Tocotrienols reverse IKAP and monoamine oxidase deficiencies in familial dysautonomia

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Abstract

Familial dysautonomia (FD), a recessive neurodegenerative disease, is caused by mutations in the IKBKAP gene that result in the production of nonfunctional IKAP protein. Manifestations of FD include autonomic crises characterized by hypertension, tachycardia, diaphoresis, and vomiting. Elevated plasma levels of norepinephrine (NE) and dopamine observed during autonomic crises and an exaggerated hypertensive response to low doses of NE prompted an examination of monoamine oxidase (MAO) levels, key isoenzymes responsible for degrading biogenic and dietary monoamines, in individuals with FD. Fetal tissue homozygous for the common FD-causing mutation and peripheral blood cells of individuals with FD have reduced MAO A mRNA levels. FD-derived cells, stimulated with tocotrienols or EGCG to produce increased levels of functional IKAP, express increased amounts of MAO A mRNA transcript and protein. Administration of tocotrienol to individuals with FD results in increased expression of both functional IKAP and MAO A transcripts in their peripheral blood cells. These findings provide new insight into the pathophysiology of FD and demonstrate the value of therapeutic approaches designed to elevate cellular levels of functional IKAP and MAO A.

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Familial dysautonomia (FD), an autosomal recessive disorder primarily confined to individuals of Ashkenazi Jewish descent, affects the development and survival of sensory, sympathetic, and some parasympathetic neurons [1,2]. Individuals with FD manifest a variety of symptoms including oropharyngeal incoordination, delayed developmental milestones, unsteady gait, poor balance, decreased perception of pain and temperature, an absence of overflow tears, and dysautonomic “crises”, which are characterized by protracted periods of nausea and vomiting accompanied by hypertension, diaphoresis, and tachycardia [3]. FD is caused by mutations in the IKBKAP gene which encodes a protein termed IKAP (IkB kinase complex associated protein) [4,5]. The most common FD-causing mutation, IVS20+6T→C, is a T → C transition in the sixth base of the donor splice site of intron 20, resulting in aberrant splicing that generates an IKAP transcript lacking exon 20. Translation of this transcript generates a truncated protein lacking the amino acids encoded by exons 20–37.

Characterization of the IKAP transcript in cells and tissues from individuals homozygous for the IVS20+6T→C mutation reveals the presence of a small amount of exon 20-containing (wild-type) IKAP transcript and full-length protein [6,7]. The ability of these cells to produce wild-type transcript prompted investigators to seek agents capable of increasing its production, with the hope that such compounds could be used to ameliorate the symptoms associated with FD. We have demonstrated that tocotrienols, a form of vitamin E, and epigallocatechin gallate (EGCG), a component of
green tea, increase the levels of wild-type transcript and full-length protein produced in FD-derived cells [6,8]. In a recent study, Slaugenhaupt et al. [9] demonstrated the ability of the synthetic plant cytokinin, kinetin, to increase production of the wild-type transcript. The ready availability of tocotrienols and EGCG as dietary supplements has allowed for their use by individuals with FD.

Monoamine oxidases (MAOs) A and B are key iso-enzymes that degrade biogenic and dietary monoamines. MAO A preferentially oxidizes norepinephrine (NE) and serotonin [10], and MAO B preferentially degrades phenylethylamine [11]. Dopamine (DA) and tyramine are oxidized by both forms of MAO. Because of MAO’s role in degrading neurotransmitters, MAO inhibitors (MAOIs) have been developed and used as antidepressants [12,13]. Individuals taking MAOIs may experience hypertensive crises following the ingestion of, for example, cheeses, aged or cured meats or red wine [14–19]. The tyramine present in these foods, which is normally metabolized by MAO A in the gastro-intestinal tract, is not degraded in those taking MAOIs and thus enters the systemic circulation [20–22]. As tyramine is a good substrate for the high-affinity adrenergic uptake system, it is concentrated in the adrenergic neurons where it causes the release of NE from the intracellular vesicles. The released NE triggers a hypertensive crisis which is characterized by hypertension, tachycardia, nausea, vomiting, diaphoresis, and sometimes intracranial hemorrhage [23,24]. Because of their inability to process biogenic amines, individuals taking MAOIs also exhibit hypersensitivity to a variety of sympathomimetic agents.

In individuals with FD, hypertensive crises are often triggered following emotional or physical stress that results in marked elevation of plasma NE and DA levels [3]. The elevated levels of NE and DA during stress and hypertensive crises and the observed exaggerated hypertensive response in FD patients to infusion by low doses of NE [25,26] prompted us to consider whether individuals with FD have reduced levels of MAO and whether many of the “spontaneous” hypertensive crises observed in those with FD may be occurring as a result of the ingestion of tyramine-containing foods. As interviews with parents following a child’s dysautonomic crisis often revealed the ingestion of tyramine-containing foods such as aged cheese, herring, and over-ripe bananas, we examined MAO gene expression in FD-derived tissues and studied the impact of substances that elevate functional IKAP levels on MAO levels. We report a reduced presence of the MAO A in FD-derived tissues and an elevation of this gene product, in FD-derived cells and in FD patients, in response to substances that elevate functional IKAP levels. These findings, as well as the observation that tocotrienol administration to individuals with FD resulted in a reduced frequency of dysautonomic crises (Rubin et al., manuscript in preparation), suggest that these crises may be attributable to the reduced presence of MAO A. More importantly, alleviation of some of the symptoms of FD might be accomplished by raising MAO A levels.

Materials and methods

Cells, tissues, and reagents. The GM00850 and GM04663 cell lines, homozygous for the IVS20\(^{\text{ST}–\text{AC}}\) FD-causing mutation, and the GM02912 cell line, derived from an unaffected individual, were obtained from the NIGMS Human Genetic Mutant Cell Repository. Tissues were harvested from an approximately 22-week-old fetus that had been diagnosed as homozygous for the IVS20\(^{\text{ST}–\text{AC}}\) FD-causing mutation. Control fetal protein and RNA were purchased from Clontech/BD Biosciences; according to the manufacturer, tissue was isolated from pools of 34–63 individuals approximately 20–40 weeks (for the preparation of liver and kidney protein, and liver RNA) or 12–31 weeks (for the preparation of kidney RNA) after gestation. EGCG and γ-tocotrienol were purchased from Calbiochem. Tocotrienol supplements, MaxiLIFE Rice Tocotrienols, were purchased from TwinLab. A monoclonal antibody generated against a peptide encoded by exons 23–28 of \(\text{IKBKAP}\) was purchased from BD Biosciences. Rabbit polyclonal antibody to MAO A was purchased from Santa Cruz Biotechnology, Inc. Tyramine testing was performed by Great Lakes Scientific.

RNA preparation and real-time RT-PCR. For tissue culture experiments, 4000 cells per well seeded in 96-well plates were treated in triplicate with either 5 μM γ-tocotrienol or with 50 μM EGCG for 48 h. After treatment, the cells were washed with PBS and then lysed in 50 μl Cells-to-cDNA lysis buffer (Ambion) at 75 °C for 12 min. After one freeze-thaw cycle, 4 μl lysate was used in each 20 μl real-time RT-PCR. RNA from tissue was prepared as previously described [6]. Blood was collected into PAXgene Blood RNA Tubes (PreAnalytiX) and the RNA was purified using a PAXgene Blood RNA Kit (PreAnalytiX) according to the manufacturer’s directions. All blood samples were collected with informed consent and with approval from the Institutional Review Board of Fordham University. Twenty nanograms of tissue- or blood-derived RNA were used in each 20 μl real-time RT-PCR.

The Quantitect SYBR Green RT-PCR Kit (Qiagen) was used for real-time RT-PCR analysis of the relative quantities of the IKAP, MAO A, and MAO B transcripts. Real-time RT-PCR conditions and IKAP primer sequences were as previously described [6]. Primers used to amplify MAO transcripts were as follows: for MAO A, 5’-TG GAGAATCAAGAGAAGG-3’ and 5’-TCTTCTCCCAACCCCTGTC-3’; for MAO B, 5’-ACTGGAAGCTCTGCTGGACTG-3’ and 5’-GCTCACTCATTGGACACAAG-3’. Real-time RT-PCR conditions were exactly the same as that used for IKAP. To control for the differences in the amount of RNA present in the samples, RT-PCR amplification of β-actin RNA was performed on all RNA preparations using Quagen’s QuantiTect Actin Gene Expression Assay and Probe RT-PCR Kit as per the manufacturer’s protocol.

Protein extraction and Western blot analysis. Tissue was homogenized in T-Per Protein Extraction Reagent (Pierce) containing a protease inhibitor cocktail (Sigma) according to the manufacturer’s directions. Samples were then centrifuged for 5 min at 10,000g and the supernatants were harvested. For tissue culture experiments, cells were treated as described above in six-well plates, and then washed once in PBS, followed by lysis in 150 μl NuPAGE LDS sample buffer (Invitrogen). Cells were then homogenized by passing the lysates through 25 gauge needles. Western blot analysis was performed as described [8]. All blots were probed with an anti-actin antibody (Oncogene) to confirm equal protein loading.
Results

As: (1) elevated levels of NE and DA are noted in individuals with FD during stress and hypertensive crises [3]; (2) an exaggerated hypertensive response is observed in individuals with FD following infusion of low doses of NE [25,26]; and (3) there are similarities between the symptoms manifested in individuals with FD during a dysautonomic crisis [3] and those observed in individuals taking MAOIs who have ingested tyramine [23,24], we examined, using real-time RT-PCR analysis, the levels of the wild-type, exon 20-containing IKAP, and MAO-encoding transcripts in liver and kidney tissues derived from control and FD-affected fetuses. A reduced presence of functional IKAP and MAO A, but not of MAO B, mRNA was observed in the FD-derived tissues examined (Fig. 1A). Western blot analysis performed on tissues derived from normal and FD-affected individuals revealed a reduction in the level of MAO A protein in the FD-derived tissues (Fig. 1B). This finding and a report demonstrating the expression of MAO A in peripheral monocytes [27] prompted an examination of MAO A and IKAP mRNA levels in peripheral blood of normal and FD-affected individuals. Using real-time RT-PCR analysis, we found that individuals with FD have less than 20% of both MAO A and IKAP mRNA levels present in normal individuals (Fig. 2).

The ability of tocotrienols and EGCG to elevate production of the full-length IKAP transcript and protein in FD-derived fibroblast cells [6,8] prompted an examination of the impact of tocotrienol and EGCG on the expression of MAO A. FD-derived cells treated with 5 μM γ-tocotrienol or 50 μM EGCG for 48 h exhibited a coordinate increase in the amount of wild-type IKAP and MAO A transcripts, while levels of MAO B transcripts were unaffected (Fig. 3A). MAO A protein levels were also correspondingly elevated in the tocotrienol- and EGCG-treated cells (Fig. 3B). EGCG treatment of fibroblast cell lines derived from unaffected individuals, which, because EGCG alters the splicing process in IVS20+6T→C containing cells and has no effect on wild-type IKAP levels in normal cells, does not result in the increased production of MAO A (data not shown).

As IKAP and MAO A mRNA and protein levels are elevated in FD-derived cells following treatment with tocotrienols, we examined functional IKAP and MAO A mRNA levels in peripheral blood cells of individuals with FD before and up to 6 months following the daily ingestion of 100 mg tocotrienol. Real-time RT-PCR analysis revealed a clear elevation in the relative levels of wild-type IKAP and MAO A mRNAs following tocotrienol supplementation. The mean increase in IKAP mRNA levels was greater than twofold and the

![Graph A](image1.png)

Fig. 1. Functional IKAP and MAO A levels are reduced in FD fetal tissue. (A) Control fetal RNA and RNA purified from FD-derived fetal tissue were subjected to real-time RT-PCR analysis as described under Materials and methods. By calculating the change in threshold cycle (ΔCₜ) between the results observed for the respective tissues, relative levels of IKAP, MAO A, and MAO B mRNA in FD and control fetal tissue were obtained. FD tissue mRNA levels are plotted as a percentage of the levels observed in control fetal tissue. Experiments were repeated three times, each time in triplicate. Results presented are typical. (B) Protein from FD fetal tissue was prepared or purchased (control fetal protein) and subjected to Western blot analysis using a rabbit polyclonal antibody to MAO A as described under Materials and methods. Actin was used as a loading control (not shown).

![Graph B](image2.png)

Fig. 2. Functional IKAP and MAO A mRNA levels are deficient in individuals with FD. Real-time RT-PCR, using primers designed to detect MAO A and wild-type IKAP transcripts, was performed on mRNA isolated from the peripheral blood cells of seven individuals with FD before and six unaffected individuals. Mean relative MAO A and IKAP RNA levels were calculated.
increase in MAO A mRNA levels was greater than threefold (Fig. 4). No effect was observed on the levels of the MAO B-encoding transcript (data not shown). Thus, in this population of individuals, tocotrienol supplementation raises functional IKAP and MAO A levels from less than 20% of the normal value (Fig. 2) to approximately 40% and 50% of normal values, respectively.

Discussion

Autonomic crises in individuals with FD are life-threatening events that often require significant medical intervention. While the onset of crisis can sometimes be attributed to an intense emotional occurrence, the triggering event often cannot be identified. As the precipitating events leading to crises are not fully understood, treatment of individuals with FD has focused on controlling the symptoms exhibited during crisis. The study presented herein reveals a clear reduced presence of the MAO A gene product in fetal tissue and peripheral blood cells from individuals homozygous for the common FD-causing mutation. A reduced presence of this biogenic amine-metabolizing enzyme in individuals with FD could explain the onset of dysautonomic crises following emotional events that trigger the release of NE and DA [3], the hypertensive response to infusion by low doses of NE [25,26] and the precipitation of dysautonomic crises following the ingestion of tyramine-containing foods. The recent onset of crises in individuals with FD following the consumption of canned cranberries and enteral formula (personal communications) might be explained by the tyramine content of these products. The cranberries contained 143 g of tyramine/g and the enteral formula had 76 g/ml. Considering that 10 mg tyramine can induce a severe hypertensive crisis in an adult taking an MAO A inhibitor [13], the levels present in these products might be the cause of the dysautonomic crises experienced by these individuals.

As tocotrienol treatment of FD-derived cells increases IKAP levels in vitro [6], we studied the impact of this supplement on functional IKAP and MAO A levels, and on symptoms experienced by individuals with FD. Tocotrienol supplementation was observed to increase the expression of both IKAP and MAO A mRNA in blood cells and reduce the frequency of dysautonomic crises in over 80% of the participants in our study (Rubin et al., manuscript in preparation). While these findings do not definitively demonstrate that increased levels of the MAO A result in a reduction in the number of dysautonomic crises experienced by those with FD, the
observation that crises can occur in response to elevated levels of NE and DA certainly suggests that increased levels of MAO A, which oxidize these biogenic amines, could lessen their impact on the autonomic nervous system and reduce the number of crises. The underlying mechanism by which coordinate expression of IKAP and MAO A occurs is currently under investigation. The reports that IKAP is a component of the Elongator complex [28] and/or a Jun N-terminal kinase (JNK) associated protein [29] suggest that IKAP may be capable of regulating MAO A gene expression.

It is interesting to note that individuals taking MAOIs as well as those with FD often experience postural hypotension which results in episodes of lightheadedness, dizziness, and occasionally syncope [3,30,31]. Orthostatic hypotension occurs when the sympathetic reflexes responsible for arterial constriction in the legs and splanchnic region are blocked [30]. In those taking MAOIs, the hypotensive response is believed to be due to the generation of tyramine-derived octopamine which, by binding to the nerve terminals, acts as a false neurotransmitter and renders sympathetic nerve transmission less functional [13,30]. It is possible that the orthostatic hypotension observed in individuals with FD may be mediated, in part, by the same mechanism.

In 1996, Axelrod et al. [32] demonstrated an altered pattern of plasma levels of catecholamine metabolites in individuals with FD, which included elevated levels of norepinephrine, a reduced presence of dihydroxyphenylglycol (DHPG), an elevated dihydroxyphenylalanine (DOPA)/DHPG ratio, and a normal dihydroxy-phenylacetic acid (DOPAC)/DHPG ratio. As MAO is responsible for the conversion of NE to DHPG, the elevated level of NE and the reduced presence of DHPG could be the result of a reduced presence of MAO. As the conversion of DA to DOPAC is mediated by MAO, the observed reduction in DOPAC levels could also be the result of a reduced presence of this enzyme. Furthermore, the reduced presence of DOPAC and DHPG can explain the altered ratios of the catecholamine metabolites observed in individuals with FD. It is interesting to note that Axelrod et al. [32] suggested that the neurochemical anomalies observed in individuals with FD may be due to “limited oxidative deamination.” We suggest that these anomalies may in fact result from the reduced presence of MAO A in individuals with FD.

It is clear from post-mortem examinations that deficits exist in the neuronal populations in individuals with FD and it is likely that these deficiencies are responsible for many of the symptoms experienced by those with FD [33,34]. It is nevertheless interesting to note that many of the symptoms associated with FD are controlled by regions of the brain to which MAO A has been localized. Topographical localization studies have revealed the presence of MAO A in the substantia nigra, locus coeruleus, nucleus subcoeruleus, and the periventricular region of the hypothalamus [35,36]. The substantia nigra is critical for communication between the motor cortex and the somatosensory cortex and plays an important role in gait control, maintaining posture, coordinated movement, and muscle tone [37,38]. All of these functions are compromised in those with FD [3,39]. The locus coeruleus is involved in the baroreceptor reflexes, control of blood pressure, cardiovascular homeostasis, and hypoxia responsiveness [40–43]. Individuals with FD exhibit baroreflex dysfunction as well as abnormalities in the control of blood pressure, cardiac function, and responsiveness to hypoxia [44–47]. The locus coeruleus/nucleus subcoeruleus region plays a significant role in nociceptive processing, colonic motor function, and control of micturition [48–50], which are all compromised in individuals with FD [3,51]. Neurons in the periventricular region of the hypothalamus manufacture peptides that control the secretion of a variety of hormones by the anterior pituitary [52]. It has been suggested that production of growth hormone, which occurs in the anterior pituitary [53], may be deficient in individuals with FD [54]. Whether the reduced presence of MAO A in these brain regions plays a role in the symptoms manifested by those with FD awaits further study. It is, however, interesting to note that altered neurotransmission has been observed in the locus coeruleus of knockout mice lacking MAO A [55,56] and that MAO A inhibition causes a significant depression of the firing rate of neurons in the locus coeruleus [57].

To date, with the exception of tocotrienols and EGCG, treatments for individuals with FD have been supportive and directed towards alleviating the symptoms. The findings presented herein provide a new perspective on the pathophysiology of FD and demonstrate the value of therapeutic approaches designed to elevate the levels of IKAP and MAO A in these individuals. Our findings further indicate that avoidance of exposure to tyramine as well as serotonergic and sympathomimetic agents will likely improve the quality of life and increase the lifespan of these individuals.

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References


