UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 328

Nitrate, Nitrite and Nitric Oxide in Gastric Mucosal Defense

JOEL PETERSSON

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2008

ISSN 1651-6206
ISBN 978-91-554-7152-1
urn:nbn:se:uu:diva-8624

Dissertation presented at Uppsala University to be publicly examined in the main building of Uppsala University, Room IV, Friday, May 9, 2008 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract
Petersson, J. 2008. Nitrate, Nitrite and Nitric Oxide in Gastric Mucosal Defense. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 328. 90 pp. Uppsala. ISBN 978-91-554-7152-1.

The human stomach normally contains high levels of bioactive nitric oxide (NO). This NO derives from salivary nitrate (NO_3^-) that is converted to nitrite (NO_2^-) by oral bacteria and thereafter non-enzymatically reduced in the acidic gastric lumen to NO. Nitrate is a common component in vegetables, and after ingestion it is absorbed in the small intestine. Interestingly, circulating nitrate is then concentrated by the salivary glands. Hence, intake of nitrate-rich vegetables results in high levels of NO in the stomach. The physiological effects of the high concentration of NO gas normally present in the gastric lumen have been hitherto unknown, and the present investigations were therefore conducted to address this issue.

NO produced in the gastric lumen after nitrate ingestion increased gastric mucosal blood flow and the thickness of the firmly adherent mucus layer in the stomach. The blood flow and mucus layer are essential defense mechanisms that protect the mucosa from luminal acid and noxious agents. Nonsteroidal antiinflammatory drugs (NSAID) are commonly prescribed and effective drugs for treating pain and inflammation, but are associated with severe gastrointestinal side effects. We demonstrated that a nitrate-rich diet protects against NSAID-induced gastric damage, as a result of the increased formation of NO in the stomach. We also showed that the gastroprotective effect attributed to nitrate depended completely on conversion of nitrate to nitrite by the bacterial flora colonizing the tongue, and that the oral microflora is therefore important in regulating physiological conditions in the stomach.

In summary, this thesis challenges the current dogma that nitrate intake is hazardous, and on the contrary suggests that dietary nitrate plays a direct role in regulating gastric homeostasis. It is likely that a sufficient supply of nitrate in the diet together with the oral microflora is essential for preventing pathological conditions in the gastrointestinal tract.

Keywords: dietary nitrate, mucus gel layer, mucosal blood flow, mucus thickness, laser-Doppler flowmetry, mucosal damage, intra-vital microscopy, mucosal permeability

Joel Petersson, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden

ISSN 1651-6206
ISBN 978-91-554-7152-1
urn:nbn:se:uu:diva-8624 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8624)

For a greener diet

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Björne, H., **Petersson, J**., Phillipson, M., Weitzberg, E., Holm, L., and Lundberg, J.O. 2004. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. *J Clin Invest* 113:106-114.
Björne and Petersson contributed equally to this work

II. **Petersson, J**., Phillipson, M., Jansson, E., Patzak, A., Lundberg, J.O., and Holm, L. 2007 Dietary Nitrate increases gastric mucosal blood flow and mucosal defense. *Am. J. Physiol Gastrointest. Liver Physiol.* 292:G718-24

III. Jansson, E., **Petersson, J**., Reinders, C., Sobko, T., Björne, H., Phillipson, M., Weitzberg, E., Holm, L., Lundberg, J.O. 2007 Protection from NSAID-induced gastric ulcers by dietary Nitrate. *Free Rad. Biol. Med.* 42 510-518

IV. **Petersson, J**., Schreiber, O., Phillipson, M., Christoffersson, G., Jägare, A., Roos, S., Jonsson, H., Jansson, E., Lundberg, J.O., and Holm, L., 2008 Oral bacteria regulate gastric mucosal defense via bioactivation of dietary nitrate in rats and mice. *Manuscript*

Contents

Abbreviations

CFU	colony-forming units
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
^{51}Cr-EDTA	^{51}chromium-labeled ethylenediaminetetraacetate
HCl	hydrochloric acid
LDF	Laser-Doppler flowmetry
i.p.	intraperitoneal
i.v.	intravenous
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
MAP	mean arterial pressure
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
cNOS	constitutive NOS
eNOS	endothelial NOS
iNOS	inducible NOS
NSAID	nonsteroidal anti-inflammatory drugs
ODQ	1H-[1,2,4]Oxadiazolo-[4,3-a]quinoxalin-1-one
PCR	polymerase chain reaction
PU	perfusion units
ppb	parts per billion
ppm	parts per million
SNAP	S-nitroso-N-acetyl-penicillamine
S-NO	S-nitrosothiol
VR	vascular resistance

Introduction

Nitric oxide (NO) is an important signaling molecule in the mammal, which is formed in the body from the amino acid L-arginine.[1, 2] The reaction is catalyzed by a family of NO synthases (NOS).[3] The different nitric oxide synthases, namely neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) were formerly believed to be the only way in which the body produced NO.[4] Nitrate (NO_3^-) and nitrite (NO_2^-) were long known predominantly as inert oxidative end products of NO metabolism. However, research conducted during the last decade has changed our way of viewing nitrate, nitrite and NO.[5-8]

In 1994, another important pathway for NO production was discovered, when it was found that NO can be formed nonenzymatically in the stomach after swallowing of nitrite.[9-11] Nitrite is rapidly reduced to NO in the acidic environment in the stomach. The nitrite originates from nitrate in the saliva that is converted to nitrite by oral bacteria. Nitrate was identified in saliva in 1890,[12] and it has been known for over 30 years that nitrate is actively taken up from the plasma and concentrated in the salivary glands and secreted into the oral cavity,[13] but a reason for the nitrate accumulation in the saliva has never been suggested.

The above discovery in 1994 revealed that the stomach normally contains high concentrations of NO as a result of swallowing of nitrite-rich saliva.[9-11]

This thesis therefore focuses on the question of how the bioactive NO present in the gastric lumen influences the normal functions of the stomach and especially the gastric mucosa.

Gastric Mucosal Defense

Hydrochloric acid (HCl) is produced and secreted by the parietal cells located in the gastric glands in the corpus of the stomach. When stimulated, the parietal cells can secrete acid at a concentration of 155 mM (pH 0.8).[14] Chief cells in the glands secrete digestive enzymes into the gastric lumen with the purpose of chemically degrading ingested proteins. In this acidic, hostile environment it is very important for the gastric mucosa to have a well developed defense against acid, pathogenic bacteria and digestive enzymes. An effective barrier against the luminal contents will prevent the mucosa from digesting itself. The defense

consists of different mechanisms at different levels, and under normal physiological conditions these mechanisms maintain an effective protective barrier.

1. The first, pre-epithelial defense consists of an adherent mucus layer that covers the entire mucosa.
2. The second line of defense is a tight epithelium.
3. The third line is the gastric mucosal blood flow.

The efficacy of these different mechanisms in protecting the stomach from digesting itself is obvious in situations where this system is disrupted. It has been known for more than two centuries that after death, the stomach digests itself as a result of absence of gastric mucosal protection while acid and digestive enzymes are still present in the stomach lumen.[15] These different defense mechanisms are summarized in Figure 1.

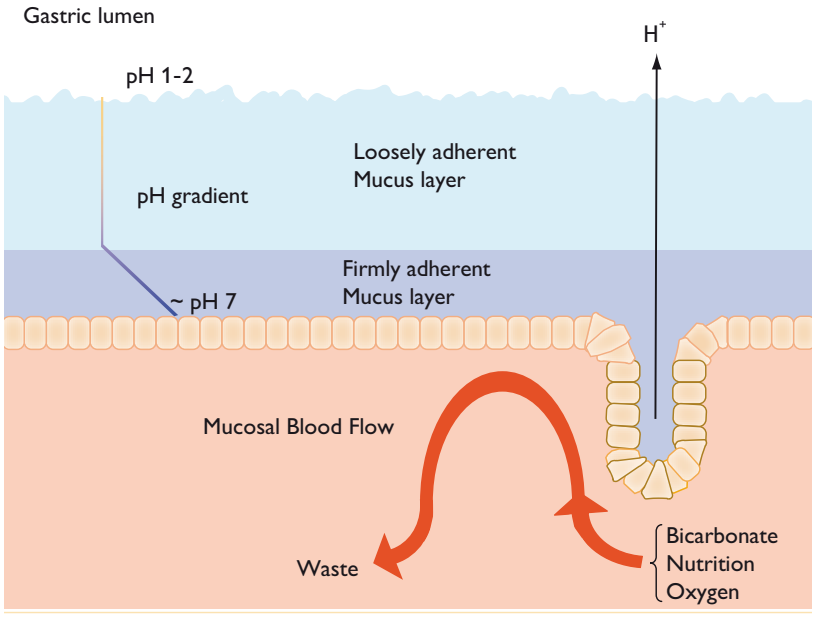


Figure 1. A schematic drawing of the gastric mucosa showing the different mucus layers, the pH gradient in the mucus and the mucosal blood flow.

The Gastric Mucus Layers

The gastric mucosa is covered by a continuous mucus layer. The mucus consists of mucins (glycoproteins) and water and is secreted by the surface epithelial cells and the mucus neck cells.[16, 17] The mucus in the stomach mainly consists of MUC5AC and MUC6 mucins.[18] The mucus gel serves as an important physical barrier against luminal contents.[15] The mucus layer covering the gastric mucosa can be divided into two distinct layers: one outer layer which can easily be removed by suction and is referred to as the loosely adherent mucus layer, and one inner layer that adheres strongly to the gastric mucosa and cannot be removed without destroying the underlying mucosa.[19, 20]

A protective pH gradient is created in the firmly adherent mucus layer when bicarbonate is secreted from the epithelium into the mucus, where it neutralizes back-diffused acid.[21, 22] This gradient maintains a neutral pH at the epithelial surface (juxtamucosal pH), while the luminal pH can be very low. In this way the mucus also provides an important means of chemical neutralization of the acidic stomach content.

If the firmly adherent mucus layer becomes too thin, the ability to maintain a neutral juxtamucosal pH is reduced.[23] A thick firmly adherent mucus layer therefore serves as an important pre-epithelial protector of the gastric mucosa. Both NO and prostaglandins have been shown to be involved in gastric mucus secretion.[24-26] Prostaglandins stimulate secretion of both mucus layers, whereas NO only stimulates secretion of the firmly adherent mucus layer.[27]

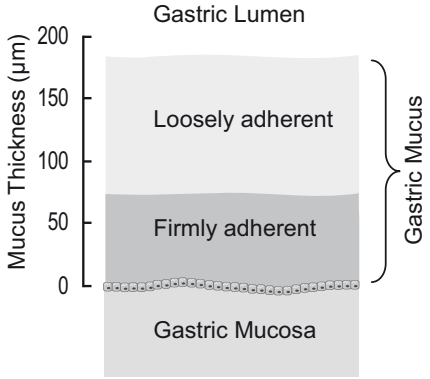


Figure 2. The thickness of the different mucus layers in the gastric mucosa in rats, based on results from *in vivo* measurements at our laboratory.

The Gastric Epithelium

The cells in the gastric epithelium live in a very harsh environment and their life-cycle is only a few days.[28, 29] Despite this quick turnover, the gastric epithelium is very tight. The cell membrane on the apical side of the gastric surface cells has a very low permeability for protons,[30, 31] and the tight junctions that

connect the surface cells to one another are even less conductive for ions than the pathway through the cells.[32] These properties protect these cells from destruction in this milieu. Despite this well developed defense, substances such as nonsteroidal anti-inflammatory drugs (NSAIDs), ethanol and bile acids may damage the surface cells and break this important barrier. However, the gastric surface epithelium has a capacity for very rapid repair. Within minutes after an injury, cells from the gastric pits begin to migrate toward the epithelial surface,[33] resulting in a new epithelium after just a few hours.[34, 35] But if deeper parts of the mucosa are damaged, the restitution is inhibited,[33] and the healing process continues for some months.[36]

The Gastric Mucosal Blood Flow

The arteries that enter the muscle layer of the stomach give off branches that supply the muscles with oxygen and nutrition.[37] Further up, in the submucosa, the arteries form a vascular plexus. Arterioles arising from the submucosal vascular plexus split up in the base of the mucosa and form capillaries which supply the mucosa with blood. These capillaries run around the gastric glands toward the surface of the mucosa, with numerous connections between them. In this way a hexagonal network of capillaries is formed around the openings of the gastric crypts.[38] The capillaries empty into large collecting veins which run perpendicularly from the lumen to the submucosa. The stomach wall has a high blood flow and owing to this blood vessel arrangement the mucosa receives approximately 70% of the total gastric blood flow.[39]

The circulation in the mucosal capillaries is regulated by the densely innervated submucosal arterioles.[40] The blood flow in the arterioles is controlled by a complex combination of the central and enteric nervous systems, hormones, and production of prostaglandins and NO.[39, 41-43] Several studies suggest that endogenously produced NO increases the mucosal blood flow and hence protects the mucosa from injury, as reviewed by Kawano and Tsuji.[44]

An adequate gastric mucosal blood flow is an important mechanism for maintaining the integrity of the gastric mucosa. The blood supplies the mucosa with oxygen and nutrition and dilutes and flushes away any back-diffused acid[45, 46] and cellular waste products.[47] The blood also supplies the surface cells with bicarbonate for neutralization to maintain the pH gradient in the overlaying mucus layer.[48, 49] Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of the gastric mucosa against injury.[40, 44, 46, 50-54] The mucosa is more vulnerable to noxious agents if the blood flow is reduced.[46, 50, 55] It is also known that an increase in blood flow protects the mucosa from damage caused by ulcerogenic substances.[45, 56] Furthermore, the blood flow increase induced by barrier breakers has been shown to be important for rapid repair of the mucosa.[57]

The Biological Chemistry of Nitrate, Nitrite and NO

The radical NO has an extremely short life (less than 1 s) in circulating blood.[58] The NO generated by endogenous NOS is oxidized in the blood to form nitrate and nitrite.[3] NO and nitrite react with oxyhemoglobin, yielding nitrate and methemoglobin.[59, 60] NO together with oxygen can also be oxidized to nitrite in the plasma with the help of different oxidases.[61] The half-life of nitrite in the blood is 20-30 minutes,[62] whereas nitrate has a circulating half-life of several hours.[63, 64] Thus plasma nitrate and nitrite do not only reflect dietary sources of nitrate and nitrite, but also the endogenous NOS activity. Under fasting conditions with low intake of nitrate, NO produced by nNOS, eNOS and iNOS can have a major impact on the levels of circulating nitrate and nitrite.[65] This is also obvious in animals lacking NOS, where the amount of circulating nitrite are substantially reduced.[66] On the other hand, during systemic inflammatory processes, with a substantial upregulation of iNOS, the levels of circulating nitrate and nitrite are greatly increased.[3, 67] Nevertheless, a diet rich in nitrate does have a substantial influence on the circulating levels of nitrate and nitrite.[5] Under normal conditions, a nitrate-rich diet makes a much larger contribution to circulating nitrate than the amount formed by all three different NOS isoforms together during a whole day.[6, 68 69]

There are several pathways for oxidation and reduction of nitrate, nitrite and NO in the body, and some of them are summarized in Figure 3.
Nitrate is reduced to nitrite by oral bacteria.[70-73] Bacterial nitrate reductase reduces the nitrate to nitrite by using nitrate as an alternative electron acceptor to oxygen under hypoxic conditions.[6] This is the main pathway for nitrate reduction and the reaction is described in Table 1.

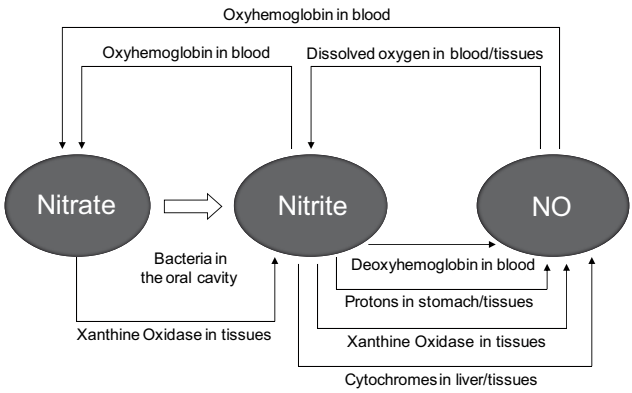


Figure 3. Different pathways for oxidation and reduction of nitrate, nitrite and NO in the human body.

Previously, the bacterial conversion of nitrate to nitrite was believed to be the only way in which nitrate could be reduced to nitrite in mammals, but in a recent study in mammals another pathway was discovered, when xanthine oxidase was shown to possess the ability to reduce nitrate to nitrite.[74]

Nitrite, on the other hand, can easily be reduced to NO in many different ways. Deoxyhemoglobin in the blood reduces nitrite to NO and methemoglobin.[67] Xanthine oxidase, deoxymyoglobin, and cytochromes in the liver and tissues also reduce nitrite to NO.[75-78] Reduction of nitrite occurs extensively in the stomach, with its very high concentration of protons from the gastric juice in combination with high levels of nitrite from the saliva.[9, 10, 79] Nitrite reacts with protons to form nitrous acid (pKa 3.4), which is rapidly dissociated to dinitrogen trioxide and water.[80] Dinitrogen trioxide then forms nitric oxide and nitrogen dioxide.[7] The chemical reaction is explained in Table 1. The reduction of nitrite to NO in the presence of protons is greatly enhanced by compounds such as vitamin C and polyphenols.[64, 66] In the presence of vitamin C (ascorbic acid), nitrous acid is reduced to NO without yielding nitrogen dioxide as an end product. Most vegetables, in addition to being rich in nitrate, also contain large amounts of vitamin C and polyphenols.[80, 81] The gastric mucosa also actively secretes vitamin C.[82] This taken together ensures a very efficient reduction of nitrite to NO in the stomach after ingestion of vegetables.[81]

Bacterial Nitrate reductase

$NO_3^- + 2e^- + 2\,H^+ \rightarrow NO_2^- + H_2O$

Nitrate — Nitrite

Reduction of Nitrite to NO under acidic conditions

$NO_2^- + H^+ \rightarrow HNO_2$ (Nitrite — Nitrous acid)

$2\,HNO_2 \rightarrow N_2O_3 + H_2O$ (Dinitrogen trioxide)

$N_2O_3 \rightarrow NO_2 + NO$

Dinitrogen trioxide — Nitrogen dioxide — Nitric Oxide

Nitrite reduction to NO in the presence of protons and Vitamin C (Ascorbic Acid)

$NO_2^- + H^+ \rightarrow HNO_2$

Nitrite — Nitrous acid

$2\,HNO_2 + \text{Ascorbic Acid} \rightarrow 2\,NO + \text{dehydro Ascorbic Acid} + H_2O$

Nitrous acid — Nitric Oxide

Table 1. Examples of some reactions of nitrate and nitrite in the human body

The Entero-Salivary circulation of Nitrate

Nitrate undergoes an entero-salivary circulation in humans:

1. Ingested nitrate is absorbed in the small intestine and mixed with endogenously produced nitrate in the blood.[65, 83] The endogenously produced nitrate in the body originates from NO and nitrite, which is oxidized to nitrate with the help of oxyhemoglobin in the blood, as described earlier.[59-61]
2. Most of the plasma nitrate is excreted through the kidneys,[64] but 25% is actively taken up and concentrated in the salivary glands.[13] This results in an up to twenty times higher concentration of nitrate in the saliva, compared to that in the plasma.
3. The nitrate-rich saliva is then secreted into the oral cavity, where nitrate-reducing bacteria reduce some of the nitrate to nitrite.[71] This reduction is dependent on the oral bacterial flora, since no mammalian enzymes seem to be involved. The nitrate-reducing oral bacteria are concentrated at the rear of the dorsal surface of the tongue both in humans and rats. [70, 73] In humans, these bacteria can be differentiated into strict anaeorobes, where *Veillonella atypical* and *Veillonella dispar* are dominant, and facultative anaerobes, where *Actinomyces dispar* and *Rothia mucilaginosa* are dominant.[70] In rats, one bacterial strain, *Staphylococcus scuri*, is clearly dominant.[73] Bacterial nitrate reductase reduces the nitrate to nitrite by using nitrate as an alternative electron acceptor to oxygen under hypoxic conditions, as mentioned earlier.[6]
4. After swallowing, the nitrite is rapidly reduced to NO and other nitrogen oxides via nitrous acid in the acidic environment in the stomach (Table 1).[80] This enterosalivary circulation of nitrate is summarized in Figure 4.

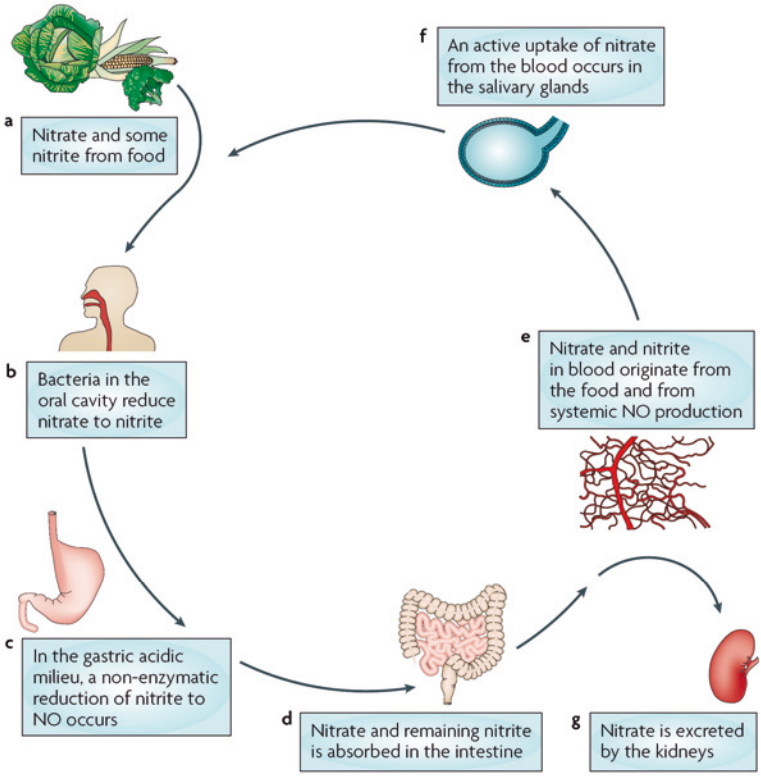


Figure 4. The enterosalivary circulation of nitrate in humans. The figure is adapted from reference 7.

The basal level of nitrate in the plasma in humans is 20-30 μM, but this can increase 20- to 40-fold after nitrate intake.[11, 69] Nitrate intake rapidly increases the nitrate concentration in saliva.[72, 84] The concentrations of nitrate in saliva and plasma remain high for several hours after nitrate ingestion, since the half-life of circulating nitrate is 5-6 hours.[63, 64]

Nitrate in the Diet

Eighty percent of nitrate intake in humans originates from fruits and vegetables.[85] The other 20% derives from drinking water, meat and meat products, and grain products.[86] During the evolution, vegetables and fruits have always been a natural source of food for humans, and the American Institute for Cancer Research now recommends a daily intake of 600 g non-starchy vegetables as a preventive measure against cancer.[87] If we follow this recommendation, the total amount of nitrate intake will increase and fruit and vegetables will account for way over 95% of all ingested nitrate.

Plants take up nitrate through their root system, and use nitrate for synthesis of amino acids and proteins.[86] Nitrate is present in most plants, since a nitrate reserve is necessary for continuous growth, irrespective of the nutrient supply in the soil. If the nitrate is not used immediately by the plant, it is stored in vacuoles.[88] The nitrate remains in the vacuoles if the plants are supplied with more nitrate than they can use in the photosynthesis. Nitrate will therefore be stored in vegetables with low photosynthesis. This phenomenon is typical in vegetables harvested in greenhouses, during periods of low light intensity, as in northern Europe during the winter period. Vegetables harvested in wintertime therefore have a higher nitrate content than those harvested in the summer.[86]

There is also a difference in the distribution of nitrate throughout the lettuce plant, with higher concentrations of nitrate in the central heart, a part of the plant without direct contact with sunlight, compared with the leaves surrounding the heart. Root crops such as beet and beetroot are specialized in nitrate storing and therefore contain large amounts of nitrate. Green leafy vegetables such as lettuce and spinach, as well as radish and cabbage, contain as much as 1-4 g nitrate per kg.[89]

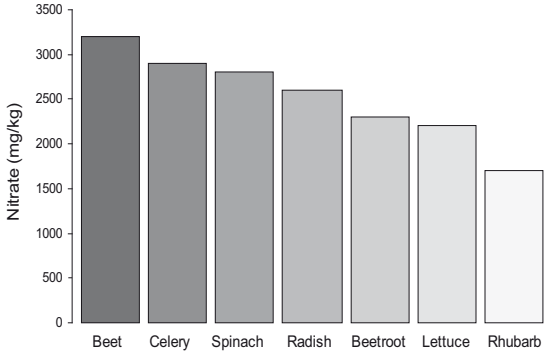


Figure 5. Average nitrate content in some nitrate-rich vegetables. Values are adapted from reference 89.

Nitrate occurs naturally in soil and is therefore also present in groundwater. Normally, the level of nitrate in drinking water is low, but in agricultural areas with great use of nitrogen fertilizers, the nitrate levels in the water are increased. Drinking water from private wells often has a higher nitrate content, since these wells are usually shallower and closer to the nitrate-rich soil, whereas public wells are deeper.[90]

Nitrite is used in small amounts for preserving meat products. A significant amount of research has been focused on the antibacterial effects of nitrite, as reviewed by Pierson and Smoot.[91] Nitrite has been shown to inhibit anaerobic bacteria, and is particularly effective against *Clostridium botulinum*, an anaerobic bacterium that produces a deadly neurotoxin. [91]

Nitrate and Cancer

In the late 1960s it was suggested that the nitrous acid and dinitrogen trioxide that is formed when nitrite reacts with protons may nitrosate secondary amines from the food and produce nitrosamines.[92] Some of these nitrosamines have been found to be carcinogenic in animal studies.[93] However, a direct carcinogenic effect of nitrosamines or nitrate intake has never been shown in humans.

Over the past 30 years, numerous research groups have debated on whether nitrite and nitrate are good or bad for human health. Attempts have been made in numerous epidemiological studies to establish a causal relationship between dietary nitrate and gastrointestinal cancer in humans. While some initial findings in the 1970s and early 1980s supported a positive correlation, numerous later studies have shown no such relation, as reviewed by McKnight in the late 1990s.[94]

It is a paradoxical fact that over 80% of our nitrate intake comes from vegetables, a food group that is associated with a lower incidence of cancer.[87] As mentioned earlier, vegetables contain large amounts of vitamin C. Vitamin C, or ascorbic acid, is known to decrease the formation of nitrosamines and instead favor the formation of NO, as described earlier.[80] Vitamin C is therefore an important antioxidant, which is believed to reduce cancer.[82] In a recently published case-control study it was found that nitrate intake was not associated with gastric cancer.[95] That study highlights the importance of combining nitrate intake with vitamin C. The key here may be the formation of NO instead of nitrosamines in the upper gastrointestinal tract. Indeed, in contrast to nitrous acid and dinitrogen trioxide, NO itself is not a nitrosating agent.

Aim

The overall aim of this research was to investigate how the large amount of non-enzymatic NO produced in the stomach influences the physiology of the gastric mucosa. More specifically, the following questions were addressed:

- How does intragastric NO influence the gastric mucosal blood flow?
- Does intragastric NO influence the mucus secretion in the stomach?
- Does a nitrate-rich diet increase the levels of NO produced in the gastric lumen?
- How does a nitrate-rich diet affect the gastric mucosa?
- Does the intraluminal nonenzymatic production of NO protect the mucosa against ulcerogenic substances?
- How important is the commensal oral flora for conversion of nitrate to nitrite?

Methods

This section describes the different methods used in the present investigation. For detailed information about the methods used in the different studies, see the individual papers.

Experimental Animals

The studies were performed on a total of 323 rats and 42 mice. All experiments were approved by the Uppsala University Ethical Committee for Animal Experiments or by the Ethical Committee for Animal Experiments at the Karolinska Institute.

Rats

Male Sprague-Dawley rats (B&K Universal, Stockholm, Sweden), weighing 165-320 g, were kept under standardized conditions of temperature (21-22°C) and illumination (12:12-h light/darkness) at the Animal Department at the Biomedical Center, Uppsala, Sweden or at the Animal Department at the Karolinska Institute, Stockholm, Sweden. They were allowed to adjust to this environment in cages with mesh bottoms with free access to tap water and pelleted food for at least 7 days before the experiment began. The rats were deprived of food for 18-20 h before anesthesia but had free access to water. They were anesthetized with 120 mg/kg body weight of 5-ethyl-5- (1-methylpropyl)-2-thiobutabarbital sodium (Inactin® Sigma-Aldrich, St. Louis, Missouri, USA) or 120 mg/kg body weight of sodium pentobarbital injected intraperitoneally. In most experiments the rats were tracheotomized with a short PE-200 cannula to facilitate spontaneous breathing. Body temperature was maintained at 37-38°C with a heating pad regulated by a rectal thermistor probe. A PE-50 cannula containing Heparin (Leo Pharma AB, Sweden, 12.5 IU ml^{-1}) dissolved in isotonic saline was placed in the right femoral artery to monitor blood pressure, and the left common carotid artery was cannulated for blood sampling in some animals. The right femoral vein was cannulated for continuous infusion of a Ringers solution (25 mM $NaHCO_3$, 120 mM NaCl, 2.5 mM KCl and 0.75 mM $CaCl_2$) at a rate of 1.0 ml h^{-1} and the left femoral vein was cannulated for drug infusions.

Mice

The following strains of mice were used in these studies:

- Male C57BL/6J mice (B&K Universal, Stockholm, Sweden)
- Male NMRI mice (B&K Universal, Stockholm, Sweden and germ-free animal facility, Karolinska Institute, Stockholm, Sweden)
- Male germ-free NMRI mice (germ-free animal facility, Karolinska Institute, Stockholm, Sweden)
- Male eNOS-deficient mice (The Jackson Laboratory, Maine). These mice (background C57/BL/6J-Nos3^{tm1Unc}) were generated by gene targeting in embryonic stem cells as previously described.[96]

The mice weighed 20-37g, and were kept under the same standard conditions as the rats, but they were not fasted before the experiments. The mice were anesthetized by inhalation of ~2.4% isoflurane (Forene®, Abbot Scandinavia AB, Kista, Sweden) via an anesthesia unit (Univentor 400 Anaesthesia unit, AgnTho's AB, Lidingö, Sweden), through a breathing mask. Body temperature was maintained at 37-38°C with a heating pad regulated by a rectal thermistor probe. In the blood flow experiments, a pulled PE-200 catheter containing a Ringer solution was placed in the left carotid artery to monitor blood pressure and to give a continuous infusion at a rate of 0.35 ml h^{-1}.

Animal Treatments

Nitrate Supplementation

In addition to the conventional chow, many of the animals in the studies were given nitrate in the drinking water (8-10 mM $NaNO_3$, Sigma-Aldrich, Steinheim, Germany) for 7 days before the experiment. Our rats consume ~60 mg water/g body weight daily,[97] and the daily intake of nitrate in the nitrate-supplemented rats is therefore about 0.8-1.0 mmol/kg body weight and day or 7.5- 9.5 mg/day.

Nitrite Supplementation

Some rats were given nitrite in the drinking water (1.0 mM $NaNO_2$, Sigma-Aldrich, Steinheim, Germany) for 7 days before the experiment.

Induction of Gastric Damage

In study III (paper III), the rats were deprived of food for 18-20 h before the ulcer was induced, but had free access to water. On the day of the ulcer induc-

tion, a single dose of 30 mg kg^{-1} diclofenac was given via gavage and the rats were left in their cages for 4 hours.

In study IV (paper IV), the rats were given food before the ulcer was induced and had free access to water. On the day of the ulcer induction, a single dose of 30 mg kg^{-1} diclofenac was given via gavage and the rats were left in their cages for 17-18 hours with no access to food but free access to water.

Chlorhexidine Mouth Spray

In study IV, we suppressed the oral flora in some animals. Rats were treated with 0.3 ml chlorhexidine mouth spray (2 mg/ml, Corsodyl®, GlaxoSmith-Kline, Brentford, England) twice daily for a period of 7 days with or without nitrate supplementation. Chlorhexidine was sprayed on the dorsal aspect of the tongue in the morning and evening. The last chlorhexidine mouth spray was given in the morning of the day of an experiment.

Animal Model

To be able to study the gastric mucosa *in vivo*, an animal model in which the mucosa is exposed was used. In the setup, the gastric mucosa is constantly covered with warm (37°C) solutions. This preparation enables the central and enteric nervous regulation of the stomach to remain intact and allows a stable blood flow in the mucosa. This model is therefore very suitable for *in vivo* studies of the gastric mucosa, both in rats and in mice. This unique animal model was used in the blood flow experiments, the mucus measurement experiments, and the mucosal permeability studies.

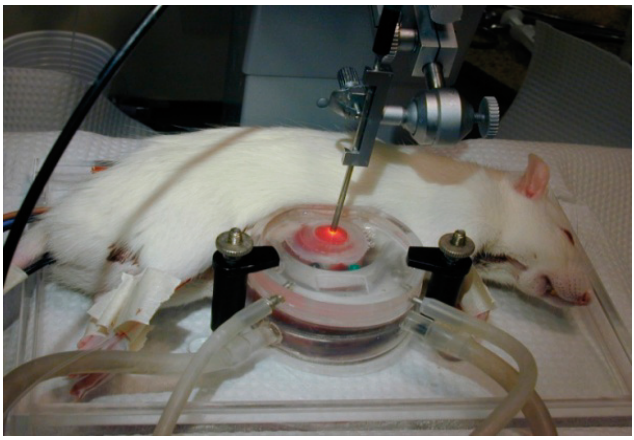


Figure 6. The animal model for *in vivo* studies of the gastric mucosa. The mucosal chamber covers the mucosa with warm saline. A laser Doppler probe is fixed over the mucosa for measurements of mucosal blood flow.

Surgical Protocol

The abdomen is opened through a midline incision. The gastro-hepatic ligaments are cut and the stomach is gently exteriorized and kept moistened and warm with 37°C saline during the preparatory procedure. The short gastric artery and vein are ligated and cut. An incision is made along the greater curvature in the forestomach. The animal is placed on a heating pad on a Lucite microscope stage and the stomach is everted through the incision and loosely draped over a truncated cone with the luminal side up. A double-bottom mucosal chamber with a hole in the bottom is fitted over the mucosa, exposing the mucosa through the hole (exposed area: rats 0.8 cm^2; mice 0.2 cm^2). The junction was sealed with silicon grease (Dow Corning high vacuum grease, Dow Corning GmbH, Weisbaden, Germany). The chamber is filled with warm (37°C) unbuffered 0.9% saline solution to keep the tissue moist and prevent the mucus gel from dehydration. The technique has been described in detail previously.[20, 38]

Mucus Thickness Measurements

Since the gastric mucus is highly hydrated, the use of conventional in vitro and histological methods is limited when it comes to measuring the thickness of the mucus layer. In vitro studies of the thickness result in a very thin or discontinuous mucus layer, as the mucus layer becomes dehydrated and eroded.[98] The only accurate way to study the gastrointestinal mucus thickness is therefore by *in vivo* methods. Our laboratory has long experience in measuring mucus thickness in vivo in the gastrointestinal tract in both rats and mice.[19, 20, 24]

Briefly, the method is as follows: Mucus thickness is measured with micropipettes connected to a micromanipulator (Leitz, Wetzlar, Germany). The micropipettes are pulled to a tip diameter of 1-3 µm with a pipette puller (pp-83; Narishige Scientific Instrument Laboratories, Tokyo, Japan). To prevent mucus from adhering to the glass, the tip of the micropipette is siliconized. The tip is dipped into a silicon solution (MS1107, 25% acetone) and dried at 100ºC for 30 minutes.

To visualize the luminal surface of the mucus gel, a suspension of carbon particles (activated charcoal, extra pure, Merck, Germany) is applied on the mucosa. The epithelial cell surface is visible through the microscope.

The method of mucus measurements is illustrated in Figure 7. The micropipette is pushed into the mucus gel at an angle (a) of 25-35º to the epithelial cell surface and the distance traveled by the micropipette from the luminal surface of the mucus gel to the epithelial cell surface (l) is measured with a digimatic indicator (IDC Series 543, Mitutoyo Corp, Tokyo, Japan) connected to the micromanipulator. The mucus gel thickness (T) is then calculated.

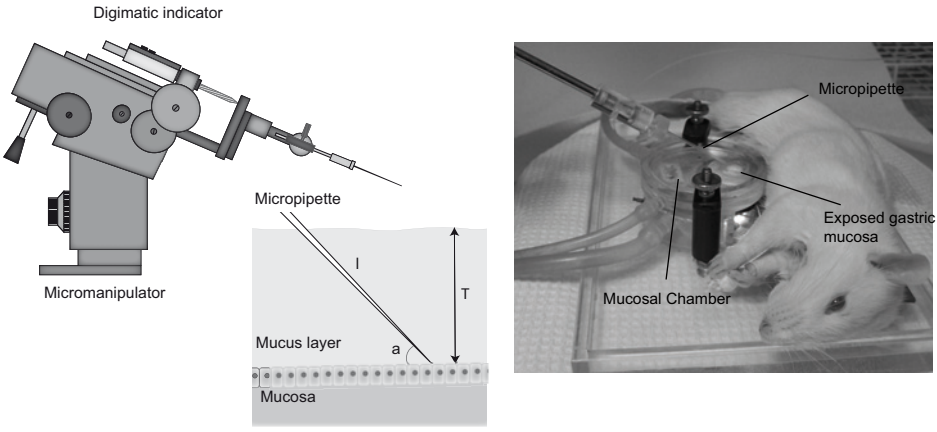


Figure 7. Left: A schematic drawing describing the mucus thickness measurements. The mean value from four to five measurements at different locations was used as one observation. Right: A rat prepared for mucus measurements.

Mucosal Permeability Measurements

One good way of measuring the permeability of the gastric mucosa is to measure the blood to lumen clearance of the tracer molecule ^{51}Cr-EDTA.[99] ^{51}Cr-EDTA is a chemically stable, non-toxic molecule, with a small radius of 7 Å.[100, 101] The cellular uptake of ^{51}Cr-EDTA is minimal and the molecule is not metabolized in the tissue.[102, 103] The transport from blood to lumen is dependent on the properties of the mucosa, and a change in mucosal blood flow does not affect this transport.[104, 105]

The permeability studies were performed in rats. After completion of surgery and 60 minutes before the start of the experiment, 50-75 μCi of ^{51}chromium-labeled EDTA (^{51}Cr-EDTA, NEN chemicals, Du Pont, Mass) was injected as a bolus dose intravenously. The bolus dose was followed by a continuous infusion of ^{51}Cr-EDTA (in the Ringer solution) at a rate of 1.0 ml h^{-1} (10-30 μCi h^{-1}) giver throughout the experiment. Three 0.2 ml blood samples were drawn during the experiment. The first was taken 60 min after the bolus injection of ^{51}Cr-EDTA. After each blood sample had been drawn the volume loss was compensated for with an injection of an equal volume of 7 % bovine serum albumin solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The blood sample was centrifuged, and 50 μl of the plasma was removed for measurements of radioactivity in counts per minute (cpm). The gastric mucosa was covered with 5 ml isotonic saline or 5 ml 10 mM HCl during the experiments. The 10 mM HCl solution is made from a stock solution of 1 M HCl (Titrisol® Merck KGaA, Darmstadt, Germany) and adjusted to isotonicity by the addition of sodium chloride. The luminal solution and the blood plasma were analyzed for activity in a gamma counter (1282 Compugamma Cs; Pharmacia, Uppsala, Sweden). Each clearance value was calculated by dividing each individual cpm value by a corresponding plasma cpm value (Table 2). If there was a deviation of <10% between the different blood plasma counts, a mean plasma cpm per milliliter value was calculated and used for all clearance samples, and if there was >10% deviation between the different blood plasma counts, the activity was plotted against time, and a straight line was drawn between the two nearest values. The part of the stomach that had been exposed in the chamber was cut out and weighed after the experiment. Clearance is expressed as milliliters per minute per 100 g wet tissue weight.

$$\text{Clearance} = \frac{\text{Lumen sample (cpm ml}^{-1}) \times \text{sample volume (ml)} \times 100}{\text{Plasma (cpm ml}^{-1}) \times \text{tissue weight (g)} \times \text{time (min)}}$$

Table 2. Calculation of blood to lumen clearance.

Mucosal Blood Flow Measurements

Laser-Doppler flowmetry (PeriFlux 4001 Master and PeriFlux Pf 3; Perimed, Stockholm, Sweden) was used for blood flow measurements. The nature of the Doppler shift from an illuminated tissue depends on the velocity and number of moving red blood cells.[106] The laser light (wavelength 635 nm, helium neon laser) is guided to the tissue by an optical fiber. The backscattered light is picked up by a pair of fibers with a fiber separation of 0.25-0.5 mm, and blood flow is recorded as the changes in frequency, the Doppler shift. With this technique, blood flow is determined as a voltage output and expressed as perfusion units (PFU). The blood flow is recorded continuously from the mucosal side of the gastric mucosa, with a probe fixed to a micromanipulator and kept at a distance of 0.5-1 mm above the surface of the mucosa, in the solution.

Owing to the thinness of the gastric wall, we probably measure blood flow through the entire gastric wall. Since 70% of the total blood flow in the gastric wall is mucosal,[39] and the probe records blood flow exponentially to the distance, the recorded blood flow is mainly mucosal. This technique for measuring the gastric mucosal blood flow by laser Doppler flowmetry has been well evaluated previously.[107, 108]

Figure 8 summarizes the method of blood flow measurement with the laser-Doppler technique.

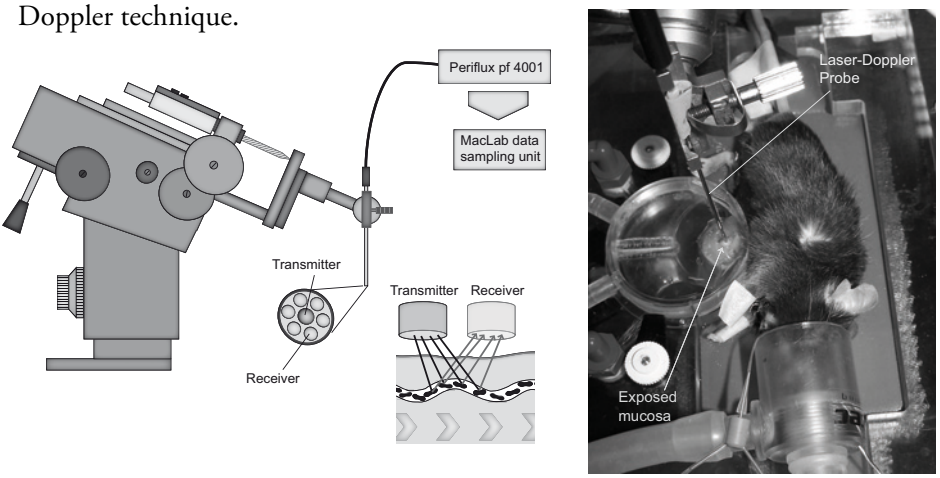


Figure 8. Left: A schematic drawing of a micromanipulator holding the laser-Doppler probe and an illustration explaining the laser Doppler technique. Right: A mouse in the experimental setup for measuring mucosal blood flow.

P-selectin Antibody Experiments

P-selectin is one of many cell adhesion molecules on the endothelium involved in the recruitment of leukocytes, and upregulation of P-selectin is an early event in the inflammatory cascade.[109] Thus, measurements of P-selectin expression in

the vasculature are a good way to determine the presence of inflammation. We used the dual-labeled monoclonal antibody technique for this purpose.

The rats were anesthetized with Inactin® as described, and the left jugular vein and right carotid artery were cannulated for injection of antibodies and for blood sampling, respectively. To measure P-selectin expression, a mixture of 10 μg of ^{125}I -labeled P-selectin MAb (RMP-1) and 5-10 μg of ^{131}I-labeled non-binding MAb (P-23) was injected. After the injection, the animals were heparinized (3,000 IU/kg), and blood samples were taken via the carotid artery catheter after 2.5 and 5.0 min. At 5.0 min, the animal was exsanguinated via the carotid artery with an infusion of PBS via the jugular vein. The vena cava was then severed, and the circulation was flushed via the carotid artery with ≈60 ml of buffer. The organs for study were taken out, washed, dried, weighed, put into test tubes, and counted for ^{125}I and ^{131}I activity; see below. The selected organs were: Left and right kidneys, stomach (divided into superficial mucosa (scraped off with a scalpel), deeper part of the mucosa, submucosa, and muscular layers) heart, aorta, and vena cava.

Labeling of Antibodies with ^{125}I and ^{131}I

Monoclonal antibodies directed against P-selectin [RMP-1[110]] were labeled with ^{125}I (DuPont NEN; Boston,MA), whereas isotype-matched nonbinding antibodies [P23 [111]] were labeled with ^{131}I. (Antibodies were kindly supplied by Dr. D.Neil Granger, Louisiana State University, Shreveport, LA.) Radioiodination was performed using the iodogen method, as previously described,[112] The labeled antibodies were stored in 0.5-ml aliquots at 4°C for a maximum of 3 weeks.

Expression of P-selectin Activity

The activity of ^{125}I and ^{131}I was determined using an LKB 1282 Compugamma (Wallac Oy, Turku, Finland). Samples were counted for 10 minutes. The total activity injected (and the total nanograms of antibody injected) in each experiment was calculated by counting a 5-μl sample of the injectate. The activity remaining in the injection syringe was subtracted from the total injected counts. The accumulated activity in the tissue was expressed as percent antibody bound per gram tissue and was calculated as follows:

$$\%\ \text{antibody bound g}^{-1} = (\%\text{ID} \times \text{g}^{-1}\ \text{for}\ ^{125}\text{I}) - [(\%\text{ID} \times \text{g}^{-1}\ \text{for}\ ^{131}\text{I}) \times (\%\text{ID}\ ^{125}\text{I in plasma})/(\%\text{ID}\ ^{131}\text{I in plasma})$$

where %ID is percentage of injected dose.
Nanograms of antibody bound per gram were calculated as follows:

$$\text{ng antibody bound g}^{-1} = (\text{corrected \% injected}\ ^{125}\text{I bound g}^{-1}) \times (\text{total ng}\ ^{125}\text{I antibody injected}) \times (100)^{-1}$$

Measuring Nitric Oxide

For measurements of nitric oxide, a chemiluminescence NO analyzer (Aerocrine AB, Stockholm, Sweden.) was used. Calibration with cylinder gas (NO 10 ppm, AGA AB, Lidingö, Sweden) was performed continuously.

Headspace Nitric Oxide

To determine the formation of NO derived from saliva and sodium nitrite in the mucosal chamber, we used an *in vitro* model in study I (paper I). A plastic cup (100 ml) was placed over the mucosal chamber to collect headspace NO. The same concentrations and pH as in the *in vivo* experiments were used for sodium nitrite and saliva. For the sodium nitrite experiments, the chamber was filled with 5 ml isotonic HCl, pH 2, and nitrite (100 mM), added through a hole in the base of the cup, to reach final concentrations of 0.1, 0.5, 1 and 5 mM. For the saliva experiments, the chamber was filled with 2.5 ml saliva, whereafter 2.5 ml of 32 mM HCl was added to reach pH 2. In addition, we studied NO formation from saliva at pH 5.5. Headspace NO was measured through a sample tube connected to a chemiluminescence analyzing system. The sample flow rate was 100 ml/min, and the peak NO concentration was measured after adding the compounds. The 8 mm Ø hole in the base of the cup secured circulation of air during measurements.

Intragastric Nitric Oxide Generation

Intragastric NO levels after pretreatment with sodium nitrate were measured in rats. The rats were anesthetized (Inactin or sodium pentobarbital injected intraperitoneally) and a laparotomy was performed. In studies II, III, and IV (papers II, III, and IV), fasting rats were given sodium nitrate in the drinking water for 7 days before the experiment. Control animals were given regular nitrate-free drinking water. The rats were anesthetized and a laparotomy was performed. A fine needle was inserted intragastrically via the stomach wall and the stomach was inflated with 4 ml of NO-free air. Passage of air into the esophagus or duodenum was prevented by external clamps. After 15 s the air was aspirated and immediately injected into a chemiluminescence NO analyzer. This method was first described by Sobko and colleagues.[113]

Measuring Nitrate, Nitrite and S-nitrosothiols

For measurements of nitrite, nitrate, and S-nitrosothiols we used a chemiluminescence method. This method was first described independently by Feelisch et al.[114] and Gladwin et al.[115] The ozone-based chemiluminescence assay is of

general utility for quantifying physiological levels of NO and NO-derived metabolites in biological materials, as extensively reviewed by Bryan and Grisham.[116]

Nitrate, Nitrite, and S-nitrosothiols in Saliva

In study I, nitrite, nitrate, and S-nitrosothiols were measured in human saliva. Human saliva was collected before and after ingestion of sodium nitrate as described below. After centrifugation at 2,500 rpm for 10 min, saliva was mixed (1/1) with isotonic hydrochloric acid to reach pH 2 or pH 5.5. The solutions were kept for 15 min at room temperature before analysis. The nitrite and nitrate contents in the saliva were measured on the day of the experiment with the chemiluminescence method.

Nitrate and Nitrite in Gastric Mucus

In study II, we measured the amount of nitrite and nitrate in loosely and firmly adherent gastric mucus. The loosely adherent mucus layer was removed under a microscope by suction with a catheter connected to a syringe, and saved for analyses. The remaining, firmly adherent mucus layer was scraped off the mucosa with a scalpel. The total volume of the different mucus layers was estimated by mucus measurements and the samples were stored at -70°C.

Nitrate and Nitrite in Plasma and Gastric Tissue

In study III we measured the concentration of nitrate and nitrite in plasma and gastric tissue and in study IV we measured nitrite in plasma. Blood samples were transferred to tubes containing 250 mM EDTA (study III). In study IV, plasma was collected without addition of EDTA. Plasma was then collected following centrifugation at 3,000 rpm for 5 min. A tissue sample from the stomach was snap-frozen and stored at -70°C. A 200 mg sample of frozen tissue was then homogenized, using a polytron, in ice cold EDTA in PBS 2:1 v/w and centrifuged at 18,000 rpm for 20 min at 4°C. The concentrations of nitrite and nitrate were then measured in the supernatant.

Nitrate in Chow

In study IV we measured nitrate levels in chow. All animals were given conventional chow (Ewos; Södertälje, Sweden). The amounts of nitrate in the chow were quantified with chemiluminescence. The chow was homogenized, using a polytron (Kinematica, Switzerland), in distilled nitrate-free water (1g/ml). Samples were then centrifuged at 2,000 rpm for 10 min in order to remove any

residual grains of chow. The contents of nitrite and nitrate in the resulting supernatant were measured.

Quantification of Bacteria on the Tongue

The amounts of bacteria present on the dorsal part of the tongue were quantified in study IV. The tongue was removed and frozen in liquid nitrogen and was kept at -70°C until analyzed. The dorsal part of the tongue was disaggregated in 1 ml sterile PBS containing 0.05% Tween 20, using a mortar. Serial dilutions were then made in the same buffer. A medium described by Doel et al.[70] (tryptone 2% w/v, horse serum 2% v/v, agar 1.2% w/v, hemin (5 μg ml^{-1}), sodium chloride 0.5% w/v, yeast extract 0.5% w/v, glucose 0.5% w/v, cysteine hydrochloride 0.05% w/v) was used and the plates were incubated either aerobically or anaerobically (BD GasPak EZ Anaerobe Container System) for 48 h, whereafter the colonies were counted. The detection limit of the determination was 10^2 cfu per tongue.

Collection of Human Saliva

To obtain saliva with different nitrite contents, saliva was collected from overnight fasting volunteers before and one hour after ingestion of sodium nitrate (0.1 mmol/kg). Previous studies have shown that salivary nitrite/nitrate is greatly increased one hour after a nitrate load.[117] The saliva was centrifuged (2,500 rpm, 10 min) and stored at –20° C until immediately before use. All experiments were approved by the Local Ethics Committee for Human Research at the Karolinska Institute, Stockholm, Sweden.

Real-time PCR for studies of Mucin Gene Expression

Stomach tissue was obtained from rats pretreated with nitrate. Snap-frozen stomach tissue was homogenized in Trizol® solution and total RNA was extracted according to instructions from the manufacturer (Invitrogen, Scotland,UK). Real-time polymerase chain reaction (PCR) was carried out in a 25-μl amplification mixture containing 0.5 μl of template cDNA, 10 μl of 2x SYBR Green I Master Mix, and 40 pmol sense and antisense primers. The PCR conditions included a polymerase activation step at 95°C for 15 min followed by 45 cycles at 95°C for 10 s, 50°C for 15 s and 72°C for 1 s and run on an Opticon (Bio-Rad Laboratories). The gene expression level from the untreated control was set as 1 to compare the relative gene expression levels in the experimental groups. All samples were run in triplicate. The forward primer used to quantify the mucin-6 gene was 5′- ACTACTGCAACCCCCATCAG-3′, and the re-

verse primer was 5′- TGTGGGTGTTGACTTCGGTA-3′.The forward primer used to quantify the housekeeping gene RPLPO was 5′-TGTTGAACATCTCCCCCTTC-3′ and the reverse primer was 5′-TGATGGAGTGAGGCACTGAG-3′.

Scoring Gastric Lesions

Sodium pentobarbital anesthetic, 120 mg kg^{-1}, was administered intraperitoneally. The abdomen was opened through a midline incision and the stomach was removed and opened along the greater curvature. After a gentle wash in PBS the stomach was spread for subsequent photography. The ulcer index was expressed as mm and reflected the total length of gastric lesions per stomach as judged by two independent researchers blinded to the protocol. A sample of the stomach was excised and snap-frozen for subsequent analysis.

Myeloperoxidase assay

In study III, the amount of myeloperoxidase (MPO) present in the gastric tissue was measured. This was done as described in detail previously.[118] A 50-100 mg specimen of gastric tissue from the central part of the gastric antrum was excised. The specimen was placed in 50 mmol/l Tris HCl (pH 8.4) buffer and then vortexed and centrifuged at 10,000 *g* for 15 s. After removal of supernatants the remaining tissue was homogenized with a Polytron in 1.0 ml of 50 mmol/l phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, Sweden), and then subjected to three sessions of freezing-thawing. Subsequently, the homogenates were centrifuged at 1600 *g* for 10 min at 4 °C. 5 ml aliquot of each supernatant was mixed with 145 ml of phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma-Aldrich, Sweden) and 0.0005% $H2O2$, whereafter the change in the rate of absorbance at 450 nm was measured with a microplate reader (Spectra max Plus384, Molecular Devices). The MPO activity was expressed as Units of MPO as defined by Bradley et al.[119]

Immunohistochemistry for iNOS expression

After the excision of the stomach, a specimen from the central part of the gastric antrum was placed in TissueTec (Sakura Finetek Europe B.V, The Netherlands) and snap-frozen for subsequent immunohistochemistry. Sections 4-5 µm thick were then pretreated by deparaffination and blocking of endogenous peroxidase with H_2O_2 (Merck, Darmstadt, Germany) for 15 min. The sections were incubated with primary antibody (polyclonal iNOS, Santa Cruz Biotech-

nology, sc-651) overnight at +4 ºC, and subsequently with 2 % normal goat serum (Jackson Immuno Research, Baltimore, USA), and were further incubated with the secondary biotin-conjugated antibody in the presence of 2 % goat serum. The secondary antibody used was biotinylated anti-rabbit IgG (Jackson Immuno Research, Baltimore, USA). Thereafter, avidin biotin enzyme reagent was applied, followed by DAB (Vector Laboratories Inc., Burlingame, CA) and the sections were subsequently counterstained with hematoxylin. No immunostaining was observed when the secondary antibody was replaced by preimmune serum. Expression of iNOS was scored with use of a scale graded from 0-2, reflecting the intensity of staining, as judged by a blinded researcher.

Statistical Analysis

Differences between groups of animals were evaluated by analysis of variance (one-way ANOVA, followed by the Fisher protected least significant difference test) or by the two-tailed Mann-Whitney test. For comparison within groups, we used ANOVA for repeated measures followed by the Fisher protected least significant difference test. For statistical calculations different software systems were used (STATISTICA, version 6, StatSoft, Tulsa, OK; Statview II SE Graphics software, Abacus Concepts Inc., Berkeley, CA; Minitab Inc. State College, PA, USA; GraphPad Prism 4.0 Software, San Diego, CA, USA). All data are presented as means ± SEM or as median (range). A p value < 0.05 was considered significant.

Experimental Protocols

Study I

In this study we investigated the effects of acute luminal application of nitrite or nitrite rich saliva on the gastric mucosa.

- Nitrite in different concentrations (0.1, 0.5, 1.5 and 5.0 mM) or saliva (nitrite-rich saliva and fasting saliva) was applied to the gastric mucosa together with HCl, pH 2, in a 10 min period and the mucosal blood flow was continuously measured.
- Some animals were pretreated with an NOS inhibitor or a COX inhibitor intravenously, with continuous measurement of the effect of saliva and nitrite on the blood flow.
- Animals were also pretreated luminally with the guanylyl cyclase inhibitor ODQ (1.0 mM) for 10 min before addition of nitrite (1.0 mM) and the NO donor SNAP (0.3 mM), and the mucosal blood flow was measured.
- Gastric mucus thickness was measured after 60 min of luminal exposure to nitrite (1.0 mM) or nitrite-rich saliva.
- In addition, we measured the amount of NO formed when nitrite-rich saliva or nitrite (1.0 mM) was added together with HCl, pH 2, to the mucosa.

Study II

Gastric permeability and mucosal blood flow were studied before, during, and after a mucosal provocation. The experiments lasted for 170 min and the solutions to which the gastric mucosa was exposed were changed every 10 min. The mucosa was exposed to saline for the first 20 min, then to HCl for 130 min, and finally to 20 min of saline. Forty minutes after the start the COX inhibitor diclofenac was given intravenously, and 30 min later 20 mM taurocholate was added to the acidic luminal solution for 40 min (Fig. 9).

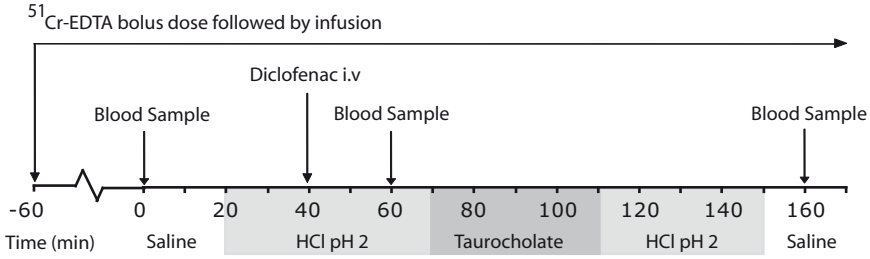


Figure 9. The experimental protocol in the permeability measurements in study II.

To investigate whether a high nitrate diet had gastro-protective effects, one group of rats was pretreated with sodium nitrate daily in the drinking water for 7 days before the experiments. The experimental protocol described above was then followed.

To mimic the effect of swallowing nitrite-rich saliva, another group of rats was submitted to continuous luminal administration of sodium nitrite to the mucosa 30 min after the start of the experiment.

We also measured the mucosal blood flow continuously and removed the loosely adherent mucus layer in control and nitrate-pretreated animals and measured the levels of nitrite in the different mucus layers.

Study III

1. In a first set of experiments (*part one*) the effects of dietary nitrate supplementation on the expression of mucin genes in the gastric mucosa, the thickness of the mucus layer, the levels of NO in the gastric lumen, and the nitrate/nitrite levels in gastric tissue and plasma were studied. The experimental protocol is described in Figure 10.

2. In a second set of experiments (*part two*) the effects of nitrate pretreatment on diclofenac-induced gastric injury were studied by measuring the damaged area (ulcer index) and the inflammatory reaction caused by diclofenac administration (iNOS expression and MPO) (Fig. 10). Diclofenac (30 mg/kg) was given to fasted animals 4 hours prior to the experiments.

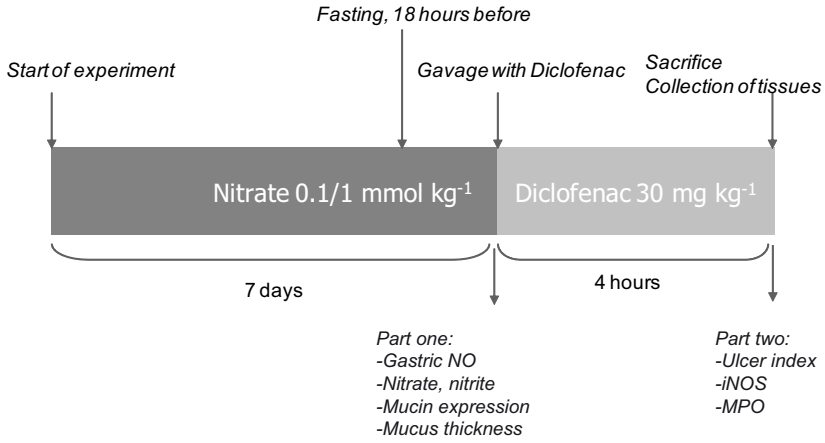


Figure 10. Protocol used for studying the effects of dietary nitrate on physiological and pathological processes in the rat stomach

Study IV

1. In a first set of experiments the effect of chlorhexidine mouth spray (2 mg/ml) on the levels of nitrate-reducing bacteria in the oral cavity was evaluated. This was done by measuring the number of colony-forming units present on the dorsal rat tongue in control rats and rats treated with chlorhexidine mouth spray twice daily for one week. We also measured the intragastric NO formation and plasma nitrite in rats treated with nitrate and mouth spray for one week.

2. In a second set of experiments the effect of nitrate supplementation on the gastric mucus thickness was investigated. The gastric mucus thickness was measured *in vivo* in control and in chlorhexidine mouth-sprayed rats with or without nitrate and nitrite supplementation. We also performed mucus measurements in conventional and germ-free mice.

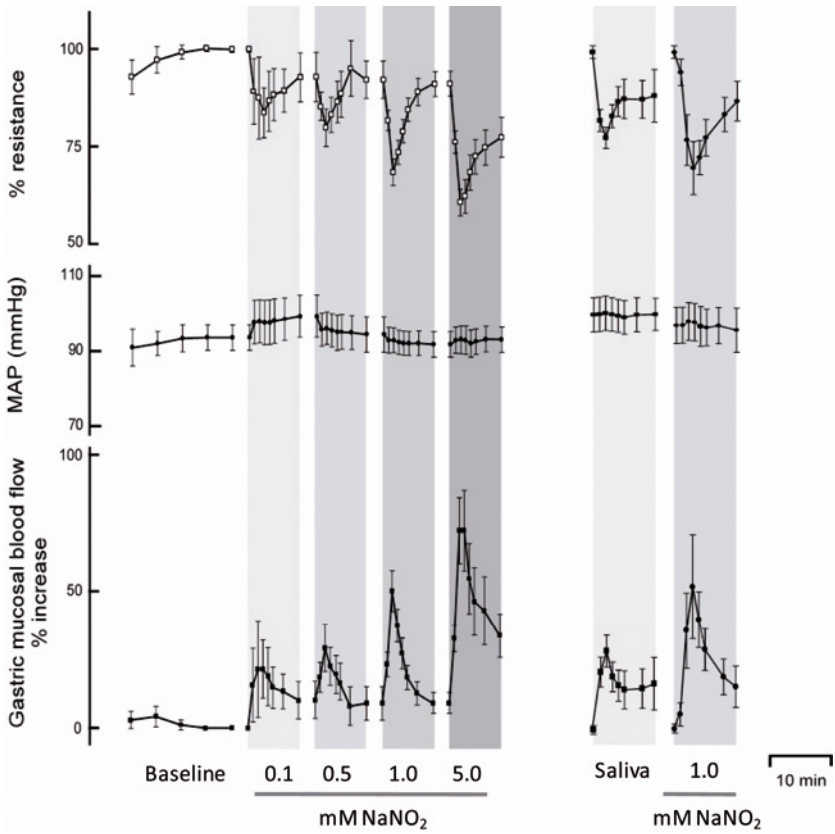
3. In a third set of experiments gastric damage was induced with diclofenac, and the levels of the adhesion molecule P-selectin in the gastric vasculature were measured as a marker of inflammation. This was performed in control and chlorhexidine mouth-sprayed rats with or without nitrate supplementation. Diclofenac (30 mg/kg) was given 18 hours prior to the experiments.

Results

Study I

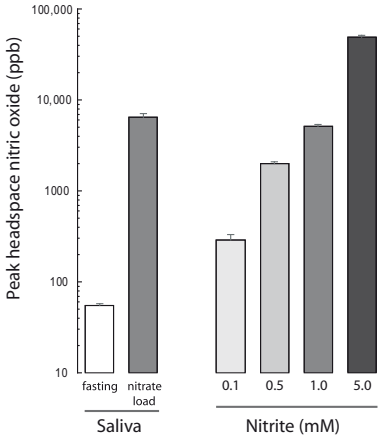
Luminal acidified nitrite or nitrite-rich saliva increased gastric mucosal blood flow

Luminally applied nitrite in HCl, pH 2, dose-dependently increased the gastric mucosal blood flow in rats without changing the mean arterial blood pressure (MAP). Saliva collected from healthy volunteers after ingestion of 0.1 mmol/kg nitrate also increased the mucosal blood flow when applied luminally in HCl, pH 2, (Fig. 11). This increase in blood flow was not altered by pretreatment with either the COX inhibitor indomethacin or the unselective NOS inhibitor L-NNA.



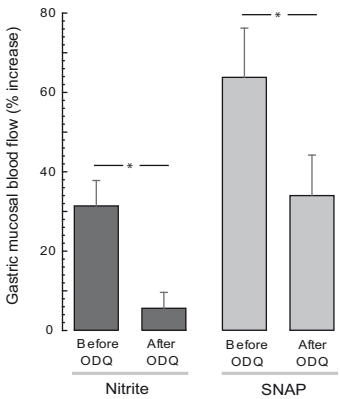
Acidified nitrite and nitrite-rich saliva increased NO formation

The increase in mucosal blood flow that occurred when nitrite was applied luminally was well correlated to the NO generation over the mucosal chamber (Fig. 12, right). Fasting saliva, low in nitrite, did not change the blood flow, while the non-thiol-containing NO donor DETA/NO also increased the mucosal blood flow (31 ± 8 %).



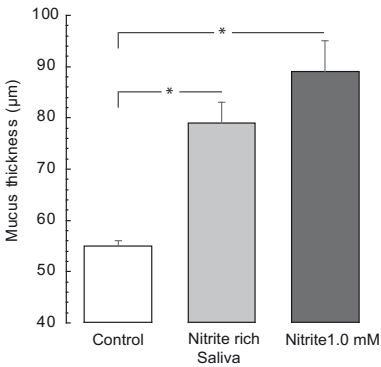
The blood flow increase was NO dependent

Luminal pretreatment with the guanylyl cyclase inhibitor ODQ (1 mM) reduced the effect of acidified nitrite (1 mM, pH 2) and the NO donor SNAP (0.3 mM) on the mucosal blood flow (Fig. 13, right).



Luminal acidified nitrite or nitrite-rich saliva increased thickness of firmly adherent mucus layer

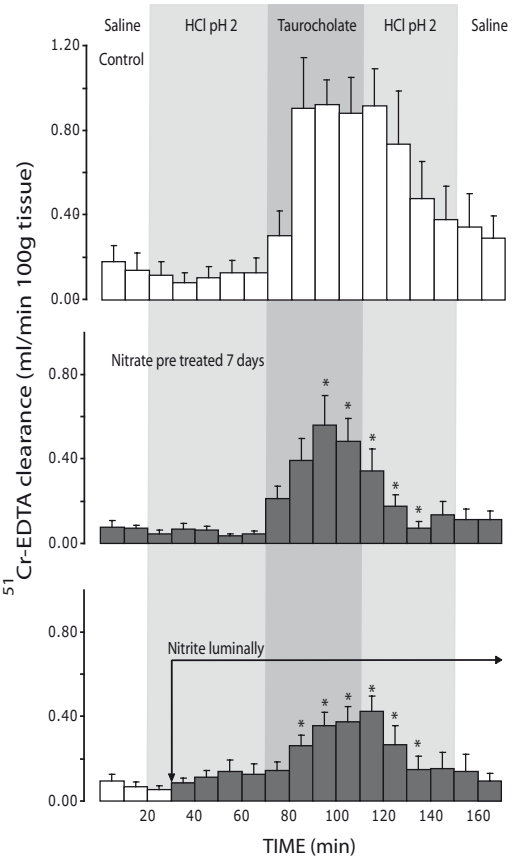
We also found that luminal application of 1 mM sodium nitrite or nitrite-rich saliva for 60 min increased the thickness of the firmly adherent mucus layer in the rat stomach (Fig. 14, right).



Study II

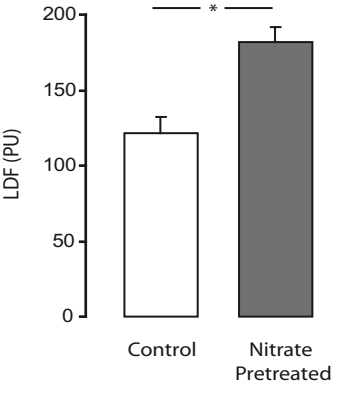
Dietary nitrate and nitrite luminally - effects of diclofenac and taurocholate on mucosal permeability

Mucosal permeability, expressed as blood to lumen clearance of ^{51}Cr-EDTA, increased during diclofenac and taurocholate provocation in all three groups. The increase was significantly greater in the control animals, however, than in animals treated with nitrate or nitrite (Fig. 15, right) Nitrite was applied continuously to the lumen to mimic the effect of swallowing nitrite-rich saliva and this resulted in an increased blood flow, which increased even further after application of taurocholate. The blood flow also increased in the control animals when taurocholate was applied luminally.



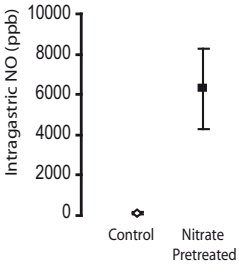
Dietary nitrate increased gastric mucosal blood flow

The nitrate-pretreated animals had a higher blood flow than the controls from the start of the experiment (Fig. 16, right) and this decreased slowly during the experiment and was not affected by taurocholate. MAP did not differ between the groups and diclofenac resulted in a significant reduction of MAP in all groups.



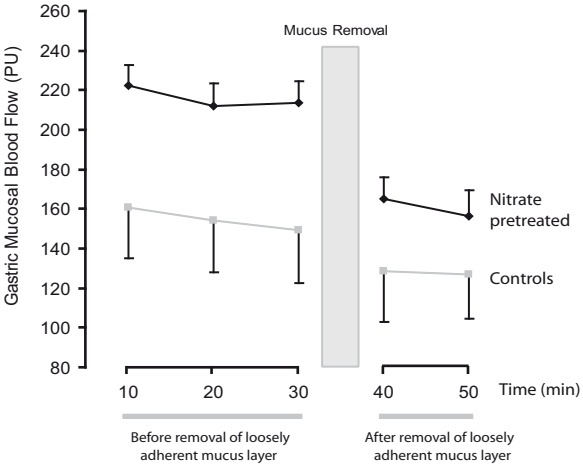
Dietary nitrate increased intragastric levels of NO gas

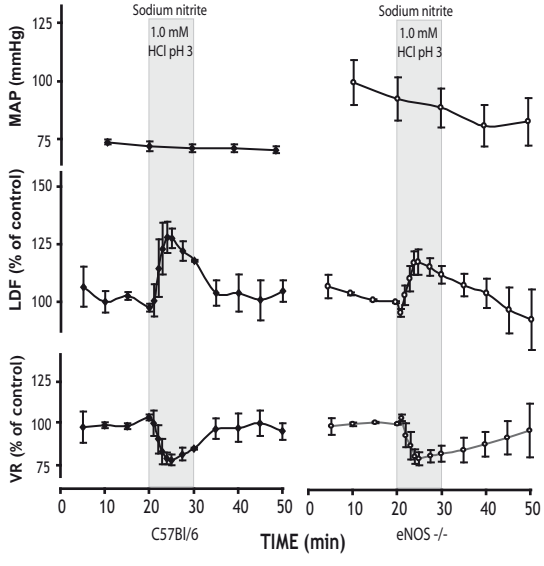
Pretreatment with nitrate in the drinking water resulted in high intragastric levels of NO (Fig. 17, right).



Dietary nitrate increased the nitrite content in loosely adherent gastric mucus

Pretreatment with nitrate resulted in an increased concentration of nitrite in the loosely adherent mucus gel layer (8.0 ± 2.0 µM), compared to that in control animals (1.4 ± 0.6 µM). When the loosely adherent mucus layer was removed from the nitrate-pretreated animals, the mucosal blood flow decreased significantly, in contrast to that in the controls (Fig. 18, right).





Luminal acidified nitrite increased gastric mucosal blood flow in eNOS-deficient mice

In addition to the findings in the rat experiments, we also showed that acidified nitrite (1.0 mM in HCl, pH 3) increased gastric mucosal blood flow both in C57Bl/6 mice and eNOS-/- mice (Fig. 19, Left).

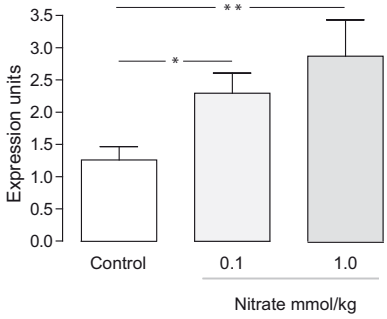
Study III

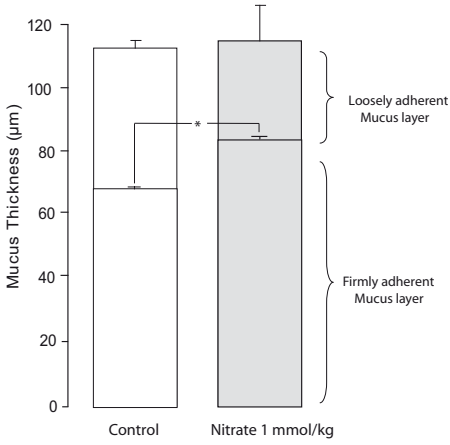
Dietary nitrate increased levels of nitrate and nitrite in plasma and the gastric tissue

Nitrate-pretreated animals had elevated levels of nitrate and nitrite both in plasma and in homogenized stomach tissue. The nitrate content in the plasma increased from 52 (20-57) µM in control animals to 783 (409-1010) µM in the group receiving 1 mmol kg^{-1} dietary nitrate, and the nitrite level increased from 0.4 (0.2-0.5) µM to 2.6 (1.1-3.1) µM. In the homogenized stomach tissue similar changes were seen, the nitrate content increasing from 43 (24-52) µM in control animals to 463 (292-1029) µM following the dietary supplementation of nitrate, and the nitrite level correspondingly increasing from 0.6 (0.4-1.1) µM to 1.5 (1.2-2.7) µM.

Dietary nitrate resulted in increased MUC6 expression

The expression of the MUC6 gene in the gastric mucosa increased dose-dependently in animals receiving nitrate as compared to controls (Fig. 20, right). However, the level of expression of the MUC5AC gene was not affected (data not shown).



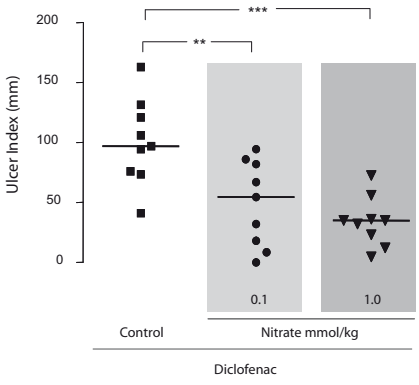


Dietary Nitrate increased firmly adherent gastric mucus thickness

The total mucus thickness did not differ between the controls and the nitrate-treated animals, but the inner, firmly adherent mucus layer was significantly thicker in the nitrate-treated group compared to than in the control group (Fig. 21, Left).

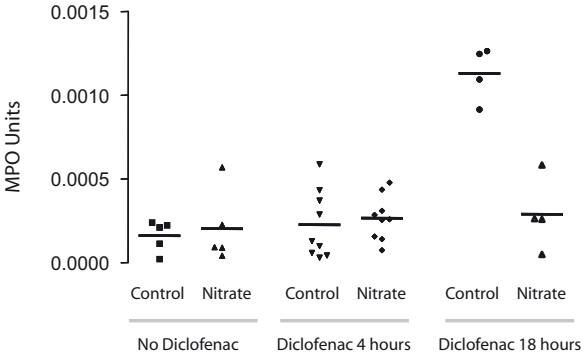
Dietary nitrate reduced the acute gastric injury caused by administration of diclofenac

Animals receiving nitrate supplementation for 7 days in the drinking water showed a significantly reduced ulcer index, reflecting a reduction in macroscopically visible ulcer lesions. This decrease in ulcer index was dose-dependent ranging from 97 (41-163) mm in the control group to 55 (0-95) mm in the low dose group (0.1 mmol kg^{-1}) and to 35 (5-73) mm in the high dose group.



Inflammatory markers were reduced after treatment with dietary nitrate

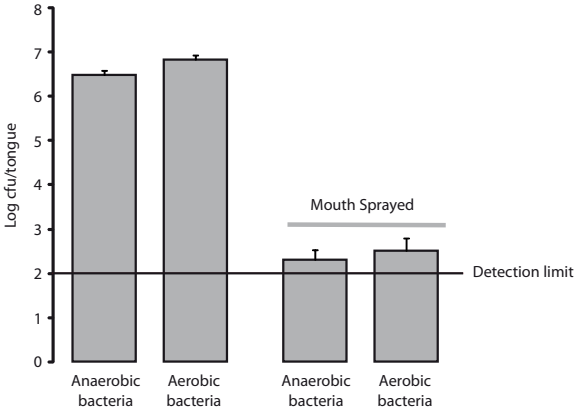
As the MPO activity in the homogenized gastric tissue was not significantly increased in the control group 4 h after diclofenac challenge, additional experiments were run to determine MPO activity 18 h after challenge. At this time point MPO activity was greatly increased in the control group but remained low in the nitrate-treated rats.



Staining for another inflammatory marker, iNOS, using immunohistochemistry, showed that its expression in the gastric mucosa was markedly increased after diclofenac challenge. This expression was dose-dependently prevented by dietary supplementation with nitrate as compared with controls. At the higher nitrate dose, iNOS expression did not differ from that found in control animals not receiving diclofenac. Nitrate pretreatment did not significantly affect the mucosal expression of iNOS in non-diclofenac-treated animals.

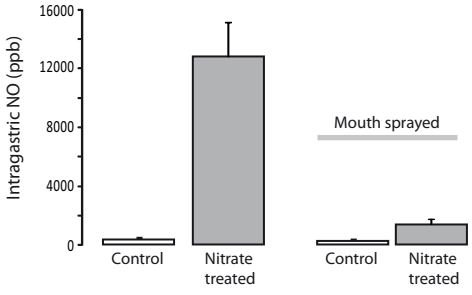
Study IV

Chlorhexidine mouth spray eliminated oral bacteria



In nitrate-treated animals 6.5 ± 0.1 log cfu anaerobic and 6.8 ± 0.1 log cfu aerobic bacteria were detected per tongue, while the nitrate-treated animals that received mouth spray showed a drastic reduction in the bacterial count. No anaerobic bacteria were detected (< 2 log cfu per tongue) in three of the five samples, and no aerobic bacteria in two of the five samples. The maximum counts of anaerobic and aerobic bacteria in the mouth-sprayed, nitrate-treated animals were 3.2 and 3.3 log cfu per tongue, respectively.

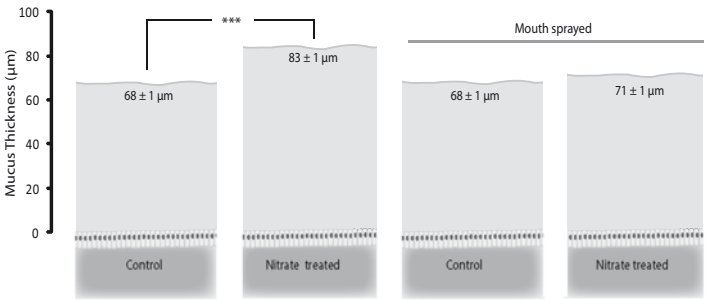
Dietary nitrate did not greatly increase intragastric levels of NO gas or plasma nitrite when the oral flora was eliminated



Nitrate supplementation resulted in increased levels of NO in the stomach. This effect was markedly reduced in the mouth-sprayed rats (Fig. 25, Left). Nitrate addition to the drinking water resulted in an almost tenfold increase in plasma nitrite (control 0.18 mM; nitrate-treated 1.73 mM) When the oral flora was suppressed with mouth spray, only a small increase in plasma nitrite was observed after nitrate supplementation (control 0.17 mM; nitrate-treated, 0.43 mM).

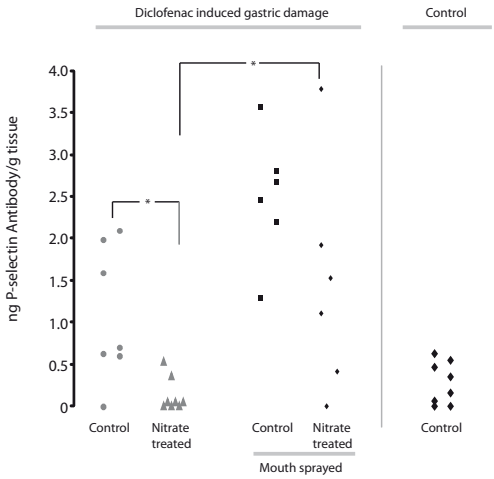
Dietary nitrate did not increase firmly adherent gastric mucus thickness when the oral flora was eliminated

Nitrate supplementation resulted in a 20% increase in the thickness of the firmly adherent mucus layer. This increase was totally absent in the rats in which the oral microflora was reduced with the antiseptic mouth spray. In mouth-sprayed animals that received nitrate in the drinking water, the firmly adherent mucus layer was not significantly altered. In the experiments where rats were given nitrite (1.0 mM, one tenth of the nitrate dose) in the drinking water for one week, the thickness of the firmly adherent mucus layer increased in both control (100 ± 1 µm) and mouth-sprayed animals (100 ± 2 µm) (not shown in figure).



Dietary nitrate did not reduce the acute gastric injury caused by administration of diclofenac when the oral flora was eliminated

In control animals the damage caused by diclofenac was detected by the finding of upregulated levels of P-selectin, expressed as ng P-selectin antibody per gram tissue, in the deeper layer of the gastric mucosa and in the submucosa and muscle layers. This upregulation was totally abolished in animals that were given nitrate for one week prior to the experiment. In mouth-sprayed animals nitrate supplementation had no protective effect, and administration of diclofenac resulted in an upregulation of P-selectin in the stomach in both the control and nitrate-treated animals.



Discussion

Why would the human evolution result in ten times higher levels of nitrate in the salivary glands compared with those in plasma if this did not have a purpose? There must be a physiological reason for this active uptake and concentration of nitrate in the salivary glands.

And why is the human stomach normally filled with NO gas? The bioactive NO present in the stomach must play a physiological role.

Both the concentrations of nitrate and nitrite in the saliva, and the level of NO in the stomach, increase after ingestion of vegetables, a historically fundamental source of nutrition for humans.

This thesis provocatively suggests that dietary nitrate, its oral conversion to nitrite, and the reduction to NO in the stomach, play an important part in regulating the gastric mucosal defense.

This doctoral research is based on the theory that bioactive NO present in the gastric lumen penetrates the mucus gel into the superficial mucosa and passes through the mucosa to the submucosal arterioles, with various biological effects. It is most probable that NO generated in the lumen is able to penetrate to the submucosa, since the gaseous and lipophilic properties of NO facilitate its diffusion through tissue membranes.[120]

The nitrate dose used in the present studies was chosen for its physiological relevance. We compensated for the differences in nitrate metabolism between rats and humans. The active transport of nitrate from blood to saliva that occurs in humans leads to a ten times higher concentration of nitrate in the saliva than in the plasma.[13] This active transport has never been shown in rodents.[121] The active uptake of nitrate by saliva in humans may explain the difference in intragastric NO formation between humans and rats. In rats the NO level can reach to about 15 ppm, as shown here and earlier,[113, 122] while in humans the level is at least ten times higher after ingestion of the same amount of nitrate.[123] In fact, the small amount of nitrate added to the drinking water in the present studies resulted in intragastric levels of NO in the rats that corresponded to levels of intragastric NO that are normally present in humans. Thus it is very likely that the daily dose of nitrate used here was comparable to that readily achievable in humans through a high intake of nitrate-rich vegetables.

It is concluded from these studies that intake of nitrate, its reduction to nitrite, and the generation of NO in the gastric lumen results in enhancement of

gastric mucosal defense mechanisms to such an extent that this process actually protects the mucosa from provoked damage.

Gastric Mucosal Blood Flow

Luminally applied nitrite-rich saliva and nitrite in an acidic environment increase the protective gastric mucosal blood flow. This increase in blood flow is probably caused by NO that forms from nitrite in the gastric lumen under these conditions. A clear dose-dependent relationship was found between the increase in mucosal blood flow and the NO generation in the mucosal chamber. Luminal NO affects the submucosal arterioles, resulting in a cGMP-dependent blood flow increase in the mucosa. This was clearly a direct effect of luminally produced NO, since the results showed that the increase in blood flow caused by nitrite was unaltered by both prostaglandin inhibition and inhibition of endogenous NO production via eNOS. The same increase in blood flow was caused by acidified nitrite in eNOS-deficient mice. Furthermore, the fact that this nitrite-mediated blood flow increase was markedly attenuated after luminal application of the guanylyl cyclase inhibitor ODQ supports the view that the increased blood flow was due to cGMP mediated relaxation of the smooth muscle cells in the arterioles caused by NO.

To further investigate the effect of nitrite on the blood flow, an in vivo study was designed in rats where small amounts of nitrite were applied continuously to the acidic mucosa to mimic the effect of constant swallowing of nitrite-rich saliva in humans. When nitrite was added to the acidic gastric mucosa the blood flow increased immediately. This increased blood flow was maintained as long as the nitrite was continuously applied to the acidic mucosa. When the luminal HCl was changed to neutral saline at the end of the experiment, the reduction of nitrite to NO ceased and the blood flow decreased to the control level. This shows that NO not only increases the blood flow when acidified nitrite is given once as a bolus dose, but is also able to maintain a high blood flow over time when nitrite is given continuously.

Blood flow was also measured in animals pretreated with nitrate for a week and we found that these animals had higher mucosal blood flow than untreated rats. Nitrate-treated animals had very high levels of intragastric NO, as a direct consequence of the nitrate intake. The observed increase in mucosal blood flow is probably not dependent on the high intragastric NO levels, since the stomach is opened during the animal preparation, which results in a quick reduction of the NO concentration.

Pretreatment with nitrate resulted in elevated levels of nitrite in the loosely adherent mucus in the stomach. The combination of nitrite accumulation in the mucus and ongoing acid production in the mucosa probably results in slow,

sustained NO formation, which could explain the persistent increase in blood flow in the nitrate-treated animals even when the stomach was opened. This theory was confirmed by the finding that removal of the loosely adherent gastric mucus decreased the mucosal blood flow in the nitrate-treated animals to the same level as was observed in the controls.

These results suggest, for the first time, that the loosely adherent mucus gel layer in the stomach has an important protective function. The accumulation of nitrite in this mucus layer will be especially important under conditions with no ongoing acid secretion but when the stomach still contains acid. This is true for times in between meals and, especially, during sleep. We have shown earlier that the juxtamucosal pH is much better maintained in the acid-secreting than in the non-secreting stomach.[21, 22, 124] This is due to delivery of bicarbonate from the acid-secreting parietal cells via the blood to the epithelial cells for transport to the mucus. Under non-secreting circumstances, but still with an acidic lumen, the accumulated nitrite in the loosely adherent mucus gel might compensate for the reduced protection caused by decreased bicarbonate delivery. NO will be continuously produced when the nitrite in the loosely adherent mucus layer reacts with protons present in the stomach and will lead to increased blood flow levels.

Another possible cause of the increase in blood flow may be formation of S-nitrosothiols in the gastric mucus. S-nitrosothiols can function as stable carriers of NO, e.g. in the blood, thereby increasing the half-life of NO.[125] The stomach appears to be an ideal milieu for generation of S-nitrosothiols, since the powerful nitrosylating agent N_2O_3 is generated from acidified nitrite[126] and nitrate ingestion has been shown to increase gastric S-nitrosothiols.[127] It is likely that gastric thiols, e.g. from sulfur-containing glycoproteins in the mucus or from glutathione produced by gastric epithelial cells,[128] are S-nitrosylated by acidified nitrite. This is an interesting theory but the exact nature of the thiols in the different mucus layers remains to be determined.

Gastric Mucus Thickness

Application of nitrite-rich saliva and nitrite to the gastric lumen increased the thickness of the important inner, firmly adherent mucus layer. Addition of nitrate to the drinking water for one week also increased this mucus layer. However, dietary nitrate did not influence the growth and accumulation of the outer, loosely adherent mucus layer. We believe that the positive effect on the firmly adherent mucus layer is related to intragastric formation of NO. In the stomach, mucus formation is primarily controlled by the mucin genes MUC6 and MUC5AC.[18, 129-131] Our results indicate that effects of NO are mediated primarily by induction of the MUC6 gene, as the mRNA of this gene was increased dose-dependently by nitrate while that of MUC5AC remained un-

changed. MUC6 forms an essential part of the inner firmly attached mucus layer and it is predominantly secreted by the mucus neck cells.[18]
Several studies have shown that NO donors increase the mucus thickness.[25, 26] Data from our laboratory show that NO is important in regulating the firmly adherent gastric mucus layer, since rats treated with NOS inhibitors and iNOS-deficient mice have a thinner firmly adherent mucus layer.[27] The observation in the present studies that the high luminal concentration of NO increases only the firmly adherent mucus thickness supports the findings in another study that luminal NO donors only had an impact on the firmly adherent gastric mucus layer.[27]

The studies summarized in this thesis showed that intraluminal NO increased the mucus thickness by approximately 20 %. One question that must be raised is: How do we know that this increase in the thickness of the firmly adherent gastric mucus layer is protective? The answer is that we cannot know this with certainty. Under physiological conditions, when the different gastric barriers function as they should, this increase is probably of less importance. But it may be of greater importance in pathological situations when the normal regulation of gastric mucus secretion or mucosal blood flow is disturbed, for instance during prostaglandin inhibition. Prostaglandins interact with NO in the regulation of both mucus secretion and mucosal blood flow.[42] In conditions where the levels of prostaglandins decrease in the gastric mucosa, for instance during NSAID treatment, NO generated from nitrite in the gastric lumen might play a more significant role in regulating mucus secretion.

Bactericidal effects of Nitric Oxide

Yet another important function of NO in the stomach is to reduce different strains of pathogenic bacteria.[132-135] Several groups have shown that many enteropathogens can resist acid alone, but that addition of nitrite results in effective killing.[9, 132, 134-136] This effect is attributed to actions of NO and other reactive nitrogen oxides on different bacterial targets such as DNA, proteins, and cell wall components.[6, 137]
This indicates that dietary nitrate has an important role in protection against ingested pathogens.

Figure 28 summarizes the different gastroprotective effects of luminal NO.

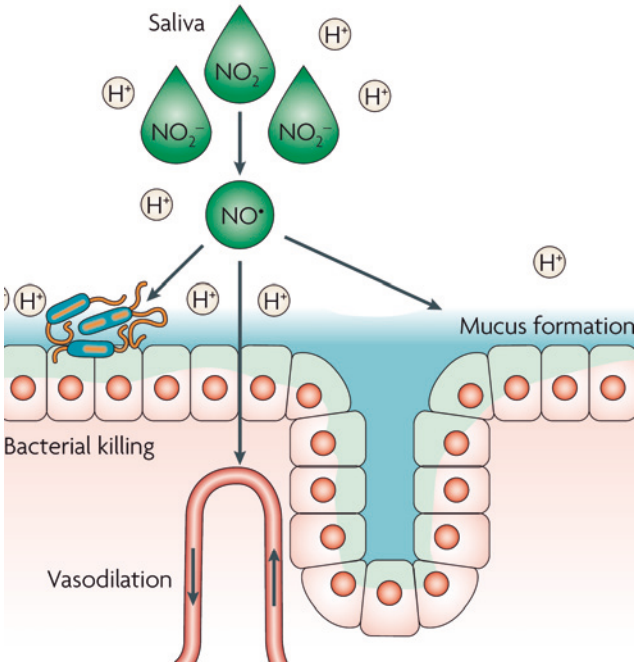


Figure 28. NO generated non-enzymatically in the gastric lumen from nitrite in saliva helps to kill pathogenic bacteria, stimulates mucosal blood flow and mucus generation, and thereby enhances gastric mucosal protection. The illustration is adapted from reference 7.

Gastric Damage

After establishing the positive effect of nitrite on the gastric mucosal blood flow and mucus thickness, we addressed the question whether dietary nitrate or nitrite applied luminally protected the gastric mucosa against injury. Intravenous injection of diclofenac (an NSAID) was combined with luminal application of the bile salt taurocholate, to challenge the gastric mucosa. Diclofenac has been shown to decrease gastric PGE_2 levels and blood flow and increase gastric damage,[138, 139] and taurocholate has been reported to increase gastric mucosal permeability.[140] Both pretreatment with nitrate in the drinking water and luminal application of acidified nitrite significantly reduced the permeability increase caused by taurocholate compared with the findings in untreated animals. These results suggest that the gastric mucosa increases its ability to resist gastric irritants when treated with high levels of luminal NO.

In addition, an acute gastric injury was induced in rats by oral administration of diclofenac, which resulted in severe gastric bleeding. The damage was dose-dependently reduced by pretreating the animals with nitrate in the drinking water for one week. In addition to the decrease in macroscopic lesions after NSAID challenge, we also noted other signs of reduced inflammation in the treated animals. The expression of iNOS, for example, was dose-dependently decreased by nitrate, reflecting decreased inflammatory activity in the mucosa. In fact, at the highest dose of nitrate iNOS expression was not different from the control level. Moreover, dietary nitrate seemed to decrease leukocyte infiltration in the mucosa, as evident by the lower levels of MPO in the treated animals.

Other studies have also shown that dietary nitrate prevents gastric damage,[141-143] but the studies presented in this thesis offer for the first time a concrete physiological explanation for this. We reason that the reduced mucosal damage observed after nitrate pretreatment is a result of enhanced mucosal protection, with increased mucosal blood flow in combination with an increased thickness of the firmly adherent mucus layer. There may, however, be yet another explanation for the mucosal protection after nitrate pretreatment. We found that nitrate intake also resulted in elevated levels of both nitrate and nitrite in the gastric tissue. Nitrite has been shown to exert potent cytoprotective effects in ischemia-reperfusion injuries in the heart and liver.[144] We cannot rule out the possibility that nitrite present in the gastric mucosa exerts cytoprotective effects in that tissue during an injury.

The Importance of Oral Bacteria in Converting Nitrate to Nitrite

When the oral flora was suppressed by antiseptic mouth spray, the gastroprotective effect of dietary nitrate was abolished. Nitrate in the diet normally results in increased levels of plasma nitrite and large amounts of intragastric NO. This was not observed in animals lacking an oral flora. Nitrate supplementation did not increase the thickness of the firmly adherent mucus layer when the rats were treated with bactericidal mouth spray. The amount of NO present in the stomach under these conditions was probably insufficient to stimulate mucus secretion. When the animals were given nitrite instead of nitrate in the drinking water, the mucus thickness increased irrespective of whether they were mouth-sprayed or not. Under this condition, the oral flora is of less importance, since the nitrate conversion is not necessary. In this case the nitrite was supplied directly from the drinking water, resulting in high levels of NO in the acid-producing stomach, stimulating mucus secretion. Germ-free animals have very low levels of NO in the stomach.[113] This is not surprising, since these animals totally lack nitrate-reducing bacteria on the tongue, and conversion of nitrate to

nitrite is therefore absent.[79] Remarkably, germ-free mice have a very thin firmly adherent mucus layer, and this layer was totally unaffected when these mice were given nitrate.

The gastroprotective effect of nitrate was not observed when the rats were mouth-sprayed. This result supports a previous finding by Miyoshi and colleagues that local oral administration of antibiotics abolished the gastroprotective effect of dietary nitrate.[143] In the present study, gastric NO formation decreased when the oral flora was reduced. The nitrate-reducing bacteria in the mouth, by conversion of dietary nitrate to nitrite, therefore play an important physiological role in gastric mucosal defense. The significance of oral bacteria is perhaps even more important in humans, with normally higher levels of NO present in the stomach. These results show that our symbiosis with the commensal oral flora is essential and influences other organ systems than the oral cavity.

Chlorhexidine is often used as an active ingredient in mouthwash designed to kill oral bacteria for prevention of gingivitis or to improve bad breath. In these animal studies we used a chlorhexidine-based mouth spray, for its ability to kill nitrate-reducing bacteria in the oral cavity. The risk associated with everyday use of chlorhexidine-based mouth wash in humans needs to be further investigated. A substantial reduction of oral bacteria over time may influence the physiological protection of the gastric mucosa. This is important to highlight today, when manufacturers of dental care products are eager to encourage us to reduce the oral flora with antiseptic mouthwash.

The Influence of Intragastric NO formation on the Epithelium

Since the stomach and the gastroesophageal junction are exposed to high concentrations of NO following nitrate intake, the question arises whether NO generated intraluminally will have a negative effect on the epithelium. From our studies in which animals were treated with a nitrate-rich diet for one week, we concluded that the levels of NO consequently formed in the gastric lumen did not influence the gastric or esophageal epithelium in a harmful way. This conclusion was based on our observations that no increase occurred in the MPO level or iNOS expression in gastric tissue and that there was no upregulation of P-selectin in the esophageal or gastric tissue after the nitrate pretreatment, suggesting the absence of pathological conditions.

Theories have been proposed concerning possible carcinogenesis associated with NO generated in the stomach. McColl and colleagues believe that NO generated in the gastric lumen, with the aid of ascorbic acid, diffuses into the esophageal epithelium, where it may induce production of mutagenic N-nitrosamines.[145] This theory must be strongly questioned, since N-nitrosamines,

if formed in large quantities in the epithelium, would probably cause inflammation, and we observed no signs of inflammatory processes in the epithelium after nitrate supplementation in our animal studies, as mentioned above. The reported N-nitrosamine formation has only been shown to occur in an in situ model of the epithelium.[145] From a recently published study, the same group reports that NO has the potential to induce double-strand DNA breaks and proposes that NO generated luminally may therefore be carcinogenic.[146] A major and important drawback of the latter study is that acid alone caused the same damage as when acid was combined with nitrite. The above studies suggesting an association between NO generated luminally and carcinogenesis have had a great impact in the field of gastroenterology, but the authors have had difficulty in proving their hypotheses. With these hypotheses as a background the daily intake of fruits and vegetables ought to be restricted, a recommendation that does not have any evidential support. By contrast, there are studies suggesting that a vegetarian diet in fact protects against cancer in the gastrointestinal tract.[147, 148]

Clinical Aspects

In critically ill patients, the entero-salivary nitrate cycle may be disturbed by sedation and endotracheal intubation, resulting in low levels of intragastric NO. It has been suggested that the insufficient levels of gastric NO contribute to the gastric lesions and to the bacterial overgrowth commonly found in these patients. The low NO level can be restored by intragastric supplementation with nitrite.[123]

Gastric ulcers associated with the use of NSAIDs remain a major clinical problem,[149-153] especially as NSAIDs are today the most commonly used group of drugs.[154] The adverse effects of NSAID cause considerable pain for the patients and entail a high cost for the health care system. The pharmaceutical industry has modified NSAIDs and has developed different NO-donating NSAIDs with the aim of attenuating the side effects. The claimed beneficial effects of the NO-NSAIDs have been attributed to a release of NO and its effect in increasing gastric mucosal blood flow and protecting gastric epithelial cells from necrosis.[155, 156] But as yet no NO-donating NSAIDs have reached the patients.

The development of the cyclooxygenase -2 (COX-2) selective inhibitors was another attempt to reduce the side effects of the NSAIDs. COX-2 inhibitors are a form of NSAIDs that directly targets the COX-2 enzyme, and selectivity for COX-2 reduces the gastrointestinal side effects. The COX-2 inhibitors have been introduced clinically but studies revealed an association between the COX-2 inhibitor Rofecoxib and an increased risk of cardiovascular disease,[157, 158] and this drug was therefore taken off the market in 2004.

A new approach to the vast problems of adverse gastrointestinal effects of NSAID treatment may instead be to recommend an increased intake of vegetables. If we reach the daily intake of non-starchy fruit and vegetables recommended by the American Institute of Cancer Research (600g per day)[87] it will probably make a huge difference. This quantity of vegetables will greatly increase the gastric NO formation, especially since the general consumption of vegetables in a westernized diet is very low.

The present studies have demonstrated that our diet plays a direct role in regulating gastric homeostasis. The NO formation in the stomach, which follows adequate nitrate intake, improves the gastric mucosal defense by increasing both the blood flow and the thickness of the mucus layer. In addition, the studies have shown that the oral flora plays a crucial role, as it has a key task in reducing nitrate to nitrite.

In summary, this thesis challenges the current dogma that nitrate intake is hazardous, and on the contrary suggests that dietary nitrate is important for health. It is in fact quite possible that a sufficient supply of nitrate in the diet together with the oral microflora is essential for preventing pathological conditions in the gastrointestinal tract.

Conclusions

- Ingestion of inorganic nitrate results in a rapid accumulation of nitrite in saliva, through conversion by the oral bacterial flora
- In the acidic stomach, nitrite-containing saliva generates NO.
- This NO causes a cGMP-dependent increase in the defensive mucosal blood flow.
- NO formed in the stomach from nitrite also increases the thickness of the protective firmly adherent gastric mucus layer.
- Nitrate in the diet thus leads to an increase in the gastric mucosal blood flow and the firmly adherent gastric mucus layer.
- The NSAID diclofenac and the bile salt taurocholate increase the permeability of the gastric mucosa. Nitrate in the diet or nitrite applied luminally reduces this increase in permeability.
- Nitrate in the diet protects the gastric mucosa from the acute injury caused by oral administration of diclofenac
- Suppression of the oral flora strongly reduces the conversion of nitrate to nitrite in the oral cavity
- The gastroprotective effect of dietary nitrate is abolished if the oral flora is reduced, since there is no oral conversion to nitrite and consequent reduction to NO in the stomach.
- Oral bacteria hence play a crucial role for the gastro-protection provided by Nitrate, Nitrite and Nitric Oxide.

Future studies will reveal whether a diet rich in nitrate can offer an additional nutritional approach to prevention and treatment of peptic ulcer disease.

Future Perspectives

We are interested in measuring the amounts of S-nitrosothiols in the different mucus layers in this model. It has previously been shown that S-nitrosothiols form in the stomach after ingestion of nitrate[127] and that these thiols release NO slowly over a relatively long period of time, and this process might be involved in the observed increase in blood flow following nitrate intake.

It has been suggested that NO may stimulate the secretion of bicarbonate into the gastric mucus gel. The question has never been studied whether NO generated in the lumen influences bicarbonate secretion.

To mimic the clinical situation of low-dose long-term NSAID treatment in a study of the gastroprotective effect of nitrate, a low dose of NSAID could be given daily by gastric gavage for a relatively long period of time during administration of nitrate in the drinking water, and the gastric mucosal physiology and damage could then be evaluated.

One important factor that should be investigated is the long-term effects of high nitrate intake. How will a high nitrate diet for 6 months to one year affect the animals? Most probably the positive effects of dietary nitrate will remain. This is a key question in the discussion concerning a possible correlation of nitrate with cancer in the gastrointestinal tract. Does long-term exposure to nitrate affect cell proliferation? Can histological changes be observed?

Another interesting and relevant question is that of the effects of a suppressed oral flora over time. Can constant, intense use of antiseptic oral dental care products weaken the gastric mucosal defense?

Effects of Dietary Nitrate on the Intestine

Dietary nitrate also increases the concentrations of NO in the duodenum. This is probably a spillover effect from the stomach. Intake of nitrate in the diet can result in exposure of the duodenum to high levels of NO. An interesting field would be to study how intraluminal NO influences the physiology of the duodenum Is bicarbonate secretion or motility influenced by these high levels of NO?

We have investigated the effects of a high nitrate diet on NSAID-induced damage in the small bowel. Preliminary data have shown that dietary nitrate, and elevated levels of circulating nitrate and nitrite, protect against NSAID-

induced damage in the small bowel, and the specific mechanisms for this protection need to be elucidated.

Dietary nitrate has been shown to protect against ischemia reperfusion damage in several different organs.[7] Whether and how such protection occurs in the gastrointestinal tract has never been studied. This is a very important area. Bowel ischemia is a serious condition, since a reduced blood flow rapidly destroys the defense barrier against luminal bacteria. An ischemic bowel can therefore rapidly lead to sepsis due to bacterial translocation from the bowel into the blood.

Sammanfattning på svenska

Bakgrund

Grönsaker har, under evolutionens gång, varit en huvudsaklig näringskälla för människor. I grönsaker som spenat, sallad, rädisor och rödbetor finns förutom näringsämnen stora mängder nitrat. När vi äter nitratrika grönsaker tas nitrat, som merparten av våra näringsämnen, upp i tunntarmen och frisätts i blodbanan. Nitrat tas sedan *aktivt* upp från blodet och koncentreras i våra spottkörtlar. Vår saliv innehåller därför stora mängder nitrat efter intag av nitratrik kost. Den nitratrika saliven utsöndras kontinuerligt ut i munhålan där munhålans bakterier reducerar delar av nitraten till nitrit. När vi sväljer nitriten kommer den i kontakt med sur magsaft och reduceras då till det biologiskt aktiva ämnet kväveoxid (NO). Detta resulterar i att vi efter att ha ätit grönsaker kommer få höga nivåer av NO i magsäcken.

Magsäcken innehåller mycket sur magsaft och olika proteinnedbrytande enzymer för att kunna bryta ner vår föda. För att skydda sig från denna ogästvänliga miljö har magslemhinnan utvecklat olika komplexa försvarsmekanismer. Utan ett kraftigt skydd skulle magsäcken, som består av proteiner, förstöras av den starka syran och enzymerna som finns inne i magsäcken. Detta är särskilt tydligt efter döden, då magsäcken snabbt skadas i brist på skyddande funktioner. Att ständigt förnya ett slemskikt som täcker slemhinnan samt att upprätthålla ett stabilt blodflöde i magslemhinnan är två mycket viktiga försvarsmekanismer. Blodflödet syresätter slemhinnan och neutraliserar eventuella indiffunderade syrajoner med basiska bikarbonatjoner. Ett tunt slemskikt eller låga blodflöden i slemhinnan kan leda till skador på ytepitelcellerna, något som kan resultera i magsår.

I magslemhinnan spelar NO en lokal kärldilaterande roll. Denna kärlvidgning ökar magslemhinnans blodflöde och därmed förmågan att skydda sig. Det har även visat sig att NO är involverat i regleringen av utsöndringen av det viktiga slem som utgör magslemhinnans slemskikt.

Man har länge vetat att NO produceras av olika enzym i den mänskliga kroppen och fungerar som en betydande signalmolekyl, något som belönades med Nobelpriset i Medicin och Fysiologi 1998. Men upptäckten att NO kan bildas i magsäcken, helt utan involvering av enzym, från nitrit i saliven är rela-

tivt ny. Därför är också kunskapen om hur magsäcken påverkas av dessa höga nivåer av NO som vi normalt har i våra magar mycket liten.

Detta avhandlingsarbete bygger på studier kring hur dessa höga NO-nivåer i magen, som är en direkt konsekvens av intag av nitratrik föda, påverkar magslemhinnan.

Metoder

Arbetet är till stor del utfört i en djurmodell med råttor och möss. Djuren sövs och katetrar sätts för övervakning av blodtryck och infusion av saltlösning och läkemedel. För att underlätta spontan andning i djuren, öppnas också luftstrupen men ett kort snitt och en plastslang förs in. Därefter öppnas buken och ett 1 cm långt snitt görs i övre delen av magsäcken. Djuret läggs sedan på en tempererad värmeplatta där djurets kroppstemperatur hålls konstant med hjälp av en rektaltermometer kopplad till värmeplattan. Magen vänds sedan ut och in över en tempererad plexiglaskon och fixeras med nålar. Över konen sätts ett tempererat lock med ett runt hål för den exponerade magslemhinnan. Detta bildar ett utrymme, där vätska kan tillsättas och komma i kontakt med den levande magslemhinnan. Genom att byta och samla den vätska som tillsätts kan man, genom att mäta pH på vätskan, följa om djuret producerar sur magsaft samt exponera slemhinnan för olika lösningar. Här tillsättes tempererad fysiologisk koksaltlösning och saltsyra. Blodflödet i slemhinnan mäts med laserdoppler flödesteknik. Slemtjockleken mäts med en mikropipett som är kopplad till en mikromanipulator. Slemhinneskador detekteras både mikro- och makroskopiskt och skademarkörer i vävnaden mäts med molekylärbiologiska metoder. Modellen är utarbetad på vårt laboratorium och finns tidigare beskriven i detalj.[38]

Resultat

- I studien; ***"Nitrite in saliva increases gastric mucosal blood flow and mucus thicknes"*** visas att nitrit i saliven ökar det skyddande blodflödet och slemtjockleken och att detta är en direkt effekt av det NO som bildas i magsäcken när nitritrik saliv sväljs. När surgjord nitrit eller nitritinnehållande saliv läggs direkt på råttans magslemhinna ökar blodflödet och slemlagret blir tjockare. Detta beror på att nitriten reduceras till NO i närvaro av syran i magen. NO vidgar blodkärlen som leder till blodflödesökningen och stimulerar till slemsekretion.

- I studien; ***"Dietary Nitrate increases gastric mucosal blood flow and mucosal defense"*** visas att ett nitrattillskott i kosten i en vecka ökar blodflödet i magslemhinnan. Magslemhinnan utsätts för en mild provokation genom att gallsalter läggs på magsleminnan i kombination med att läkemedlet diclofenac ges intravenöst och permeabiliteten i magslemhinnan mäts. Diclofenac är ett så kallat NSAID (icke steroida antiinflammatoriska läkemedel), känt för att ge skador i magtarmkanalen. Permeabiliteten ökar av den milda skadan, men djur som fått nitrat i kosten i en vecka får inte denna kraftige ökning av permeabiliteten. Slutsatsen dras att nitrat i kosten ökar magslemhinnans blodflöde i sådan utsträckning att detta skyddar vid en mild provokation.

- I studien; ***"Protection from NSAID-induced gastric ulcers by dietary Nitrate"***, visas vi att nitratrik föda skyddar mot akuta NSAID-inducerade magsår. Nitrat tillförs till djurens vatten i en vecka och sedan matas djuren med en hög dos av diclofenac för att inducera magsår. Djurens magslemhinna undersöks därefter genom att mäta skadad yta och olika inflammatoriska parametrar. Nitratmatade djur har ett tjockare slemlager och att dessa djur inte får lika svåra skador av diclofenac. Vi drar slutsatserna att nitrat i kosten ökar magslemhinnans slemtjocklek i kombination med blodflödet i sådan utsträckning att detta skyddar mot kraftigare skador.

- I studien; **"*Oral bacteria regulate gastric mucosal defense via bioactivation of dietary Nitrate"***, visas att bakterierna i munhålan är av yttersta vikt för det skydd som nitrat i födan har på magslemhinnan. Råttor ges nitrat i födan samtidigt som de munsprayas med en antibakteriell munspray. Resultaten visar att munsprayade råttor har lägre nivåer av NO i magsäcken och inte får ett tjockare slemlager trots att de får nitrat i kosten. Vidare ses att nitrattillskott i kosten i en vecka inte skyddar

vid en diclofenacinducerad skada om djuren är munsprayade. Vi konkluderar att bakterierna i munhålan är nödvändiga för omvandlingen av nitrat till nitrit i saliven. Utan dessa bakterier uteblir NO bildning i magsäcken och dess positiva, skyddande effekter.

Diskussion

Studierna i denna avhandling visar att det NO som bildas efter intag av nitratrik kost stimulerar magslemhinnans skyddsmekanismer. NO bildat i magsäcken vidgar blodkärlen och ökar slemhinnans blodflöde. Detta NO stimulerar också utsöndringen av slem, något som resulterar i ett tjockare slemlager som täcker magslemhinnan. Detta tillsammans leder till en motståndskraftigare magslemhinna.

Icke steroida antiinflammatoriska läkemedel är en grupp mycket vanligt förskrivna läkemedel vid värk och inflammation. En stor nackdel med dessa läkemedel är de många och allvarliga biverkningarna de ger upphov till i magtarmkanalen i form av blödningar och sår. Här presenteras studier som visar att en nitratrik diet ökar blodflödet och slemtjockleken i sådan grad att magen motstår skadorna som normalt orsakas av dessa läkemedel.

Bakterierna i munhålan har en betydande roll genom att de omvandlar nitrat i saliven till nitrit. Utan dessa bakterier sker ingen omvandling och NO-bildningen i magsäcken uteblir. Den sista studien i denna avhandling trycker på betydelsen av en intakt munflora, och om denna slås ut med antibakteriella munvatten kan det ha betydelse för den normala funktionen i magsäcken, då de skyddande nivåerna av NO kraftigt minskar.

Slutsats

De forskningsresultat som presenteras här ger en ny syn på vikten av frukt och grönsaker i vår diet. Om vi ökar vi intaget av frukt och grönt, till de av livsverket rekommenderade 500 g per person och dygn, skulle säkert våra magar må bättre.

Att våra bakterier i munnen inte bara är involverade i vår munhygien, utan också spelar en betydande roll för de normala funktionerna i magtarmkanalen är något helt nytt. Detta kan uppfattas provokativt i en tid då vi är överösta med reklam för tandborstar med tungskrapor och munvatten som ska eliminera bakterier i munnen.

Acknowledgments

Research is a collective endeavor and many people have contributed to the accomplishment of this thesis. I owe them all my deepest gratitude.
My special thanks are due to:

Professor *Lena Holm*, my perfect supervisor. Thank you for introducing me to research and brilliantly guiding me through the physiology of the gastrointestinal tract. During these years you have always, no matter what, given me time for discussing results. Without your strong belief in me and constant encouragement, I would never have reached this far.

Professor *Jon Lundberg*, my co-supervisor, for sharing your vast knowledge in the field of NO physiology and for everything I have learned from you. I so appreciate your endless optimism on everything I do. I have really enjoyed working with a real giant in science like you.

Assistant Professor *Mia Phillipson*, my co-supervisor, for being an excellent role model when it comes to science. You have convinced me that hard core science can be combined with a fabulous life outside the lab. Your unfailing support in science and life in general makes you a brilliant colleague and good friend.

My close PhD-student colleague*, Olof Schreiber*, for always, always, always being positive and helpful when I ask for help and for always, always, always supporting and encouraging me. You are a special person, an excellent friend and a true rock.

Annika Jägare, for all your excellent help with experiments, and for your positive and helpful attitude even when time has been against us. To have you as a collaborator has really been a privilege.

Johanna Henriksnäs, for giving me an outstanding start at the department as a graduate student in 2002. Your invaluable help with everything in and outside the lab is very much appreciated.

My co-authors for fruitful collaboration:
Håkan Björne, for fantastic fun and instructive collaboration in study I. Your thesis has been my Nitrite/Nitrate Bible.
Emmelie Jansson, for sharing your wide expertise in molecular biology and for being such a nice person. Your positive *"those experiments are no problem to perform"* attitude makes collaboration with you so joyful.
Eddie Weitzberg, for your constant flow of ideas and your never-ending enthusiasm when it comes to science. It is a true pleasure to work with you.
Andreas Patzak and *Andreas Steege* for letting me come to your lab in Berlin and explore the world of PCR.
Stefan Roos and *Hans Jonsson* for imparting some of your wide knowledge in microbiology and for your great efforts in study IV.

Professor *Erik Persson,* for sharing your extensive medical knowledge and for your bright sense of humor.
Professor *Olof Nylander,* for your strong belief in me regarding both science and teaching.
Professor *Michael Perry*, for mentoring me in the techniques of radiolabeling antibodies and P-selectin antibody experiments.

The rest of the faculty at the division of Integrative Physiology:
Professor *Örjan Källskog*, Professor *Mats Sjöquist*, Professor *Peter Hansell* and Assistant Professor *Fredrik Palm*, for creating a relaxed scientific environment by combining coffee break laughter with constructive criticism during Thursday seminars.
Angelica Fashing and *Britta Isaksson* for adding your decades of experimental skills in the field of *in vivo* physiology to the corridor, and for giving the corridor the continuity it needs when it comes to important traditions.

My former and present PhD student colleagues:
Mattias Carlström, for continuity of friendship during our graduate and postgraduate studies.
Johan Sällström for your invaluable -not only computer -support at any time, even during acute situations after office hours.
Lina, Johan O, Gustav, Åsa, Ulrika, Sara, Nina, Jenny, Malou, Louise, Markus, Sara R, Micke, Andrei, Russell, EnYin, Zufu, for making lunches and coffee breaks much more fun.

Gunno Nilsson and *Birgitta Klang,* for making teaching so much easier. Special thanks to Gunno for giving my computer problems high priority.
Göran Ståhl, for your quick assistance whenever I need help.
Agneta Bäfwe, Marianne Ljungkvist and *Karin Öberg*, for running the important administration.

Carina Nihlén, Annika Olsson and *Margareta Stensdotter,* for all technical assistance with the nitrate and nitrite measurements.
Maud Marsden, for excellent linguistic review.
Maud, Jonna, Martina, Ann-Charlotte, Lena L and the rest of the staff working in the animal facility at BMC, for always giving me VIP treatment and for taking good care of my animals at all times.

All the wonderful people in my close surroundings who give me a vibrant life outside the lab.

Björn, for your invaluable friendship and solid loyalty.
Christian, for being the amusing person you are; your company adds humor to my everyday life.
Martin and Lisa for your relaxed way of viewing life and for always being there when needed.
Josefine, for 15 years of unfailing back-up.

And all my other friends who are all an important part of my vital social safety net; *Anders F, Niclas, Elin A, Malin & Peter, Henrik, Lisa A, Niklas E, Kristina D, Sofie, Ullacarin, Anna, Åsa, Helene, Kristin K, Johanna N* (often with the contributions from your partner, who I hereby also thank).

My family, my strength in life.
Axel, *Olle* and *Klara* for constantly giving me all the space and admiration that shaped me to the person I am today. I am proud to have siblings like you.

My parents, *Marie* and *Anders*, for raising me to be the efficient and creative person I sometimes am. I know it was not easy. I admire you enormously. Countless thanks for your unconditional love and solid support.

Uppsala, March 2008

Financial support for these studies was provided by grants from;

The Swedish Research Council (08646)+(12585)+(12586), EU's 6th Framework Program (Eicosanox LSHM-CT-2004-005033), The Swedish Nutrition Foundation, Smålands Nation and The AnnaMaria Lundin Foundation, The Swedish Pharmaceutical Society, The Ekhaga Foundation, The Wallenberg Foundation, The Medical Faculty, Uppsala University, The National Institute of Health, The American Gastroenterological Association, The Scandinavian Physiological Society, The Royal Swedish Academy of Science, The Anna Cederberg Foundation, The Golje Memory Foundation, The Sven Brolin Foundation, The Jeanssen Foundation, The Swedish Heart and Lung Foundation, and The Ruth and Richard Juhlin Foundation.

Special thanks go to the Microsoft Corporation, with the Windows operating system Vista and software Office 2007, for testing my ability to handle acute stress.

Reprints of the figures were made with permission from the publisher.

References

1. **Moncada S**. The 1991 Ulf von Euler Lecture. The L-arginine: nitric oxide pathway. *Acta Physiol Scand* 1992;145:201-27.

2. **Moncada S, Palmer RM, Higgs EA**. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol* 1989;38:1709-15.

3. **Moncada S, Higgs A**. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;329:2002-12.

4. **Moncada S, Higgs A, Furchgott R**. International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol Rev* 1997;49:137-42.

5. **Bryan NS**. Nitrite in nitric oxide biology: cause or consequence? A systems-based review. *Free Radic Biol Med* 2006;41:691-701.

6. **Lundberg JO, Weitzberg E, Cole JA, Benjamin N**. Nitrate, bacteria and human health. *Nat Rev Microbiol* 2004;2:593-602.

7. **Lundberg JO, Weitzberg E, Gladwin MT**. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 2008;7:156-67.

8. **Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO, 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR, Jr., Feelisch M, Lundberg JO**. The emerging biology of the nitrite anion. *Nat Chem Biol* 2005;1:308-14.

9. **Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H**. Stomach NO synthesis. *Nature* 1994;368:502.

10. **Lundberg JO, Weitzberg E, Lundberg JM, Alving K**. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 1994;35:1543-6.

11. **McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N**. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 1997;40:211-4.

12. **Tannenbaum SR, Sinskey AJ, Weisman M, Bishop W**. Nitrite in human saliva. Its possible relationship to nitrosamine formation. *J Natl Cancer Inst* 1974;53:79-84.

13. **Tannenbaum SR, Weisman M, Fett D**. The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol* 1976;14:549-52.

14. **Niv Y, Fraser GM**. The alkaline tide phenomenon. *J Clin Gastroenterol* 2002;35:5-8.

15. **Allen A, Flemstrom G, Garner A, Kivilaakso E**. Gastroduodenal mucosal protection. *Physiol Rev* 1993;73:823-57.

16. **Allen A, Bell A, Mantle M, Pearson JP**. The structure and physiology of gastrointestinal mucus. *Adv Exp Med Biol* 1982;144:115-33.

17. **Allen A, Cunliffe WJ, Pearson JP, Sellers LA, Ward R**. Studies on gastrointestinal mucus. *Scand J Gastroenterol Suppl* 1984;93:101-13.

18. **Ho SB, Takamura K, Anway R, Shekels LL, Toribara NW, Ota H**. The adherent gastric mucous layer is composed of alternating layers of MUC5AC and MUC6 mucin proteins. *Dig Dis Sci* 2004;49:1598-606.

19. **Atuma C, Strugala V, Allen A, Holm L**. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G922-9.

20. **Henriksnas J, Phillipson M, Petersson J, Engstrand L, Holm L**. An in vivo model for gastric physiological and pathophysiological studies in the mouse. *Acta Physiol Scand* 2005;184:151-9.

21. **Schade C, Flemstrom G, Holm L**. Hydrogen ion concentration in the mucus layer on top of acid-stimulated and -inhibited rat gastric mucosa. *Gastroenterology* 1994;107:180-8.

22. **Phillipson M, Atuma C, Henriksnas J, Holm L**. The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G211-9.

23. **Henriksnas J, Phillipson M, Storm M, Engstrand L, Soleimani M, Holm L**. Impaired mucus-bicarbonate barrier in Helicobacter pylori- infected mice. *Am J Physiol Gastrointest Liver Physiol* 2006.

24. **Sababi M, Nilsson E, Holm L**. Mucus and alkali secretion in the rat duodenum: effects of indomethacin, N omega-nitro-L-arginine, and luminal acid. *Gastroenterology* 1995;109:1526-34.

25. **Brown JF, Hanson PJ, Whittle BJ**. Nitric oxide donors increase mucus gel thickness in rat stomach. *Eur J Pharmacol* 1992;223:103-4.

26. **Brown JF, Keates AC, Hanson PJ, Whittle BJ**. Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. *Am J Physiol Gastrointest Liver Physiol* 1993;265:G418-22.

27. **Phillipson M, Johansson MEV, Henriksnäs J, Petersson J, Gendler JS, Sandler S, Persson AEG, Hansson GC, Holm L.** The gastric mucus layers; its constituents and regulation of accumulation, 2008. *Submitted Manuscript*

28. **Helander HF**. The cells of the gastric mucosa. *Int Rev Cytol* 1981;70:217-89.

29. **Eastwood GL**. Epithelial renewal in protection and repair of gastroduodenal mucosa. *J Clin Gastroenterol* 1991;13 Suppl 1:S48-53.

30. **Ashley SW, Soybel DI, Moore CD, Cheung LY**. Intracellular pH (pHi) in gastric surface epithelium is more susceptible to serosal than mucosal acidification. *Surgery* 1987;102:371-9.

31. **Kivilaakso E, Kiviluoto T**. Intracellular pH in isolated Necturus antral mucosa in simulated ulcerogenic conditions. *Gastroenterology* 1988;95:1198-205.

32. **Hirst B.** The gastric mucosal barrier. In: Forte J, ed. Handbook of physiology; The gastrointestinal system. Volume 3. Baltimore: Waverly Press Inc, 1989:539-554.

33. **Lacy ER**. Prostaglandins and histological changes in the gastric mucosa. *Dig Dis Sci* 1985;30:83S-94S.

34. **Ito S, Lacy ER**. Morphology of rat gastric mucosal damage, defense, and restitution in the presence of luminal ethanol. *Gastroenterology* 1985;88:250-60.

35. **Ito S, Lacy ER, Rutten MJ, Critchlow J, Silen W**. Rapid repair of injured gastric mucosa. *Scand J Gastroenterol Suppl* 1984;101:87-95.

36. **Bennett A**. Some new aspects of gastric mucosal protection and damage. *Acta Physiol Hung* 1989;73:179-83.

37. **Gannon B, Browning J, O'Brien P**. The microvascular architecture of the glandular mucosa of rat stomach. *J Anat* 1982;135:667-83.

38. **Holm-Rutili L, Obrink KJ**. Rat gastric mucosal microcirculation in vivo. *Am J Physiol Gastrointest Liver Physiol* 1985;248:G741-6.

39. **Guth PH, Leung FW.** Physiology of the gastric circulation. In: R. JL, ed. Physiology of the gastrointestinal tract. New York: Raven Press, 1987:1031-1053.

40. **Guth PH**. Current concepts in gastric microcirculatory pathophysiology. *Yale J Biol Med* 1992;65:677-88.

41. **Pique JM, Whittle BJ, Esplugues JV**. The vasodilator role of endogenous nitric oxide in the rat gastric microcirculation. *Eur J Pharmacol* 1989;174:293-6.

42. **Whittle BJ, Lopez-Belmonte J, Moncada S**. Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. *Br J Pharmacol* 1990;99:607-11.

43. **Elliott SN, Wallace JL**. Nitric oxide: a regulator of mucosal defense and injury. *J Gastroenterology* 1998;33:792-803.

44. **Kawano S, Tsuji S**. Role of mucosal blood flow: a conceptional review in gastric mucosal injury and protection. *J Gastroenterol Hepatol* 2000;15 Suppl:D1-6.

45. **McGreevy JM, Moody FG**. Protection of gastric mucosa against aspirin-induced erosions by enhanced blood flow. *Surg Forum* 1977;28:357-9.

46. **Cheung LY, Chang N**. The role of gastric mucosal blood flow and H+ back-diffusion in the pathogenesis of acute gastric erosions. *J Surg Res* 1977;22:357-61.

47. **Holzer P LE, Guth PH.** Neural, metabolic, physical and endothelial factors in the regulation of the gastric circulation. In: Johnson L, ed. Physiology of the Gastrointestinal Tract. 2nd ed. New York: Raven Press, 1994:1311-1330.

48. **Kivilaakso E**. High plasma HCO3-protects gastric mucosa against acute ulceration in the rat. *Gastroenterology* 1981;81:921-7.

49. **Starlinger M, Schiessel R**. Bicarbonate (HCO3) delivery to the gastroduodenal mucosa by the blood: its importance for mucosal integrity. *Gut* 1988;29:647-54.

50. **Leung FW, Itoh M, Hirabayashi K, Guth PH**. Role of blood flow in gastric and duodenal mucosal injury in the rat. *Gastroenterology* 1985;88:281-9.

51. **Stein HJ, Bauerfeind P, Hinder RA, Koerfer J, Blum AL**. Luminal acid reduces gastric mucosal blood flow in the ischemic stomach. *J Surg Res* 1989;46:616-9.

52. **Sato N, Kawano S, Tsuji S, Ogihara T, Yamada S**. Gastric blood flow in ulcer diseases. *Scand J Gastroenterol Suppl* 1995;208:14-20.

53. **Whittle BJ, Kauffman GL, Moncada S**. Vasoconstriction with thromboxane A2 induces ulceration of the gastric mucosa. *Nature* 1981;292:472-4.

54. **Abdel-Salam OM, Czimmer J, Debreceni A, Szolcsanyi J, Mozsik G**. Gastric mucosal integrity: gastric mucosal blood flow and microcirculation. An overview. *J Physiol Paris* 2001;95:105-27.

55. **Mersereau WA, Hinchey EJ**. Effect of gastric acidity on gastric ulceration induced by hemorrhage in the rat, utilizing a gastric chamber technique. *Gastroenterology* 1973;64:1130-5.

56. **Esplugues J, Lloris JM, Marti-Bonmati E, Morcillo EJ**. Effects of beta-adrenoceptor drug stimulation on various models of gastric ulcer in rats. *Br J Pharmacol* 1982;76:587-94.

57. **Guttu K, Sorbye H, Gislason H, Svanes K, Gronbech JE**. Role of bicarbonate in blood flow-mediated protection and repair of damaged gastric mucosa in the cat. *Gastroenterology* 1994;107:149-59.

58. **Kelm M**. Nitric oxide metabolism and breakdown. *Biochim Biophys Acta* 1999;1411:273-89.

59. **Doyle MP, Hoekstra JW**. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem* 1981;14:351-8.

60. **Doyle MP, Pickering RA, DeWeert TM, Hoekstra JW, Pater D**. Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. *J Biol Chem* 1981;256:12393-8.

61. **Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE**. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A* 1993;90:8103-7.

62. **Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO, 3rd, Schechter AN, Gladwin MT**. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation* 2007;116:1821-31.

63. **Tannenbaum SR**. Nitrate and nitrite: origin in humans. *Science* 1979;205:1332, 1334-7.

64. **Green LC, Ruiz de Luzuriaga K, Wagner DA, Rand W, Istfan N, Young VR, Tannenbaum SR**. Nitrate biosynthesis in man. *Proc Natl Acad Sci U S A* 1981;78:7764-8.

65. **Walker R**. The metabolism of dietary nitrites and nitrates. *Biochem Soc Trans* 1996;24:780-5.

66. **Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Godecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, Kelm M**. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med* 2003;35:790-6.

67. **Crawford JH, Chacko BK, Pruitt HM, Piknova B, Hogg N, Patel RP**. Transduction of NO-bioactivity by the red blood cell in sepsis: novel mechanisms of vasodilation during acute inflammatory disease. *Blood* 2004;104:1375-82.

68. **Wennmalm A, Benthin G, Edlund A, Kieler-Jensen N, Lundin S, Petersson AS, Waagstein F**. Nitric oxide synthesis and metabolism in man. *Ann N Y Acad Sci* 1994;714:158-64.

69. **Lundberg JO, Govoni M**. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 2004;37:395-400.

70. **Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP**. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci* 2005;113:14-9.

71. **Spiegelhalder B, Eisenbrand G, Preussmann R**. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food Cosmet Toxicol* 1976;14:545-8.

72. **Bos PM, Van den Brandt PA, Wedel M, Ockhuizen T**. The reproducibility of the conversion of nitrate to nitrite in human saliva after a nitrate load. *Food Chem Toxicol* 1988;26:93-7.

73. **Li H, Duncan C, Townend J, Killham K, Smith LM, Johnston P, Dykhuizen R, Kelly D, Golden M, Benjamin N, Leifert C**. Nitrate-reducing bacteria on rat tongues. *Appl Environ Microbiol* 1997;63:924-30.

74. **Jansson EÅ, Huang L, Malkey R, Govoni M, Nihlen C, Olsson A, Stensdotter M, Petersson J, Holm L, Weitzber E, Lundberg JO.** A mammalian functional nitrate reductase activity that regulates nitrite and nitric oxide homeostasis, 2008. *Submitted Manuscript*

75. **Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A**. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci U S A* 2004;101:13683-8.

76. **Kozlov AV, Staniek K, Nohl H**. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett* 1999;454:127-30.

77. **Rassaf T, Flogel U, Drexhage C, Hendgen-Cotta U, Kelm M, Schrader J**. Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetics and function. *Circ Res* 2007;100:1749-54.

78. **Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM, Gladwin MT**. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration *Circ Res* 2007;100:354-61.

79. **Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, Benjamin N**. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* 1995;1:546-51.

80. **Weitzberg E, Lundberg JO**. Nonenzymatic nitric oxide production in humans. *Nitric Oxide* 1998;2:1-7.

81. **Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M**. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? *Free Radic Biol Med* 2005;39:668-81.

82. **Schorah CJ, Sobala GM, Sanderson M, Collis N, Primrose JN**. Gastric juice ascorbic acid: effects of disease and implications for gastric carcinogenesis. *Am J Clin Nutr* 1991;53:287S-293S.

83. **Bartholomew B, Hill MJ**. The pharmacology of dietary nitrate and the origin of urinary nitrate. *Food Chem Toxicol* 1984;22:789-95.

84. **van Maanen JM, van Geel AA, Kleinjans JC**. Modulation of nitrate-nitrite conversion in the oral cavity. *Cancer Detect Prev* 1996;20:590-6.

85. **White JW, Jr.** Relative significance of dietary sources of nitrate and nitrite. *J Agric Food Chem* 1975;23:886-91.

86. **Vogtmann H, Biedermann R**. The nitrate story--no end in sight. *Nutr Health* 1985;3:217-39.

87. Food, Nutrition, Physical activity and the Prevention of Cancer: a Global Perspective. World Cancer research Fund, American Institute for Cancer research. Washington D.C., 2007.

88. **Martinoia E**. Vacuoles as storage compartments for nitrate in barley leaves. *Nature* 1981;289:292-294.

89. **Chung SY, Kim JS, Kim M, Hong MK, Lee JO, Kim CM, Song IS**. Survey of nitrate and nitrite contents of vegetables grown in Korea. *Food Addit Contam* 2003;20:621-8.

90. **Manassaram DM, Backer LC, Moll DM**. A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes. *Environ Health Perspect* 2006;114:320-7.

91. **Pierson MD, Smoot LA**. Nitrite, nitrite alternatives, and the control of Clostridium botulinum in cured meats. *Crit Rev Food Sci Nutr* 1982;17:141-87.

92. **Sen NP, Smith DC, Schwinghamer L**. Formation of N-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Food Cosmet Toxicol* 1969;7:301-7.

93. **Low H**. Nitroso compounds: safety and public health. *Arch Environ Health* 1974;29:256-60.

94. **McKnight GM, Duncan CW, Leifert C, Golden MH**. Dietary nitrate in man: friend or foe? *Br J Nutr* 1999;81:349-58.

95. **Ja Kim H, Lee SS, Choi BY, Kim MK**. Nitrate intake relative to antioxidant vitamin intake affects gastric cancer risk: a case-control study in Korea. *Nutr Cancer* 2007;59:185-91.

96. **Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O**. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 1996;93:13176-81.

97. **Carlstrom M, Brown RD, Edlund J, Sallstrom J, Larsson E, Teerlink T, Palm F, Wahlin N, Persson AE**. Role of nitric oxide deficiency in the development of hypertension in hydronephrotic animals. *Am J Physiol Renal Physiol* 2008;294:F362-70.

98. **Allen A, Flemstrom G**. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *Am J Physiol Cell Physiol* 2005;288:C1-19.

99. **Crissinger KD, Kvietys PR, Granger DN**. Pathophysiology of gastrointestinal mucosal permeability. *J Intern Med Suppl* 1990;732:145-54.

100. **Jenkins RT, Bell RA**. Molecular radii of probes used in studies of intestinal permeability. *Gut* 1987;28:110-1.

101. **Ahrens FA, Aronson AL**. A comparative study of the toxic effects of calcium and chromium chelates of ethylenediaminetetraacetate in the dog. *Toxicol Appl Pharmacol* 1971;18:10-25.

102. **Volf V, Vladar M, Seidel A**. Distribution of labelled calcium-, yttriumand chromium-chelates of EDTA in rats. *Arch Int Pharmacodyn Ther* 1971;190:110-23.

103. **Bjarnason I, Smethurst P, Levi AJ, Peters TJ**. Intestinal permeability to 51Cr-EDTA in rats with experimentally induced enteropathy. *Gut* 1985;26:579-85.

104. **Nylander O, Sababi M, Bark J**. Characterization of 51Cr-EDTA as a marker of duodenal mucosal permeability. *Acta Physiol Scand* 1991;143:117-26.

105. **Nylander O, Kvietys P, Granger DN**. Effects of hydrochloric acid on duodenal and jejunal mucosal permeability in the rat. *Am J Physiol Gastrointest Liver Physiol* 1989;257:G653-60.

106. **Nilsson GE, Tenland T, Oberg PA**. Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. *IEEE Trans Biomed Eng* 1980;27:597-604.

107. **Holm-Rutili L, Berglindh T**. Pentagastrin and gastric mucosal blood flow. *Am J Physiol Gastrointest Liver Physiol* 1986;250:G575-80.

108. **Kvietys PR, Shepherd AP, Granger DN**. Laser-Doppler, H2 clearance, and microsphere estimates of mucosal blood flow. *Am J Physiol* 1985;249:G221-7.

109. **Wan MX, Riaz AA, Schramm R, Wang Y, Vestweber D, Menger MD, Thorlacius H**. Leukocyte rolling is exclusively mediated by P-selectinin colonic venules. *Br J Pharmacol* 2002;135:1749-56.

110. **Walter UM, Ayer LM, Wolitzky BA, Wagner DD, Hynes RO, Manning AM, Issekutz AC**. Characterization of a novel adhesion function blocking monoclonal antibody to rat/mouse P-selectin generated in the P-selectin-deficient mouse. *Hybridoma* 1997;16:249-57.

111. **Ma L, Raycroft L, Asa D, Anderson DC, Geng JG**. A sialoglycoprotein from human leukocytes functions as a ligand for P-selectin. *J Biol Chem* 1994;269:27739-46.

112. **Panes J, Perry MA, Anderson DC, Manning A, Leone B, Cepinskas G, Rosenbloom CL, Miyasaka M, Kvietys PR, Granger DN**. Regional differences in constitutive and induced ICAM-1 expression in vivo. *Am J Physiol Gastrointest Liver Physiol* 1995;269:H1955-64.

113. **Sobko T, Reinders C, Norin E, Midtvedt T, Gustafsson LE, Lundberg JO**. Gastrointestinal nitric oxide generation in germ-free and conventional rats. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G993-7.

114. **Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourd'Heuil D, Kelm M**. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *Faseb J* 2002;16:1775-85.

115. **Gladwin MT, Wang X, Reiter CD, Yang BK, Vivas EX, Bonaventura C, Schechter AN**. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O2/NO-linked allosteric function. *J Biol Chem* 2002;277:27818-28.

116. **Bryan NS, Grisham MB**. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic Biol Med* 2007;43:645-57.

117. **Zetterquist W, Pedroletti C, Lundberg JO, Alving K**. Salivary contribution to exhaled nitric oxide. *Eur Respir J* 1999;13:327-33.

118. **Takahashi S, Keto Y, Fujita H, Muramatsu H, Nishino T, Okabe S**. Pathological changes in the formation of Helicobacter pylori-induced gastric lesions in Mongolian gerbils. *Dig Dis Sci* 1998;43:754-65.

119. **Bradley PP, Priebat DA, Christensen RD, Rothstein G**. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;78:206-9.

120. **Liu X, Miller MJ, Joshi MS, Thomas DD, Lancaster JR, Jr.** Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci U S A* 1998;95:2175-9.

121. **Fritsch P, de Saint Blanquat G, Derache R**. [Absorption kinetics of nitrate and nitrite in the intestine of rats (author's transl)]. *Toxicol Eur Res* 1979;2:141-7.

122. **Bjorne HH, Petersson J, Phillipson M, Weitzberg E, Holm L, Lundberg JO**. Nitrite in saliva increases gastric mucosal blood flow and mucus thickness. *J Clin Invest* 2004;113:106-14.

123. **Bjorne H, Govoni M, Tornberg DC, Lundberg JO, Weitzberg E**. Intragastric nitric oxide is abolished in intubated patients and restored by nitrite. *Crit Care Med* 2005;33:1722-1727.

124. **Synnerstad I, Johansson M, Nylander O, Holm L**. Intraluminal acid and gastric mucosal integrity: the importance of blood-borne bicarbonate. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G121-9.

125. **Foster MW, McMahon TJ, Stamler JS**. S-nitrosylation in health and disease. *Trends Mol Med* 2003;9:160-8.

126. **Keshive M, Singh S, Wishnok JS, Tannenbaum SR, Deen WM**. Kinetics of S-nitrosation of thiols in nitric oxide solutions. *Chem Res Toxicol* 1996;9:988-93.

127. **Richardson G, Hicks SL, O'Byrne S, Frost MT, Moore K, Benjamin N, McKnight GM**. The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans. *Nitric Oxide* 2002;7:24-9.

128. **Body SC, Sasame HA, Body MR**. High concentrations of glutathione in glandular stomach: possible implications for carcinogenesis. *Science* 1979;205:1010-2.

129. **Reid CJ, Harris A**. Developmental expression of mucin genes in the human gastrointestinal system. *Gut* 1998;42:220-6.

130. **Nam SY, Kim N, Lee CS, Choi KD, Lee HS, Jung HC, Song IS**. Gastric mucosal protection via enhancement of MUC5AC and MUC6 by geranylgeranylacetone. *Dig Dis Sci* 2005;50:2110-20.

131. **Nordman H, Davies JR, Lindell G, de Bolos C, Real F, Carlstedt I**. Gastric MUC5AC and MUC6 are large oligomeric mucins that differ in size, glycosylation and tissue distribution. *Biochem J* 2002;364:191-200.

132. **Bjorne H, Weitzberg E, Lundberg JO**. Intragastric generation of antimicrobial nitrogen oxides from saliva--physiological and therapeutic considerations. *Free Radic Biol Med* 2006;41:1404-12.

133. **Duncan C, Li H, Dykhuizen R, Frazer R, Johnston P, MacKnight G, Smith L, Lamza K, McKenzie H, Batt L, Kelly D, Golden M, Benjamin N, Leifert C**. Protection against oral and gastrointestinal diseases: importance of dietary nitrate intake, oral nitrate reduction and enterosalivary nitrate circulation. *Comp Biochem Physiol A Physiol* 1997;118:939-48.

134. **Dykhuizen RS, Fraser A, McKenzie H, Golden M, Leifert C, Benjamin N**. Helicobacter pylori is killed by nitrite under acidic conditions. *Gut* 1998;42:334-7.

135. **Dykhuizen RS, Frazer R, Duncan C, Smith CC, Golden M, Benjamin N, Leifert C**. Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob Agents Chemother* 1996;40:1422-5.

136. **Anyim M, Benjamin N, Wilks M**. Acidified nitrite as a potential antifungal agent. *Int J Antimicrob Agents* 2005;26:85-7.

137. **Fang FC**. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 1997;99:2818-25.

138. **Wallace JL, Reuter B, Cicala C, McKnight W, Grisham M, Cirino G**. A diclofenac derivative without ulcerogenic properties. *Eur J Pharmacol* 1994;257:249-55.

139. **Gambero A, Becker TL, Zago AS, de Oliveira AF, Pedrazzoli J, Jr.** Comparative study of anti-inflammatory and ulcerogenic activities of different cyclooxygenase inhibitors. *Inflammopharmacology* 2005;13:441-54.

140. **Birkett D, Silen W**. Proceedings: The pH-dependent effect of sodium taurocholate on increasing gastric mucosal permeability. *Gut* 1974;15:336.

141. **Larauche M, Anton PM, Garcia-Villar R, Theodorou V, Frexinos J, Bueno L, Fioramonti J**. Protective effect of dietary nitrate on experimental gastritis in rats. *Br J Nutr* 2003;89:777-86.

142. **Larauche M, Bueno L, Fioramonti J**. Effect of dietary nitric oxide on gastric mucosal mast cells in absence or presence of an experimental gastritis in rats. *Life Sci* 2003;73:1505-16.

143. **Miyoshi M, Kasahara E, Park AM, Hiramoto K, Minamiyama Y, Takemura S, Sato EF, Inoue M**. Dietary nitrate inhibits stress-induced gastric mucosal injury in the rat. *Free Radic Res* 2003;37:85-90.

144. **Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ**. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest* 2005;115:1232-40.

145. **Winter JW, Paterson S, Scobie G, Wirz A, Preston T, McColl KE**. N-nitrosamine generation from ingested nitrate via nitric oxide in subjects with and without gastroesophageal reflux. *Gastroenterology* 2007;133:164-74.

146. **Clemons NJ, McColl KE, Fitzgerald RC**. Nitric oxide and acid induce double-strand DNA breaks in Barrett's esophagus carcinogenesis via distinct mechanisms. *Gastroenterology* 2007;133:1198-209.

147. **Rao DN, Sanghvi LD, Desai PB**. Epidemiology of esophageal cancer. *Semin Surg Oncol* 1989;5:351-4.

148. **Hirayama T**. Mortality in Japanese with life-styles similar to Seventh-Day Adventists: strategy for risk reduction by life-style modification. *Natl Cancer Inst Monogr* 1985;69:143-53.

149. **Hawkey CJ, Langman MJ**. Non-steroidal anti-inflammatory drugs: overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. *Gut* 2003;52:600-8.

150. **Allison MC, Howatson AG, Torrance CJ, Lee FD, Russell RI**. Gastrointestinal damage associated with the use of nonsteroidal antiinflammatory drugs. *N Engl J Med* 1992;327:749-54.

151. **Wolfe MM, Lichtenstein DR, Singh G**. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. *N Engl J Med* 1999;340:1888-99.

152. **Wallace JL**. Nonsteroidal anti-inflammatory drugs and gastroenteropathy: the second hundred years. *Gastroenterology* 1997;112:1000-16.

153. **Hawkey CJ**. Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* 2000;119:521-35.

154. **Abraham NS, Graham DY**. NSAIDs and gastrointestinal complications: new clinical challenges. *Expert Opin Pharmacother* 2005;6:2681-9.

155. **Holm L, Phillipson M, Perry MA**. NO-flurbiprofen maintains duodenal blood flow, enhances mucus secretion contributing to lower mucosal injury. *Am J Physiol Gastrointest Liver Physiol* 2002;283:G1090-7.

156. **Fiorucci S, Del Soldato P**. NO-aspirin: mechanism of action and gastrointestinal safety. *Dig Liver Dis* 2003;35 Suppl 2:S9-19.

157. **Scott PA, Kingsley GH, Smith CM, Choy EH, Scott DL**. Non-steroidal anti-inflammatory drugs and myocardial infarctions: comparative systematic review of evidence from observational studies and randomised controlled trials. *Ann Rheum Dis* 2007;66:1296-304.

158. **Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C**. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ* 2006;332:1302-8.

159. **Gago B, Lundberg JO, Barbosa RM, Laranjinha J**. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. *Free Radic Biol Med* 2007;43:1233-42.

Additional Materials- for a greener diet

Min avhandling fokuserar ju på de positiva effekter som nitrat i grönsaker har på skyddet av magslemhinnan. Mat är en stor passion i mitt liv. Jag älskar att både äta och laga god mat och i min matlagning spelar grönsaker en stor och viktig roll. Med devisen ”för en grönare diet” vill jag därför som tillägg till mina forskningsresultat ta tillfället i akt att bjuda på några av mina guldkorn bland recept. Naturligtvis mycket grönt och mycket nitrat, med tillagningsmetoder där näringsämnen och nitraten stannar i grönsakerna. Drick gärna ett glas rött till, det finns visat att rödvin ökar bildningen av skyddande kväveoxid i magsäcken om det intas i samband med nitrat![159] Laga och njut, med riktigt gott samvete! Alla recept är för 4 personer.

Krämig Kräftsallad med Selleri och Avokado

Kräftstjärtar, färsk dill, citron, mjuk avokado och krispig selleri passar oerhört bra ihop. Den diskreta basilikasmaken ger också en extra touch. Snabbt och mycket enkelt.

1 burk (170g) kräftstjärtar
1 selleristjälk skuren i små bitar
½ dl finhackad färsk dill
2 msk majonäs
Saft och rivet skal från ½ citron
Flingsalt och svartpeppar från kvarn
½ finhackad rödlök
1 avokado skuren i bitar
1 dl lättcremefraiche
2 msk basilikaolja

Blanda allt utom avokadon (tillsätts innan servering) och låt stå och dra i några timmar. Perfekt på toast som förrätt eller som fyllning i bakad potatis!

Ångkokta Juliennegrönsaker

Jag älskar Juliennegrönsaker, eller grönsaker skurna i tunna, späda stavar. Ångkokas de behålls all krispighet. Enkelt gott och mycket vackert. Jag brukar kombinera följande grönsaker;

2 stora morötter (gärna både gul och orange)
1 röd paprika
1 purjolök
1 gul paprika
100g sockerärtor
2 stjälkar selleri

Skär allt i 5 cm långa och mycket tunna stavar. Absolut snyggast är att skära för hand. Lägg grönsakerna i kallt vatten i kylen i några timmar så de drar åt sig vatten och blir härligt krispiga. Ångkoka sedan med flingsalt i 3 minuter och servera de varma krispiga grönsakerna till fisk eller grillat kött!

Rostade rotsaker

Jag serverar ofta rostade rotsaker på höst och vinterhalvåret. En idealiskt rätt till alla slags kötträtter eftersom vare sig separata grönsaker eller sås faktiskt inte behövs.

6 stora fasta potatisar
1 palsternacka
4 rödbetor
½ dl god olivolja
Flingsalt och svartpepper från kvarn
2 stora morötter
½ rotselleri
1 purjolök
½ msk timjan

Skala och tärna rotsakerna och purjolök i stora kuber. Blanda allt i en stor ungssäker form med olja och kryddor. Rosta i ugnen 175° i upp till en timma. Vill man så kan man smula chevre över grönsakerna och chevregratinera de sista 15 minuterna.

Spenatlasange

En mustig köttfärssås med morot och rotselleri perfekt som bas i en lasagne, eller som den är till nykokt spagetti.

500g nötfärs
En liten bit (100g) rotselleri
2 gula lökar
2 msk kinesisk soja
150 g färsk babyspenat
2 dl rött vin
Lasagneplattor (vanliga och spenat)
3 msk vetemjöl
Olivolja att steka i
1 tsk torkad basilika
Salt, och svartpeppar från kvarn
2 morötter
1 burk krossade tomater
2 msk tomatpuré
2 vitlöksklyftor
1 köttbuljongtärning
1 grönsaksbuljongtärning
4 dl mjölk
100g färsk riven parmesan
1 msk socker
1 tsk italiensk salladsdressingskrydda

Hacka lök och riv morot och rotselleri grovt. Fräs köttfärs och lök, tillsätt den rivna moroten och sellerin. Häll över krossade tomater, tomatpure, soja, riven vitlök, buljongtärningar, kryddor och rött vin. Låt koka ihop i minst 30 minuter på låg värme och späd eventuellt med lite vatten. Smaka av med salt, socker och peppar. Gör under tiden bechamelsåsen; Blanda mjöl och fett i botten på en kestrull fyll på med mjölk och koka upp och rör tills det tjocknar. Riv osten

och blanda hälften i berchamelsåsen. Varva de olika lasagneplattorna med köttfärssås och färsk spenat. Bred såsen och riv extra ost mellan lagren. Gratinera i 30 minuter i 175°C.

Ljummen kycklingsallad med tagliatelle

Stekt kycklingfilé med mycket oregano och citrondressing tillsammans med nykokt pasta och alla grönsaker gör den här salladen till en suverän vårrätt med medelhavskänsla.

4 kycklingfiléer
2 paprikor, blandade färger
½ gurka
1 dl kalamataoliver
500 g tagliatelle (gärna med spenat)
250 g körsbärstomater
200 g fetaost
en ask ruccolasallad
4 msk färsk, grovhackad oregano eller 1 msk torkad, olivolja, salt och svartpeppar från kvarn

Dressing

½ dl olivolja
1 tsk torkad oregano
Salt och svartpeppar från kvarn
½ dl pressad citronsaft

Blanda skuren gurka och paprika med ruccola, kuber av fetaost, oliver och tomater på ett stort fat. Stek kycklingen i bitar med oregano, salt och peppar. Blanda ingredienserna till dressingen. Koka pastan al dente och häll av vattnet. Blanda ner dressingen i den varma pastan och stjälp över grönsakerna. Blanda ordentligt med kycklingen och servera ljummen.

Rabarberkaka

En mjuk mandelkaka med tydlig rabarbersmak. Enkelt och snabbt att göra och mycket god.

100 g smör
4 ägg
½ dl vetemjöl
200 g sötmandel
2 ½ dl socker
1 rabarberstång (färsk eller frusen)

Skär rabarbern i tunna slantar. Skålla, skala (eller köp skalad mandel) och mal mandeln. Vispa ägg och socker pösigt. Rör ner malda mandlar, smör och vetemjöl i socker och äggsmeten. Häll smeten i en smord och bröad form med löstagbar kant. Fördela rabarberslantarna överst. Grädda i nedre delen av ugnen 45 min i 175°. Låt kakan svalna och pudra över florsocker

Uppifrån från vänster till höger; Juliennegrönsaker, spenatlasagne, rotsaker, kräftsallad på toast, rabarberkaka och ljummen kycklingsallad med tagliatelle.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 328

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-8624

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2008