The Nitrate-Nitrite-NO Pathway

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Two dominant sources of nitrate and nitrite in mammals

- NO synthases
- Diet

> NO → NO₂⁻ / NO₃⁻
The two "dogmas" about nitrate ($\text{NO}_3^-$)

1. Inert end product of NO metabolism
2. Unwanted residue in the food chain
Rapid inactivation of NO by oxidation to nitrite and nitrate

- **eNOS** (endothelial nitric oxide synthase)
  - Converts L-arginine to NO
- **NO**
- **Oxy-Hb** (oxyhemoglobin)
- **NO$_3^-$** (nitrate)
- **NO$_2^-$** (nitrite)
- **O$_2$** (oxygen)
- **sGC** (soluble guanylate cyclase)
  - Activated by GTP, produces cGMP
  - cGMP↑ (increase in cyclic guanosine monophosphate)

**Blood**

**Endothelium**

**Smooth muscle**
The two "dogmas" about nitrate (NO$_3^-$)

1. Inert end product of NO metabolism

2. Unwanted residue in the food chain
The dark side of dietary nitrate

- Promotes formation of potentially carcinogenic nitrosamines (gastric cancer)

Nitrate content in food and drinking water is strictly regulated!
Biovive

0% de Nitrate*

Eau Minérale Naturelle

12,5 cl
Todays main message on nitrate

- Unwanted residue in the food chain
- Inert end product of NO

Desirable

Substrate for
Two parallel systems for NO generation in mammals

\[ \text{L-Arginine} \rightarrow \text{NO} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^- \]

Normoxia: NOS-dependent pathway

Hypoxia: NOS-independent pathway
Nitric oxide: Role in the cardiovascular system

L-arginine + $O_2$ → NO synthase → NO → Vasodilation

Leucocyte adhesion ↓
Platelet aggregation ↓
Mitochondrial function

Reduced NO bioavailability is a central event in many cardiovascular disorders
(Hypertension, atherosclerosis, stroke, myocardial infarction)
Nobel Prize in Physiology or Medicine 1998

Robert F. Furchgott
Louis J. Ignarro
Ferid Murad

"For their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system"
NO research in our lab
Background

Measurements of exhaled NO

[Diagram showing a person exhaling into a device with a graph indicating NO levels (in ppb)]
EXPERIMENTAL SETTINGS

Carbonated Water

+5 min

NO Analyzer
NO levels in expelled stomach gas

Lundberg et al Gut 1994
Nitrite is reduced to NO under acidic conditions

\[
\text{NO}_2^- + H^+ \rightarrow \text{HNO}_2 \ (\text{pKa} \sim 3.3)
\]

\[
2 \text{HNO}_2 \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow \text{NO} + \text{NO}_2
\]

Gastric pH is 1-2 in fasting humans

Saliva is very rich in nitrite (high µM-mM)
Why is saliva so rich in nitrite?

- In mammals 25% of circulating nitrate ($\text{NO}_3^-$) is actively taken up by the salivary glands and secreted in saliva.

- Nitrate in saliva is 10-25 times higher than in plasma.

- Bacteria on the tongue effectively reduce nitrate to nitrite.

Spiegelhalder Food Cosmet Toxicol 1976
Salivary nitrate/nitrite levels after an oral nitrate intake

Sodium nitrate 10 mg/kg
The entero-salivary circulation of nitrate, its reduction to nitrite in the mouth and then to NO in the stomach

1. Nitrate and some nitrite from food
2. Bacteria in the oral cavity reduce nitrate to nitrite
3. In the gastric acidic milieu, a non-enzymatic reduction of nitrite to NO occurs
4. An active uptake of nitrate from the blood occurs in the salivary glands
5. Nitrate and nitrite in blood originate from the food and from systemic NO production
6. Nitrate is absorbed in the intestine
7. Nitrate is excreted by the kidneys

Does intragastric NO have any biological effects?
Antibacterial effects of gastric NO

E. coli

1-2 h

24 h

Benjamin et al.  Nature -94
Antibacterial effects of gastric NO

E. coli

"No growth"

Benjamin et al. Nature -94
Does gastric NO affect the host mucosa?
Salivary nitrite increases gastric mucosal blood flow and mucus generation

- Effects were cGMP mediated (inhibited by ODQ)
- Intragastric NO gas increased
- L-NAME/Indomethazine had no effect
Are these effects associated with gastroprotection?
Dietary nitrate, aspirin-like drugs and ulcers

Nitrate dose corresponds to 100-500 gr nitrate-rich vegetable/day in a human.
Dietary nitrate and NSAID-induced Gastric Ulcers

Control

Nitrate

Ulcer Index (mm)

Diclofenac 30 mg/kg
Control
Nitrate 0.1 mmol/kg
Nitrate 1 mmol/kg

0
25
50
75
100
125
An antiinflammatory role of dietary nitrate?

Nitrate

Nitrite

Tissue nitrite reductases

NO

P-selectin

ICAM-1

Jädert FRBM 2012
Intragastric NO formation from inorganic nitrate

Lundberg, Weitzberg, Cole, Benjamin
Nature Reviews Microbiology 2004
Will some of the nitrite formed in the oral cavity survive GI passage and enter the systemic circulation?
Dietary nitrate is a source of systemic nitrite

10 healthy volunteers ingesting sodium nitrate 10 mg/kg
What happens if we kill the oral nitrate reducing bacteria?
Salivary nitrate reduction is abolished by an antiseptic mouthwash

Oral nitrate load 10 mg /kg
Change in plasma nitrite after an oral nitrate load

Δ Nitrite (nM)

-50 0 50 100 150 200

0 50 100 150 200 250 300 350

Time (min)

Control
Mouth Wash

Govoni NO Biol Chem 2009
Conclusions from these studies....

- Dietary nitrate can be a major source of \textit{systemic} nitrite

- This requires enterosalivary circulation of nitrate and reduction by commensal bacteria in the mouth
So, why is it interesting that dietary nitrate increases systemic nitrite levels?
NOS-independent NO production in the ischemic heart

- NO production increased 10-fold after global ischemia

- This NO was not inhibited by NOS inhibitors

- Using isotopically labeled nitrite it was shown that nitrite is reduced to NO in the ischemic heart
Nitrite can be further reduced to NO in blood and tissues.

- **Diet** interacts with NOS to produce NO.

  - **NO$_3^-$** can be reduced by bacterial nitrate reductases (Xanthine oxidase).
  - **NO$_2^-$** can be reduced by deoxygenated Hb, Mb, Xanthine oxidase, respiratory chain enzymes, protons, vitamin C, and polyphenols.

  - **NO** is produced under conditions of low oxygen and low pH.

References:
- Webb PNAS 2004
- Cosby Nature Med 2003
- Modin Acta Physiol 2001
- Zweier Nat Med 1995
- Lundberg Gut 1994
- Benjamin Nature 1994
Is nitrite-derived NO doing anything?

In ischemic tissues oxygen tension and pH are low - ie conditions that favour nitrite reduction to NO
Nitrite and myocardial Infarction

Figure 2A

Murine in vivo Myocardial Ischemia-Reperfusion Protocol

- Control
- LCA Ischemia
- Reperfusion

Nitrite or Nitrate (48 nmoles, i.v.)

*In Vivo area-at-risk
- Evans Blue injection
Infarct Size
- 1.0% 2,3,5-TTC

Figure 2C

Nitrite Dose (nmoles)

<table>
<thead>
<tr>
<th>Nitrite Dose</th>
<th>AAR per Left Ventricle (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>placebo</td>
<td>2.4 6 4.8 48 960 1920</td>
</tr>
</tbody>
</table>

Figure 2D

Infarct per Area-at-Risk (%)

<table>
<thead>
<tr>
<th>Nitrite Dose</th>
<th>Infarct per Area-at-Risk (%)</th>
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</thead>
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Duranski JCI 2005
Nitrite and Stroke

Jung et al. Stroke 2006
Low dose nitrite prevents ischemia-reperfusion injury in several organs

Systemic administration, (rats, mice, dogs)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>Webb, PNAS 2004</td>
</tr>
<tr>
<td></td>
<td>Duranski, JCI 2005</td>
</tr>
<tr>
<td></td>
<td>Dezfulian Circulation 2008</td>
</tr>
<tr>
<td>Liver</td>
<td>Duranski, JCI 2005</td>
</tr>
<tr>
<td>Stroke</td>
<td>Jung, Stroke 2006</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>Dias-Junior, FRBM 2007</td>
</tr>
<tr>
<td>Chronic limb ischemia</td>
<td>Kumar PNAS 2007</td>
</tr>
</tbody>
</table>
How does nitrite protect in IR injury?

- Nitrite interactions with mitochondria -
What about dietary nitrate and cardiovascular function?
Nitrite and cardioprotection - In vivo

![Graph showing the effect of nitrite on infarct size.](image)
How much nitrite can be made systemically from nitrate in vegetables?

The dose of nitrite needed for maximal cardioprotection in mice (48 nmoles or 3 µg) corresponds to an oral nitrate load of around 200-300 mg in a human.

This is less than one beetroot or half a plate of spinach!
Vegetables and fruit protect against stroke and coronary heart disease

”Of the food groups analyzed, green leafy vegetable intake showed the strongest inverse association with cardiovascular disease”
So, does dietary nitrate have NO-like effects in the cardiovascular system?
Dietary Nitrate and Blood Pressure

- 17 healthy volunteers age 20-25 years
- Nitrate supplementation (NaNO$_3$ 0.1 mmol/kg/day) or placebo (NaCl)
- Double blind placebo-controlled cross-over design
- Nitrate amount corresponds to about 150-200 g spinach, lettuce or beetroot
Effects of Dietary Nitrate on Blood Pressure

- 4 mmHg
Is nitrate bioactivation dependent on oral bacteria?
Antibacterial mouthwash, nitrate and blood pressure

![Graph showing MAP (mmHg) with control and nitrate periods, indicating a statistically significant difference (p < 0.05).]

no mouthwash
Beetroot juice and blood pressure

- 14 healthy volunteers
- Crossover design
- 500 ml fresh beetroot juice (22 mmoles nitrate)

Webb et al. Hypertension 2008
Is nitrate the active ingredient in beetroot juice?

- 6 healthy volunteers - spit/swallow
- 500 ml fresh beetroot juice (22 mmoles nitrate)

Webb et al. Hypertension 2008
Hypertension?
Dietary nitrate in a model of chronic hypertension with renal dysfunction

- Rats are uninephrectomized (UNX) at a young age and put on a high-salt diet (HS).
- Animals develop hypertension and renal/cardiac dysfunction
- Nitrate in chow for 10 wks
- Low dose (0.1 mmol/kg/day), High dose (1 mmol/kg/day)
- BP, NOx, markers of oxidative stress, histology etc measured
Results: Telemetric Blood Pressure Measurements

![Graph showing mean arterial pressure over days and nights with different conditions: Control, UNX+HS, UNX+HS+nitrate (low), and UNX+HS+nitrate (high). The graph indicates significant differences (*) between conditions.](image)
Histological evaluation of renal tissue

**Tubular Changes**

- Controls
- UNX+HS
- UNX+HS nitrate (low)
- UNX+HS nitrate (high)

**Kidney Inflammation**

- Controls
- UNX+HS
- UNX+HS nitrate (low)
- UNX+HS nitrate (high)

**Kidney Fibrosis**

- Controls
- UNX+HS
- UNX+HS nitrate (low)
- UNX+HS nitrate (high)

**Glomerular Changes**

- Controls
- UNX+HS
- UNX+HS nitrate (low)
- UNX+HS nitrate (high)

**Arterio-Arteriolar Sclerosis**

- Controls
- UNX+HS
- UNX+HS nitrate (low)
- UNX+HS nitrate (high)
eNOS-deficient mice
What is the Phenotype?

- Hypertension
- Increased weight
- Increased visceral fat accumulation
- Higher circulating triglycerides
- Insulin resistance

The Metabolic Syndrome

In a situation with reduced generation of endogenous NO and nitrate, what will be the result of restoration with inorganic nitrate?
How much nitrate should we give?
NOS activity in mice:
How much NO is being produced?

- **0.2 mmol/kg/24 h**
  (Wickman NO Biol Chem, 2003)
- inhalation of a stable oxygen isotope, $^{18}\text{O}_2$
- plasma nitrate analyzed by GC/MS
- 91% reduction after L-NAME

- Around 50-70% of this is from eNOS
  (Kleinbongard FRBM, 2003)

So, eNOS makes around 0.1 mmol/kg/day
Study design: Dietary Nitrate in eNOS-deficient Mice

- Aged eNOS-null mice (16 Mo)
- Nitrate in drinking water for 8-10 weeks
- Nitrate dose 0.1 mmol/kg/day
- Endpoints: Body weight, TGs, visceral fat, glucose homeostasis, Blood pressure

This dose corresponds to intake of 100-300 g/day of a nitrate rich vegetable in humans.
Dietary Nitrate Increases Levels of Potentially Bioactive Nitrogen Oxides

![Graphs showing changes in nitrate and nitrite levels in plasma, liver, muscle, and fat under acute and chronic dietary nitrate intake.](Image)

Carlstrom Proc Natl Acad Sci USA. 2010
Inorganic nitrate reduces body weight, triglycerides and adipose tissue
Inorganic nitrate improves glucose homeostasis

Carlstrom Proc Natl Acad Sci USA. 2010
Interpretations

- Inorganic nitrate seems capable of functionally replacing some of the metabolic effects of eNOS

- This could support the idea that NOS-derived nitrate has a physiological role as a stable reservoir of NO-like bioactivity
A physiological role for nitrate and nitrite?

- Exogenously administered nitrate and nitrite clearly has biological NO-like effects even with very modest doses

- But is there a role for endogenous nitrate/nitrite (derived from NOS) in control of NO homeostasis?

- The classical way of studying the physiological significance of an endogenous messenger is to use a pharmacological inhibitor or to delete the gene, and then observe the phenotype.

How can we study the role of endogenous nitrate?
Recycling of endogenous nitrate?

Diet

NOS

NO

NO$_2^-$

NO$_3^-$
Overall Summary

- Dietary nitrate is converted to NO and other bioactive nitrogen oxides in our bodies

- Oral symbiotic bacteria help to bioactivate nitrate

- Nitrate modulates cardiovascular and metabolic functions

- Nitrate in vegetables may explain some of the well-known health effects of this food group