Nicotinamide nucleotide transhydrogenase: a link between insulin secretion, glucose metabolism and oxidative stress

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Abstract

This paper reviews recent studies on the role of Nnt (nicotinamide nucleotide transhydrogenase) in insulin secretion and detoxification of ROS (reactive oxygen species). Glucose-stimulated insulin release from pancreatic β-cells is mediated by increased metabolism. This elevates intracellular [ATP], thereby closing KATP channels (ATP-sensitive potassium channels) and producing membrane depolarization, activation of voltage-gated Ca2+ channels, Ca2+ influx and, consequently, insulin secretion. The C57BL/6J mouse displays glucose intolerance and reduced insulin secretion, which results from a naturally occurring deletion in the Nnt gene. Transgenic expression of the wild-type Nnt gene in C57BL/6J mice rescues the phenotype. Knockdown of Nnt in the insulin-secreting cell line MIN6 with small interfering RNA dramatically reduced Ca2+ influx and insulin secretion. Similarly, mice carrying ENU (N-ethyl-N-nitrosourea)-induced loss-of-function mutations in Nnt were glucose intolerant and secreted less insulin during a glucose tolerance test. Islets isolated from these mice showed impaired insulin secretion in response to glucose, but not to the KATP channel blocker tolbutamide. This is explained by the fact that glucose failed to elevate ATP in Nnt mutant islets. Nnt is a nuclear-encoded mitochondrial protein involved in detoxification of ROS. β-Cells isolated from Nnt mutant mice showed increased ROS production on glucose stimulation. We hypothesize that Nnt mutations enhance glucose-dependent ROS production and thereby impair β-cell mitochondrial metabolism, possibly via activation of uncoupling proteins. This reduces ATP production and lowers KATP channel activity. Consequently, glucose-dependent electrical activity and insulin secretion are impaired.

Type 2 (non-insulin-dependent) diabetes mellitus is a serious metabolic disease that is reaching epidemic proportions in Western societies and is predicted to affect over 300 million people worldwide by 2025 [1]. Impaired glucose tolerance, which precedes diabetes and is a risk factor for the disease, currently affects a further 200 million. The disease has considerable human and economic health costs: for example, in the U.K., the National Health Service spends >£14 million each day on treating diabetes and its complications (http://www.diabetes.org.uk; extrapolated from [2]). Type 2 diabetes is characterized by elevation of the blood glucose concentration, usually presents in middle age, and is exacerbated by age and obesity. It is associated with impairment of both insulin secretion and insulin action, which results from a naturally occurring deletion in the Nnt gene. Transgenic expression of the wild-type Nnt gene in C57BL/6J mice rescues the phenotype.

Increased mitochondrial metabolism promotes insulin secretion via downstream events, as can be observed when the β-cell membrane is depolarized by other means [7]. Sulfonylurea drugs stimulate insulin secretion by binding to, and closing, KATP channels, thus by-passing the metabolic steps in stimulus-secretion coupling [8]. Their effectiveness in treating Type 2 diabetes suggests that events downstream of KATP channel closure are largely intact. Furthermore, mutations in KATP channel genes that reduce its sensitivity to metabolic inhibition [9,10], or in genes involved in glucose metabolism [11–13] or mitochondrial function [14,15] cause rare monogenic forms of diabetes. Taken together, these studies suggest that defects in β-cell metabolism, and/or metabolic regulation of KATP channels, may underlie the
Figure 1 | Insulin secretion is triggered by an increase in \([\text{Ca}^{2+}]_i\), which in turn is regulated by the electrical activity of the pancreatic \(\beta\)-cell

When metabolism is low (A), \(K_{\text{ATP}}\) channels are open, keeping the membrane hyperpolarized and \(\text{Ca}^{2+}\) channels closed, so that \([\text{Ca}^{2+}]_i\) remains low. When metabolism increases (B), \(K_{\text{ATP}}\) channels shut, depolarizing the \(\beta\)-cell membrane potential and thereby eliciting electrical activity and opening of voltage-gated \(\text{Ca}^{2+}\) channels. This leads to \(\text{Ca}^{2+}\) influx and an increase in \([\text{Ca}^{2+}]_i\), that triggers exocytosis of insulin granules.

impaired insulin secretion found in Type 2 diabetes [5,15,16]. Studies of human Type 2 diabetic islets support this idea [17–20].

C57BL6J mice lack Nnt (nicotinamide nucleotide transhydrogenase)

The C57BL/6J mouse is an inbred laboratory strain that is widely used in many studies. It exhibits an impaired glucose tolerance that is independent of obesity. This results from a reduction in both first and second phase insulin release [21,22]. When fed a high-fat diet, C57BL/6J mice develop obesity, diabetes and insulin resistance [23,24]. Their phenotype is therefore reminiscent of that of human Type 2 diabetes.

Quantitative trait mapping identified Nnt as a strong candidate gene for the glucose intolerance and reduced insulin secretion of C57BL/6J mice [21]. Further analysis revealed a spontaneous in-frame 5-exon deletion in Nnt that led to a complete loss of the protein [21]. Transgenic expression of the entire Nnt gene in C57BL/6J mice rescued the phenotype, thereby demonstrating directly that their glucose intolerance and impaired insulin secretion is indeed due to lack of Nnt [25]. Analysis of 22 other mouse strains, including an additional seven C57BL strains, showed that only the C57BL/6J strain had the naturally occurring deletion in Nnt [25]. As it is not known precisely when the gene was deleted in the stock, it is possible that some C57BL/6J strains may still retain Nnt. Nevertheless, the widespread use of C57BL/6J mice for genetic studies suggests that caution should be exercised when attributing their disease entirely to imposed genetic manipulations, as the Nnt background may make a significant contribution [26].

Lack of Nnt impairs insulin secretion

A range of studies in both mutant mice and \(\beta\)-cell lines have revealed that loss of Nnt causes a substantial reduction in glucose-stimulated insulin secretion because it impairs \(\beta\)-cell metabolism and thereby prevents \(K_{\text{ATP}}\) channel closure and \(\text{Ca}^{2+}\) influx. Thus, although \(K_{\text{ATP}}\) channels in C57BL/6J \(\beta\)-cells have normal ATP sensitivity, glucose fails to close them, or to elevate \([\text{Ca}^{2+}]_i\) and stimulate insulin secretion [21]. Similarly, mice possessing ENU (N-ethyl-N-nitrosourea)-induced point mutations in Nnt (mNnt mice) were glucose intolerant, and their islets secreted substantially less insulin [27]. Knockdown of Nnt by siRNA (small interfering RNA) in the insulin-secreting \(\beta\)-cell line MIN6 mimicked these results, preventing the glucose-dependent rise in \([\text{Ca}^{2+}]_i\), and reducing insulin secretion dramatically [27]. However, the \(K_{\text{ATP}}\) channel blocker tolbutamide remained an effective secretagogue in C57BL/6J and mNnt islets, as well as in Nnt-siRNA-transfected MIN6 cells [21,27]. Taken together, these data suggest that the effects of loss of Nnt function on insulin secretion lie upstream of \(K_{\text{ATP}}\) channel closure. In support of this idea, glucose failed to elevate [ATP], in mNnt islets [27], pinpointing a possible defect in \(\beta\)-cell metabolism.

Nnt is a mitochondrial inner membrane protein

Mitochondrial metabolism accounts for more than 95% of ATP required for insulin secretion [15]. It is therefore relevant that Nnt is a nuclear-encoded protein located in the inner mitochondrial membrane. It functions as a redox-driven proton pump, catalysing the reversible reduction of NADP\(^+\) by NADH and the conversion of NADH into NAD\(^+\) [28–30]. Under physiological conditions, i.e. where a proton
**Figure 2** | Suggested mechanism for the effect of Nnt mutations on insulin secretion

A region of the inner mitochondrial membrane is shown. Activity of the electron transport chain leads to generation of superoxide (O$_2^-$), much of which is released into the mitochondrial matrix. This is detoxified by SOD2 to H$_2$O$_2$, which either diffuses into the cytoplasm or remains in the mitochondrial matrix. In the latter case, it is converted into water by the activity of glutathione peroxidase and glutathione reductase, which require NADPH as a cofactor. Nnt generates NADPH. Thus, when Nnt is non-functional, superoxide and its metabolites increase, leading to activation of UCP2, dissipation of the mitochondrial membrane potential and reduced ATP synthesis. Reprinted from Cell Metabolism, vol. 3, H. Freeman, K. Shimomura, E. Horner, R.D. Cox and F.M. Ashcroft, ‘Nicotinamide nucleotide transhydrogenase: a key role in insulin secretion.’, pp. 35–44, copyright 2006, with permission from Elsevier.

Gradient exists across the mitochondrial membrane, Nnt is a very efficient generator of intramitochondrial NADPH.

In eukaryotes, Nnt is a homodimer [28]. Each subunit is composed of three principal domains: the first and third domains lie within the mitochondrial matrix and contain the NAD- and NADPH-binding domains, respectively. The second consists of 14 transmembrane-spanning helices and harbours the proton-conducting pore.

Nnt is present in most prokaryotes and eukaryotes, although interestingly it is not found in either Drosophila or yeast DNA sequences (Ensemble). In the mouse, Nnt is ubiquitously expressed, with highest densities in heart, kidney, pancreas and liver, and lesser levels in brain [31]. It lies on chromosome 13 (D2, 64 cM, 120.24 Mb). There is only one copy of the gene in humans and it lies on chromosome 5p13.1-5cen.

**Nnt, ROS and insulin release**

Electron flow through the electron transport chain leads to the establishment of a proton gradient and an associated potential (estimated as 150–200 mV negative to the cytosol) across the inner mitochondrial membrane. This proton-motive force is harnessed by the F$_1$F$_0$-ATPase to generate ATP.

Although mitochondrial electron transport is essential for ATP generation, it also produces damaging ROS (reactive oxygen species), which result in lipid peroxidation, formation of reactive aldehydes, protein damage and mitochondrial DNA mutations [32]. Oxidative damage leading to mitochondrial dysfunction is considered to be responsible for many degenerative diseases, including Type 2 diabetes, cardiomyopathy, Parkinson’s disease and even normal aging [32,33].

Nnt plays an important part in the detoxification of ROS. A significant fraction of the superoxide generated by the electron transport chain is released into the mitochondrial matrix and, as it is membrane-impermeant, must be detoxified in situ. As Figure 2 illustrates, ROS are first converted into H$_2$O$_2$ by the mitochondrial form of superoxide dismutase (SOD2). This is subsequently converted into H$_2$O and O$_2$ by glutathione peroxidase and glutathione reductase in an enzyme cascade that requires NADPH provided by Nnt. Thus, when Nnt is non-functional, intra-mitochondrial NADPH will be low and removal of ROS will be impaired. The crucial role of Nnt in ROS detoxification is revealed by the fact that ablation of Nnt renders Caenorhabditis elegans more susceptible to oxidative stress and impairs the cellular GSH/GSSG ratio [34]. Furthermore, mice deficient in SOD2 die much earlier if they also lack Nnt [26]. When SOD2 is deleted on the C57BL/6J background, mice die during gestation from dilated cardiomyopathy, whereas on the DBA/2J background they have an average lifespan of 8 days and die of
metabolic acidosis. Genetic mapping determined that this difference results from lack of Nnt in C57BL/6J mice [26].

The rate of ROS production increases steeply with the mitochondrial electromotive force [32] and thus with the metabolic rate. Measurement of ROS is notoriously difficult and no fully satisfactory assay exists. Nevertheless, despite these limitations, several studies suggest that high glucose causes a small but significant increase in ROS [27,35] (although this has been contested [36]). In β-cells from mice carrying loss-of-function mutations in Nnt, however, glucose causes a dramatic increase in ROS, consistent with a role for Nnt in ROS detoxification.

Activation of UCP2 (uncoupling protein 2) is postulated to provide the link between ROS and ATP generation (Figure 2). Activation of UCP2 enhances proton leakage across the inner mitochondrial membrane, reducing the mitochondrial membrane potential and thereby ATP synthesis [37,38]. This provides a feedback mechanism that is postulated to limit ROS generation when metabolic flux is high. However, insulin secretion is tightly coupled with mitochondrial ATP synthesis and even ‘mild’ uncoupling causes reduced release [15,27]. Consequently, enhanced UCP2 activity impairs insulin secretion [39], whereas reduced UCP2 activity increases insulin release [40].

One hypothesis, therefore, is that when Nnt is non-functional, intramitochondrial NADPH is low. Consequently, ROS increases, producing UCP2 activation, a fall in mitochondrial membrane potential and decreased ATP synthesis. In support of this idea, there was an increase in glucose utilization in Nnt mutant islets, as expected if elevated ROS induces mild uncoupling [27]. Definitive proof, however, will require the demonstration that concomitant ablation of UCP2 and Nnt restores the wild-type phenotype. An alternative way in which deletion of Nnt might regulate UCP2 is via an increase in UCP2 expression, since mice that overexpress UCP2 exhibit impaired insulin secretion [40]. However, there is circumstantial evidence that at least part of the effect of Nnt deletion might be mediated via direct activation of UCP2. This is because exogenous H$_2$O$_2$ depolarizes the mitochondrial membrane potential, decreases ATP production and activates K$_{ATP}$ channel activity within a few minutes of application [41,42] (too fast for protein synthesis to take place).

Although Nnt is widely expressed [31], the β-cell seems especially sensitive to loss of Nnt function [27]. The reason for this is unclear. However, pancreatic β-cells have unusually low levels of most antioxidants and antioxidant enzymes [43], which means that they will be particularly susceptible to oxidative stress. This may be one reason why C57BL/6J mice do not appear to suffer from any major problems other than glucose intolerance. In addition, whereas most cells maintain a constant ATP level, β-cell metabolism is unusual in that [ATP], changes in response to glucose. It remains possible, however, that lack of Nnt may affect the function of other tissues under pathological conditions that enhance oxidative stress (for example, obesity or insulin resistance).

**Role of Nnt in disease**

The discovery that Nnt influences insulin secretion is likely to be of relevance for human Type 2 diabetes. Islets isolated from patients with Type 2 diabetes exhibit an increase in oxidative stress that correlates with the degree of impairment in glucose-stimulated insulin release [18]. This may underlie their reduced metabolism and insulin secretion, as 24 h exposure to the antioxidant glutathione improves their secretory response [18]. Thus strategies to reduce ROS production/damage may be of benefit in Type 2 diabetes [33,44]. Clearly, it will also be of interest to determine if polymorphisms in the Nnt gene are associated with human Type 2 diabetes.

Obesity leads to enhanced levels of circulating NEFAs (non-esterified fatty acids), which are metabolized within the β-cell to LC-CoAs (long chain fatty acyl CoAs). Chronic exposure to LC-CoAs suppresses insulin secretion. NEFA and LC-CoAs have many effects, including increasing expression of UCP2 [45,46] and direct activation of the K$_{ATP}$ channel [47]. However, LC-CoAs also strongly inhibit Nnt. Palmitoyl-CoA is particularly effective ($K_i = 0.15$ μM; [48]). In this respect, it is interesting that palmitate causes a partial depolarization of the mitochondrial membrane potential [49]. In our model (Figure 1), inhibition of Nnt by palmitate would be expected to depolarize the mitochondria.

Type 1 diabetes is caused by immune-mediated destruction of the pancreatic β-cells. It is widely thought that oxidative stress resulting from ROS generation by infiltrating T- and B-cells contributes to cell death in Type 1 diabetes [50]. Apoptosis is also induced by agents that generate ROS, such as alloxan and H$_2$O$_2$ [51]. Thus it is possible that mice or humans with loss-of-function mutations in Nnt may be more susceptible to Type 1 diabetes. Furthermore, agents that enhance Nnt activity, by contributing to increased ROS detoxification, may be of value in treating both Type 1 and Type 2 diabetes.

**Conclusion**

The last year has seen considerable advances in our understanding of the functional role of Nnt. It is now clear that it plays a key role in the detoxification of ROS and of insulin secretion. Nevertheless, these advances serve mainly to highlight our ignorance of this mitochondrial protein. The precise details of the mechanism by which it regulates insulin release remains to be established. Its role in insulin secretion in human β-cells and whether it plays any part in the development of diabetes (Types 1 and 2) are still unclear. The importance of Nnt in other tissues and pathological conditions also requires exploration. It seems possible, for example, that tissues in mNnt mice that have a high metabolic rate may have greater susceptibility to oxidative stress. Indeed, the fact that SOD2-knockout mice on a C57BL/6J background die prematurely of cardiac hypertrophy suggests that this is likely. Whether normal aging is affected also remains to be established.
References

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