Vitamin E as a Potentiator of Vitamin K Inadequacy

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Vitamin E: Thromboembolism Prevention

- Women’s Health Study (WHS)
- WHS reported that vitamin E supplementation decreased venous thromboembolism by 21%
- 24% reduction in cardiovascular death
  - Largely attributable to fewer sudden deaths in the vitamin E group (38) compared to placebo group (51)
- Decreased sudden death and decreased thromboembolism may arise from α-tocopherol’s pharmacologic effects on vitamin K that result in decreased clot formation

Glynn et al., Circulation 116, 1497-503, 2007
Vitamin K: Health Significance

- Blood Clotting
  - Hemorrhaging
  - Interference with Warfarin
- Bone Health
  - Bone Mineral Density (BMD)
Vitamin K: Structure

Phylloquinone (K1) is converted to menadione (K3) in the body, which is then converted to menaquinone (MK-4). However, the mechanism for this metabolic change is not yet known!
Vitamin K: Cofactor Role

- Vitamin K serves as an essential cofactor for \( \gamma \)-glutamyl carboxylase
  - \( \gamma \)-glutamyl carboxylase catalyzes the carboxylation of glutamic acid residues on vitamin K-dependent proteins

- Glutamic acid (Glu) is carboxylated to \( \gamma \)-carboxyglutamic acid (Gla) and vitamin K is oxidized by \( \gamma \)-glutamyl carboxylase
Vitamin K Dependent Proteins

- The key vitamin K-dependent proteins include:
  - Coagulation Proteins
    - Factors II (prothrombin)
    - Factor VII
    - Factor IX
    - Factor X
  - Anticoagulation Proteins
    - Protein C
    - Protein S
    - Protein Z
  - Bone Proteins
    - Osteocalcin
    - Matrix-Gla Protein
    - Certain Ribosomal Proteins
Vitamin K Dependent Proteins

- The key vitamin K-dependent proteins include:
  - **Coagulation Proteins**
    - Factors II (prothrombin)
    - Factor VII
    - **Factor IX**
    - Factor X
  - **Anticoagulation Proteins**
    - Protein C
    - Protein S
    - Protein Z
  - **Bone Proteins**
    - Osteocalcin
    - Matrix-Gla Protein
    - Certain Ribosomal Proteins
Vitamin E: Physiological Role

Vitamin E = α-tocopherol

- Antioxidant
  - Cell Membrane Protection
  - Low Density Lipoprotein (LDL) Protection
**Structure: Vitamin E & Vitamin K**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Structure</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylloquinone (K1)</td>
<td><img src="image1" alt="Phylloquinone Structure" /></td>
<td><img src="image2" alt="Phylloquinone Metabolite" /></td>
</tr>
<tr>
<td>Menaquinone (MK-4)</td>
<td><img src="image3" alt="Menaquinone Structure" /></td>
<td><img src="image4" alt="Menaquinone Metabolite" /></td>
</tr>
<tr>
<td>α-Tocopherol (E)</td>
<td><img src="image5" alt="α-Tocopherol Structure" /></td>
<td><img src="image6" alt="α-Tocopherol Metabolite" /></td>
</tr>
</tbody>
</table>
Possible Vitamin E Actions:

- Interference with the conversion of vitamin K1 to MK-4 by an unknown enzyme
- Up-regulation of xenobiotic metabolism to increase vitamin K breakdown metabolites
- Increased excretion of all vitamin K forms

Vitamin E & Vitamin K

Liver

- Phylloquinone (K1)
- Menadione (K3)
- Vitamin E Stimulates?
- Vitamin K Metabolites

Extrahepatic Tissues

- Menadione (K3)
- MK-4

Excretion

Plasma
Vitamin E & Vitamin K

Liver

Vitamin E Inhibits?

Phylloquinone (K1)

Vitamin E Stimulates?

Vitamin K Metabolites

Menadione (K3)

MK-4

Plasma

Excreted

Menadione (K3)

MK-4

Extrahepatic Tissues
Hypotheses

- Related Gene Transcription Levels
  - Elevated $\alpha$-tocopherol concentrations alter vitamin K status through:
    1. Xenobiotic Metabolism
    2. Transporters for Excretion

- Vitamin K Activity
  - Elevated tissue $\alpha$-tocopherol concentrations decrease the availability of vitamin K for vitamin K-dependent $\gamma$-glutamylcarboxylation, thus resulting in under-$\gamma$-carboxylation of vitamin K-dependent proteins.
Objective: Determine if α-tocopherol is able to alter the transcriptional levels of cytochrome P450 enzymes in vitamin K metabolism or transporters involved in its excretion
Vitamin K & Vitamin E: Animal Experiment

- Two different diets administered to male Sprague-Dawley rats:
  - K1: Phylloquinone diet
  - K3: Menadione diet
- 2.0 μmol K1 or K3 per kg diet
- Rats were injected daily with vitamin E (E) or a vehicle (V)
- 10 mg α-tocopherol vitamin E injections per 100 grams of body weight

<table>
<thead>
<tr>
<th>Week</th>
<th>Injections</th>
<th>Rats on K1 Diet</th>
<th>Rats on K3 Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>---</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>E</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>5</td>
<td>5</td>
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</tbody>
</table>
Methods: Gene Expression

- Vitamin E Effects
  - Gene Transcription Levels
    - Real Time RT-PCR
      - CYP4Fs
      - FIX
      - Bile Transporters
CYP Genes

- CYP enzymes (encoded by CYP genes) have various physiological roles:
  - Synthesis of steroid hormones, cholesterol, bile acids, and other fatty acids
  - Metabolism of fatty acids and vitamins
  - Metabolism of xenobiotics (i.e. medications or toxins)

- Human CYP4F2 Enzyme: ω-hydroxylation of vitamins E and K

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reason for Interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP4F4</td>
<td>Rat Homologue of Human CYP4F2</td>
</tr>
<tr>
<td>CYP4F1</td>
<td>Similar Long Chain Fatty Acid Substrates</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Drug Metabolism &amp; Previous Studies</td>
</tr>
</tbody>
</table>
Real Time PCR Results

Gene Expression Data

Fold Change Over Vehicle

- K1 Diet
- CYP4F1
- K3 Diet

Vehicle

α-T
Real Time PCR Results

Gene Expression Data

Fold Change Over Vehicle

* indicates statistically significant difference

- K1 Diet
- K3 Diet

Vehicle
a-T

CYP4F4
Real Time PCR Results

Gene Expression Data

Fold Change Over Vehicle

K1 Diet
K3 Diet
CYP3A

Vehicle
a-T
Factor IX Gene

- Factor IX (FIX): Coagulation Precursor
- Vitamin K Dependent Protein
- Down regulation would result in lowered blood clotting efficiency

The FIX protein and its activated form (FIXa) are an integral part of the coagulation cascade!
Real Time PCR Results

Gene Expression Data

Fold Change Over Vehicle

K1 Diet  | K3 Diet
---|---
Vehicle | a-T

FIX
Bile Transporters

- **OATP (Organic Anion-Transporting Polypeptide)**
  - Solute Carrier Family
  - Mediates Organic Anion Transport Across Cell Membrane
    - Bile Acids

- **BCRP1 (Breast Cancer Resistance Protein)**
  - Xenobiotic Transporter
  - ATP-Binding Cassette (ABC) Transporter Superfamily
    - Molecule Transport Across Extra- and Intra-Cellular Membrane
Real Time PCR Results

Gene Expression Data

Fold Change Over Vehicle

- **OATP**
  - K1 Diet
  - K3 Diet

- **BCRP1**
  - K1 Diet
  - K3 Diet

**Vehicle**

**a-T**

* Indicates significant change.
Objective: Determine the severity of molecular under-$\gamma$-carboxylation of vitamin K caused by vitamin E
Methods: Vitamin K Activity

- Vitamin E Effect
  - Vitamin K Activity
    - Hydroxyapatite Assay
      - Total OC ELISA
        - Osteocalcin Carboxylation Levels
Determining Vitamin K Activity Levels: Osteocalcin

Osteocalcin (a.k.a. Vitamin K-dependent Ca\(^{2+}\) binding protein): a bone matrix protein with three carboxylated glutamic acid residues (Gla) at positions 17, 21, and 24 (carboxylated by vitamin K-dependent $\gamma$-carboxylase)

- Found in Bone and Dentin
- Pro-osteoblastic
  - “Bone Building”
Vitamin K: Determining Activity Levels

- Hydroxyapatite Assay
- Total OC ELISA
- Quantify levels of undercarboxylated OC in the rat plasma samples

Remember: Vitamin K is a cofactor for $\gamma$-glutamyl carboxylase, which $\gamma$-carboxylates vitamin-K dependent proteins.
Hydroxyapatite Assay

Gla-OC Binds Hydroxyapatite

Centrifugation Forms Hydroxyapatite Pellet

Pellet Contains Gla-OC
Supernatant Contains Glu-OC

Total OC EIA Plate:
Glu-OC Supernatant
Glu-OC and Gla-OC Serum Samples

Gla-OC: Carboxylated OC
Glu-OC: Undercarboxylated OC
Conclusions

- The K1 and K3 diets, had no effect on xenobiotic metabolism, thus the change of vitamin K source had no effect on this parameter.
- Vitamin E “excess” decreased the gene expression of cytochrome P450s involved in xenobiotic metabolism.
- OATP and BCRP1, two ABC transporters in the hepatic biliary membrane, that transport bile acids and xenobiotics respectively, showed changes in transcription levels with “excess” vitamin E:
  - OATP expression decreased
  - BCRP1 expression increased over 7-fold
- Neither the varying vitamin K source nor the vitamin E status changed FIX gene expression.
- These findings are important because high levels of vitamin E supplements in humans decrease blood clotting. Also, our data suggests that vitamin E may increase vitamin K excretion in bile. However, further studies are needed to test this hypothesis.
Acknowledgements

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- Linus Pauling Institute
- Dr. Maret Traber
- Sherry Farley
- Traber Labites
- Dr. Kevin Ahern
Hydroxyapatite Assay

1. Carboxylated OC Binds Hydroxyapatite
2. Centrifugation Forms Hydroxyapatite Pellet
3. Pellet Contains Gla-OC; Supernatant Contains Glu-OC
4. Total OC EIA Plate: Glu-OC Supernatant, Glu-OC and Gla-OC Serum Samples
BTI’s Rat Osteocalcin EIA Kit
- Sandwich ELISA
- Quantifies both Gla-OC and Glu-OC
- Selectively recognizes intact OC

ELISA: Enzyme-Linked Immunosorbent Assay
EIA: Enzyme Immunoassay
Methods: Quantitative Real Time RT-PCR

- DNA Amplification
- TOPO Cloning
  - *E. coli*
- *E. coli* Colony PCR
- DNA Purification
- Real Time PCR
  - CYP4F1
  - CYP4F4
  - CYP3A
  - FIX
  - GAPDH
Vitamin E: Heart Disease Prevention

- Women’s Health Study
- 40,000 women aged 45 y and older randomly assigned:
  - vitamin E (600 IU every other day) or placebo
  - aspirin or placebo
  - study lasted 10 y
- 24% reduction in cardiovascular death
  - largely attributable to fewer sudden deaths in the vitamin E group (38 vs. 51 in the placebo group)
- No reduction in stroke rate was observed
- No effect of vitamin E on total mortality

Lee et al., JAMA 294, 56-65 (2005).
Vitamin E: Heart Disease Prevention

- Women’s Health Study Subgroup Analysis
- In women aged at least 65 y (10% of study participants) assigned to vitamin E
  - 26% reduction in major cardiovascular events
  - 34% reduction in myocardial infarction
  - 49% reduction in cardiovascular deaths
- Vitamin E efficacy was not evaluated with biomarkers, but with mortality or heart attacks, etc.
- Study authors concluded that vitamin E provided no overall benefit and do not support recommending vitamin E supplementation for cardiovascular disease prevention among healthy women.

Lee et al., JAMA 294, 56-65 (2005).