensity throughout the cell image (Goldstein et al., 2000; Toescu and Verkhratsky, 2000; Duchen et al., 2003). In basal conditions the pancreatic insulin secreting cell has $\sim$ 70 mV across the

cell membrane and an additional ~200 mV across the inner mitochondrial membrane. At low TMRM concentrations (50 nM) in the extracellular solution this will lead to a slight increase in dye concentration inside cell cytosol and a much larger increase in dye concentration in the mitochondrial matrix (Figure 9 A,B,C). This distribution of the fluorescent probe results in a high standard deviation of the signal intensity.

Hyperpolarization of the mitochondrial membrane attracts additional amounts of TMRM into the matrix increasing the intensity of the signal from mitochondria and decreasing it in the cytosol (Figure 9B). Such redistribution of fluorescent signal will be reflected in an increase of the overall standard deviation. In contrast, depolarization of the mitochondrial membrane results in the loss of dye from the mitochondria to the cell cytosol and consequently, the standard deviation of the fluorescent signal decreases.

To account for different initial mean signals in cells, the standard deviation is usually scaled by the mean of the signal intensity.

9 Measuring nicotinamide nucleotide transhydrogenase activity

Nicotinamide nucleotide transhydrogenase (NNT) is located in the mitochondrial inner membrane, with its active sites facing the matrix (Pederson *et al.* 2008 *in press*). The enzyme is thus not accessible to its hydrophilic substrates NAD(H) and NADP(H) (or the analogue 3-acetyl-pyridine-NAD⁺, APAD) added externally. Normally, therefore, NNT is assayed in submitochondrial particles (SMP) where the topology of the membrane is inverted.

However, the sonication procedure involved in making these SMP requires relatively large amounts of mitochondria. A protocol describing the assay for NNT in large mitochondrial preparations from beef heart has been reported (Rydstrom 1979). In the present procedure, which involves recovering much smaller amounts of mitochondria from mouse tissues (pancreas, heart, lungs etc). NNT is assayed in isolated mitochondria that have been made permeable to its substrates by the presence of the detergents Brij 35 and lysolecithin. Lysolecithin has earlier been shown to work both as an effective detergent as well as maintaining NNT in an active state, presumably through its property as a phospholipid (Rydstrom *et al.* 1976). Brij 35 is a mild detergent, the weak denaturing effect of which is counteracted by lysolecithin. The composition of the detergent mixture was optimized regarding activity using purified *E. coli* NNT, and regarding permeability using intact mouse heart mitochondria (J. Rydström, unpublished). Pure *E. coli* NNT is prepared as described (Meuller *et al.* 1997).

9.1 Microassay of nicotinamide nucleotide transhydrogenase (NNT)

To prepare the mitochondria the procedure described in "Protocol: Isolation of Mitochondria from Tissues using the Qproteome™ Mitochondria Isolation Kit" (Qiagen) is followed in detail, until step 11a (standard preparation of mitochondria), using 1.5-2ml Eppendorf tubes, a cooled Eppendorf centrifuge, a TissueRuptor (Qiagen), and a 2ml dounce homogenizer (teflon/glass). It does not appear to be necessary to continue with step 12a, i.e. a final wash. As applied to mouse heart and pancreas, acceptable modifications include doubling the amount of starting tissue material (100mg instead of 60mg wet weight). After the final centrifugation step, the mitochondrial pellet is about 2-3mm in diameter (starting with a 3

weeks old BALB/c mouse heart of about 100mg wet weight). Following suspension of the pellet with 20ul of mitochondria storage buffer (Qproteome™ kit), the final volume of the mitochondrial suspension (using 100mg of tissue) is 25-30ul, giving an estimated protein concentration of 5-10mg/ml, which is enough for at least 4 assays and a protein determination. The suspension should be kept on ice as freezing will result in a loss of activity.

The assay of NNT in isolated mitochondria is carried out using a dual wavelength Shimadzu UV-3000 spectrophotometer, or an equivalent spectrophotometer with accurate signal to noise filters, with a minimal distance between cuvette and photomultiplier (in order to minimise noise due to the turbid sample). A normal split beam spectrophotometer is usually not sufficient, because it is too sensitive to the turbidity changes at the wavelengths used. At this wavelength, oxidation of NADPH in the reaction will contribute to an increased extinction coefficient. For the dual wavelength instrument, wavelengths are normally 375-400nm (375nm is the wavelength chosen for reduced APAD, and 400nm is the reference, isosbestic, wavelength for APAD/APADH(NADP⁺/NADPH) (under these conditions, the extinction coefficient is 7.63mM⁻¹,cm⁻¹). A 1ml cuvette with black sides is used to avoid stray light.

The assay medium is composed of 50mM Tris-HCl (pH 8.0), 0.5% Brij 35, 1 mg/ml of lysolecithin, 300uM each of APAD and NADPH. Stock solutions are 0.5M Tris-HCl, 10% Brij-35, 10mg/ml lysolecithin, and 20mM each of APAD and NADPH. A drop of the Tris buffer (pH 8.0) is added to the stock NADPH (but not to the stock APAD) in order to maintain its preservation at 4°C (NADPH is sensitive to low pH). Both substrate stock solutions are stable for at least a couple of days at 4°C (but not in the freezer).

Full scale sensitivity of the spectrophotometer is set to at least 0.05 OD (can be lower, i.e. a higher sensitivity, but not higher). The assay is carried out with 1ml of assay buffer and 10ul of the mitochondrial suspension. A slight delay in the activity (max. 30 seconds) has occasionally been noted, probably reflecting the fact that it may take time for the mitochondria to become permeable to the substrates. However, after this lag phase, the activity is essentially linear for several minutes. An example of the linear trace observed when measuring NNT activity in mouse pancreas mitochondria is shown in Figure 10. The specificity of the assay is most apparent by the lack of activity observed in mitochondria from C57BL/6J tissue, which has a deletion in the NNT gene resulting in a lack of function. The slopes from these traces can be used to generate values for the rate of activity per mg of protein. By introducing a known amount of (AcPy)NADH at the end of the reaction the corresponding change in OD can be used as an indicator to calculate the change in (AcPy)NADH over time.

9.2 Assay considerations

The reason for choosing a higher assay pH is to minimise non-specific interactions of NADH-linked reductases/dehydrogenases with NADPH. For the same reason it is best to avoid rotenone or other respiratory chain inhibitors, because these tend to have the same effect. The reduced APAD is not significantly oxidised due to its high redox potential.

It might be possible to also assay NNT by using a fluorometer, but again, the required high sensitivity combined with the turbidity will produce a noisy signal.

Whilst it is preferable to use a dual wavelength spectrophotometer, the availability of this equipment is increasingly scarce. We have successfully used a Varian Cary 4000 that can measure separate wavelengths almost simultaneously and therefore provides an accurate subtraction of the turbid background.

The concentrations of the substrates have been chosen based on the earlier estimated K_m -values for NAD^+ and NADPH, which are between 20 and 30 μM . Assay concentration should be at least 10 times K_m . It should be noted though that permeabilized mitochondria are used, and traditional K_m values may therefore not apply. To be certain, one should double the substrate concentrations. Too high substrate concentrations (and thus too high absorbance) tend to decrease the sensitivity of the spectrophotometer.

Conclusions

This chapter provides the background and techniques that were used in studying NNT and its role in insulin secretion. The importance of NNT was originally identified by its effect on glucose homeostasis in C57Bl/6J that carries a natural deletion of *Nnt*. To validate our findings, we have since combined a range of *in vitro* techniques including the use of ENU mutagenesis and BAC transgenesis to generate a number of *Nnt*-deficient alleles, as well as *in vivo* methodologies such as RNAi silencing in insulinoma cell lines which can be more readily performed. The mouse models that we have generated allow us to further investigate NNT function by using islets extracted from wild-type and mutant mice, and treating them with various reagents. To further dissect the mechanism of NNT function within the mitochondria, we have developed imaging techniques that quantify a range of intracellular and intra-mitochondrial variables in testing our hypothesis on NNT function. Finally, the NNT activity assay on isolated mitochondria gives a more direct measure of NNT function, and when combined with the other assays will provide us with a strong basis for validating drugs that target NNT, which may have important medical potential in treating patients with Type2 diabetes.

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Figure 1.

Absorbance trace of 2 pooled PCR products on the WAVE. Samples containing an ENU mutation cause the formation of heteroduplexes (dotted line), which are eluted from the WAVE column quicker than homoduplexes (solid line) and so appear as additional peaks appearing before the main sample peak. Natural polymorphisms between the inbred strains used to create the archive will also produce traces containing multiple peaks and may reduce the ability to detect an ENU induced mutation.

Figure 2. Melt profile of an ENU induced mutation on the LightScanner. LCGreen saturates heteroduplexes (dotted line, left) and causes an early decrease in fluorescence so the melt curve shifts to the left compared to homoduplexes (solid line, right). PCR must be optimal and the melt profile of the exon must be narrow or variants may not be detected.

Figure 3.

Expression of NNT in mice transgenic for the *Nnt* BAC transgene. (A) RT-PCR analysis of *Nnt* mRNA from C3H, C57Bl/6J, a transgenic line rederived from the original rescue line, and the original rescue line. One of the RT primers is located within the deleted region of the transcript (exons 7-11) and the other at the 3' end including the NADP motif. These primers amplify a 1.3kb product from C3H but not in Bl/6J. The BAC rescue line re-derived from frozen embryos stored shortly after the line was created (Nnt-BACline1-RED) also expresses *Nnt* at level similar to C3H. In contrast, the original line (Nnt-BACline1) that had been maintained by breeding over several years has lost *Nnt* expression, possibly due to genome rearrangement. (B) Proteins were separated by SDS-PAGE and the proteins transferred to a membrane for immunoblotting with a rabbit anti-NNT polypeptide antibody. C3H and 'Nnt-BACline1-RED' mice express NNT as expected, whereas Bl/6J and 'Nnt-BACline1' mice do not express NNT. Note that the polyclonal antibody detects some non-specific bands in both C3H (intact) and Bl/6J (deleted) tissues.

Figure 4.
Injection of liberase into the bile duct in order to inflate the pancreas.

Figure 5. Schematic of the siRNA protocol.

MIN6 cells were treated with 4 different silencing RNAs, *Nnt1*, *Nnt2*, *Gck* and a nonsense siRNA

Figure 6. Typical calcium transient of a pancreatic islet stimulated with 10mM glucose. Pancreatic islets exhibit a robust elevation of $[Ca^{2+}]_i$ when stimulated with glucose. This calcium peak occurs after a delay which can last for a couple of minutes. There is usually a decrease in $[Ca^{2+}]_i$ immediately before the calcium peak which is probably caused by Ca^{2+} sequestration in the ER driven by metabolism (Chow et al., 1995; Tengholm et al., 2001). Oscillations, which may follow the sharp rise in $[Ca^{2+}]_i$, have typically three components: a faster component with a period of tens of seconds (as seen in the figure), a slower component with a period of 4–6 min and a combination of both, which shows faster oscillations superimposed on the slower plateaus (Bertram et al., 2007). Application of tolbutamide, which blocks the K_{ATP} channel, triggers a robust increase in $[Ca^{2+}]_i$.

Figure 7. Changes in mitochondrial H_2O_2 concentration in si*Nnt* silenced MIN6 cells (70% knockdown efficiency) stimulated with 20mM glucose added at 5min. Representative traces of siRNA transfected MIN6 cells (grey) and nonsense siRNA transfected MIN6 cells (black) are depicted.

Figure 8. MIN6 cells co-transfected with anti-*Nnt* siRNA duplexes (white bars) or nonsense siRNA duplexes (black bars) and pHyPer-dMito were measured for ROS following treatment with glucose or menadione. Cells with reduced *Nnt* mRNA expression have higher levels of ROS after addition of 2mM and 20mM glucose. The experiment was performed in triplicate and the bars represent the mean and standard error for one experiment.

Figure 9.

The visualization of the mitochondrial potential in MIN6 cells. Cells were loaded with TMRM at 50 nM concentration. Images were obtained on Zeiss confocal microscope LSM510 META. Column A: top panel – a confocal image of a loaded Min6 cell at 0 glucose; mitochondria are seen as elongated red dots; bottom panel – the intensity profile inside the image of the cell; sharp peaks represent mitochondria; the signal initiated in the cytosol is seen as low level line between peaks. Column B: – a confocal image of a loaded MIN6 cell at 20 mM glucose; the intensity of signal initiated in mitochondria is increased in comparison to image in column A; the cytosolic signal has decreased; this indicates the hyperpolarization of the mitochondrial membrane. Column C: – a confocal image of a loaded MIN6 cell after application of FCCP; the peaks representing mitochondria are essentially diminished, the overall level of the signal is increased; this indicates depolarization of the mitochondrial membrane. The dark region in all three confocal images is the cell nucleus.

Figure 10.

Raw traces of NNT activity in mitochondria from the pancreas of different mouse strains. C57BL6/J, which has a deletion in the NNT gene, shows minimal activity most of which is likely due to non-specific reduction of (AcPy)NAD⁺.