



## Tocotrienols induce *IKBKAP* expression: a possible therapy for familial dysautonomia

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### Abstract

Familial dysautonomia (FD), a neurodegenerative genetic disorder primarily affecting individuals of Ashkenazi Jewish descent, is caused by mutations in the *IKBKAP* gene which encodes the I $\kappa$ B kinase complex-associated protein (IKAP). The more common or major mutation causes aberrant splicing, resulting in a truncated form of IKAP. Tissues from individuals homozygous for the major mutation contain both mutant and wild-type IKAP transcripts. The apparent leaky nature of this mutation prompted a search for agents capable of elevating the level of expression of the wild-type IKAP transcript. We report the ability of tocotrienols, members of the vitamin E family, to increase transcription of IKAP mRNA in FD-derived cells, with corresponding increases in the correctly spliced transcript and normal protein. These findings suggest that in vivo supplementation with tocotrienols may elevate *IKBKAP* gene expression and in turn increase the amount of functional IKAP protein produced in FD patients.

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Familial dysautonomia (FD), also known as “Riley–Day Syndrome” or “hereditary sensory neuropathy type III” (MIM 223900), is an autosomal recessive disorder primarily confined to individuals of Ashkenazi Jewish descent. FD affects the development and survival of sensory, sympathetic, and some parasympathetic neurons [1–3] and is caused by mutations in the gene termed *IKBKAP* which encodes a protein termed IKAP (I $\kappa$ B kinase complex-associated protein) [4,5]. IKAP was initially reported to be a scaffold protein involved in the assembly of the I $\kappa$ B kinase complex [6], but subsequently was reported to have no association with this complex [7]. IKAP is homologous to the Elp1 protein of the *Saccharomyces cerevisiae* Elongator complex [8] and is a component of the human Elongator complex [9]. IKAP has recently been reported to be a c-Jun N-terminal kinase (JNK)-associated protein capable of JNK stress kinase activation [10]. The multiple biological activities of IKAP and their roles in FD-mediated neurological deficits remain to be elucidated.

Two FD-causing mutations have been identified in individuals of Ashkenazi Jewish descent. The more common, or major, FD-causing mutation occurs in the donor splice site of intron 20, resulting in aberrant splicing that produces an IKAP transcript lacking exon 20. Translation of this mRNA results in a frameshift that generates a truncated protein lacking all of the amino acids encoded in exons 20–37. The less common, or minor, mutation is a G  $\rightarrow$  C transversion that results in an arginine to proline substitution of amino acid residue 696 of IKAP [4,5].

Mutations that affect RNA splicing are a major cause of human genetic diseases. These diseases may occur as a result of mutations in the splice donor or splice acceptor sequences or in exons or introns, generating cryptic splice junctions. While many of these mutations result in what appears to be an absolute absence of the appropriately spliced gene product, in some cases mutations that affect splicing result in a milder form, or an adult onset form, of the disease in which “leaky” alternative mRNA splicing is observed that produces both mutant (skipped exon) and wild-type (full-length) transcripts [11–16]. The major FD-causing mutation, termed

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2507 + 6T → C or IVS20<sup>+6T→C</sup>, changes the sequence of the splice donor element of intron 20 from the consensus GTAAGT to a non-consensus GTAAGC, resulting in the generation of a transcript lacking exon 20. This mutation appears to be somewhat leaky as both the mutant and wild-type transcripts are detected in lymphoblasts of individuals homozygous for this FD-causing mutation [5].

As FD-derived cells produce the full-length IKAP transcript, in an effort to generate elevated levels of functional IKAP protein, experiments were performed to identify agents that either promote splicing that generates the exon 20-containing transcript or upregulate IKAP transcription which, due to the somewhat leaky nature of this mutation, could generate increased levels of the correctly spliced transcript and, thereby, more functional IKAP protein.

In this study, we report that tocotrienols, members of the vitamin E family, upregulate IKAP transcription, resulting in elevated levels of the full-length transcript as well as elevated levels of functional, full-length IKAP protein in normal and FD-derived cells.

## Materials and methods

**Cells and reagents.** The GM00850 and GM04663 cell lines, homozygous for the IVS20<sup>+6T→C</sup> FD-causing mutation, and the GM02912 cell line, derived from a healthy individual, were obtained from the NIGMS Human Genetic Mutant Cell Repository. LA1-55n cells were kindly provided by Dr. Robert A. Ross. Post-mortem tissue samples were obtained from the University of Miami Brain and Tissue Bank for Developmental Disorders. Purified tocotrienols and tocopherols were purchased from Calbiochem. A monoclonal antibody generated against a peptide encoded by exons 23–28 of *IKBKAP* was purchased from BD Biosciences.

**RT-PCR.** Frozen tissue was homogenized with a Tissue Tearor (Biospec Products) in Lysis/Binding Solution (Ambion) and the RNA was purified using the RNAqueous Total RNA Isolation Kit (Ambion). Twenty-five nanograms of RNA was amplified in 20 µl RT-PCRs using the GeneAmp EZ rTth RNA PCR Kit (Applied Biosystems). One-step RT-PCR was carried out as follows: one cycle of 58 °C × 45 min and 94 °C × 2 min, followed by 45 cycles of 94 °C × 30 s, 58 °C × 30 s, and 72 °C × 30 s, and then a final extension of 72 °C × 7 min. PCR products were analyzed on a 2% agarose gel.

**RNA preparation and real-time RT-PCR.** Cells seeded in 96-well plates at a concentration of 4000 cells/well for at least one day prior to

treatment were treated in triplicate for approximately 48 h unless noted otherwise. After treatment, the cells were washed once with PBS and then lysed in 50 µl of Cell-to-cDNA lysis buffer (Ambion) at 75 °C for 12 min. After one freeze–thaw cycle, 4 µl of lysate was used in 20 µl RT-PCRs. The Quantitect SYBR Green RT-PCR Kit (Qiagen) was used for real-time RT-PCR analysis of the relative quantities of the exon 20-containing transcript (wild-type), the exon 20-lacking transcript (mutant), as well as an exon 34–35-containing transcript that is unaffected by the FD-causing mutation (total). An ABI PRISM 7000 Sequence Detection System (Applied Biosystems), programmed as follows, was used to perform the real-time RT-PCR and analysis: 50 °C × 30 min and 95 °C × 15 min for one cycle, followed by 40 cycles of 94 °C × 15 s, 57–60 °C × 30 s, and 72 °C × 30 s. Primers, used at a concentration of 0.5 µM, are shown in Table 1. To present relative amounts of PCR product obtained, results are expressed as changes in the threshold cycle ( $\Delta C_T$ ) compared to untreated cells. The threshold cycle refers to the PCR cycle at which the fluorescence of the PCR is increased to a calculated level above background. A change of 1.0 in  $C_T$ , assuming 100% PCR efficiency, would reflect a twofold change in the starting amount of the RNA template that was amplified.

To control for the amount of RNA present in the samples, RT-PCR amplification of ribosomal 18S RNA was performed on all cell lysates. The use of the ribosomal 18S RNA has been shown to be an effective control for the quantity of RNA present in samples [17]. For this analysis, TaqMan Ribosomal RNA Control Reagents were used with the TaqMan EZ RT-PCR Kit (Applied Biosystems) as per the manufacturer's protocol.

**Measurement of transcription rate.** Isolation of nuclei and transcription in the presence of [ $\alpha$ -<sup>32</sup>P]UTP (Perkin–Elmer Life Sciences) were performed essentially as described [18]. Briefly, confluent monolayers of cells in 175 cm<sup>2</sup> flasks were incubated for 48 h in the presence or absence of 12.5 µg/ml  $\delta$ -tocotrienol. The cells were then lysed by Dounce homogenization and the resultant nuclei were incubated with 250 µCi [ $\alpha$ -<sup>32</sup>P]UTP. The radiolabeled RNA was DNase treated, purified with Trizol LS reagent (Invitrogen), and then hybridized to 5 µg of plasmid containing either a full-length cDNA encoding IKAP or a cDNA encoding GAPDH that was immobilized on Nytran Super-Charge nylon membrane (Schleicher & Schuell) using a spot-blot apparatus. The amount of radiolabeled RNA bound was detected by autoradiography and then quantitated by densitometric scanning of the X-ray film.

**Western blot analysis.** Cells treated for 48 h with 12.5 µg/ml of  $\delta$ -tocotrienol were washed twice with PBS and lysed in 0.5 M Tris–HCl, pH 6.8, containing 1.4% SDS. Western blot analysis was performed essentially as described [19]. Equal amounts of protein fractionated on a 7% NuPAGE Tris–Acetate Gel (Invitrogen) were blotted onto nitrocellulose (Bio-Rad) and probed overnight with a monoclonal antibody (BD Biosciences) directed against the carboxyl end (exons 23–28) of IKAP. The blot was then washed and probed with a goat anti-mouse antibody conjugated to alkaline phosphatase (Promega), followed by detection with Western Blue Substrate solution (Promega).

Table 1  
Sequences of the oligonucleotide primers used in RT-PCR and real-time RT-PCR analysis

IKAP amplicon	Annealing temperature (°C)	Primer	Sequence (5' → 3')	Position (GenBank Accession No. AK001641)
Wild-type	57	Forward	AGTTGTTTCATCATCGAGC	2459–2476
		Reverse	CATTTCCAAGAAACACCTTAGGG	2607–2585
Mutant	60	Forward	CAGGACACAAAGCTTGTATTACAGACTT	2415–2438, 2513–2516
		Reverse	CATTTCCAAGAAACACCTTAGGG	2607–2585
Total	60	Forward	GAGATCATCCAAGAATCGC	3881–3899
		Reverse	GGTAGCTGAATTCTGCTG	4160–4143

## Results

The ability of FD-derived lymphoblasts to produce exon 20-containing (wild-type) *IKBKAP* transcript [5] prompted an examination of RNA isolated from a variety of tissues of FD-affected individuals for the presence of wild-type transcript. RT-PCR, using a primer recognizing sequence encoded in exon 20 of the *IKBKAP* gene and a primer that spans exons 21 and 22, revealed the presence of exon 20-containing transcript in all of the FD-derived tissues studied (Fig. 1). Cuajungco and co-workers [20] also recently reported the presence of wild-type transcript in a variety of tissues derived from FD-affected individuals.

The ability of the IVS20<sup>+6T-C</sup> containing allele to produce the wild-type transcript prompted a study of agents that may modulate the level of this transcript produced in FD-derived cells, either by increasing transcription or modulating splicing. For this analysis, RNA isolated from FD-derived fibroblast cells treated with a variety of biological agents was subjected to real-time RT-PCR analysis using primers specific for the wild-type *IKAP* transcript. While most of the reagents examined failed to modulate expression of this transcript,  $\delta$ -tocotrienol treatment was observed to mediate a concentration-dependent elevation in the level of this transcript (Fig. 2). To determine whether  $\delta$ -tocotrienol treatment stimulates correct splicing or elevates the cellular level of the *IKAP* RNA which, in turn, results in elevated levels of the wild-type transcript, quantitative RT-PCR was performed using primers recognizing a region of the *IKAP* RNA not altered by the FD-causing mutation, as well as primers which are specific for the mutant transcript. Levels of both the truncated and wild-type transcripts were elevated in a concentration-

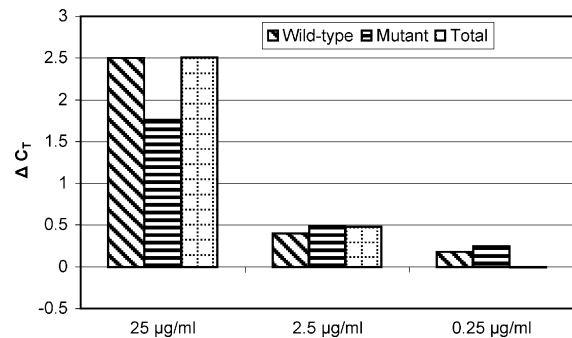


Fig. 2. Real-time RT-PCR analysis of the *IKAP* RNA. cDNA was generated from FD-derived human fibroblast (GM00850) cells incubated for 48 h in the presence of varying concentrations of  $\delta$ -tocotrienol. Real-time RT-PCR analysis was performed to measure the relative amounts of the *IKAP* RNAs produced by these cells. Results, the mean of three experiments, each done in triplicate, are expressed as changes in the threshold cycle ( $\Delta C_T$ ) relative to results from untreated cells (see Materials and methods).

dependent manner (Fig. 2), with as much as a sixfold increase ( $\Delta C_T = 2.5$ ) in the experiment shown, at the highest concentration tested, demonstrating a tocotrienol-mediated increase in the cellular level of the *IKAP*-encoding RNAs.

To characterize the kinetics of the  $\delta$ -tocotrienol-mediated accumulation of the *IKAP* transcript, cells were exposed to 12.5  $\mu\text{g}/\text{ml}$  of  $\delta$ -tocotrienol for periods up to 48 h and the amount of the *IKAP*-encoding transcripts was determined. As can be seen in Fig. 3, significantly elevated levels of these transcripts are detected 24 h following the start of  $\delta$ -tocotrienol treatment, as compared to untreated cells, and continue to accumulate through 48 h of treatment.

The  $\delta$ -tocotrienol-mediated accumulation of the *IKAP*-encoding transcripts is either the result of an

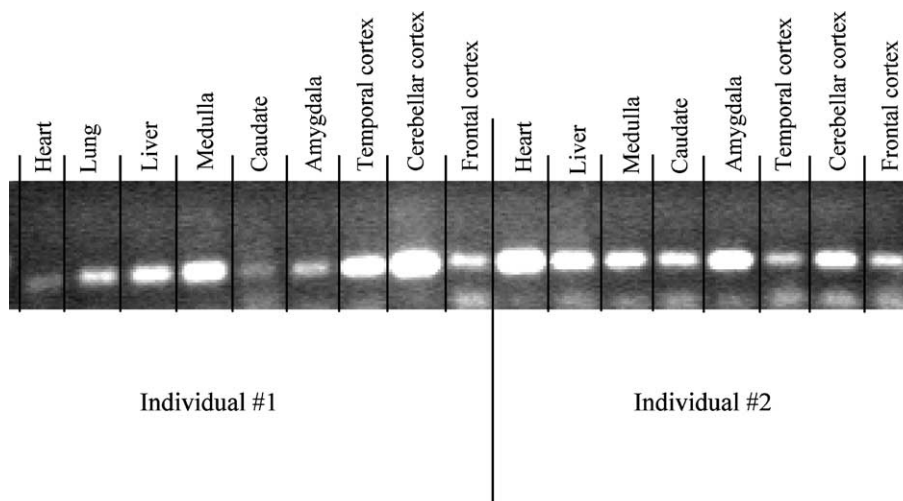


Fig. 1. Expression of *IKAP* RNA in post-mortem tissue samples. RT-PCR, using primers located in exon 20 and spanning exons 21 and 22, was performed on RNA isolated from post-mortem tissue samples from two individuals with FD. The resulting amplified products were fractionated on a 2% agarose gel.

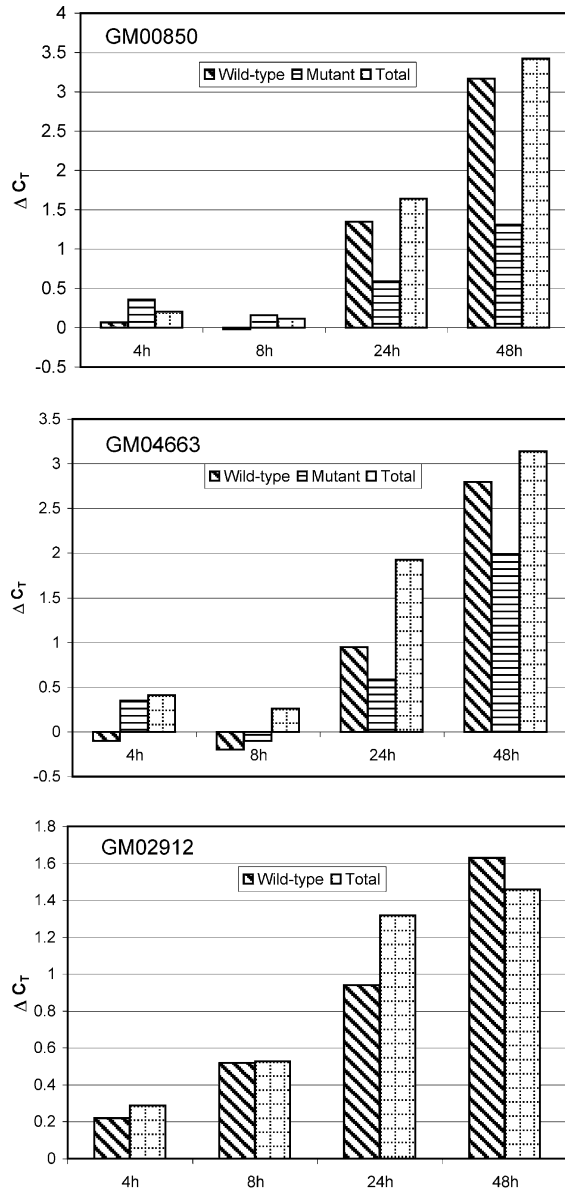


Fig. 3. Time kinetics of the  $\delta$ -tocotrienol mediated accumulation of the IKAP RNAs. Cultures of FD-derived (GM00850 and GM04663) and normal (GM02912) fibroblast cells were treated for varying times with 12.5  $\mu$ g/ml of  $\delta$ -tocotrienol. The relative amounts of the IKAP RNAs were determined by real-time RT-PCR. The results presented represent mean values obtained in three experiments, each done in triplicate.

elevation in the level of gene transcription or the stabilization of this RNA. To distinguish between these possibilities, nuclear run-on transcription assays were performed on cells incubated in either the presence or absence of  $\delta$ -tocotrienol. As can be seen in Fig. 4,  $\delta$ -tocotrienol treatment results, in the experiment shown, in an approximate 3.5-fold increase in *IKBKAP* gene transcription.

Western blot analysis was performed on cellular extracts prepared from  $\delta$ -tocotrienol-treated FD-derived and normal fibroblasts, using a monoclonal antibody

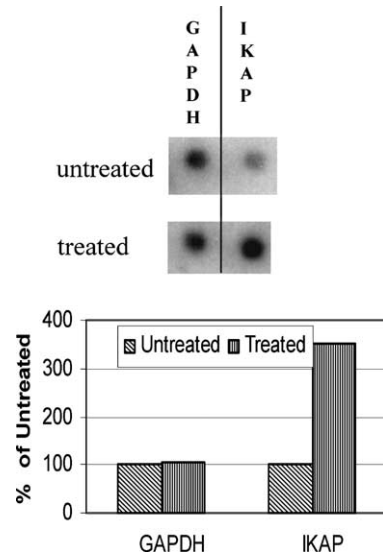


Fig. 4. Nuclear run-on transcriptional analysis of the IKAP RNA in response to  $\delta$ -tocotrienol treatment. Radiolabeled RNA was purified from nuclei prepared from GM04663 cells incubated for 48 h in the presence or absence of 12.5  $\mu$ g/ml of  $\delta$ -tocotrienol. The RNA was hybridized for 48 h to a membrane to which cDNAs for IKAP and GAPDH were spotted and crosslinked. The relative amount of radioactivity associated with the cDNA was determined densitometrically and is expressed as a percentage of the signal generated in the untreated cells. The experiment depicted was a typical result obtained.

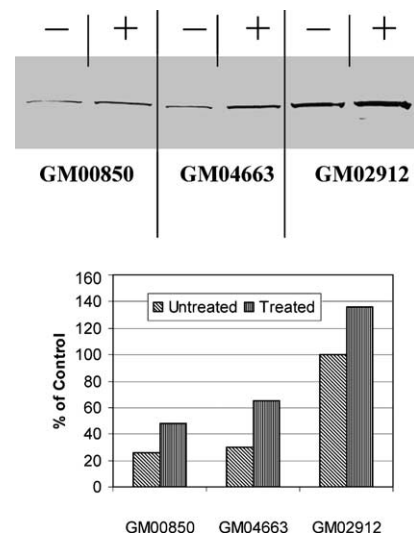


Fig. 5. The induction of full-length IKAP in FD-derived (GM00850 and GM04663) and normal (GM02912) cells. Lysates prepared from cells incubated in the presence or absence of 12.5  $\mu$ g/ml of  $\delta$ -tocotrienol for 48 h were fractionated and characterized by Western blot analysis. The presence of IKAP was detected using a monoclonal antibody recognizing a fragment of the IKAP protein encoded by exons 25–28 of *IKBKAP*. Relative amounts of IKAP produced were determined densitometrically. Values expressed are as a percentage of the IKAP levels present in the untreated normal (GM02912) cells. Following immunological screening, the blot was stained with Coomassie brilliant blue to confirm that equal amounts of protein were loaded on the gel. The Western presented is representative of the experiments performed.

generated against a peptide corresponding to exons 23–28 of IKAP that only recognizes the full-length IKAP.  $\delta$ -Tocotrienol treatment results in an approximate two-fold increase, on average, in the amount of full-length IKAP produced in the FD-derived fibroblasts. The tocotrienol treatment of the FD-derived cells, in the experiment shown, elevates IKAP levels from 26–30% of the level present in untreated normal cells to 48–65% of the level present in these cells (Fig. 5).

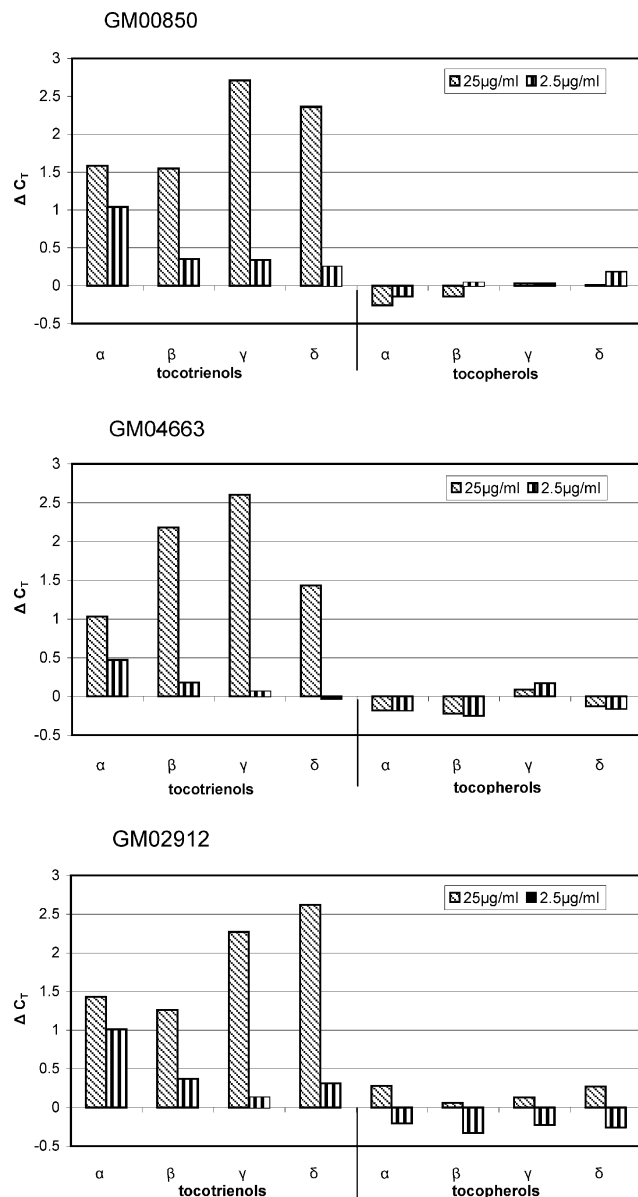


Fig. 6. Wild-type IKAP RNA levels in cells incubated in the presence of members of the vitamin E family of molecules. cDNA was prepared from GM00850, GM04663, and GM02912 cells incubated for 48 h in the absence or presence of 25 and 2.5  $\mu\text{g/ml}$  of either  $\alpha$ ,  $\beta$ ,  $\gamma$  or  $\delta$  tocopherols or tocotrienols. The relative amounts of IKAP RNA present in these cells were determined by real-time PCR. The results presented are mean values obtained over three experiments, each done in triplicate.

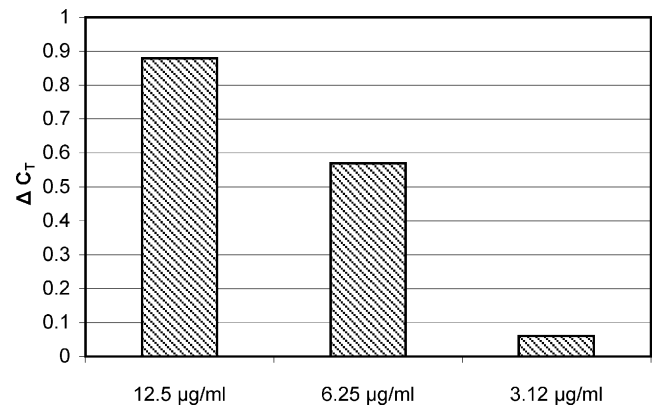


Fig. 7. Real-time RT-PCR analysis of wild-type IKAP RNA in LA1-55n cells. cDNA was prepared from LA1-55n cells incubated for 48 h in the absence or presence of varying concentrations of  $\delta$ -tocotrienol. The relative amounts of IKAP RNA present in these cells were determined by real-time RT-PCR. The results presented represent mean values obtained in three experiments, each done in triplicate.

$\delta$ -Tocotrienol is a member of a group of eight molecules that make up the vitamin E family. To evaluate the IKAP mRNA-inducing ability of these related molecules, cells were treated with varying concentrations of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  tocopherols and tocotrienols. As can be seen in Fig. 6, treatment with all four forms of tocotrienol elevates cellular levels of the IKAP transcripts as much as sixfold, while the tocopherols had no effect on IKAP transcript levels.

As FD affects the development and survival of sensory, sympathetic, and parasympathetic neurons and, as the ability of the tocotrienols to elevate the level of functional IKAP protein suggests their possible use as a therapeutic modality for individuals with FD, we examined the response of the neuroblastoma-derived LA1-55n cell line, which has characteristics of sympathetic neurons [21,22], to treatment with  $\delta$ -tocotrienol. As can be seen in Fig. 7,  $\delta$ -tocotrienol induced almost a twofold increase in IKAP transcript levels in these cells.

## Discussion

The recent discovery that a mutation in the sixth base of the splice donor sequence of intron 20 of *IKBKAP* is responsible for most of the cases of FD and the observation that cells derived from individuals with FD are capable of producing the wild-type form of the *IKBKAP*-encoded RNA suggested a novel therapeutic approach. Effective modalities could either modulate the splicing event to facilitate the production of more wild-type RNA or cause the production of more of the *IKBKAP*-encoded transcript at the transcriptional level which, because of the leakiness of the mutation, would result in an elevated level of the wild-type transcript. To identify agents capable of either of these activities, we

examined the effect of a variety of compounds on the cellular levels of the exon 20-containing, or wild-type, IKAP transcript in FD-derived fibroblasts. We report that treatment of FD-derived human fibroblasts with  $\delta$ -tocotrienol, a member of the vitamin E family, results in an elevation of *IKBKAP* transcription, multi-fold increases in the levels of both mutant and wild-type IKAP transcripts and substantially elevated levels of the full-length IKAP protein. Furthermore, tocotrienol treatment of neuronally derived cells also results in an elevation of the level of the *IKBKAP*-encoded transcript.

Vitamin E is a generic term that includes two groups of closely related fat-soluble compounds termed tocopherols and tocotrienols. Tocopherols have a saturated side chain while the tocotrienols have an unsaturated isoprenoid side chain. The tocopherols and tocotrienols exist in one of four analogues termed  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , which are so designated based on the number and position of the methyl groups on the benzene ring. Both tocopherols and tocotrienols are found in various components of the human diet. Tocopherols are present primarily in vegetable oils, while the tocotrienols are minor plant constituents especially abundant in rice bran, cereal grain, and palm oil.

Despite the similarities in their molecular structures, the tocopherols and tocotrienols clearly have different biological activities. For example, tocotrienols have more potent antioxidant properties [23,24], more effectively suppress the activity of hydroxy-3-methylglutaryl coenzyme A reductase [25,26], more effectively prevent glutamate-induced signal transduction pathways leading to neurodegeneration [27], and more effectively activate gene expression via the pregnane X receptor [28]. We report here the ability of the tocotrienols, but not the tocopherols, to induce expression of the *IKBKAP*-encoded transcript.

The reduced presence of the wild-type IKAP transcript in neuronal tissue of individuals with FD [5,20] and the observed ability of  $\delta$ -tocotrienol to elevate the level of IKAP transcripts in neuronal cells suggest that individuals with FD might benefit from tocotrienol supplementation. The data presented suggest the value of a clinical protocol that will assess the efficacy of this supplementation on individuals with FD.

It is worth noting that in cell lines derived from heterozygous carriers of the *IVS20*<sup>+6T-C</sup> allele who possess a normal phenotype, there are decreased levels, compared to normal cell lines, of the wild-type IKAP transcript. This would suggest that increasing the amount of the wild-type transcript, even to levels below that of normal individuals, could result in a substantially improved phenotype in individuals with FD.

The observed ability of the tocotrienols to significantly elevate expression of IKAP transcripts as well as IKAP protein further suggests the value of an extensive investigation into other therapeutic modalities for indi-

viduals with FD that can either elevate IKAP transcription or facilitate the correct splicing of the IKAP transcript produced by the *IVS20*<sup>+6T-C</sup> bearing allele and thereby ameliorate the effect of the major FD-causing mutation.

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