Vitamin K₂ Is a Mitochondrial Electron Carrier That Rescues Pink1 Deficiency

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Human UBIAD1 localizes to mitochondria and converts vitamin K₁ to vitamin K₂. Vitamin K₂ is best known as a cofactor in blood coagulation, but in bacteria it is a membrane-bound electron carrier. Whether vitamin K₂ exerts a similar carrier function in eukaryotic cells is unknown. We identified *Drosophila* UBIAD1/Heix as a modifier of *pink1*, a gene mutated in Parkinson's disease that affects mitochondrial function. We found that vitamin K₂ was necessary and sufficient to transfer electrons in *Drosophila* mitochondria. *Heix* mutants showed severe mitochondrial defects that were rescued by vitamin K₂, and, similar to ubiquinone, vitamin K₂ transferred electrons in *Drosophila* mitochondria, resulting in more efficient adenosine triphosphate (ATP) production. Thus, mitochondrial dysfunction was rescued by vitamin K₂ that serves as a mitochondrial electron carrier, helping to maintain normal ATP production.

Parkinson's disease (PD) is a common neurodegenerative disorder, and genetic causes of the disease allow us to elucidate the molecular pathways involved (1, 2). Mutations in *pink1*, encoding an evolutionarily conserved

mitochondrial kinase, cause PD in humans and mitochondrial defects in model organisms (3–6). To understand Pink1 function in vivo, we performed a genetic modifier screen in *Drosophila*. Because PD affects the nervous system we

screened 193 chemically induced recessive lethal mutants that were selected for defects in neurocommunication (7–9). We tested dominant modification of *pink1*^{B9} null mutant flight defects (fig. S1A). Although none of the chemically induced mutants showed dominant flight defects when crossed to a wild-type *pink1*^{RV} allele, 24 mutants suppressed and 32 enhanced the *pink1*^{B9} flight defect, such that *pink1*^{B9} flies failed to fly (fig. S1A).

To reveal the mechanism by which the modifiers affected Pink1, we mapped one of the strongest enhancers that, in combination with *pink1*^{B9}, results in enhanced lethality to *heix* (fig. S1B). We named this allele *heix*² and identified several additional *heix* alleles (fig. S1, B to E) (10). To test whether loss of *heix* specifically exacerbated *pink1* phenotypes, we assessed flight, adenosine triphosphate (ATP) levels, and neuronal mitochondrial membrane potential (Ψ_m) (10). Heterozygosity for *heix* combined with

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Fig. 1. Identification of *heix* as modifier of *pink1^{B9}*. Enhancement of *pink1^{B9}* phenotypes by heterozygosity for *heix* (pink) compared with *pink1^{RV}* controls heterozygous for *heix* (black) and rescue with UAS-*heix* (*k11403/+; da>heix*); flight ($n > 50$) (A), ATP levels ($n = 10$ assays) (B), and ψ_m measurements using the potentiometric dye JC-1. Red aggregate/green monomeric fluorescence ratio measured at third instar neuromuscular boutons; aggregates accumulate

at negative ψ_m ($n = 20$ synapses) (C). Suppression of *pink1^{B9}* and *park^{1/Δ21}* phenotypes by overexpression of Heix (*da>heix*); flight ($n > 50$) (D), ATP levels ($n = 10$ assays) (E), and JC-1 red/green fluorescence ratio ($n = 20$ synapses) (F). Data are percentage [(A) and (D)] and mean normalized to control [(B), (C), (E), and (F)]. Error bars indicate SEM. Analysis of variance (ANOVA)/Dunnett: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. *Heix* is involved in vitamin K_2 production. (A) Improved survival of first instar *heix* mutants placed on MK-4, on ubiquinone (Q10), or on menadione (black) shown as the difference in the percentage of control larvae that survive on control medium ($n = 10$ experiments, each with 10 larvae); that is, about 20% more *heix* mutant larvae survive with vitamin K_2 compared with *heix* mutants on control medium. Error bars, SEM. ANOVA Dunnett: * $P < 0.05$; ** $P < 0.01$. ns, not significant. (B) Schematic of the biochemical conversion of menadione to vitamin K_2 .

pink1^{RV} did not display defects, whereas it strongly enhanced defects in *pink1^{B9}* null mutants that are rescued by overexpressing Heix (Fig. 1, A to C, and fig. S2). Conversely, overexpression of Heix rescued *pink1^{B9}*-associated defects and those in *parkin^{1Δ21}*, which also encodes a PD-associated gene affecting mitochondria (Fig. 1, D and E) (4, 5). Although Heix overexpression does not fully rescue flight, suggesting Pink1 acts in different pathways, the effect is similar in magnitude to previously identified suppressors (4, 5, 11). Thus, Heix is a dosage-sensitive modifier of *pink1*.

Heix is evolutionary well conserved (fig. S3A), and its bacterial and human homologs, MenA and UBIAD1, harbor a prenyltransferase domain involved in synthesis of vitamin K₂/menaquinone (MK-*n*, *n* indicating the number of prenyl groups) (fig. S3A) (12, 13). In a dendrogram, the three

proteins cluster closely, suggesting the enzymes are functionally conserved from bacteria to human (fig. S3B). Feeding *heix* mutants during development with MK-4 but not with menadione, a synthetic precursor that is prenylated to form vitamin K₂, significantly improved their survival rate (Fig. 2, A and B) and other cellular defects (below), whereas ubiquinone (Q-10) feeding did not (Fig. 2A), indicating specificity (10). Thus, similar to UBIAD1 and MenA, Heix produces vitamin K₂ in *Drosophila*. We thus tested whether supplementing *pink1^{B9}* with vitamin K₂ alleviated its phenotypes. Because *Escherichia coli* produces vitamin K₂ (MK-8) (14), we supplemented minimal medium with living *E. coli* and then placed 1-day-old adult control and *pink1^{B9}* mutant flies on this medium. A significant improvement in flight and ATP levels was observed in *pink1^{B9}* (Fig. 3, A to C). In contrast, *pink1^{B9}*

flies on medium with *menA* mutant *E. coli* that barely produce vitamin K₂ (fig. S4A) (12) were not rescued (Fig. 3, A to C), whereas *pink1^{B9}* on medium with *menA* mutant *E. coli* complemented with a wild-type *menA* gene (fig. S4A) showed a significant improvement in their flight and ATP defects (Fig. 3, A to C). Thus, vitamin K₂ produced in *E. coli* alleviates *pink1*-related defects.

Next, we placed 1-day-old adult *pink1^{B9}* mutants and controls on MK-4-supplemented or on control medium. Control flies flew normally and did not show altered ATP levels or Ψ_m after feeding with various concentrations of MK-4 (100 to 1000 μ M) (Fig. 3, D, F, and G, and fig. S4, B and C). *Pink1^{B9}* on MK-4 showed a dose- and time-dependent improvement of flight, increased ATP amount, and a more negative Ψ_m (Fig. 3, E to G, and fig. S4, B and C). Similarly,

Fig. 3. Vitamin K₂ rescues *pink1^{B9}* mutant phenotypes. Flying ability of *pink1^{RV}* (A) and *pink1^{B9}* (B) and ATP levels in *pink1^{RV}* and *pink1^{B9}* flies (C) that were placed on control medium (black), on medium with *E. coli* that produce vitamin K₂ (MK-8, yellow), or on medium with *menA* mutant *E. coli* that do not produce vitamin K₂ (green) or with *menA* mutant *E. coli* transformed with wild-type *menA* that produce vitamin K₂ (blue) (fig. S4). Flying ability (D and E), ATP levels (F), Ψ_m at neuromuscular boutons determined by using JC-1 red/green ratio (G), and mitochondrial morphology in third instar larval muscles (H to J) in control *pink1^{RV}* and in *pink1^{B9}* on MK-4 containing medium (gray) or control medium (black) [(D) to (I)] or upon overexpression of Heix (*da>heix*) (J). The amount of rounded mitochondria is quantified (H). Arrows are normal mitochondrial structure in (I) and (J). Error bars, SEM. *n* > 50 flies, and data are percentages in (A), (B), (D), and (E); 10 assays in (C) and (F); 20 synapses or muscles in (G) to (J); and mean normalized to control in (C) and (F) to (H). ANOVA/Dunnett: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

park^{1/Δ21} mutants showed improved flight, increased ATP levels, and a more negative Ψ_m when placed on MK-4 compared with *park^{1/Δ21}* mutants on control medium (fig. S5, A to D). Thus, MK-4 acutely rescued PD-gene-associated mitochondrial and systemic defects in flies.

Larval and adult *pink1^{B9}* and *parkin^{1/Δ21}* muscles show enlarged, clumped mitochondria, a defect that is modified by mitochondrial re-

modeling (15). This mitochondrial morphology defect was partially rescued by placing *pink1^{B9}* or *park^{1/Δ21}* on MK-4 (Fig. 3, H and I, and figs. S6 and S5, E to I) (10) and by overexpressing Heix (Fig. 3J and fig. S5J), indicating that this rescue of mitochondrial morphology was because of a *heix*-dependent vitamin K₂ deficit. We believe that this effect of vitamin K₂ is the result of improved mitochondrial function and

not because of an important role for vitamin K₂ in mitochondrial remodeling, because feeding MK-4 to *drp1^{1/2}* or *pink1^{B9};drp1/+* mutants that are defective in mitochondrial fission did not rescue their mitochondrial morphological defects (fig. S7, A and B). Conversely, mitochondrial morphological defects observed in animals expressing RNA interference (RNAi) to electron transport chain (ETC) complex I subunits were alleviated when placed on MK-4 (fig. S7C). Thus, morphological defects that arise because of functional defects in mitochondria can be rescued by vitamin K₂.

In bacteria, vitamin K₂ transfers electrons in the ETC, establishing a proton motive force across the membrane (16). Could vitamin K₂ contribute to electron transport in the ETC in mitochondria? Heix-His expressed in fly cells was present in mitochondria (Fig. 4, A and B) (10), similar to the human ortholog UBIAD1 (17), and vitamin K₂ has previously been detected in mitochondrial fractions (18, 19). We tested the consequence of loss of *heix* on mitochondrial function (10) and found that, compared with controls, *heix* mutants showed a less negative Ψ_m and lower ATP levels that could be significantly rescued by rearing *heix* mutants on MK-4 or by reexpression of Heix (Fig. 4C and fig. S8, A and B). Similarly, severe Ψ_m defects in *pink1^{B9};heix/+* were rescued almost to control levels when placed on MK-4 or reexpression of Heix (fig. S8, C and D), and intact mitochondria purified from *heix* mutants showed a significantly reduced capacity to produce ATP in vitro (Fig. 4D). Thus, MK-4 in *heix* mutants is necessary to maintain a negative Ψ_m and to produce ATP.

To determine whether vitamin K₂ is sufficient to facilitate electron transport in mitochondria, we prepared mitochondrial fractions and measured reduction of an artificial electron acceptor, 2,6-dichlorophenolindophenol (DCPIP), downstream of complex II/succinate dehydrogenase (EC 1.3.5.1). In this reaction, succinate is the electron donor and Q-10 the electron carrier (fig. S9) (20). When we added MK-4 rather than Q-10, we found that it was also effective at reducing DCPIP, and increasing concentrations of MK-4 resulted in more efficient electron transport (Fig. 4E) (10). Compared with MK-4, Q-10 was more effective at reducing DCPIP (Fig. 4F); however, when we placed 1-day-old adult *pink1^{B9}* animals on Q-10 medium, we observed a systemic and mitochondrial rescue that did not exceed that obtained when mutants were reared on MK-4 (fig. S10 and Fig. 3). Direct application of MK-4 to mitochondria purified from *pink1^{B9}* significantly facilitated ATP production (Fig. 4G). Furthermore, time-dependent oxygen consumption of intact mitochondria prepared from *pink1^{B9}* mutants was increased when the flies were first placed on MK-4 (Fig. 4H) (10). Thus, vitamin K₂ is sufficient for electron transport downstream of a eukaryotic ETC complex, resulting in improved mitochondrial oxygen

Fig. 4. Vitamin K₂ acts as a mitochondrial electron carrier. Immunolabeling of S2 *Drosophila* cells (A) and Western blot of fractionations (B) transfected with Heix-His and labeled with anti-His and anti-ATP synthase β (A). (C) ATP levels of *heix¹/Df* and *heix¹/Df;da>heix* ($n = 8$). (D) Time-dependent ATP production in mitochondria isolated from *heix¹/Df* mutant larvae ($n = 8$). (E) Measurements of complex II activity [normalized to citrate synthase activity (CS)] using a dose-response of MK-4 as electron carrier ($n = 4$). (F) Measurements of complex II activity using ubiquinone (0.13 mM; Q-10, light gray) or MK-4 (0.35 mM, gray) as electron carrier ($n = 4$). (G) Time-dependent ATP production in mitochondria isolated from *pink1^{B9}* mutant flies. Reactions are either not supplemented with MK-4 (black) or supplemented with 0.15 mM (dark gray) or 0.35 mM MK-4 (light gray) ($n = 8$). (H) Adenosine diphosphate-stimulated complex I-driven respiration rate (oxygen consumption) in mitochondria isolated from *pink1^{RV}* controls and *pink1^{B9}* mutants placed for 72 hours on MK-4 medium or on control medium. Mitochondria from vitamin K₂-fed mutant flies consume oxygen faster. Data are mean normalized to controls in (C) and (H) or mean in (D) to (G). Error bars, SEM. ANOVA/Dunnett: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

consumption and energy production, particularly in *pink1*^{B9} mutants.

Our data predict that vitamin K₂ may also alleviate the defects in other conditions that impair mitochondrial function. We thus placed flies that express RNAi to different complex I components, *sbo* mutants that produce less ubiquinone, and rotenone-treated animals on control medium or on MK-4 medium and assessed Ψ_m and ATP levels. MK-4 rescued the mitochondrial defects in all of these flies (fig. S11, A and B). These results thus support a role for vitamin K₂ in the transport of electrons in eukaryotic mitochondria to produce ATP, similar to its role in prokaryotic membranes (16), suggesting that vitamin K₂ serves a conserved function in prokaryotes and mitochondria. Because mitochondria are involved in aging (21), we reared aged flies on MK-4 or overexpressed Heix but did not observe a significant rescue in mobility (fig. S11C). We surmise that long-lasting compensatory changes may be more important in these situations.

Human *UBIAD1* mutations may also affect mitochondrial function. Electron microscopic analyses of corneal samples from Schnyder's crystalline corneal dystrophy patients who harbor mutations in the *UBIAD1* gene (22–24) indicate cystic swelling of mitochondria, but the nature for this defect is unknown (25). Heix/*UBIAD1* produces vitamin K₂, and in our studies vitamin K₂ rescued mitochondrial defects in numerous conditions that affect mitochondrial function.

Vitamin K₂ was even effective at improving systemic locomotion defects in fully developed adult *pink1* and *parkin* mutant flies. Vitamin K₂ did not affect mitochondrial remodeling directly, but, by increasing ETC efficiency, it contributed to the proton motif force that facilitates ATP production, similar to ubiquinone (26). Vitamin K₂ may thus constitute a promising compound to treat mitochondrial pathology, also in PD patients suffering from *Pink1* or *Parkin* deficiency.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S11

References (27–44)