DIETARY NITRATE INCREASES GASTRIC MUCOSAL BLOOD FLOW AND MUCOSAL DEFENSE

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Running Head: DIETARY NITRATE IN GASTRIC MUCOSAL DEFENSE

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Abbreviations:

HCl Hydrochloric acid
LDF Laser-Doppler flowmetry
NSAID Nonsteroidal antiinflammatory drug
PU Perfusion unit
ppb Parts per billion
Salivary nitrate from dietary or endogenous sources is reduced to nitrite by oral bacteria. In the acidic stomach nitrite is further reduced to bioactive nitrogen oxides including NO. In this study we investigated the gastro-protective role of nitrate intake and of luminally applied nitrite against provocation with diclofenac and taurocholate. Mucosal permeability (\(^{51}\text{Cr-EDTA} \) clearance) and gastric mucosal blood flow (laser-Doppler flowmetry) were measured in anesthetized rats, either pretreated with nitrate in the drinking water or given acidified nitrite luminally. Diclofenac was given i.v. and taurocholate luminally to challenge the gastric mucosa. Luminal NO and nitrite content in the gastric mucus were determined by chemiluminescence. The effect of luminal administration of acidified nitrite on the mucosal blood flow was also investigated in eNOS-deficient mice. Rats pretreated with nitrate or given nitrite luminally had higher gastric mucosal blood flow than controls. Permeability increased more during the provocation in the controls than in the nitrate- and nitrite-treated animals. Dietary nitrate increased luminal NO levels 50 times compared to controls. Nitrate intake also resulted in nitrite accumulation in the loosely adherent mucus layer and after removal of this mucus layer blood flow was reduced. Nitrite administrated luminally in eNOS-/- mice increased mucosal blood flow. We conclude that dietary nitrate as well as direct luminal application of acidified nitrite decrease diclofenac- and taurocholate-induced mucosal damage. The gastro-protective effect likely involves a higher mucosal blood flow caused by non-enzymatic NO production. These data suggest an important physiological role of nitrate in the diet.

Keywords: Laser-doppler Flowmetry, \(^{51}\text{Cr-EDTA} \) clearance, Nitrite, Rats, eNOS-/-, Gastric mucus
INTRODUCTION

The role of nitrate in our diet has been a subject of great controversy over the years. The anxiety concerning nitrate consumption is related to the presumed association with gastrointestinal cancer. Today however there are more studies contradicting than supporting such a correlation (36). Several recent studies have in fact suggested that dietary nitrate has a gastro-protective role (6, 16, 31, 32, 35, 38). Most of the nitrate in our diet originates from green leafy vegetables, a natural source of food for humans. Bacteria in the oral cavity reduce parts of the nitrate to nitrite, and human saliva therefore contains both these anions (49). A large amount of nitric oxide (NO) is produced in the upper gut when swallowed nitrite react with hydrogen ions (6, 35, 37).

The gastric mucosa is covered with a continuous viscous mucus layer, which acts as an important physical pre-epithelial level of the gastric mucosal defense (4, 48). The mucus also provides a chemical barrier, where the epithelium secretes bicarbonate into the mucus and neutralizes back-diffused acid (3, 43). Earlier studies have shown that acidified nitrite and NO donors increase mucus thickness in the rat (9-11). The second layer of defense, the gastric epithelial barrier, consists of tight junctions. Noxious agents have been shown to disrupt this barrier and increase the clearance of large molecules from blood to lumen (14, 26). Different research groups have shown that inhibition of endogenous NO production leads to an increase in mucosal permeability (21, 28). Several studies have also shown that nonsteroidal antiinflammatory drugs (NSAIDs) increase the mucosal permeability (15).
There is a current opinion that an adequate mucosal blood flow plays an important role in maintaining the mucosal integrity. Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of the gastric mucosa against injury (1, 12, 20, 27, 33, 42, 47, 52). The blood flow carries bicarbonate to neutralize acid and removes cellular waste products. We have recently shown that acidified nitrite and acidified nitrite-rich saliva triggers this defense mechanism and increases the gastric mucosal blood flow (9).

In this study we addressed the question whether dietary nitrate and locally administered nitrite might upregulate the mucosal blood flow to such an extent as to protect the gastric mucosa from injury. We wanted to investigate the role of ingested nitrate and its subsequent products nitrite and NO in protecting the stomach from injury produced by NSAID and bile salts. For this purpose we measured the mucosal blood flow and epithelial permeability in vivo in rats and mice and challenged the gastric mucosa by giving the rats the NSAID diclofenac intravenously in combination with luminal administration of the bile salt taurocholate.
METHODS

Animal Preparation

All experiments were approved by the Uppsala University or Karolinska Institute Ethical Committee for Animal Experiments.

Rats

Male Sprague-Dawley rats (B&K Universal, Stockholm, Sweden), weighing 170-280 g, were kept under standardized conditions of temperature (21-22°C) and illumination (12:12-h light/darkness). 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 anesthetized with 120 mg kg⁻¹ body weight of 5-ethyl-5-(1-methylpropyl)-2-thiobutabarbitral sodium (Inactin®, Sigma St. Louis, MO) injected intraperitoneally. 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. The right femoral vein was cannulated for continuous infusion of a modified Ringer’s solution (25 mM NaHCO₃, 120 mM NaCl, 2.5 mM KCl, and 0.75 mM CaCl₂) at a rate of 1.0 ml h⁻¹ and the left femoral vein was cannulated for drug infusions.

Mice

Male C57BL/6 mice (B&K Universal, Stockholm, Sweden) and male mice deficient in endothelial nitric oxide synthase (eNOS) (The Jackson Laboratory, Maine) weighing 24-37 g
were used for studies of mucosal blood flow in some experiments. The eNOS-/ mice (background C57/BL/6J-Nos3^tm1Unc) were generated by gene targeting in embryonic stem cells as previously described.\(^{(44)}\) The mice were kept under the same standardized conditions as the rats, but they were not fasted before the experiments. The mice were anesthetized by inhalation of ~2.2% isoflurane (Forene\(^{®}\), Abbot Scandinavia AB, Kista, Sweden) via an anesthesia unit (Univentor 400 Anesthesia 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. A pulled PE-200 catheter containing heparin (12.5 IU ml\(^{-1}\)) dissolved in isotonic saline was placed in the left carotid artery and connected to a pressure transducer to monitor blood pressure. The jugular vein was cannulated (pulled PE-200) for continuous infusion of a modified Ringer’s solution at a rate of 0.35 ml h\(^{-1}\).

**Tissue Preparation**

The abdomen was opened through a midline incision. The gastro-hepatic ligaments were cut and the stomach was gently exteriorized and kept moistened and warm with 37°C saline during the preparation procedure. The short gastric artery and vein were ligated and cut. An incision was made along the greater curvature in the forestomach. The animal was placed on its left side on a Lucite microscope stage on a heating pad, and the stomach was 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 was fitted over the mucosa, exposing the mucosa through the hole (exposed area: rats 0.8 cm\(^2\), mouse 0.2 cm\(^2\)). The junction was sealed with silicon grease (Dow Corning high vacuum grease. Dow Corning GmbH, Weisbaden, Germany). The chamber was 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 (22, 25).
**Mucosal Permeability**

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. This dose was followed by a continuous infusion of $^{51}$Cr-EDTA (in the Ringer’s solution) at a rate of 1.0 ml h$^{-1}$ (10-30 µCi h$^{-1}$) 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 the volume loss was compensated for by an injection of an equal volume of 7% bovine 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 was made from a stock solution of 1 M HCl (Titrisol® Merck KGaA, Darmstadt, Germany) and adjusted to isotonicity by 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. If there was a difference of <10% between the consecutive blood plasma counts, a mean plasma cpm per ml value was calculated and used for all clearance samples. If there was a difference of >10% deviation between the blood plasma counts, the activity was plotted against time, and a straight line was drawn between the 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.

**Mucosal Blood Flow**

Laser-Doppler flowmetry (LDF) (PeriFlux 4001 Master and PeriFlux Pf 3; Perimed, Stockholm, Sweden) was used for blood flow measurements in all experiments. The nature of
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the Doppler shift from an illuminated tissue depends on the velocity and number of moving red blood cells (40). The laser light (wavelength 635 nm, helium neon laser) is guided to the tissue by an optic fiber. The backscattered light is picked up by a pair of fibers separated by a distance of 0.25-0.5 mm. With this technique, blood flow is determined as a voltage output and expressed as perfusion units (PU). The blood flow was 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. This technique for measuring the gastric mucosal blood flow with laser Doppler flowmetry has been well evaluated previously (24, 29).

**Experimental Protocol**

**Diclofenac and taurocholate: Effects on mucosal permeability and blood flow –Control group**

Blood to lumen clearance of $^{51}$Cr-EDTA and mucosal blood flow were studied before, during, and after a challenge to the gastric mucosa in rats. The animals were allowed at least 1 h to stabilize after the operation, so that the systemic blood pressure and luminal blood flow were at steady state for at least 20 min before the experiment began. The experiments lasted for 170 min and the solutions to which the gastric mucosa was exposed to were changed every 10 min. The pH in the removed solutions was measured with a pH electrode (Autobürette ABUp1; Radiometer, Copenhagen, Denmark) and the applied solution was saved for measurements of $^{51}$Cr-EDTA activity. The mucosa was exposed to saline for the first 20 min, then to 10 mM isotonic HCl for 130 min, and finally to 20 min of saline. Forty minutes after the start diclofenac (Voltaren$^{®}$ Novartis Sverige AB, Täby, Sverige) was given intravenously at a dose of 5 mg kg$^{-1}$, and 30 min later 20 mM taurocholate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to the acidic luminal solution for 40 min (Fig.1). Mucosal blood flow and
mean arterial blood pressure (MAP) were recorded continuously during the experiments, and vascular resistance was calculated as the ratio of MAP to mucosal blood flow \((n = 7)\).

**Nitrate pretreatment**

To investigate whether a high nitrate diet had gastro-protective effects, one group of rats was pretreated with sodium nitrate 0.8 mmol kg\(^{-1}\) daily in the drinking water for 7 days before the experiments \((n = 5)\). The experimental protocol described above was then followed (Fig. 1).

**Nitrite administered luminally**

To mimic the effect of swallowing nitrite-rich saliva, another group of rats \((n = 8)\) was submitted to continuous luminal administration of sodium nitrite (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to the mucosa 30 min after the start of the experiment. Fifty µl of a 100 mM sodium nitrite solution was first applied to the mucosal chamber, containing 5 ml of HCl or saline, resulting in a concentration of 1.0 mM sodium nitrite. Since the system is open to air and the NO that forms from nitrite in the acidic environment declines rapidly, sodium nitrite was thereafter slowly and continuously applied to the mucosa over a period of 10 min \((0.5 \text{ ml of a 10 mM sodium nitrite solution was infused to the mucosal chamber filled with 5 ml HCl or saline, which results in 1.0 mM nitrite in the chamber over the 10 min})\). This procedure was repeated every 10 min, since the luminal solution was removed for analyzes, throughout the experiment, as described (Fig. 1).

**Effects of nitrate pretreatment on intragastric NO generation**

In 12 additional rats, intragastric NO levels were measured. The rats were given sodium nitrate \((0.8 \text{ mmol kg}^{-1} \text{ daily}, n = 5)\) in the drinking water for 7 days prior to the experiment. The control animals received ordinary drinking water \((n = 7)\). The rats were anesthetized and a laparotomy was performed after they had been fasted overnight. A thin 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 seconds the air was aspirated and immediately injected into a chemiluminescence NO analyzer (Aerocrine AB, Stockholm, Sweden).

**Effects of nitrate pretreatment on mucus nitrite content**

In another 22 rats (nitrate pretreated \( n = 10 \), controls \( n = 12 \)) nitrite levels in the gastric mucus were measured. Before the removal of the mucus layers, gastric mucosal blood flow was measured for 30 min. The gastric mucosa was covered with warm (37°C) saline throughout the experiments. The loosely adherent mucus layer was removed under a microscope by suction with a catheter connected to a syringe, and saved for analyzés. The remaining, firmly adherent mucus layer was scraped off the mucosa with a scalpel. The total volume of the different mucus layers was estimated, from measurements of mucus thickness (5) and exposed area, and the samples were stored at -70°C. For measurements of the nitrite content in the mucus, we used a chemiluminescence method described in detail by Feelish et al (17).

**Effects of removal of loosely adherent mucus on mucosal blood flow**

In nitrate-pretreated rats (\( n = 7 \)) and controls (\( n = 7 \)) gastric mucosal blood flow was measured for 30 min, after which the loosely adherent mucus layer was removed as described above. The blood flow was then followed for another 20 minutes. The gastric mucosa was covered with warm (37°C) saline throughout the experiments.

**Effects of nitrite on mucosal blood flow in eNOS-deficient mice**

The effect of luminal administration of acidified nitrite on the mucosal blood flow in C57BL/6 mice (\( n = 4 \)) and in eNOS-deficient mice (\( n = 4 \)) was investigated. The gastric mucosa was
Petersson first covered with 3 ml warm saline for 20 min, followed by a 10 min period of 1.0 mM sodium nitrite in 3 ml HCl pH 3, and then another 20 min with saline. Mucosal blood flow and mean arterial blood pressure were continuously recorded during the experiments, and mucosal vascular resistance was calculated as the ratio of MAP to mucosal blood flow.

Statistics

The results are expressed as means ± SE. Differences between groups of animals were evaluated by one-way ANOVA and within groups by ANOVA for repeated measurements, followed by Fisher’s protected least significant difference test. Differences were regarded as significant if $P < 0.05$. All statistical calculations were performed with Statview II SE Graphics software (Abacus Concepts Inc., Berkeley, CA).
RESULTS

Effects of diclofenac and taurocholate on mucosal permeability

The mucosal permeability, expressed as $^{51}$Cr-EDTA clearance (ml min$^{-1} 100$ g tissue$^{-1}$) did not differ between the groups (control, nitrate-pretreated and nitrite luminally) during the first saline period, and increased during the diclofenac and taurocholate provocation in all three groups (Fig. 2). In the control group, however, clearance increased to $0.92 \pm 0.12$ ml min$^{-1} 100g^{-1}$, which was a significantly greater increase than in the nitrate- and nitrite-treated rats, where clearance only rose to $0.56 \pm 0.14$ and $0.42 \pm 0.07$ ml min$^{-1} 100g^{-1}$ respectively.

Effects of diclofenac and taurocholate on blood flow

In the control group, the blood flow was stable during the saline and HCl periods, but increased significantly by $41 \pm 12 \%$ when taurocholate was applied luminally (Fig. 3). In the nitrite group the blood flow increased after luminal application of nitrite by $47 \pm 12 \%$ and increased even further when taurocholate was applied (Fig. 3).

Diclofenac resulted in a significant reduction in MAP 40 min after the bolus dose in all groups. In the nitrate-pretreated animals the blood flow decreased slowly over time in parallel with the MAP (from $181 \pm 32$ PU to $93 \pm 5$ PU), resulting in a constantly low vascular resistance, unaffected by taurocholate. There was no difference in MAP between the groups at the beginning of the experiments (control $101 \pm 4$ mmHg, nitrate-pretreated $103 \pm 6$ mmHg, and nitrite-treated $95 \pm 2$ mmHg) or at the end of the experiments (control $80 \pm 6$ mmHg, nitrate-pretreated $73 \pm 3$ mmHg, and nitrite-treated $77 \pm 4$ mmHg).

Generation of NO

Animals pretreated with nitrate for 7 days showed greatly increased luminal NO levels ($6280 \pm 1995$ ppb), compared to control animals ($127 \pm 40$ ppb) (Fig. 4).
Effects of nitrate pretreatment on mucus nitrite content

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) (Fig. 5). There was no difference in nitrite concentration in the firmly adherent mucus layer between the groups (control: 6.0 ± 1.3 µM nitrate pretreated: 6.1 ± 2.8 µM).

Effects of removal of loosely adherent mucus on mucosal blood flow

The nitrate-pretreated animals had significantly higher basal mucosal blood flow than the control animals. When the loosely adherent mucus layer was removed from the nitrate-pretreated animals, the mucosal blood flow decreased significantly from 214 ± 11 to 156 ± 12 PU. In the control animals removal of the mucus resulted in a slight, non significant reduction from 155 ± 27 to 127 ± 22 PU (Fig. 6).

Effect of nitrate in the drinking water on mucosal blood flow

In figure 7 are presented results of baseline mucosal blood flow from control and nitrate-pretreated groups in which blood flow was measured. The results, presented in perfusion units, show that nitrate-pretreated rats had a significantly higher mucosal blood flow than the controls.

Effects of nitrite on mucosal blood flow in eNOS-deficient mice

The eNOS-/− mice had a significantly higher initial MAP than the C57BL/6 (100 ± 12 vs 74 ± 2 mmHg) The blood flow increased in both the eNOS-/− mice and the control group after application of acidified nitrite luminally (by 17 ± 6 %, and 28 ± 7 %, respectively). There was no difference in the decrease in vascular resistance between the groups during the acidified nitrite period (eNOS-/−: -22 ±3 %; C57BL/6: -21 ± 4 %) (Fig. 8).
Since vegetables during the evolution have been a natural source of food for humans, it is an appealing hypothesis that this nitrate-rich diet participates in the physiology of the gastric mucosa.

We show here that high nitrate intake for one week clearly reduced the increase in permeability of the gastric mucosa that is normally induced by diclofenac and taurocholate. The protective effect is similar if nitrite is added continuously to the mucosa. Both nitrite administered luminally and dietary nitrate pretreatment result in an increased mucosal blood flow.

The nitrate dose used (0.8 mmol kg\textsuperscript{-1} per day or 68 mg kg\textsuperscript{-1} per day) was chosen to compensate for the differences in nitrate metabolism between rats and humans (19). In humans active transport generates a 10-fold higher concentration of nitrate in saliva than in blood (49). This gradient is not observed in the rats. This difference is also clear when one compares the resulting intragastric levels of NO in rats and humans after intake of nitrate. In rats NO levels can reach up to about 10 ppm as shown here and earlier (9, 45) while in humans the levels are at least 10-fold higher after ingesting the same amount of nitrate (8). Taken together it is very likely that the daily dose of nitrate used here compares to what is readily achievable in humans through a high intake of nitrate-rich vegetables such as spinach, lettuce or beetroot which typically contain between 1800 and 4200 mg/kg (13).

The high NO levels that we have found in the stomach as a consequence of the dietary nitrate intake confirm earlier reports (8, 9, 35, 37). The ingested nitrate is absorbed in the proximal small intestine and then concentrated in the salivary glands (49). Salivary nitrate is then reduced to nitrite by oral bacteria (16) and is further reduced to NO in the acidic stomach (6,
Petersson 35). The nitrate-reducing bacteria on rat tongues, for example *Staphylococcus sciuri* and *Staphylococcus intermedius* (34), have been shown to be essential in the generation of gastric NO in nitrate-pretreated animals (45). The gastric pH in fasted rats is as low as 1.4 (39) and under these conditions nitrite is rapidly reduced to NO via formation of nitrous acid (pKₐ=3.4) (51). The NO in the stomach lumen is not produced enzymatically, since the high NO level in the stomach following nitrate intake is unaltered by NOS inhibition (46).

When nitrite was applied directly to the acidic gastric mucosa, the blood flow increased immediately. This higher blood flow level was maintained as long as the nitrite was continuously added to the acidic environment. When the luminal HCl was switched 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. Under similar conditions we have previously shown a clear correlation between the increase in gastric mucosal blood flow and the generation of NO after applying acidified nitrite (9). The high blood flow in this case is probably a direct effect of the generated NO. NO can easily diffuse through the mucus and mucosa to the submucosal arterioles where it causes vasodilatation via guanylyl cyclase activation (9). To investigate the possibility that endogenous NO production via eNOS could be involved in this blood flow increase, we administered acidified nitrite to the gastric lumen in wild-type and eNOS-deficient mice. The results showed that acidified nitrite caused exactly the same effects, vasodilatation and a reduced vascular resistance, in wild-type and eNOS-deficient mice. This clearly demonstrates that eNOS is not involved in the vasodilator effect of luminally applied nitrite.

The nitrate-pretreated animals had higher mucosal blood flow than the control groups. The blood flow level was comparable to that attained on luminal application of acidified nitrite. These animals had high intragastric NO levels as a result of the nitrate intake in the drinking water. The sustained increased mucosal blood flow could not, however, have been due to a
large amount of intragastric free NO gas, since the stomach was opened during the animal preparation, resulting in a quick reduction of the NO level. Instead the increased blood flow might be a consequence of the increased concentration of nitrite that was seen in the outer, loosely adherent mucus gel. The combination of accumulated nitrite in the outer mucus layer and an acidic lumen would result in a slow prolonged NO formation close to the gastric mucosa. Interestingly, when the loosely adherent mucus layer was removed in the nitrate-treated rats, the blood flow decreased substantially down to a level not significantly different from that in the control animals.

We have previously shown that it is the firmly adherent mucus layer that is important for gastric protection against luminal acid (3, 5, 41). Within this mucus layer a pH gradient is formed, with a neutral pH at the epithelial surface (juxtamucosal pH) in spite of an acidic lumen (41). In this present study we find for the first time an important protective function of the loosely adherent mucus gel layer. The accumulation of nitrite here will be especially important under conditions when the acid secretion is stopped but 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 stomach than in the non-secreting (41, 43, 48). 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. When the stomach is not secreting but the lumen is still acidic, the accumulated nitrite in the loosely adherent mucus gel might compensate for the reduced protection provided by bicarbonate delivery. NO will be continuously produced and lead to increased blood flow levels.

We have shown in previous studies that the firmly adherent mucus layer increases in thickness after application of acidified nitrite or human nitrite-rich saliva to the mucosa, and under those circumstances we found a large NO production (9). Other studies have shown that NO donors
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increase gastric mucus secretion (10, 11). Preliminary results from our laboratory indicate that rats treated with NOS inhibitors and iNOS-deficient mice have a thinner firmly adherent mucus layer, also suggesting that NO is regulating mucus thickness.

To investigate whether dietary nitrate might protect the gastric mucosa against injury, a combination of intravenous diclofenac and luminal acidified taurocholate was given to challenge the gastric mucosa. Diclofenac has been shown to reduce gastric PGE$_2$ levels and blood flow and increase gastric damage (50), and the bile salt taurocholate has been shown to increase gastric mucosal permeability (7). This combination is clinically relevant, since diclofenac is a widely used NSAID and bile reflux into the stomach is common. In our study this combination caused a great increase in mucosal permeability accompanied by an increase in mucosal blood flow. Both nitrate intake for one week and luminal administration of acidified nitrite significantly reduced the permeability increase during the challenge. Furthermore, in the nitrate- and nitrite-treated animals luminal clearance returned to control levels directly after the challenge, which was not seen in the control animals. These results suggest that the gastric mucosa increases its ability to resist gastric irritants when treated with high luminal NO. Further studies have to be performed to be able to answer the question if dietary nitrate can actually reduce ulcer formation after chronic treatment with diclofenac. So far we have found that nitrate in the drinking water reduces ulcers induced by a bolus dose of diclofenac given by gavage 4 hours before the experiment (unpublished observations EA Jansson et al).

Since NSAIDs are the most commonly prescribed drugs in the US and the gastrointestinal complications are an enormous clinical and economic problem (2), considerable efforts have been made to try to reduce the side effects. The gastro-protective effects of different NO-donating NSAIDs have been attributed to release of NO and its effects in increasing gastric mucosal blood flow and protecting gastric epithelial cells from necrosis (18, 23). Organic
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nitrates and nitroglycerin have also been shown to decrease the gastrointestinal side effects of
NSAIDs (30). Our results suggest that a nitrate-rich diet, resulting in accumulation of nitrite in
the loosely adherent gastric mucus and substantial non-enzymatic NO formation, with increase
in gastric mucosal blood flow, protects the gastric mucosa.

ACKNOWLEDGMENTS

The authors thank Annika Jägare for excellent technical assistant.

GRANTS

This study was supported by Swedish Research Council Grant 08646 and 12585, by European
Union 6th Framework Program (Eicosanox LSHM-CT-2004-005033)
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Figure 1 Experimental protocol
Figure 2 Mucosal permeability, expressed as blood to lumen clearance of 51Cr-EDTA, in control rats (n = 7), nitrate-pretreated rats (n = 5), and rats given nitrite luminally (n = 8), before, during and after a diclofenac and taurocholate provocation. Values are means ± SEM. *P < 0.05 compared with control animals.
Figure 3 Mean arterial blood pressure (MAP, mmHg) and mucosal blood flow (LDF); presented as % of values in the first saline period, before, during and after diclofenac and taurocholate provocation in control rats (open squares, n = 7) and rats treated with nitrite luminally (filled diamonds, n = 8) Values are means ± SEM and represents the mean value of a 10 min period.
Figure 4 Intragastric NO generation in control animals (open diamonds, n = 7) and nitrate-pretreated animals (filled squares, n = 5). Values are means ± SEM.
Figure 5 Nitrite content in loosely adherent mucus in control animals (n = 12) and nitrate-pretreated animals (n = 10). Values are means ± SEM * P < 0.05
Figure 6 Gastric mucosal blood flow, presented in perfusion units (PU), in nitrate-pretreated animals (black diamonds n = 7) and control animals (grey squares n = 7) before and after removal of the loosely adherent mucus. Values are means ± SEM. * P < 0.05 compared with control animals. # P < 0.01 compared with before mucus removal.
Figure 7 Gastric mucosal blood flow, presented in LDF perfusion units (PU), at the start of the experiments. All control animals (n = 26) and nitrate-treated animals (n = 22) in the blood flow experiments are included. Values are means ± SEM. *P < 0.001 compared with control animals.
Figure 8 Mean arterial blood pressure (MAP), gastric mucosal blood flow (LDF), and vascular resistance (VR) following topical application of sodium nitrite (1.0 mM) at pH 3 to the gastric mucosa of C57BL/6 mice (n = 4) and eNOS-deficient mice (n = 4). Values are means ± SEM.