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Mutations in *CYB561* Causing a Novel Orthostatic Hypotension Syndrome

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Rationale: **Orthostatic hypotension is a common clinical problem, but the underlying mechanisms have not been fully delineated.**

Objective: **We describe 2 families, with 4 patients in total, experiencing severe life-threatening orthostatic hypotension because of a novel cause.**

Methods and Results: **As in dopamine β-hydroxylase deficiency, concentrations of norepinephrine and epinephrine in the patients were low. Plasma dopamine β-hydroxylase activity, however, was normal, and the *DBH* gene had no mutations. Molecular genetic analysis was performed to determine the underlying genetic cause. Homozygosity mapping and exome and Sanger sequencing revealed pathogenic homozygous mutations in the gene encoding cytochrome b561 (*CYB561*); a missense variant c.262G>A, p.Gly88Arg in exon 3 in the Dutch family and a nonsense mutation (c.131G>A, p.Trp44*) in exon 2 in the American family. Expression of *CYB561* was investigated using RNA from different human adult and fetal tissues, transcription of RNA into cDNA, and real-time quantitative polymerase chain reaction. The *CYB561* gene was found to be expressed in many human tissues, in particular the brain. The CYB561 protein defect leads to a shortage of ascorbate inside the catecholamine secretory vesicles leading to a functional dopamine β-hydroxylase deficiency. The concentration of the catecholamines and downstream metabolites was measured in brain and adrenal tissue of 6 *CYB561* knockout mice (reporter-tagged deletion allele [post-Cre], genetic background C57BL/6NTac). The concentration of norepinephrine and normetanephrine was decreased in whole-brain homogenates of the *CYB561*$^{(-/-)}$ mice compared with wild-type mice ($P<0.01$), and the concentration of normetanephrine and metanephrine was decreased in adrenal glands ($P<0.01$), recapitulating the clinical phenotype. The patients responded favorably to treatment with L-dihydroxyphenylserine, which can be converted directly to norepinephrine.**

Conclusions: **This study is the first to implicate cytochrome b561 in disease by showing that pathogenic mutations in *CYB561* cause an as yet unknown disease in neurotransmitter metabolism causing orthostatic hypotension. (*Circ Res*. 2018;122:846-854. DOI: 10.1161/CIRCRESAHA.117.311949.)**

Key Words: catecholamines ■ dopamine ■ genetics ■ hypotension, orthostatic ■ sympathetic nervous system

Catecholamines—dopamine, norepinephrine, and epinephrine—play an essential role in many physiological processes, including regulation of vascular tone and circulation. Their synthesis from the amino acid precursor tyrosine involves several steps in which enzymes, cofactors, and transporters are indispensable (Figure 1). Defects in 9 enzymes in catecholamine biosynthesis and catabolism (including dopamine β-hydroxylase [DβH]) and in 2 transporters have been described.[1] Most of these lead to severe motor and mental dysfunction and present in early childhood. DβH deficiency presents as an orthostatic hypotension syndrome.[2,3]

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Novelty and Significance

What Is Known?

- Catecholamines play an essential role in regulation of vascular tone and blood pressure.
- Diseases affecting catecholamine biosynthesis and catabolism can cause orthostatic hypotension, which are often hereditary.

What New Information Does This Article Contribute?

- We report a novel autosomal recessive orthostatic hypotension syndrome in 2 families, characterized by incapacitating orthostatic hypotension, renal insufficiency, hypoglycemia, and reduced life expectancy.
- The underlying genetic defect was identified to be homozygous mutations in the gene encoding cytochrome b561 (*CYB561*). The mutations disrupt the electron shuttle necessary for the ascorbate-dehydroascorbate recycling inside catecholamine secretory vesicles and halts production of norepinephrine.
- This novel genetic cause for orthostatic hypotension is an inborn error of metabolism amenable to treatment with L-dihydroxyphenylserine, which can be converted directly to norepinephrine.

Catecholamines play an essential role in regulation of vascular tone and blood pressure. Diseases affecting catecholamine biosynthesis and catabolism can cause orthostatic hypotension. We describe 2 families, with 4 patients in total, experiencing severe life-threatening orthostatic hypotension, renal insufficiency, hypoglycemia, and reduced life expectancy. As in dopamine β-hydroxylase deficiency deficiency, concentrations of norepinephrine and epinephrine were low in our patients. Plasma dopamine β-hydroxylase activity, however, was normal, and the *DBH* gene did not carry a mutation. Homozygosity mapping, exome sequencing, and subsequent Sanger sequencing led to identification of pathogenic homozygous mutations in the gene encoding cytochrome b561 (*CYB561*). The mutations disrupted the electron shuttle necessary for the ascorbate-dehydroascorbate recycling inside catecholamine secretory vesicles. This recycling is imperative for the synthesis of norepinephrine from dopamine within these vesicles. The *CYB561* gene was found to be expressed in several human tissues, in particular the brain. The concentrations of the catecholamines and downstream metabolites were measured in brain and adrenal tissue of 6 *Cyb561* knockout mice, which recapitulated the clinical phenotype. This study is the first to implicate cytochrome b561 in human disease by showing that pathogenic mutations in *CYB561* cause a novel form of orthostatic hypotension caused by abnormalities in neurotransmitter metabolism. This novel inborn error of metabolism is amenable to treatment with L-dihydroxyphenylserine, which can be converted directly to norepinephrine.

Nonstandard Abbreviations and Acronyms

CYB561	cytochrome b561
DβH	dopamine β-hydroxylase
L-DOPS	L-dihydroxyphenylserine

We describe 2 families, with 4 patients in total, experiencing severe life-threatening orthostatic hypotension, resembling DβH deficiency. As in DβH deficiency, concentrations of norepinephrine and epinephrine were low in our patients. Plasma DβH activity, however, was normal, and the *DBH* gene was not mutated. Homozygosity mapping, exome sequencing, and subsequent Sanger sequencing revealed pathogenic homozygous mutations in the gene encoding cytochrome b561 (*CYB561*). As a consequence, the electron shuttle necessary for the ascorbate-dehydroascorbate recycling inside catecholamine secretory vesicles is disrupted. This recycling is imperative for the synthesis of norepinephrine from dopamine within these vesicles.[4] This novel genetic cause for orthostatic hypotension is an inborn error of metabolism amenable to treatment with L-dihydroxyphenylserine (L-DOPS), which can be converted directly to norepinephrine.

Methods

The authors declare that all supporting data are available within the article and its Online Data Supplement. A detailed description of methods used in this study is available in the Online Data Supplement.

Patients

Dutch Family

A 33-year-old female (patient D1) was referred to the University Medical Center Groningen because of severe orthostatic hypotension. From early childhood onwards, she had experienced dizziness and occasional fainting on standing. Her neurological examination was within normal limits. There was no ptosis. She had normal mental and physical development, and her pregnancies had been uncomplicated. Supine blood pressure was 85/50 mm Hg, but after standing up, it immediately dropped to 50/35 mm Hg without a significant change in heart rate (Figure 2). Because of the combination of orthostatic hypotension and lack of compensatory tachycardia, sympathetic dysfunction was suspected. Indeed, the concentration of norepinephrine, epinephrine, and their downstream metabolites normetanephrine and 3-methoxy-4-hydroxyphenylglycol was low in 24-hour urine and plasma. The 3-methoxy-4-hydroxyphenylglycol concentration in cerebrospinal fluid was equally low, with normal homovanillic acid and 5-hydroxyindolacetic acid concentration (Table 1; Online Table I). Sanger sequencing of *DBH*, the gene-encoding DβH, revealed no pathogenic mutations.[5] Renal function was impaired, with secondary mild anemia (hemoglobin=7.3 mmol/L). Echography of the kidneys was unremarkable. Using 125 I-iothalamate clearance, supine effective renal plasma flow was 248 mL/min and glomerular filtration rate was 56 mL/min (reference 1000–1200 and 100–120, respectively) whereas sitting effective renal plasma flow was 199 mL/min and glomerular filtration rate 38 mL/min. The patient had a 26-year-old sister (patient D2) with a comparable clinical presentation and biochemical findings (Table 1;Online Table I). D2's plasma DβH enzyme activity was normal.[6] The *DBH* gene was sequenced, but no pathogenic mutations were found. Neither the parents nor 2 other healthy siblings experienced orthostatic hypotension, and their urinary catecholamine levels were all normal (Figure 3).

American Family

A 39-year-old female (patient A1) and her 38-year-old sister (patient A2) presented at the Vanderbilt University School of Medicine because of severe orthostatic hypotension since infancy. They had episodes of symptomatic hypoglycemia during infancy. Patient A1 underwent a partial pancreatectomy at age 3 years for this reason, but she showed no improvement in these episodes. Both sisters had a normal mental and physical development and uncomplicated pregnancies. They had a brother with comparable complaints, but he died at age 16 years of an unknown cause. Both parents and 4 other sibs had no complaints (Figure 3). Plasma norepinephrine, epinephrine, and the intraneuronal norepinephrine metabolite dihydroxyphenylglycol were below the limit of detection (<0.01 nmol/L; Online Table I). Plasma DβH activity was normal.

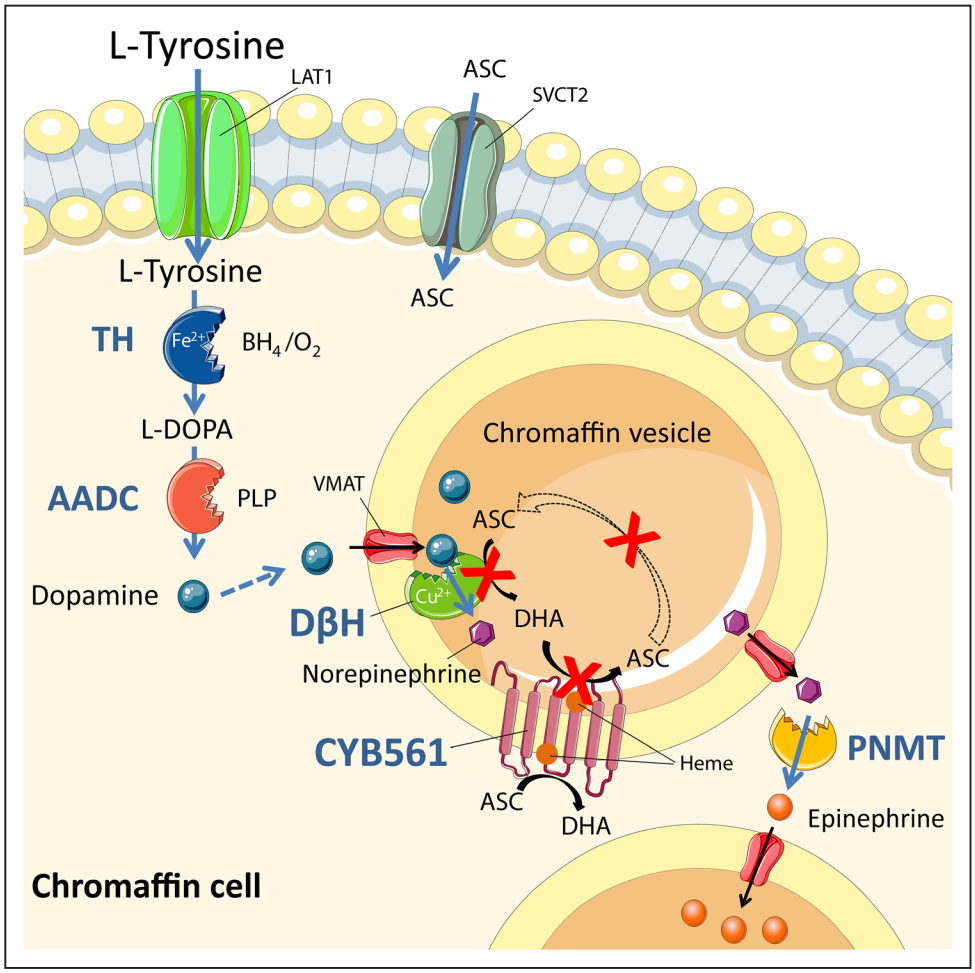

Figure 1. Catecholamine metabolism and the function of CYB561 in this pathway. The site of action is the chromaffin cell and vesicle. Tyrosine is taken up by the cell, where it is enzymatically converted by tyrosine hydroxylase (TH) to L-DOPA (rate-limiting reaction). L-DOPA is then converted to dopamine by aromatic L-amino acid decarboxylase (AADC), and the dopamine is rapidly transported by vesicular monoamine transporter (VMAT) inside the vesicle, where it is converted by dopamine β-hydroxylase (DβH) with ascorbic acid (ASC) as cofactor and resulting in norepinephrine and dehydroascorbate (DHA). Norepinephrine is transported outside the vesicle by VMAT, where it is converted to epinephrine by cytosolic phenylethanolamine N-methyltransferase (PNMT). Dehydroascorbate inside the vesicle is reduced to ascorbic acid by CYB561, which itself becomes oxidized. CYB561 is then reduced by cytosolic ascorbic acid, completing the cycle. DOPA indicates dihydroxyphenylalanine.

Molecular Genetic Analysis

To determine the genetic basis of the phenotypes, genomic DNA from the 2 affected Dutch sisters (D1, D2) and their relatives and from the 2 affected American sisters (A1, A2) was extracted from peripheral blood using standard protocols (PUREGENE; Qiagen, Venlo, the Netherlands). Homozygosity mapping and exome and Sanger sequencing were then performed (Online Data Supplement). The analyses conformed to principles defined in the Helsinki Declaration and the medical ethics committees of the 2 centers. Written informed consent was obtained from the patients or their relatives.

Results

Mutation Identification

Homozygosity mapping in patients D1 and D2 revealed 11 shared homozygous regions >2.0 cM (Online Figure I). The largest region of homozygosity was located on chromosome 17, 17q22-17q24.2, between rs11657462 and rs4968819 (g.55,463,146-g.66,742,160; UCSC Genome Browser, build hg19), spanning 550 SNPs (single nucleotide polymorphisms) and 12.26 cM, and containing 220 genes. Using different data analysis curing programs, whole-exome sequencing identified 22 689 variants in patient D1. After removal of all known variants with >1% frequency from the exome sequencing data of patient D1, we selected all the stop-gain, stop-loss, missense, splice site, frame shift, and in-frame coding indel variants that were concordant with autosomal recessive inheritance. Fifteen variants in 10 different genes fulfilled these criteria. The respective genes and variants were computationally analyzed and classified using OMIM (https://www.omim.org), GeneCards (http://www.genecards.org), and UniProt (http://www.uniprot.org; gene classification) and the Alamut software (Interactive biosoftware; Online Table II). Based on that analysis, only the variant in the *CYB561* gene was prioritized as possible candidate disease gene. Moreover, the variant in that gene was the only homozygous variant located in the longest shared homozygous region (chromosome 17q22-17q24.2) seen in patients D1 and D2, as identified by homozygosity mapping. In this chromosomal region, a homozygous missense variant c.262G>A, p.Gly88Arg in exon 3 of the *CYB561* (NM_001915.3; Figure 4)

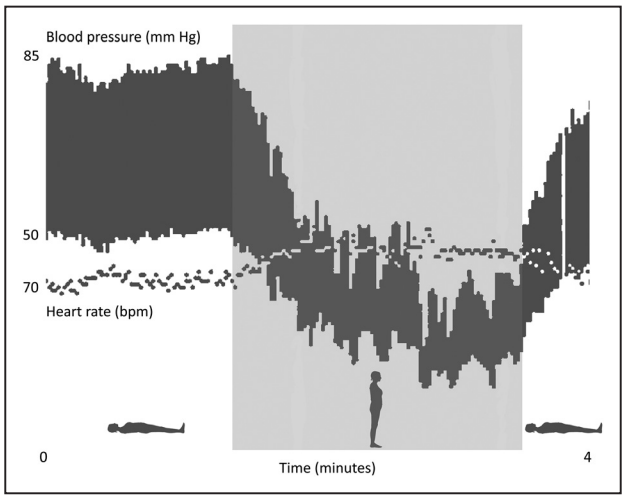

Figure 2. Blood pressure and heart rate recording. Beat-to-beat recordings of blood pressure and heart rate in the Dutch proband (patient D1) when she was supine and standing. After standing up, her blood pressure dropped profoundly without a significant change in heart rate.

was found as the most likely causative variant. Analysis of the *CYB561* closest homologs in other species showed that the nucleotide c.262 and the glycine 88 both are highly evolutionary conserved (Figure 5). The Gly88 residue is located in a crucial functional protein domain (cytochrome b561/ferric reductase transmembrane; Figure 6). The in silico pathogenicity prediction programs MutationTaster and PolyPhen-2 both suggest a pathogenic effect. Sanger sequencing confirmed the presence of the c.262G>A mutation in homozygous form in the 2 affected Dutch sisters while it was heterozygous in the parents and in an unaffected sister and wild type in the other sister (Table 1; Figure 4). Further evidence for the relationship between *CYB561* gene variants and orthostatic hypotension was obtained from the American family. Both affected sisters (A1 and A2) had a homozygous nonsense mutation (c.131G>A, p.Trp44*) in exon 2 of the gene (Table 1; Figure 4). This variant leads to reading frame interruption by a premature stop codon. We have assessed the frequencies of the CYB561 variants in the Genome Aggregation Database (http://gnomad.broadinstitute.org). The missense variant c.262G>A, p.Gly88Arg identified in the Dutch family was absent in >100 000 Non-Finnish European control alleles. Another missense variant resulting in the same amino acid change (c.262G>C, p.Gly88Arg) was found in heterozygous state 2× in 126 628 non-Finnish European control alleles. The nonsense mutation (c.131G>A, p.Trp44*) identified in the American family was found once in 110 060 Non-Finnish European control alleles.

Table 1. Clinical Signs and Symptoms, and Genetic and Biochemical Hallmarks of Patients With a *CYB561* Defect

	American Patients (n=2)	Dutch Patients (n=2)
Clinical signs and symptoms		
Severe orthostatic hypotension	+	+
Episodes of hypoglycemia in childhood	+	ND
Disturbed renal function (mild–severe)	ND	+
Mild anemia	ND	+
Early death	+	+
Genetic characteristics		
Mutation cDNA: NM_001915.3(CYB561)	c.131G>A	c.262G>A
Mutation gDNA: Chr17(GRCh37)	g.61514778C>T	g.61513454C>T
Protein change	p.Trp44*	p.Gly88Arg
Biochemical hallmarks		
Low norepinephrine in urine and plasma		
Low normetanephrine in urine and plasma		
Low MHPG in urine		
Low CSF MHPG with normal CSF HVA and 5-HIAA		
Normal dopamine and metabolites in urine and plasma		
Normal dopamine β hydroxylase enzymatic activity in plasma		

5-HIAA indicates hydroxyindolacetic acid; CSF, cerebrospinal fluid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; and ND, not documented.

CYB561 Expression in Human Tissues

After identification of the mutations, expression of *CYB561* was investigated using RNA from different human adult and fetal tissues, transcription of RNA into cDNA, and real-time quantitative polymerase chain reaction. (Online Data Supplement). The *CYB561* gene was found to be expressed in many human tissues. Expression is ubiquitous in the brain and strong, especially in the cortex and hippocampus (Online Figure II).

Catecholamine Measurements in CYB61 Knockout Mouse Tissue

To obtain additional functional evidence, tissue from *CYB561* knockout mice (reporter-tagged deletion allele [post-Cre], genetic background C57BL/6NTac) was obtained through the International Mouse Phenotyping Consortium (Release 6.1.).[7] The mouse studies in the International Mouse Phenotyping Consortium are approved by the appropriate review boards. Using liquid chromatography in combination with isotope dilution tandem mass spectrometry, we measured the concentration of the catecholamines and downstream metabolites in brain and adrenal tissue of 6 *CYB561*$^{(-/-)}$ mice and 6 wild-type mice. Mouse brain and adrenal gland tissue homogenates were prepared as previously described.[8] In short, tissue homogenates (10% weight/volume) were prepared by sonification at 11 to 12 W for 30 seconds on ice in 0.08 mol/L acetic acid with 0.8% (weight/volume) reduced glutathione. After homogenization, samples were centrifuged, and supernatant was used for analysis by isotope dilution liquid chromatography tandem mass spectrometry as previously described. Isotope-labeled standards were used for each respective analyte.[9,10] The concentration of norepinephrine and normetanephrine was profoundly and significantly decreased in whole-brain homogenates of 6 *CYB561*$^{(-/-)}$ mice compared with 6 wild type (median and range in nmol/g wet weight for

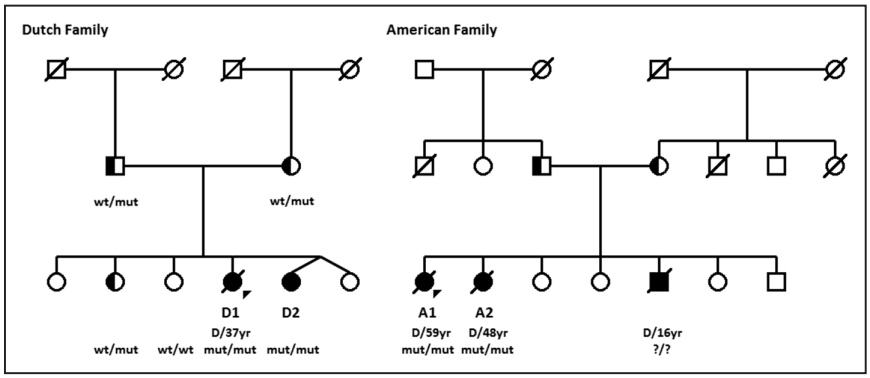

Figure 3. Pedigrees of the Dutch and the American families. Patients diagnosed with orthostatic hypotension are labeled with D1/D2 and A1/A2, respectively. Squares indicate males, and circles indicate females. Diagonal line indicates deceased, and D/XXyr the age at death. Filled symbols indicate affected individuals. Half-filled symbols indicate unaffected carriers. CYB561 mutation status is shown by wild type (wt); mutation (mut); and ?, unknown. Triangles indicate the probands of each family. No consanguinity was known and therefore not shown by a double line between the parents of the affected individuals.

norepinephrine: 0.126 [0.10–0.19] versus 2.88 [2.26–3.84]; $P<0.01$; Table 2). This nicely illustrates the consequence of the *CYB561* defect for the norepineprine presence in the brain. The concentration of the norepinephrine and epinephrine catabolites normetanephrine and metanephrine was significantly decreased in adrenal glands ($P<0.01$). Dopamine is the direct precursor of norepinephrine. Its concentration was not significantly different between wild-type and *CYB561*$^{(-/-)}$ mice in the adrenals and the brain. The concentration of the dopamine catabolite 3-methoxytyramine, however, was significantly increased in adrenal glands but not in the brain of *CYB561*$^{(-/-)}$ mice (Table 2). The normal dopamine concentration reflects that dopamine biosynthesis is fully normal in *CYB561*$^{(-/-)}$ mice. The defect affects the catecholamine biosynthesis downstream of dopamine at the level of dopamine β synthase. Low DβH enzyme activity is because of the interruption of the ascorbate cycle caused by the defect in *CYB561* (Figure 1).

Consequences at the Level of the Cytochrome b561 Protein

It is generally accepted that CYB561 has 6 transmembrane α helix domains.[4] It has 4 His-residues coordinating 2 heme-b molecules, 1 on the cytoplasmic side and 1 on the luminal side of the membrane (Figure 6). The heme groups accept electrons from ascorbate. The p.Gly88Arg change found in the Dutch family occurs in the third transmembrane domain while the c.131G>A mutation in the American family truncates the protein at Trp44 in between the first 2 transmembrane segments (Figure 6). No experimentally solved structure of the human cytochrome b561 is known. We have, therefore, built a homology model using the known cytochrome b561 structure of Arabidopsis thaliana as a template.[11] The model suggests that the introduction of a hydrophilic and positively charged arginine in the third transmembrane region might affect the interactions between the hydrophobic lipids and can alter

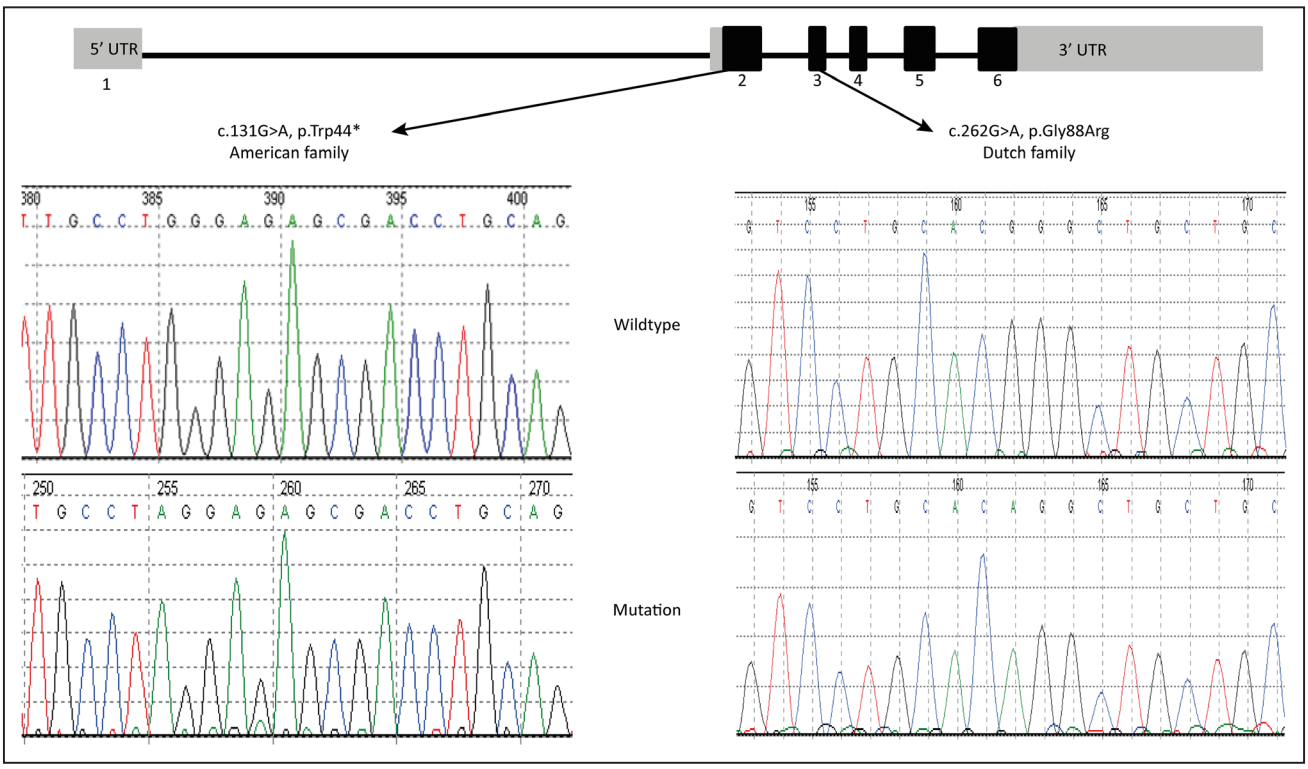

Figure 4. CYB561 mutations. The upper part shows the structure of the *CYB561* gene, with the positions of the identified mutations indicated. Gray boxes indicate 5′ and 3′ untranslated regions (UTRs), and black boxes indicate coding exon sequences. The lower part depicts spherograms showing the Sanger sequencing data of the patients (mutation) and controls (wild type).

Figure 5. Evolutionary conservation of the cytochrome b561 amino acid sequence. Part of the protein sequence of human CYB651 and the corresponding sequence in the Dutch patients (D1 and D2) with the glycine to arginine replacement at position 88 (in red) are shown. Corresponding sequences across different organisms are aligned. TM indicates transmembrane.

transmembrane anchoring of the protein (Figure 7). In addition, a smaller effect on ligand binding can be expected as the mutation occurs next to the central histidine residue, which is detrimental for heme-Fe binding.

Biochemical Phenotype

Norepinephrine and its downstream metabolites normetanephrine and 3-methoxy-4-hydroxyphenylglycol were profoundly decreased in body fluids of the patients (plasma: norepinephrine <0.05 nmol/L, normetanephrine <0.01 nmol/L; urine: norepinephrine <2 µmol/mmol creatinine). Other downstream metabolites from the catecholamine pathway were also lower than normal (Online Data Supplement). The concentration of 3-methoxy-4-hydroxyphenylglycol, the principal downstream metabolite of norepinephrine in the brain, was extremely low in cerebrospinal fluid (<3 nmol/L; reference 26–64). The downstream metabolites of dopamine and serotonin, homovanillic acid and 5-hydroxyindolacetic acid, respectively, had a normal concentration in cerebrospinal fluid. This underpins the central role of CYB561 in the brain, the normal biosynthesis and availability of dopamine and serotonin in the patients, and the profound consequences of pathogenic mutations in the *CYB561* gene for the norepinephrine availability in the brain of the patients. Plasma DβH enzymatic activity was normal in all patients. This parameter helps to discriminate between defects in *CYB561* and *DBH*. The overall biomarker profile allows the biochemical diagnosis of the *CYB561* defect in plasma or urine in other patients.

Treatment

The 4 patients were treated with L-DOPS, a synthetic precursor of norepinephrine, with dosages ranging from 200 mg once to 3× daily. This treatment led a rise blood pressure in all 4 patients: supine blood pressure invariably exceeded 100/70 mm Hg and blood pressure in the upright position usually rose to at least 90/60 mm Hg. More importantly, treatment with L-DOPS ameliorated the symptoms of orthostatic hypotension (dizziness).These beneficial effects were supported by the urinary norepinephrine levels, which we measured in the Dutch patients (D1 and D2) after initiating L-DOPS treatment; levels were markedly higher and even beyond the upper limit of normal. However, L-DOPS tolerability was hampered by nausea, headache, and pain in the lumbar region. Four years after presentation, patient D1 suddenly lost consciousness with a prolonged circulatory arrest (while she was off L-DOPS treatment). After resuscitation measures and administration of a low-dose epinephrine, her blood pressure quickly recovered, but she had incurred severe brain damage and died after being in coma for 5 days. Her sister (D2) is still alive albeit with progressive renal insufficiency (glomerular filtration rate, 26 mL/min). Both American patients (A1 and A2) were lost to follow-up and died of unknown cause at ages 59 and 48 years, respectively.

Discussion

Here, we report a novel autosomal recessive orthostatic hypotension syndrome caused by mutations in the *CYB561* gene. These mutations interfere with norepinephrine biosynthesis, a situation that resembles DβH deficiency in terms of clinical signs and symptoms and biochemical hallmarks.[2,3] DβH and CYB561 deficiency are both characterized by orthostatic hypotension, recurrent hypoglycemia, and low norepinephrine levels. Ptosis and skeletal muscle hypotonia have been described in DβH patients but were not observed in our *CYB561* patients.[2,3] Instead, the syndrome in our patients seems to be more malignant in terms of renal insufficiency and reduced life expectancy, either because of hypotensive crises or profound hypoglycemia. Fortunately, treatment with L-DOPS affords alleviation of symptoms although tolerability has been an issue.

Data on the role of *CYB561* gene in humans are limited. In a study in healthy volunteers, an SNP (rs_20582013) in *CYB561* was shown to be associated with lower norepinephrine levels.[12] A study in healthy twin pairs showed an association between another SNP (A+1485G, rs_3087776) in the microRNA motif in the 3′ untranslated regions of *CYB561* and (stress-augmented) heart rate.[13] These findings concur with

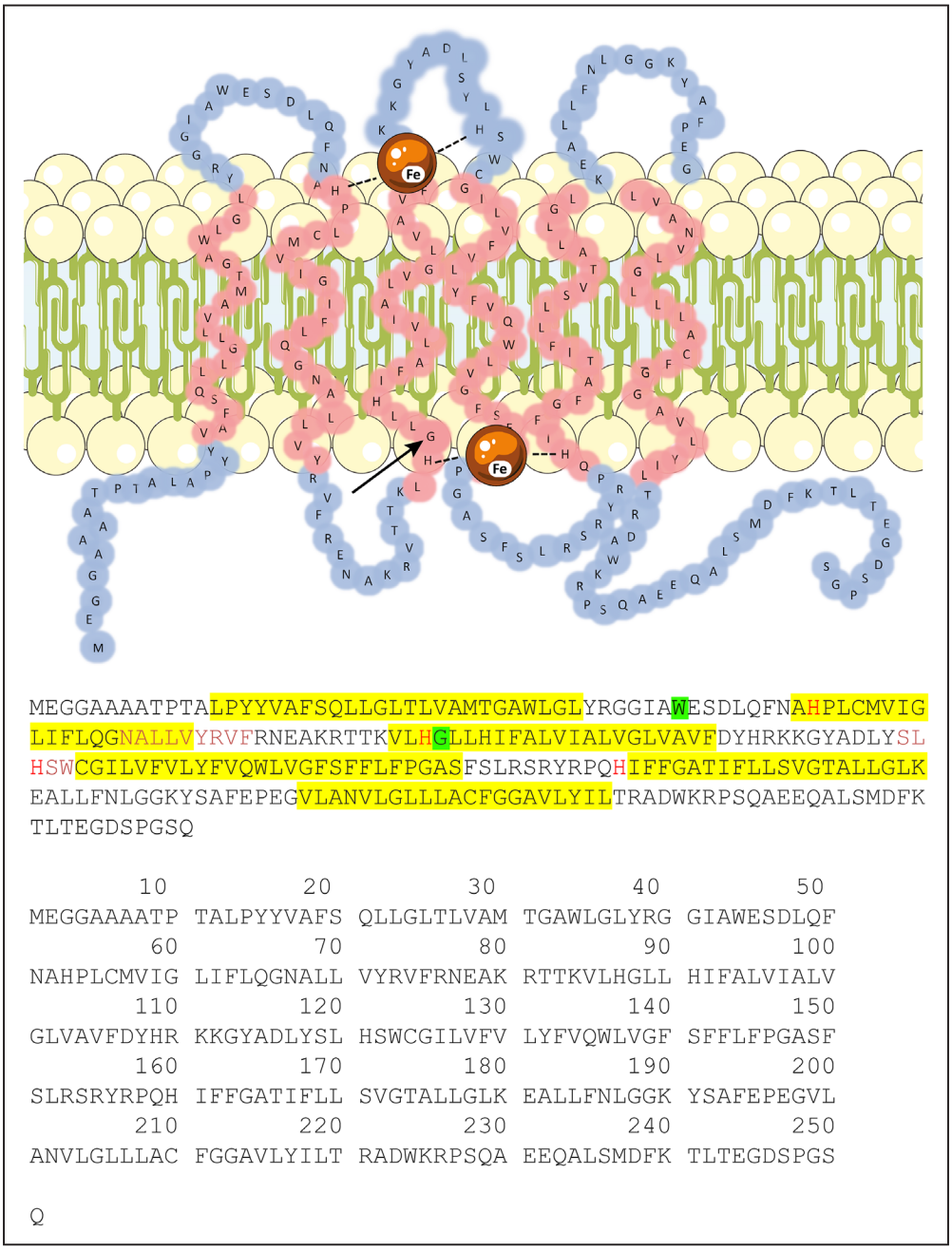

Figure 6. Cytochrome b561 protein conformation. The upper part shows the CYB561 protein conformation in the chromaffin vesicle membrane. The protein contains 2 heme groups positioned on either side of the vesicular membrane. The arrow indicates the mutated glycine in the Dutch patients (D1 and D2). The lower part shows the full protein sequence of CYB561. In yellow, the 6 transmembrane parts of the protein; in green, the positions where the mutations in the 2 families occur; in red, letters (H) the 4 Histine residues coordinating the 2 heme groups; the 2 stretches NALLVYRVF and SLHSW are the putative substrate reducing sites.

our data, implicating CYB561 in the sympathetic control of heart rate and blood pressure.[12,13] Our report is the first to actually implicate CYB561 in disease.

CYB561 encodes the protein CYB561, named as such because of its optical absorbance at 561 nm. The protein is attached to the membrane of catecholamine and neuropeptide secretory vesicles in neuroendocrine tissues (Figures 1 and 6). CYB561 is a heme-containing enzyme that is necessary for the continuous regeneration of semidehydroascorbate to ascorbate inside the chromaffin granules and neuropeptide secretory vesicles. It is widely expressed in human tissues. In the brain, it is strongly expressed in the cortex and hippocampus and to a lesser extent in other parts (Online Figure II). Its presence was demonstrated in various neuroendocrine tissues.[14] CYB561 functions as a transmembrane electron carrier, maintaining the redox state between cytosolic and intravesicular ascorbic acid (Figures 1 and 6).[15] Intravesicular ascorbic acid is required by DβH and peptidylglycine α-amidating monooxygenase for their enzymatic activity. DβH uses ascorbic acid as a cofactor to convert dopamine

Table 2. Concentrations of Catecholamines and Metanephrines for Brain- and Adrenal Gland Homogenates of Wild-Type and CYB561$^{-/-}$ Mice (n=6 Per Group)

	Wild Type	CYB561$^{-/-}$	*P* Value*
Adrenal glands			
3-Methoxytyramine	0.018 (0.010–0.020)	0.078 (0.05–0.36)	*P*<0.01
Normetanephrine	1.53 (0.96–1.89)	0.703 (0.61–0.97)	*P*<0.01
Metanephrine	1.48 (0.75–1.63)	0.530 (0.19–1.34)	*P*<0.01
Dopamine	0.431 (0.12–3.79)	1.94 (0.16–13.2)	n.s.
Norepinephrine	10.4 (2.24–139)	2.35 (0.18–35.6)	n.s.
Epinephrine	2.67 (0.84–57.9)	0.68 (0.03–37.5)	n.s.
Brain			
Normetanephrine	0.503 (0.32–0.71)	0.027 (0.02–0.05)	*P*<0.01
Norepinephrine	2.88 (2.26–3.84)	0.126 (0.10–0.19)	*P*<0.01
3-Methoxytyramine	0.898 (0.76–1.06)	1.02 (0.83–1.45)	n.s.
Dopamine	7.46 (6.51–8.12)	6.79 (4.52–8.17)	n.s.

Concentrations are in nmol/g wet weight as median values (min–max values). n.s. indicates nonsignificant.

*Mann–Whitney *U* test (2 tailed).

to norepinephrine, and peptidylglycine α-amidating monooxygenase uses ascorbic acid to amidate neuropeptides to prolong and increase activity (eg, α-melanocyte-stimulating hormone, oxytocin, vasopressin, gastrin). In the CYB561-catalyzed reaction, ascorbic acid is converted into dehydroascorbic acid. Vesicles cannot take up ascorbic acid, only semidehydroascorbate, so that semidehydroascorbate needs to be recycled inside the vesicle.[16,17] CYB561 reduces dehydroascorbate back to ascorbic acid, thereby oxidizing itself. Cytoplasmic ascorbic acid is then required to reduce CYB561, and so the cycle proceeds when enough ascorbic acid is available in the cytosol. Without CYB561 activity, norepinephrine synthesis comes to a halt because of insufficient vesicular ascorbate levels for the conversion of dopamine to norepinephrine by DβH.

The mutation in the Dutch patients introduces a change in a transmembrane domain of CYB561. Such changes are known to alter the structure and function of transmembrane proteins in general.[18] The introduction of a hydrophilic and positively charged arginine in the transmembrane region may affect the interactions between the hydrophobic lipids and can alter transmembrane anchoring of the protein. Besides that, a smaller effect on ligand binding can be expected as the mutation occurs next to the central histidine residue, which is detrimental for heme-Fe binding (Figures 6 and 7). Sequence alignment shows that Gly88 is highly conserved throughout various species. This is another indication that its substitution is likely to be detrimental for the function of CYB561 (Figure 4). Loss of CYB561 function is also likely in the American patients because their *CYB561* mutation leads to a premature stop codon.

The catecholamine measurements in tissues of *CYB561* knockout mice neatly recapitulated the clinical findings. An important consideration when using mice in ascorbic acid studies is that mice, unlike humans, can produce ascorbic acid de novo. Our biochemical findings in *CYB561*$^{-/-}$ mice are comparable to 2 studies in which mice lack the vitamin C transporter SVCT2 (sodium-dependent vitamin C transporter) and the enzyme gluconolactonase in the initial steps of ascorbic acid synthesis.[19,20] Both studies demonstrated a decreased concentration of norepinephrine while the dopamine concentration was not abnormal as was also shown in our patients and in the *CYB561*$^{(-/-)}$ mice. These results support our findings that *CYB561* mutations lead to disrupted ascorbate recycling and subsequently disturbed norepinephrine synthesis. Of note, as part of the International Mouse Phenotyping Consortium pipeline, all mice are routinely culled at 16 weeks, including the CYB561 knockout mice, and phenotyping is limited. In particular, no data on blood pressure are available, which is a limitation of our study.

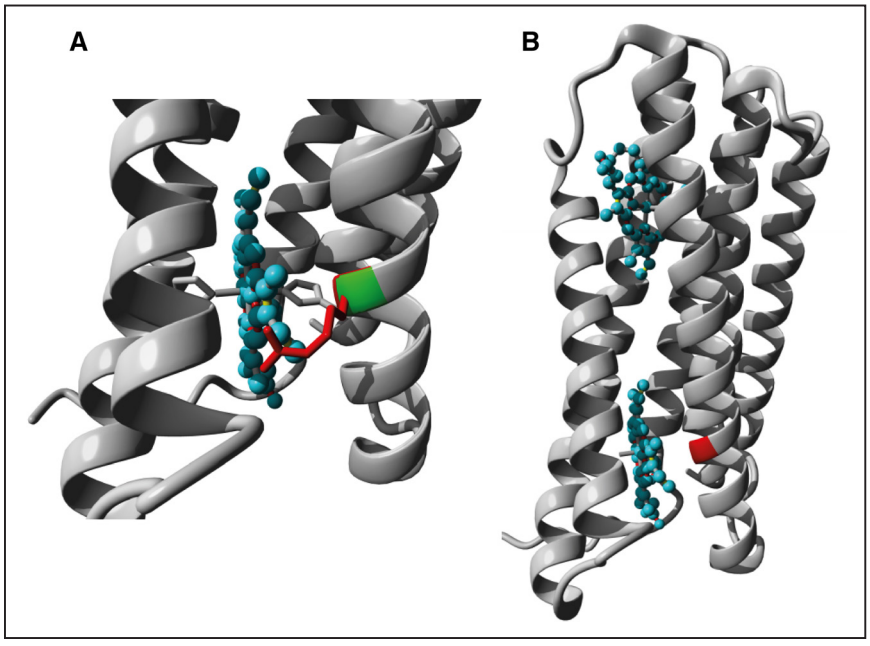

Figure 7. CYB561 homology modeling. **A**, Close-up of mutation G88R in CYB561. The protein is shown in ribbon presentation in gray, the heme ligand is shown in ball-stick presentation and colored cyan. The side chains of the mutant residue, wild-type residue, and the histidine that interacts with the Fe-atom in the heme group are all shown in stick presentation. The wild-type and mutant residues are colored green and red, respectively. **B**, Overview of the Cyb561 model shown in ribbon presentation. The 2 heme ligands are show in ball-stick presentation and colored cyan. The position of the G88R mutant is indicated in red.

In conclusion, our findings define biallelic mutations in *CYB561* as a novel cause for orthostatic hypotension in human. This novel inborn error of metabolism is amenable to treatment. Further studies are required to find an optimal medication dosing regime.

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Disclosures

None.

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