Protective effect of dietary nitrate on experimental gastritis in rats

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Nitrates have long been considered as harmful dietary components and judged responsible for deleterious effects on human health, leading to stringent regulations concerning their levels in food and water. However, recent studies demonstrate that dietary nitrate may have a major role in human health as a non-immune mechanism for host defence, through its metabolism to NO in the stomach. NO is a versatile molecule and although evidence exists showing that administration of low doses of exogenous NO protects against gastrointestinal inflammation, higher NO doses have been shown to exacerbate injury. So, the effect of an ingestion of nitrates in doses corresponding to a normal diet in human consumers on an experimental gastritis induced by iodoacetamide in rats was investigated. During gastritis one of the following compounds was given orally: water; KNO_3; the NO donor sodium nitroprusside; the NO scavenger haemoglobin given with either water or KNO_3. N(G)-nitro-L-arginine methyl ester (L-NAME), a non-specific NO synthase inhibitor, was administered with either water, iodoacetamide alone, or combined with KNO_3. After killing, the stomach was resected and microscopic damage scores, myeloperoxidase and NO synthase activities were determined. Iodoacetamide-induced gastritis was significantly reduced by KNO_3 administration, an effect which was reproduced by sodium nitroprusside and reversed by haemoglobin. L-NAME induced gastric mucosal damage in itself, and KNO_3 did not prevent the gastritis induced by iodoacetamide associated with L-NAME. In conclusion, dietary nitrate exerts a protective effect against an experimental gastritis in rats by releasing NO in the stomach but such an effect requires the production of endogenous NO.

Nitric oxide: Inflammation: Dietary nitrate: Stomach

The human diet is a source of direct (natural components) and indirect (additives, pesticides, etc) factors which are able to modulate gastrointestinal functions. Among these potentially modulating dietary factors, nitrates occupy a controversial position. The main sources of nitrates for human consumers are green and leafy vegetables, drinking water and also some meat and fish products where nitrates are widely used as preservatives in the form of Na and K salts. The total dietary intake of nitrates, excluding nitrates in the water, has been estimated as 50–80 mg/d per person (Meah *et al*. 1994; Dich *et al*. 1996). When ingested, dietary nitrate is absorbed from the stomach and proximal intestine into the plasma and then concentrated in saliva (Tannenbaum *et al*. 1976), where it is rapidly reduced to nitrites by anaerobic bacteria (Spiegelhalder *et al*. 1976; Walters & Smith, 1981). The two potentially deleterious effects of high gastric concentrations of nitrates are methaemoglobinaemia (Craun *et al*. 1981) and formation of carcinogenic N-nitroso compounds (Bruning-Fann & Kaneene, 1993). However, beyond these potential effects, nitrites are transformed into NO under the acidic conditions of the stomach (Benjamin *et al*. 1994; Duncan *et al*. 1995), an event that has been shown to participate in the protection of the organism against pathogen-induced

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gastrointestinal and oral diseases (Green, 1995; Dykhuizen *et al.* 1996; Duncan *et al.* 1997). In human subjects, ingestion of small amounts of nitrates (i.e. 2 mmol which corresponds to the amount normally found in a lettuce; Meah *et al.* 1994) has thus been found to generate high concentrations of NO in the stomach (McKnight *et al.* 1997).

Gastric NO may not be delivered only by extrinsic sources, but may also be generated by mucosal cells, which are able to produce large amounts of NO (Konturek & Konturek, 1995). Indeed, endogenous NO is produced from the amino acid L-arginine by either constitutive NO synthase (cNOS) or inducible NO synthase (iNOS) (Moncada *et al.* 1991) and both NOS have been detected in homogenates of rat gastric mucosa (Tepperman & Soper, 1994). In physiological conditions, cNOS is the only one expressed in gastric mucosa where it plays an important role in the maintenance of mucosal integrity. In contrast, iNOS, which is induced by bacterial lipopolysaccharide or cytokines such as interleukin-1 and tumournecrosis factor-a, produces large amounts of NO which are considered detrimental to the gastric mucosa (Brown *et al.* 1994) and have been found involved in the process of gastric inflammation in man as in rats (Rachmilewitz *et al.* 1994; Nishida *et al.* 1998). However, controversy still exists concerning the deleterious effects of iNOS-produced NO (Wei *et al.* 1995; McCafferty *et al.* 1997; Akiba *et al.* 1998).

Even though the effects of endogenous NO on gastrointestinal inflammation are discussed, there is substantial evidence that intragastric topical application of NO-releasing substances protects the gastric mucosa from damage induced by injurious agents (MacNaughton *et al.* 1989; Kitagawa *et al.* 1990). Nevertheless, this protective effect is dose-sensitive as high doses of exogenous NO can lead to gastric mucosal injury (Lopez-Belmonte *et al.* 1993; Lamarque & Whittle, 1995; Gurbuz *et al.* 1999) similar to what has been previously reported for endogenous NO (Brown *et al.* 1994).

Thus, considering nitrate daily consumption, controversial effects on human health as well as potentiality to induce the release of consequent quantities of NO in the stomach, and the debated role of NO in the modulation of gastric mucosal inflammation, the present study was designed to determine the effects of chronic ingestion of nitrates, in amounts that can be found in our normal daily diet, on an experimentally induced gastritis in rats.

Materials and methods

Animals

Male Wistar rats (Harlan, Gannat, France) weighing between 200 and 250 g at the time of the experiments were housed in polypropylene cages in a temperature-controlled room ($21 \pm 1^\circ$C) under controlled lighting. They were allowed free access to water and were fed during the night (19.00 to 9.00 hours) with a standard diet (A04; UAR, Epinay-sur-Orge). All protocols were approved by the local Institutional Animal Care and Use Committee.

Effect of potassium nitrate treatment on iodoacetamide-induced gastritis: role of nitric oxide

Fourteen groups of eight rats were treated for 7 d (Table 1). Solutions were administered intragastrically using a gastric feeding tube, except where otherwise indicated. The effects of KNO_3 administration on iodoacetamide-induced gastritis were investigated in groups 1 to 4. With this end in view, rats received iodoacetamide (25 mg/kg per d) or distilled water associated (groups 2 and 4) or not (groups 1 and 3) with KNO_3 (125 mg/kg twice daily). The dose of iodoacetamide used was selected from previous dose–response studies (M Larauche, P Anton, L Buéno and J Fioramonti, unpublished results). Administration of iodoacetamide was performed for the duration of 7 d according to Karmeli *et al.* (1996) who showed a maximum increase in gastric myeloperoxidase (MPO) activity and lesions for this period of administration. Groups 5 to 9 were used to assess the involvement of NO in the protective effect of KNO_3 against iodoacetamide-induced gastritis. First, groups 5 and 6, respectively received an NO donor, sodium nitroprusside (SNP; 0·7 mg/kg three times daily) associated or not with iodoacetamide. The dose of SNP used was selected from previous dose–response studies showing the near-maximum inhibition of iodoacetamide-induced gastric mucosal damage (M Larauche, P Anton, L Buéno and J Fioramonti, unpublished results). The three other groups received the NO scavenger, haemoglobin (75 mg/kg twice daily), with either distilled water (group 7), iodoacetamide (group 8), or KNO_3 and iodoacetamide (group 9). Finally, the last four groups (10 to 14) were used to investigate the interrelationships existing between endogenous gastric NO and exogenous NO brought in by KNO_3. Thus, rats received a non-specific inhibitor of NOS, N(G)-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg per d, intraperitoneally), either 30 min before distilled water (group 10), iodoacetamide (group 11), or iodoacetamide and KNO_3 (group 12), or just 5 min after L-arginine or D-arginine (300 mg/kg per d, subcutaneously; groups 13 and 14 respectively). The doses of haemoglobin and L-NAME used were in agreement with previous *in vivo* studies (Fargeas *et al.* 1996; Rouzade *et al.* 1999). Rats were killed 8 d after the beginning of

Table 1. Treatments performed in the fourteen groups of rats

Group number	Treatment
1	Water
2	Water + KNO_3
3	Iodoacetamide
4	Iodoacetamide + KNO_3
5	Water + sodium nitroprusside
6	Iodoacetamide + sodium nitroprusside
7	Water + haemoglobin
8	Iodoacetamide + haemoglobin
9	Iodoacetamide + haemoglobin + KNO_3
10	L-NAME + water
11	L-NAME + iodoacetamide
12	L-NAME + iodoacetamide + KNO_3
13	L-arginine + L-NAME
14	D-arginine + L-NAME

treatments. The stomachs were isolated, removed, and washed with saline (9 g NaCl/l). Segments (20 mm) of the stomach (antrum) were taken off in order to determine MPO and NOS activities and identify histological lesions. Tissues collected for MPO and NOS assays were stored at $-80°C$ until determination.

Collection of plasma and saliva in rats

Two groups of seven rats weighing 200–250 g at the time of the experiment were used. One group received 0·5 ml distilled water intragastrically and the other received 0·5 ml of KNO_3 at the dose of 125 mg/kg. At 30 min after oral administration, rats were anaesthetized using acepromazine (0·6 mg/kg intraperitoneally) and ketamine (120 mg/kg intraperitoneally). At 5 min after anaesthesia, rats received an injection of pilocarpine hydrochloride (0·5 mg/kg intraperitoneally, 0·2 ml). After 5–10 min, whole mixed saliva samples (250 μl) were drawn, and 500 μl of a TCA solution (25 %, v/v) were added. Samples were vigorously vortexed and centrifuged at $3000\,\boldsymbol{g}$ for 15 min at 4°C. Pellets were discarded and supernatant fractions were stored at $-20°C$ until determination. After saliva collection, 50–60 min after oral administration, the rats' body wall and peritoneum at the ventral midline were incised in order to perform needle puncture in the abdominal aorta. Thus, 1·8 ml of blood was collected using a plastic syringe filled with 0·2 ml of anticoagulant (3·8 % (w/v) sodium citrate). Immediately after collection, whole blood was centrifuged at $800\,\boldsymbol{g}$ for 15 min at 4°C, and citrated plasma was drawn. In the same way as for saliva, 500 μl of a TCA solution (25 %, v/v) was added to 250 μl citrated plasma. Samples were then vigorously vortexed and centrifuged at $3000\,\boldsymbol{g}$ for 15 min at 4°C. Pellets were discarded and supernatant fractions were stored at $-80°C$ until assay.

Morphological studies

Pieces of stomach (20 mm long) were fixed in Carnoy's solution, cleared in xylene and embedded in paraffin blocks. Transverse sections (5 μm) were cut and stained with haematoxylin and eosin, and examined by light microscopy in a blind manner. A histological grading scale (microscopic damage score) was used for the determination of the extent of the inflammatory reaction in the gastric mucosa (Fabia et al. 1993). Each of the individual variables estimated was graded from 0 to 3, according to the severity of the alterations. The variables were: ulceration; mucosal atrophy; oedema; inflammatory cell infiltration; vascular dilatation.

Myeloperoxidase activity assay

The activity of the enzyme MPO, a marker of polymorphonuclear neutrophil primary granules, was determined in gastric tissue according to a previously described technique (Bradley et al. 1982). Segments of stomach were suspended in a potassium phosphate buffer (44 mM-KH_2PO_4, 6 mM-$K_2HPO_4.3H_2O$; pH 6·0) and homogenized on ice using a Polytron (PCU-2; Kinematica GmbH, Lucerne, Switzerland), and followed by three cycles of freezing and thawing. Suspensions were then centrifuged at $9000\,\boldsymbol{g}$ for 15 min at 4°C. Supernatant fractions were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (0·5 % (w/v) in the potassium phosphate buffer), a detergent inducing the release of MPO from polymorphonuclear neutrophil primary granules. These suspensions were sonicated on ice (Büchi, Flawil, Switzerland), and again centrifuged at $9000\,\boldsymbol{g}$ for 15 min at 4°C. Pellets were discarded and supernatant fractions assayed spectrophotometrically for MPO activity and protein contents. Supernatant fractions obtained were diluted in the potassium phosphate buffer containing 0·167 mg o-dianisidine dihydrochloride/ml and 0·0005 % (v/v) H_2O_2. MPO from human neutrophils (Sigma, Saint Quentin Fallavier, France; 0·1 U/100 μl) was used as a standard. Changes in absorbance at 450 nm were recorded with a spectrophotometer (Uvikon 922; Kontron Instruments, Saint Quentin en Yvelines, France) every 10 s over 2 min. One unit of MPO activity was defined as the quantity of MPO degrading 1 μmol H_2O_2/min per ml at 25°C. Protein concentrations (g/ml) were determined with a commercial kit of the modified method of Lowry (Detergent Compatible Assay; Bio Rad, Ivry/Seine, France), and MPO activity was expressed as MPO units/g protein.

Determination of nitric oxide synthase activity

Tissue NOS activities were estimated by measuring the rate of conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline (Bush et al. 1992). Tissue samples were homogenized on ice in a buffer (pH 7·4) containing Tris-HCl (50 mM), dithiothreitol (1 mM), phenylmethylsulfonylfluoride (1 mM), EDTA (0·1 mM) and two protease inhibitors: leupeptin (23·4 μM) and pepstatin (14·6 μM). After centrifugation (13 500 $\boldsymbol{g}$, 30 min, 4°C), 100 μl of supernatant fraction was added to a reaction mixture containing 50 mM-Tris-HCl (pH 7·4), 1·58 μM-L-[^{14}C]arginine, 200 μM-NADPH as a co-substrate, 10 μM-FMN, and 10 μM-FAD as prosthetic groups of NOS, 1 mM-tetrahydrobiopterine, 1 mM-dithiothreitol and 50 mM-valine. Determination of total NOS activity was performed by adding 2 mM-$CaCl_2$. iNOS activity was determined in the presence of a Ca chelator, ethylene-glycol-bis(a-aminoethyl)-N,N,N$'$,N$'$-tetra-acetic acid (EGTA; 1 mM). cNOS activity was determined by the difference between both activities. After 30 min incubation at 37°C, the enzymic reaction was stopped by adding cold 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 5·5) containing 1 mM-EGTA and 1 mM-EDTA. L-[^{14}C]citrulline formed in the medium was separated by applying the samples to columns containing pre-equilibrated Dowex AG50W-X8, eluting them with water, and measuring the amount of radioactivity with a liquid scintillation Beta counter (Kontron Instruments, St Quentin en Yvelines, France). Protein concentrations (mg/ml) were determined with a commercial kit of the modified method of Lowry (Detergent Compatible Assay; Bio Rad, Ivry/Seine, France) Enzyme activity was expressed as pmol citrulline formed/mg protein per h.

Nitrate and nitrite assays

The levels of nitrates and nitrites were determined in rats' saliva and plasma according to the method of Griess (Grisham *et al.* 1995). Briefly, a 100 μl portion of a sample (saliva or citrated plasma) was incubated for 30 min at 37°C with 50 mM-HEPES buffer, 5 μM-FAD and 0·1 mM-NADPH in a total volume of 500 μl in the presence (nitrites+nitrates levels) or not (nitrites level) of 0·2 units nitrate reductase/ml from *Aspergillus* species. Following the incubation, 5 μl of lactic dehydrogenase (1500 units/ml) and 50 μl of 100 mM-pyruvic acid were added to each tube to oxidize unreacted NADPH. Samples were then incubated for an additional 10 min at 37°C. Premixed Griess reagent (1 ml) (0·2 % (w/v) naphtylethylenediamine+2 % (w/v) sulfanilamide in 5 % (w/v) phosphoric acid) was then added to each tube and after 10 min incubation at room temperature the absorbance of each sample was determined at 543 nm with a spectrophotometer (Uvikon 922; Kontron Instruments, St Quentin en Yvelines, France). $NaNO_3$ (1 mM) and $NaNO_2$ (1 mM) solutions were used as standards. The detection limit of the assay was 1·92 μM and the quantification limit was 6·41 μM.

Chemicals

Iodoacetamide, KNO_3, SNP, haemoglobin, L-NAME, L-arginine hydrochloride, D-arginine hydrochloride, Dowex AG50W-X8 (H^+ form) 100–200 mesh, valine, NADPH, dithiothreitol, phenylmethylsulfonylfluoride, EDTA, EGTA, leupeptin, pepstatin, FMN, FAD, $CaCl_2$, KH_2PO_4, $K_2HPO_4.3H_2O$, hexadecyl trimethyl ammonium bromide, H_2O_2, *o*-dianisidine hydrochloride, lactic dehydrogenase from bovine muscle, $NaNO_3$, $NaNO_2$, naphtylethylenediamine, sulfanilamide, and HEPES and Tris-HCl buffers were purchased from Sigma, Saint Quentin Fallavier, France. L-[^{14}C]arginine was purchased from ICN Biomedicals, Orsay, France. Nitrate reductase from *Aspergillus* species was purchased from Roche Diagnostic Biochemicals, Meylan, France. Phosphoric acid (85 %, w/v) was purchased from Carlo Erba Reactifs, Val de Reuil, France. Solutions used for oral treatments were prepared in distilled water.

Statistical analysis

MPO activity, NOS activity and histological data were analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Saliva and plasma nitrates and nitrites levels were analysed by using Student's *t* test for unpaired values using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Results are expressed as means and standard errors of the mean, and differences were considered significant at $P<0.05$.

Results

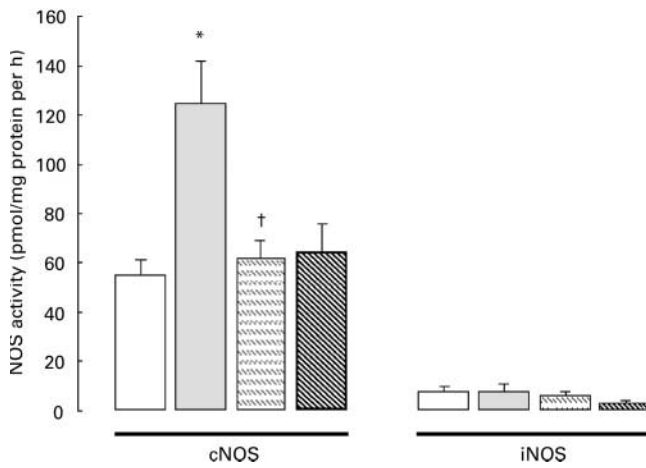### Iodoacetamide-induced gastritis in rats

Iodoacetamide administration for 7 d induced gastric mucosal damage characterized by a significant increase of tissue MPO activity (3112 (SEM 180) *v.* 1206 (SEM 134) MPO units/g protein in controls; $P<0.05$). Histologically, oedema was found in the submucosa, with vascular dilatation and a mild inflammatory cell infiltrate leading to a significant increase in microscopic damage scores (4·56 (SEM 0·50) *v.* 1·30 (SEM 0·30) units for the control group; $P<0.05$) (Fig. 1). Iodoacetamide significantly increased cNOS activity (125 (SEM 17) *v.* 55 (SEM 6) pmol/mg protein per h; $P<0.05$) but did not modify iNOS activity (Fig. 2).

Effect of potassium nitrate treatment on iodoacetamide-induced gastritis

Treatment with KNO_3 suppressed the iodoacetamide-induced increase in gastric MPO activity and in microscopic damage score (1542 (SEM 123) MPO units/g protein and 1·83 (SEM 0·54) units, respectively) (Fig. 1). The increase
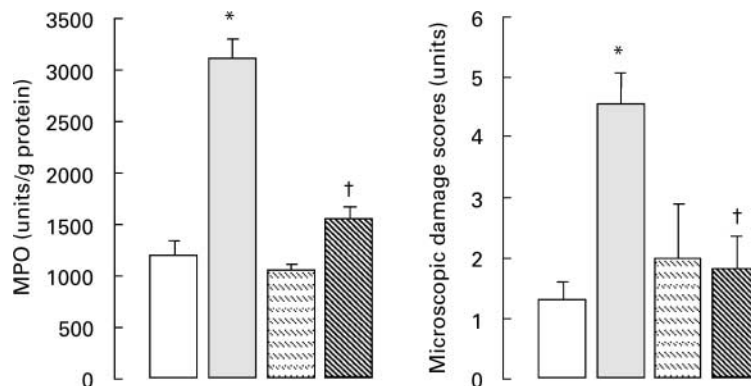
Fig. 1. Effect of KNO_3 on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). † Mean values were significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). (□), Control group; (▨ light), iodoacetamide-treated group; (▨), KNO_3-treated group; (▨), KNO_3 + iodoacetamide-treated group.
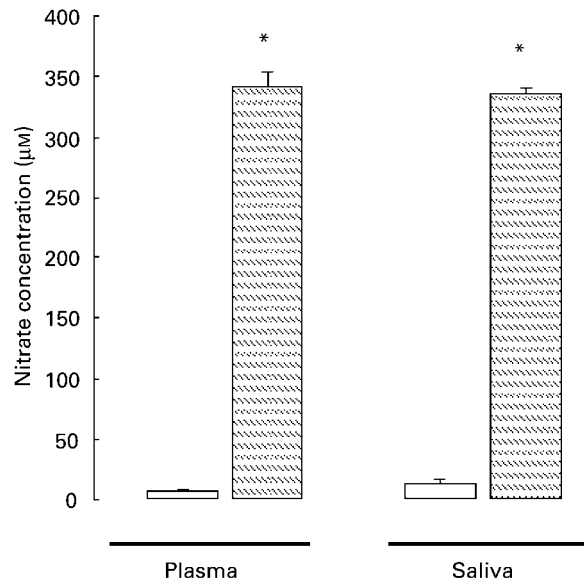
Fig. 2. Effect of KNO_3 on gastric constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) activities in iodoacetamide-induced gastritis in rats. Mean values for five to eleven rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0.05$). † Mean value was significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). (□), Control group; (▨), iodoacetamide-treated group; (▨), KNO_3-treated group; (▨), KNO_3 + iodoacetamide-treated group.

Fig. 3. Effect of KNO_3 on nitrate in the plasma and saliva of rats. Mean values for seven rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). (□), Control group; (▨), KNO_3-treated group.

in cNOS activity induced by iodoacetamide was abolished by KNO_3 treatment (Fig. 2).

### Effect of potassium nitrate treatment on nitrates and nitrites levels in plasma and saliva

In control rats, nitrate levels in plasma and saliva were 7 (SEM 2) and 13 (SEM 4) μM, respectively. Intragastric administration of KNO_3 significantly increased nitrate levels in plasma and saliva compared with control rats (342 (SEM 13) and 336 (SEM 4) μM, respectively) (Fig. 3). Nitrites were undetectable in all samples except in the saliva of all rats treated with KNO_3 where trace amounts below the detection limit of the method were observed.

Involvement of nitric oxide in the protective effect of potassium nitrate against iodoacetamide-induced gastritis

Treatment with the NO donor, SNP, suppressed the increase in gastric MPO activity and microscopic damage score induced by iodoacetamide (1818 (SEM 200) MPO units/g protein and 2·57 (SEM 0·81) units, respectively) (Fig. 4). Treatment with KNO_3 suppressed the iodoacetamide-induced increase in gastric MPO activity and microscopic damage scores, but the co-administration of haemoglobin with KNO_3 restored the increase in gastric MPO activity and damage scores induced by iodoacetamide (Fig. 5). Haemoglobin alone did not modify iodoacetamide-induced gastritis.
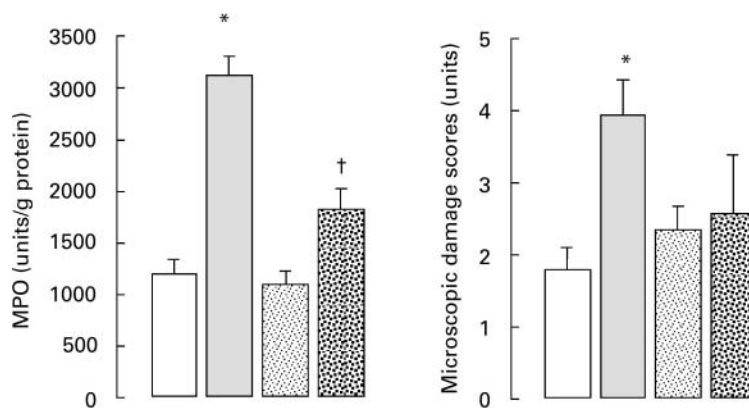
Fig. 4. Effect of sodium nitroprusside on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). † Mean value was significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). (□), Control group; (▨), iodoacetamide-treated group; (▨), sodium nitroprusside-treated group; (▨), sodium nitroprusside+iodoacetamide-treated group.
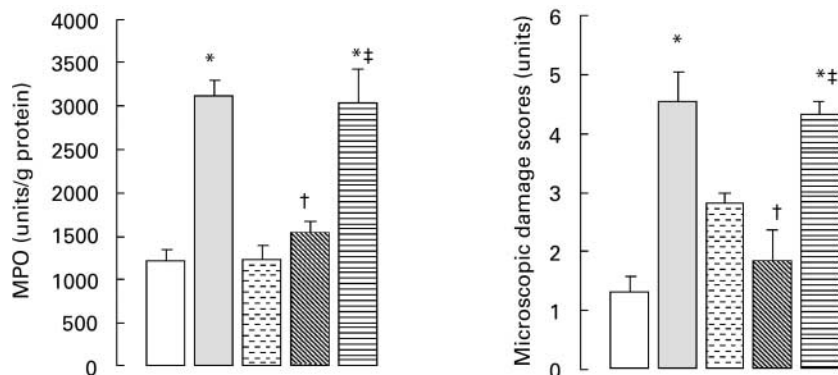
Fig. 5. Influence of haemoglobin on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats treated with KNO_3. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P < 0.05$). † Mean values were significantly different from that of the group treated with iodoacetamide alone ($P < 0.05$). ‡ Mean values were significantly different from that of the group treated with $KNO_3 +$ iodoacetamide ($P < 0.05$). (☐), Control group; (▨), iodoacetamide-treated group; (▨), haemoglobin-treated group; (▨), $KNO_3 +$ iodoacetamide-treated group; (▤), KNO_3 + iodoacetamide + haemoglobin-treated group.

Interaction between exogenous and endogenous nitric oxide in iodoacetamide-induced gastritis

Repeated administrations of L-NAME once daily for 7 d caused a significant increase in gastric MPO activity (2766 (SEM 258) *v.* 1206 (SEM 134) MPO units/g protein in controls; $P < 0.05$). This treatment tended to increase the microscopic damage scores (3·29 (SEM 0·18) *v.* 1·30 (SEM 0·30) units for the control group) but the significance level of $P < 0.05$ was not attained (Fig. 6). This effect of L-NAME on gastric MPO activity was antagonized by co-administration of L-arginine (1107 (SEM 106) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group; $P < 0.05$) but not by D-arginine (2356 (SEM 573) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group).

The co-administration of L-NAME with iodoacetamide induced greater gastric MPO activity than L-NAME given alone (4066 (SEM 543) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group) and tended to increase the microscopic damage score (5·5 (SEM 0·5)

v. 3·29 (SEM 0·18) units for the L-NAME-treated group). Treatment with KNO_3 did not prevent the L-NAME-induced aggravation of the gastritis induced by iodoacetamide (Fig. 6).

Discussion

It has been suggested for many years that dietary nitrates were potentially harmful for human health because of their ability to form nitrites and carcinogenic compounds in the stomach (Bruning-Fann & Kaneene, 1993). It has only recently been found that nitrates may play a key role in the mechanism of defence against gastrointestinal and oral pathogens in man and animals (Green, 1995; Duncan *et al.* 1997). Our results extend these later observations by showing for the first time that daily ingestion of an amount of nitrates that can be found in the normal diet protects against an experimental gastritis by releasing NO in the gastric lumen.

In our experiments, gastric mucosal damage was induced by iodoacetamide, a sulfhydryl blocker that has already
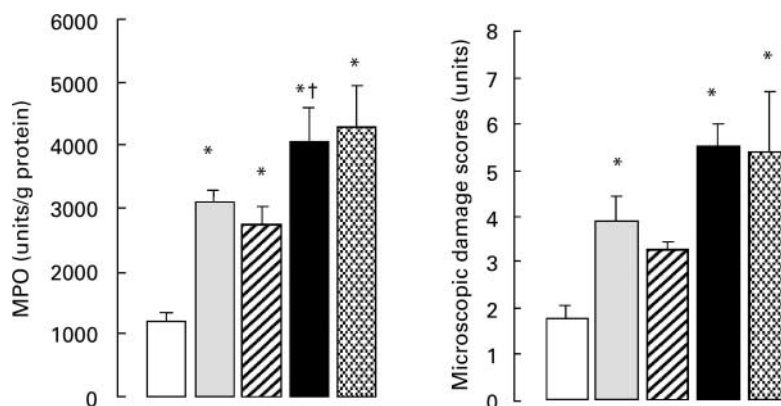
Fig. 6. Effect of N(G)-nitro-L-arginine methyl ester (L-NAME) on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats treated or not with KNO_3. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P < 0.05$). † Mean value was significantly different from that of the group treated with L-NAME alone ($P < 0.05$). (☐), Control group; (▨), iodoacetamide-treated group; (▨), L-NAME-treated group; (■), L-NAME + iodoacetamide-treated group; (▨), L-NAME + iodoacetamide + KNO_3-treated group.

been shown to induce diffuse gastritis in rats (Lalich, 1962; Yasin & Leese, 1970; Karmeli *et al.* 1996). This agent decreases the concentration of reduced non-protein sulfhydryl compounds in the gastric mucosa (Szabo *et al.* 1981), and consequently alters the structure of the mucus, which is the first barrier of protection of the gastric mucosa. Thus, in our study, daily administration of iodoacetamide for 7 d to Wistar rats induced gastric inflammation characterized by a 2·6-fold increase in gastric MPO activity and a 3·5-fold increase in microscopic damage scores. These results agree with those of Karmeli *et al.* (1996) who showed a 3-fold increase in gastric MPO activity with 7 d of iodoacetamide treatment. The severity of iodoacetamide-induced gastritis was reduced by a 7 d treatment with KNO_3 at a dose of 250 mg/kg per d. Daily nitrate consumption in European countries has been estimated to be about 90–120 mg/person (Cornee *et al.* 1992), corresponding to approximately half of the acceptable daily intake for nitrate in man (World Health Organization, 1962). Taking into account that the acceptable daily intakes for human consumers are calculated by applying a safety factor of 100 to the no-observed effect level determined in animal species (World Health Organization, 1987), it was decided to administer half of the no-observed effect level for nitrates which was estimated at 500 mg/kg per d, expressed in KNO_3 (HP Til, CF Kuper and HE Falke, unpublished results).

KNO_3 administration reduced the increase of gastric MPO activity and abolished the tissue damage induced by iodoacetamide. This reduction of gastric inflammation by KNO_3 was most probably mediated by NO since it was reproduced by an NO donor, SNP, and abolished by an NO scavenger, haemoglobin. In our experiments, nitrate was administered intragastrically to rats. In man, ingested nitrates are absorbed from the upper gastrointestinal tract into the plasma and are then concentrated in saliva (Tannenbaum *et al.* 1976) where they are rapidly reduced to nitrites by anaerobic bacteria (Spiegelhalder *et al.* 1976; Walters & Smith, 1981). It has been suggested that this active transport system of nitrates from blood to saliva is lacking in rats (Vittozzi, 1992; Walker, 1994). However, in rats, orally administered nitrate is rapidly absorbed into the bloodstream from the upper small intestine, and the presence of ^{13}N issued from $^{13}NO_x^-$ administered intravenously has been noted in the saliva of rats (Witter *et al.* 1979). Our results are in agreement with these studies. Indeed, a significant increase of nitrate levels in both plasma and saliva of rats treated intragastrically with KNO_3 was observed; moreover, nitrate levels were identical in the plasma and saliva, confirming the absence of nitrate concentration in the saliva of rats. Even if rats possess nitrate reductase on the tongue (Li *et al.* 1997), significant levels of nitrite in saliva could not be detected. This may be explained by the fact that, in rats, significant nitrite production by tongue bacteria occurs only with very high concentrations of nitrate (Li *et al.* 1997) and also by the technique used to collect saliva. Pilocarpine induces a high flow of saliva, which probably does not allow a sufficient incubation time to have a significant nitrate reduction by tongue bacteria. However, salivary nitrate reduction is not the only way to obtain nitrites in rats. Indeed, nitrate-reducing flora have been identified in the rat stomach (Bockler *et al.* 1983), and the gastric mucosa has been found able to reduce nitrate into nitrite even in germ-free rats (Ward *et al.* 1986). Finally, the possibility of mediation of the effects of nitrate on gastric mucosa by NO is supported by the fact that nitrite is readily transformed into NO in the acidic conditions of the stomach (Benjamin *et al.* 1994).

Some studies have already suggested a protective effect of exogenous NO against gastric mucosal damage (Lopez-Belmonte *et al.* 1993; Calatayud *et al.* 1999; Mourad *et al.* 2000; Potter & Hanson, 2000). However, these effects have been shown with NO donors such as glyceryltrinitrate or isoamylnitrite, systemically administered or in *in vitro* conditions but not after oral ingestion. Our results indicate for the first time a protective action of NO coming from a common dietary component that can be ingested through our daily diet.

Several mechanisms have been proposed to explain the protective role of exogenous NO on gastric mucosa. The most classical effects attributed to NO on gastric mucosa are the stimulation of blood flow and mucus secretion, and the reduction of acid secretion (Pique *et al.* 1989; Brown *et al.* 1993; Kato *et al.* 1998). These three actions can, by themselves, explain the anti-inflammatory effects of NO at the gastric level. However, other hypotheses can be proposed. Because mast cell activation is known to play a key role in gastric inflammation (Nakajima *et al.* 1996, 1997) and exogenous NO has been shown to modulate mast cell degranulation (Gaboury *et al.* 1996; Iikura *et al.* 1998), it is possible that dietary NO exerts its effects by stabilizing mast cells. However, this hypothesis requires further investigation, as mast cell degranulation has not been shown yet to be involved in iodoacetamide-induced gastritis. Another possibility could be that exogenous NO inhibited platelet and polymorphonuclear adherence (Radomski *et al.* 1987; Clancy *et al.* 1992), thus preventing their activation and the consecutive production of oxidants (Clancy & Abramson, 1995; Kubes & Wallace, 1995) whose scavenging function has been shown beneficial in iodoacetamide-induced gastritis (Karmeli *et al.* 1996). Moreover, recent studies have shown the protective role played by haeme oxygenase-1, an antioxidant enzyme, in gastrointestinal inflammation (Cavicchi *et al.* 2000; Wang *et al.* 2000). Since NO donors have been shown to increase haeme oxygenase-1 expression (Hara *et al.* 1999; Cavicchi *et al.* 2000), it could be suggested that dietary NO induced antioxidant and anti-inflammatory effects by targeting haeme oxygenase-1 (Polte *et al.* 2000).

So, considering these possible enzymic modulations of gastric inflammation, an attempt was made to determine the effect of KNO_3 treatment on gastric NOS activity. It is usually postulated that during gastrointestinal inflammation, cNOS activity which is responsible for the maintenance of mucosal integrity is diminished while iNOS activity is overexpressed and produces large quantities of NO leading to mucosal injury. It seems however that the modulation of NOS activity during inflammation differs greatly according to the experimental model used and the type of inflammation (Kimura *et al.* 1997; Nishida *et al.* 1998; Anton *et al.* 2000). A significant increase in gastric cNOS activity was observed, but no

modification of iNOS activity after 7 d of iodoacetamide treatment. cNOS has been found to account for gastric over-production of NO in uraemic rats (Mendez *et al.* 1997), and excessive levels of NO have been shown to reduce gastric cell viability, alter intracellular glutathione homeostasis and increase generation of intracellular oxidants leading to increased gastric cellular injury (Wakulich & Tepperman, 1997). Moreover, the overexpression of cNOS in portal hypertensive rats is probably responsible for the increased susceptibility of the gastric mucosa to damage (Ohta *et al.* 1997). Then, chronic administration of iodoacetamide could exacerbate the activation of gastric cNOS leading to an overproduction of NO, and then to deleterious effects on the gastric mucosa. Interestingly, KNO_3 treatment abolished the increase in gastric cNOS activity induced by iodoacetamide, suggesting a modulator role of exogenous NO on NOS. A negative feedback of NO on NOS activity has already been shown on cNOS and iNOS enzymes (Griscavage *et al.* 1993; Rengasamy & Johns, 1993), suggesting that the effect afforded by KNO_3 may also be relevant in gastric inflammation where iNOS activity is increased leading to subsequent NO overproduction such as in *Helicobacter pylori*-induced gastritis in human subjects (Franco *et al.* 1999; Fu *et al.* 1999).

So efforts were made to identify interrelations between NOS and dietary NO by using L-NAME, an NOS isoform non-selective inhibitor. L-NAME treatment for 7 d increased gastric MPO activity. This pro-inflammatory effect of L-NAME, already described in the small intestine (Miller *et al.* 1994), unveils a tonic action of NO in the maintenance of gastric mucosa integrity. L-NAME associated with iodoacetamide induced a greater inflammation than L-NAME given alone. This is in agreement with other studies showing that L-NAME aggravates gastric lesions induced by ethanol (Nahavandi *et al.* 1999), stress (Qiu *et al.* 1996) or pylorus ligation (Dixit *et al.* 1999). Exogenous NO brought in by KNO_3 was unable to reduce inflammation induced by iodoacetamide associated with L-NAME. This indicates that exogenous NO can protect the gastric mucosa only in the presence of endogenous NO production.

In conclusion, dietary nitrates exert a protective action on the rat gastric mucosa by producing NO, which can be considered as a complement of endogenous NO. These results confirm the potential therapeutic role of dietary nitrate in human health and highlight the necessity to reconsider its role in our diet.

Acknowledgements

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