Mutations in NNT encoding nicotinamide nucleotide transhydrogenase cause familial glucocorticoid deficiency

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Using targeted exome sequencing, we identified mutations in NNT, an antioxidant defense gene, in individuals with familial glucocorticoid deficiency. In mice with Nnt loss, higher levels of adrenocortical cell apoptosis and impaired glucocorticoid production were observed. NNT knockdown in a human adrenocortical cell line resulted in impaired redox potential and increased reactive oxygen species (ROS) levels. Our results suggest that NNT may have a role in ROS detoxification in human adrenal glands.

Familial glucocorticoid deficiency (FGD; MIM 202200) is a rare autosomal recessive disorder characterized by an inability of the adrenal cortex to produce cortisol in response to stimulation by adrenocorticotropic hormone (ACTH). Affected individuals typically present within the first few months of life with symptoms related to cortisol deficiency, including recurrent illnesses or infections, hypoglycemia, convulsions, failure to thrive and shock. The disease is life threatening if untreated. Of FGD cases, 50% are caused by mutations in one of two chromosome 5-linked families (Supplementary Fig. 1a). The homozygous NNT mutations c.600_601delG at the splice junction of intron 4 and exon 5 (encoding p.Tyr201Phefs*2) and c.2930T>C within exon 20 (encoding p.Leu977Pro) were discovered in the two other chromosome 5-linked families (Supplementary Fig. 1b). Eighteen further mutations in 12 kindreds were found in homozygous or compound heterozygosity in sequencing of 100 individuals with FGD of unknown cause (Table 1. Supplementary Figs. 1c,d and 2 and Supplementary Table 2). These mutations were widespread throughout the NNT gene and included a mutation that destroyed the translation-initiating methionine, two additional splice-site mutations and many missense and nonsense changes. No NNT mutations have previously been described in humans, and none of the reported variants have been annotated in any SNP or mutation database, including those of the 1000 Genomes Project and the National Heart, Lung, and Blood Institute (NHLBI) Grand Opportunity Exome Sequencing Project in which >5,000 exomes have been sequenced (see URLs).

Mutation of the initiating methionine is of unknown consequence: the next methionine is at codon 132, and initiation of translation from this codon would result in a protein lacking the whole mitochondrial targeting presequence. Many of the described mutations were nonsense and/or frameshift and would lead to premature truncation of the protein. The remaining missense mutations, with the exception of the alteration resulting in a p.His365Pro substitution, canonic of the protein. The remaining missense mutations, with the exception of the alteration resulting in a p.His365Pro substitution, were predicted to cause disruption to vital, highly conserved eukaryotic protein domains. NNT, a highly conserved gene, encodes an integral protein of the inner mitochondrial membrane. Under most physiological conditions, this enzyme uses energy from the mitochondrial proton gradient to produce high concentrations of nicotinamide adenine dinucleotide phosphate (NADPH). Detoxification in mitochondria of ROS by glutathione peroxidases depends on this NADPH for regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) to maintain a high GSH/GSSG ratio (Supplementary Fig. 3). Caenorhabditis elegans lacking nnt-1, either through mutation or RNA interference (RNAi)-mediated knockdown, were shown to be more susceptible to oxidative stress because of a lowered GSH/GSSG ratio⁶. Certain substrains of C57BL/6 mice contain a spontaneous Nnt mutation (an in-frame 5-exon deletion) and have been reported to show glucose intolerance and reduced insulin secretion⁵,⁶. Knockdown of

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Table 1  NNT mutations in 15 kindreds with FGD

| Kindred | Nucleotide change | Protein change | Exon | Protein domain and/or consequence | Zygosity |
|---------|-------------------|----------------|------|-----------------------------------|----------|
| 1<sup>a</sup> | c.[1598C>T];[1598C>T] | [Ala533Val];[Ala533Val] | 11;11 | Transmembrane | Homozygous |
| 2<sup>a</sup> | c.(600-1delG);[600-601delG] | [Tyr201Lysfs*1];[Tyr201Lysfs*1] | Intron 4/exon 5 | Premature truncation at amino acid 202 | Homozygous |
| 3<sup>a</sup> | c.[2930T>C];[2930T>C] | [Leu977Pro];[Leu977Pro] | 20;20 | Nucleotide binding | Homozygous |
| 4 | c.[1310C>T];[1669C>T] | [Pro437Leu];[Gly557*] | 10;12 | Transmembrane; truncation at amino acid 557 | Compound heterozygous |
| 5 | c.[1147C>T];[1147C>T] | [Gln383*];[Gln383*] | 9;9 | Premature truncation at amino acid 383 | Homozygous |
| 6 | c.[1094A>C];[1094A>C] | [His365Pro];[His365Pro] | 8;9 | Mitochondrial matrix | Homozygous |
| 7 | c.[2032G>A];[2585G>A] | [Gly678Arg];[Gly622Asp] | 14;17 | Transmembrane; transmembrane | Compound heterozygous |
| 8 | c.[1A>G];[1A>G] | [Met17];[Met17] | 2;2 | Initiating Met-Val | Homozygous |
| 9 | c.[1107_1110delTCAC];[3027T>G] | [His370*];[Asn1009Lys] | 9;19 | Premature truncation at amino acid 370; nucleotide binding | Compound heterozygous |
| 10 | c.[1356delA];[1355delA] | [Gln52Argfs*44];[Gln52Argfs*44] | 10;10 | Frameshift and premature truncation at amino acid 496 | Homozygous |
| 11 | c.[578G>A];[578G>A] | [Ser193Asn];[Ser193Asn] | 4;4 | Mitochondrial matrix | Homozygous |
| 12 | c.[63deG];[1864-1G>T] | [Ser22Profs*6];[Ile622Aspfs*1] | 2;14 | Frameshift and premature truncations at amino acids 28 and 623 | Compound heterozygous |
| 13 | c.[1069A>G];[2637delA] | [Thr572Ala];[Met880*] | 7;18 | Mitochondrial matrix; premature truncation at amino acid 880 | Compound heterozygous |
| 14 | c.[3022G>C];[3022G>C] | [Ala1008Pro];[Ala1008Pro] | 21;21 | Nucleotide binding | Homozygous |
| 15 | c.[1990G>A];[2293+1G>A] | [Gly664Arg];[Thr689Leufs*320] | 14;15 | Transmembrane; frameshift and premature truncation at amino acid 984 | Compound heterozygous |

<sup>a</sup>Kindreds 1, 2 and 3 had linkage to 5p13-q12. The proband from kindred 1 was subjected to targeted exome sequencing. <sup>b</sup>Predicted result if exon skipped.

NNT in human PC12 phaeochromocytoma cells resulted in decreased cellular NADPH levels, decreased GSH/GSSG ratios, increased H₂O₂ levels and, hence, impaired redox homeostasis<sup>9</sup>. Adrenal glands from 3-month-old C57BL6/J mice carrying the NNT mutation had slightly disorganized zona fasciculata with higher levels of apoptosis than wild-type C57BL6/NHsd mice (Fig. 1a,b).

**Figure 1**  NNT loss increases the levels of mitochondrial ROS and apoptosis. (a) Adrenal zonation and steroidogenesis in C57BL6/NHsd and C57BL6/J mice (genotyping as in Supplementary Fig. 6). Wild-type and Nnt mutant mice showed similar patterns of CYP11A1 (red) and CYP11B1 (green) staining, indicating no significant differences in zonation or steroidogenic capacity. The zona fasciculata (ZF) cells in C57BL6/J mice, however, were slightly hyperplastic and disorganized, were more densely packed and lacked the linear architecture seen in wild-type zona fasciculata. ZG, zona glomerulosa. (b) Increased apoptosis in C57BL6/J mice. Adrenal glands from C57BL6/J animals had a greater number of cleaved Caspase-3–positive cells (red) in zona fasciculata than C57BL6/NHsd animals. Left, low magnification; middle and right, high magnification. (c) Corticosterone levels in C57BL6/NHsd and C57BL6/J mouse serum stimulated with ACTH. Radioimmunoassay results revealed that both basal and stimulated corticosterone levels were lower in C57BL6/J mice. *P < 0.05. (d) Stable NNT knockdown in H295R cells. RT-PCR (left) and protein blot analysis (right) confirmed knockdown of NNT mRNA (71%) and protein (78%) in cells expressing NNT shRNA compared to those expressing scrambled, control shRNA. RU, relative units. (e) Detection of superoxide production with Mitosox. Quantitative analysis showed significantly higher levels of superoxide production in cells with NNT shRNA relative to those with scrambled, control shRNA. **P < 0.005. (f) Densitometric analysis showed significantly higher levels of cleaved PARP relative to actin, n = 12. ***P < 0.001. (g) The GSH/GSSG ratio was lower in cells with NNT shRNA relative to cells with scrambled, control shRNA. Oxidative stress induced by the addition of 40 µM menadione to the cells with control shRNA reduced the ratio to 0.69 ± 0.67. RU, relative light units. Error bars, s.d.
There were no observable differences in the levels of the steroidogenic enzymes CYP11A1 and CYP11B1 between the two substrains; however, the mutant mice did have lower basal and stimulated levels of corticosterone than their wild-type counterparts (Fig. 1a,c). Knockdown of NNT in the human adrenocortical H295R cell line by shRNA not only increased the levels of mitochondrial ROS and apoptosis but also lowered the GSH/GSSG ratio (29.95 ± 4.77 versus 18.82 ± 8.75 in cells receiving scrambled, control shRNA or NNT-targeted shRNA, respectively; \( P < 0.001 \)), implying that these cells also have impaired redox potential (Fig. 1d–g and Supplementary Fig. 4). We found that NNT was widely expressed in humans, with expression most readily detectable in adrenal, heart, kidney, thyroid and adipose tissues (Supplementary Fig. 5), as was seen in human and rodent expression profiles⁸⁻¹⁰.

These findings suggest that impaired adrenal steroidogenesis and the development of FGD are due to defective oxidative stress responses. Oxidative stress has been implicated in the pathogenesis of many disease conditions. Of particular relevance to individuals with FGD is Triple-A syndrome (MIM 231550)¹¹. In this condition, mutations in AAAS lead to deficiency or mislocalization of the nuclear pore protein ALADIN, resulting in impaired nuclear import of DNA-repair and antioxidant proteins¹²,¹³ and thereby rendering the cells more susceptible to oxidative stress. Aaas⁻/⁻ mice, however, showed no such phenotype¹⁴. Nnt loss in mice has been reported to lead to impaired insulin secretion and glucose intolerance because of oxidative stress in pancreatic β cells⁵. Although no adrenal phenotype has previously been shown in these mice, our studies hint at a mild deficit in adrenal steroidogenesis. In conclusion, our results suggest that, at least in humans, NNT is of primary importance for ROS detoxification in adrenocortical cells, highlighting the susceptibility of the adrenal cortex to this type of pathological damage. Over time, affected individuals may develop other organ pathologies related to impaired antioxidant defense and will therefore need careful monitoring.

**URLs.** 1000 Genomes Project, http://www.1000genomes.org; NHLBI Exome Sequencing Project (ESP) Exome Variant Server (accessed January 2012), http://evs.gs.washington.edu/EVS/.

**Note:** Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.A.M., A.J.L.C., P.J.K. and J.P.C. conceived and designed the experiments and jointly supervised the research. E.M., J.K., L.G., C.R.H., F.W. and L.A.M. performed the experiments. N.P., R.B. and H.N.S. contributed reagents and clinical information. L.A.M., A.J.L.C. and E.M. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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