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## Original Contribution

## The potent vasodilator ethyl nitrite is formed upon reaction of nitrite and ethanol under gastric conditions

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## ABSTRACT

By acting as a bioreactor, affording chemical and mechanical conditions for the reaction between dietary components, the stomach may be a source of new bioactive molecules. Using gas chromatography-mass spectrometry we here demonstrate that, under acidic gastric conditions, ethyl nitrite is formed in  $\mu\text{M}$  concentrations from the reaction of red wine or distilled alcoholic drinks with physiological amounts of nitrite. Rat femoral artery rings and gastric fundus strips dose-dependently relaxed upon exposure to nitrite: ethanol mixtures. In contrast, when administered separately in the same dose ranges, nitrite evoked only minor vasorelaxation while ethanol actually caused a slight vasoconstriction. Mechanistically, the relaxation effect was assigned to generation of nitric oxide ( $^*\text{NO}$ ) as supported by direct demonstration of  $^*\text{NO}$  release from ethyl nitrite and the absence of relaxation in the presence of the soluble guanylyl cyclase inhibitor, ODQ. In conclusion, these results suggest that ethanol in alcoholic drinks interacts with salivary-derived nitrite in the acidic stomach leading to the production of the potent smooth muscle relaxant ethyl nitrite. These findings reveal an alternative chemical reaction pathway for dietary nitrate and nitrite with possible impact on gastric physiology and pathophysiology.

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## Introduction

During the last decade, a lot of attention has been given to the biochemistry of nitrite in the human organism [1–5]. It is now clear that in the body, the nonenzymatic reduction of nitrite to  $^*\text{NO}$  can occur under acidic conditions [6] such as in the stomach lumen [7–9], in acidified urine [10], on the skin surface [11,12] and in the oral cavity [13]. The equilibrium of nitrite with nitrous acid and other reactive nitrogen oxides at low pH is described by reactions 1 to 3:



**Abbreviations:**  $^*\text{NO}$ , Nitric oxide; KH, Krebs–Henseleit; ACh, Acetylcholine; DTPA, Diethylenetriaminepentaacetic acid; GSH, Glutathione; GSNO, S-Nitrosoglutathione; SNP, Sodium nitroprusside; ODQ, [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NAME,  $\text{N}^G$ -nitro-L-arginine methyl ester; GC-MS, Gas chromatography - Mass spectrometry; NOS, Nitric oxide synthase; sGC, Soluble guanylyl cyclase; RT, Retention time.

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Nitrite, the precursor of  $\text{HNO}_2$ , derives from nutritional sources, from the reduction of dietary nitrate by oral commensal bacteria, and from oxidation of endogenous  $^*\text{NO}$  [2,5]. The stomach is continuously exposed to nitrite through the swallowing of saliva, which is particularly rich in nitrite. The general view is that nitrite is only harmful to the GI tract because it can promote the generation of carcinogenic nitrosamines [14–18]. However, with the recent discovery of *in vivo* generation of  $^*\text{NO}$  from nitrite, a number of potentially beneficial effects are being revealed. These studies suggest a role for nitrite,  $^*\text{NO}$  and its reaction products in host defense against gut pathogens [19], in regulation of gastric mucosal blood flow and mucous production [20,21], and in protection against gastric ulcers [22].

Recently, we have shown that the consumption of red wine greatly increases the *in vivo* formation of  $^*\text{NO}$  in the stomachs of healthy volunteers, via mechanisms that include the reduction of nitrite to  $^*\text{NO}$  by wine polyphenols [23]. Ethanol represents another major constituent of wine that could potentially interact with nitrite under gastric conditions. From a chemical viewpoint, nitrite-derived reactive nitrogen species can react with aliphatic alcohols to yield alkyl nitrites by O-nitrosation of the corresponding alcohol [24]. Alkyl nitrites belong to the family of organic nitrites which are known for their potent vasodilatory activity. Considering that both,  $\text{HNO}_2$  [25] and  $\text{N}_2\text{O}_3$  [26,27] may undergo such a reaction (reaction 4 and 5) they are

likely candidates for a nitrosation reaction of ethanol in the acidic stomach generating ethyl nitrite *in vivo*.



There have been a few earlier reports on the generation of ethyl nitrite *in vivo*. Macdonald and Jonsson detected ethyl nitrite in the breath of subjects who had been smoking and drinking concurrently, and the reaction of nitrogen oxides in the smoke with ethanol was suggested as its source [28]. More recently, Deng and colleagues showed that ethyl nitrite can be formed *in vivo* after intraperitoneal administration of ethanol in rats and they proposed that its production proceeded via reaction of ethanol with peroxynitrite [29].

We aimed to study if ethyl nitrite is formed from alcoholic drinks and nitrite under simulated gastric conditions, and how such generation would be influenced by the concentration of nitrite and ethanol, pH, and the type of alcoholic drink (red wine or brandy). Moreover, we attempted to confer physiological relevance of such interaction by studying the vasoactivity of a nitrite:ethanol mixture.

## Materials and methods

### Chemicals and buffer solutions

Ethanol p.a. (Ph.Eur) was obtained from Merck; ethyl nitrite (10%-20% in ethanolic solution) was obtained from Aldrich and quantified as described below; [1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was obtained from Tocris Cookson (Bristol, UK), acetylcholine (ACh), *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), phenylephrine, sodium nitrite, sodium nitroprusside (SNP), glutathione (GSH) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma Chemicals Co. Unless otherwise mentioned, pH 7.4 refers to simulated gastric juice without pepsin (USP 21). All solutions were prepared in ultrapure water. For the assays with the alcoholic drinks, single variety *Touriga nacional* red wine "Quinta de Cabriz" 2003 (14% v/v), Dão, Portugal and brandy "Macieira" (40% v/v), Portugal were used. All other reagents used were of analytical grade.

### Ethyl nitrite detection

The detection of ethyl nitrite in the nitrite:ethanol mixtures was accomplished by solid-phase microextraction and gas chromatography-mass spectrometry (GC-MS) techniques. Briefly, the reaction products of the sample (added with internal standard, 1,4-dioxane) were left to stabilize for 5 min in a sealed vial and then extracted from the headspace using a solid-phase microextraction device (SPME fiber 50/30 μm DVB/Carboxen/PDMS, Supelco, USA) at room temperature for 15 min. Identification and quantification of ethyl nitrite in the extracts were made after direct thermal desorption from the SPME fiber inside the GC-MS injector. GC-MS system was composed by an Agilent Technologies 6890N gas chromatograph equipped with an SGE Carbowax column (polyethyleneglycol 25 m × 220 μm, film thickness 0.25 μm) interfaced with a mass selective detector Agilent Technologies 5973N. GC-MS conditions were as follows: GC oven was started at 50°C for 1 min, subsequently increased to 80°C at 10°C per minute and next to 160°C at 20°C per minute, and finally held at 160°C for 1 min; Helium at 1.0 ml.min<sup>-1</sup> flow was used as carrier gas. Injector, in splitless mode, was kept at 250°C. interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 10–350 amu, 4.21 scans.s<sup>-1</sup>.

The exact concentration of ethyl nitrite in the standard (Sigma) was determined by nuclear magnetic resonance in a Bruker AMX 300 spectrometer (300.13 MHz, for <sup>1</sup>H) at 20°C, with CDCl<sub>3</sub> as solvent and TMS as internal reference. The value achieved for ethyl nitrite concentration was 6% (weight/weight). This result agrees with previously published data [29]. Ethyl nitrite quantification was achieved by one-point calibration for each of the different experimental conditions.

### Ethyl nitrite production from a nitrite:ethanol mixture

In order to characterise the production of ethyl nitrite from nitrite and ethanol in simulated gastric conditions, several reaction conditions were established, as described in figures legends. For the evaluation of ethyl nitrite production in red wine and brandy, both drinks were diluted in simulated gastric juice to reach a similar ethanol concentration and the final pH was set to 2.0. The buffer solutions at the defined pH and ethanol percentage were placed in 10 ml headspace vials and sealed with aluminium caps with a silicon septum. Nitrite solution was added through the vial cap and followed immediately by the addition of 3 mM 1,4-dioxane (internal standard). The final incubation volume was 3 ml. The mixture was let to equilibrate for 5 min and then followed by solid-phase microextraction for 15 min at room temperature of the gas-phase reaction products. The extracts were injected into the GC-MS for analysis.

### Spectrophotometric measurements

Spectra of ethyl nitrite (standard) and S-Nitrosoglutathione (GSNO) were recorded in 50 mM phosphate buffer saline at pH 7.4 between 300 to 425 nm. A Lambda 45 UV/VIS spectrophotometer (Perkin-Elmer Ltd., UK) was used at room temperature, under constant stirring and in the presence of O<sub>2</sub>.

### Nitric Oxide Measurements

\*NO in solution was measured electrochemically using the ISO-NO Mark II \*NO meter and sensor (World Precision Instruments, LTD, Hertfordshire, UK). The sensor was calibrated in KI - H<sub>2</sub>SO<sub>4</sub> (0.1M) with stock solutions of nitrite, according to the manufacturer protocol.

Gas phase \*NO was measured by chemiluminescence (CLD 77, Ecophysics, Switzerland). The instrument's detection limit for \*NO was 1 ppb. Calibration was performed with \*NO gas (10 ppm \*NO in nitrogen; AGA AB, Lidingö, Sweden).

### Nitric oxide production from ethyl nitrite standard and from nitrite:ethanol mixtures

To study the time course of \*NO release from ethyl nitrite, 50 μM ethyl nitrite (standard) was added to 50 mM phosphate buffer saline at pH 7.4 and the release of \*NO in solution was followed during 90 min. using the \*NO sensor. The influence of GSH in the release profile of \*NO from ethyl nitrite was assessed after addition of ethyl nitrite to 1 or 5 mM GSH in 50 mM phosphate saline buffer at pH 7.4. In order to verify the influence of metal traces present in the buffer solutions, \*NO release from 50 μM ethyl nitrite was also performed in the presence of 10–100 μM DTPA. All assays were performed at room temperature, in the dark, under constant stirring and in the presence of O<sub>2</sub>.

The production of \*NO from the nitrite:ethanol mixture was studied at pH 7.4 by chemiluminescence. \*NO release from 250 μM nitrite was added to simulated gastric juice at pH 2.0 in the presence and absence of 10% ethanol in a close reaction chamber from which a continuous flow of N<sub>2</sub> removed \*NO carrying it into the analyser. All the assays were made at 37°C. The concentration of nitrite and the pH were chosen to resemble normal physiological conditions in the stomach.

### Rat femoral artery rings

Male Sprague–Dawley rats (weight 200–300 g) were given a pentobarbital overdose by intraperitoneal injection. The femoral arteries were carefully dissected free from surrounding tissue, removed and put in Krebs–Henseleit (KH) solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4(\text{H}_2\text{O})$ , 25.2 mM  $\text{NaHCO}_3$ , 2.5 mM  $\text{CaCl}_2$  and 11.1 mM glucose). Circular segments (1 to 2 mm in length) of the femoral artery were mounted on two thin metal holders, one of which was connected to a force displacement transducer (model FT03, Grass Instrument, Quincy, MA) and the other to a movable device that allowed the application of a passive tension of 5 mN. The tension was recorded on a Grass polygraph (model 7B). The mounted vascular segments were kept in organ baths containing 2 ml KH solution at 37.5°C and were continuously bubbled with 6.5%  $\text{CO}_2$  in  $\text{O}_2$  to maintain a pH of 7.4. After preparation, the vascular segments were allowed to equilibrate for 60 min.

The contractile function of the vascular segments was tested by administration of phenylephrine (1  $\mu\text{M}$  final concentration) and with  $\text{K}^+$ -rich (127 mM) KH solution, prepared by replacing NaCl with equimolar amounts of KCl. Endothelium-dependent and -independent relaxations were determined by administration of acetylcholine (ACh) and the  $\text{NO}$  donor sodium nitroprusside (SNP), respectively. ACh and SNP were added to the organ baths at cumulatively increasing concentrations (1 nM to 10  $\mu\text{M}$  and 1 nM to 1  $\mu\text{M}$ , respectively) during a stable contractile tone induced by phenylephrine (1  $\mu\text{M}$ ).

After testing the contractile and relaxatory function of the vascular segment phenylephrine was re-administered 15 min later and the vascular segment developed a stable contraction.

The nitrite:ethanol mixture (250  $\mu\text{M}$  sodium nitrite in 10% ethanol at pH 2.0 followed by vortexing for 30 s) was prepared and diluted in KH solution immediately before each administration to the organ bath. This stock solution contained 100  $\mu\text{M}$  ethyl nitrite as measured by GC-MS. All solutions were kept on ice to avoid evaporation of ethyl nitrite (boiling point 17°C) and a new stock solution was prepared for each new administration. 10  $\mu\text{l}$  of the nitrite:ethanol mixture was added to the organ bath at cumulative increasing doses. The different nitrite:ethanol mixtures gave final concentrations of 50, 100, 125, 165, 250 and 510 nM ethyl nitrite in the organ bath (Fig. 6A). In separate experiments, and in order to exclude the influence of  $\text{NO}$  derived from nitric oxide synthase (NOS), the nitrite:ethanol mixture was also administered to pre-contracted vascular segments which had been incubated with the  $\text{NO}$  synthase inhibitor L-NAME at a final concentration of 1 mM. Also, to demonstrate the  $\text{NO}$ -dependent relaxatory effect of ethyl nitrite, the nitrite:ethanol mixture was administered to pre-contracted vascular segments which had been incubated with the soluble guanylyl cyclase inhibitor ODQ [30] 1  $\mu\text{M}$ . To reveal any effect of the individual compounds contained in the nitrite:ethanol mixture, control experiments with nitrite or ethanol alone were run by adding the total cumulative doses 3  $\mu\text{M}$  (nitrite) and 0.12% (ethanol) prepared at pH 2.0.

### Rat fundus stomach strips

Wistar rats (250–350 g) were killed by cervical displacement according to approved guidelines. The abdomen was opened and the stomach removed. The fundus was dissected and washed in fresh KH. Following this, longitudinal strips (1.5×15 mm) were carefully prepared and mounted vertically in the organ bath (10 ml capacity) containing KH bubbled with 6.5%  $\text{CO}_2$  in  $\text{O}_2$ . Tissues were mounted under an initial resting tension of 2 g and left to equilibrate for a period of 120 min. Tension changes were recorded using a MLT050/D isometric force transducers couple to a bridge amplifier ML224 and PowerLab 4/30 data acquisition system (AD Instruments, Australia).

Rat fundus stomach strips were pre-contracted with 5  $\mu\text{M}$  carbachol and let to equilibrate until a stable contraction was reached ( $\pm 5$  min). The nitrite:ethanol mixtures of 250 and 1000  $\mu\text{M}$  sodium nitrite in 10% ethanol at pH 2.0 were prepared immediately before administration to the organ bath. These solutions contained 100 and 500  $\mu\text{M}$  ethyl nitrite respectively as measured by GC-MS. The nitrite:ethanol mixtures gave final concentrations of 0.5 and 2.5  $\mu\text{M}$  ethyl nitrite in the organ bath. Control experiments with nitrite or ethanol alone at pH 2.0 were run with final concentrations in the organ bath of 1.25 and 5  $\mu\text{M}$  (nitrite) and 0.05% (ethanol).

### Statistical analysis and data presentation

Statistical analysis was performed by one-way Anova variance followed by Dunnett's test. In the case of two-sample comparisons, an unpaired *t* test was used. Differences were considered statistically significant at  $p < 0.05$ .

Contractions of the artery segments and the stomach strips are expressed as the percentage of phenylephrine or carbachol-induced contractions. The pre-contracted level was set to 0%, and the baseline level, corresponding to maximal relaxation, of 100%. All values were expressed as mean  $\pm$  S.E.M.

## Results

### Ethyl nitrite detection and quantification

The electron impact ionization mass spectrum for ethyl nitrite in the mixture of 250  $\mu\text{M}$  nitrite in 10% ethanol at pH 2.0 (Fig. 1) is consistent with those reported in the literature [29,31]. Mass spectrum was acquired at retention time (RT) of 1.15 min and it is also consistent with that obtained from ethyl nitrite standard at pH 2.0 (data not shown), clearly identifying the production of ethyl nitrite in the nitrite:ethanol mixture.

Quantification of ethyl nitrite in a range of concentrations from 1  $\mu\text{M}$  to 600  $\mu\text{M}$  can be achieved from extracted ion chromatograms, considering the ratios of the peak areas at RT 1.15 and at RT 3.30 when monitoring ion  $\text{CH}_2\text{ONO}^+$  ( $m/z=60$ ) characteristic for ethyl nitrite and 1,4-dioxane molecular ion ( $m/z=88$ ) in samples and standard solutions.

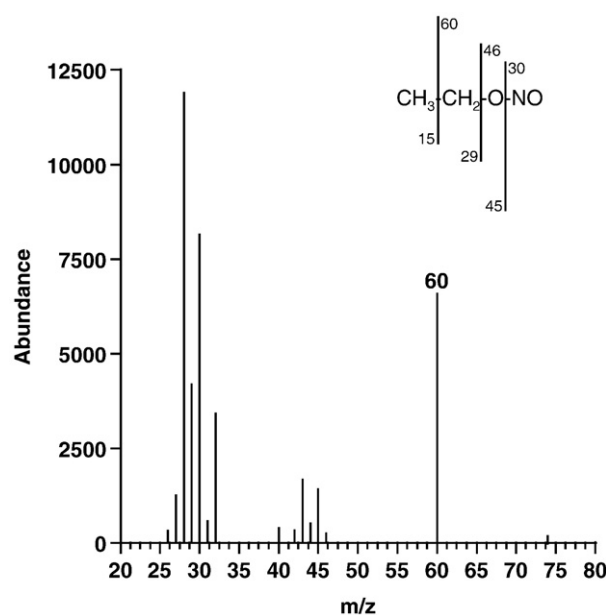


Fig. 1. GC-MS identification of ethyl nitrite. Electron ionization mass spectrum of the nitrite:ethanol mixture at RT 1.15 min. Mixture of 250  $\mu\text{M}$  nitrite in 10% ethanol at pH 2.0.

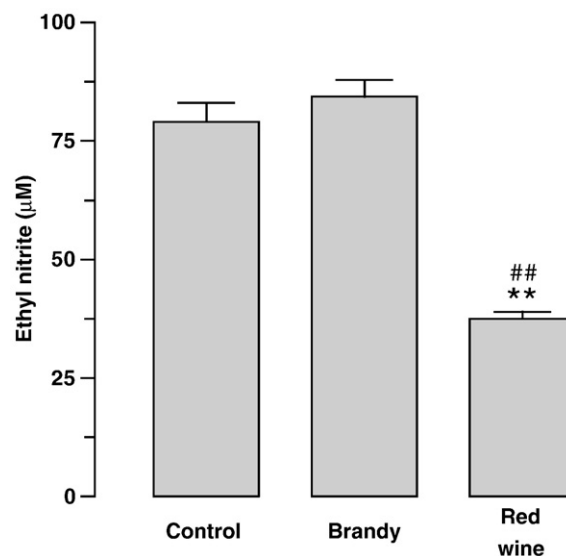
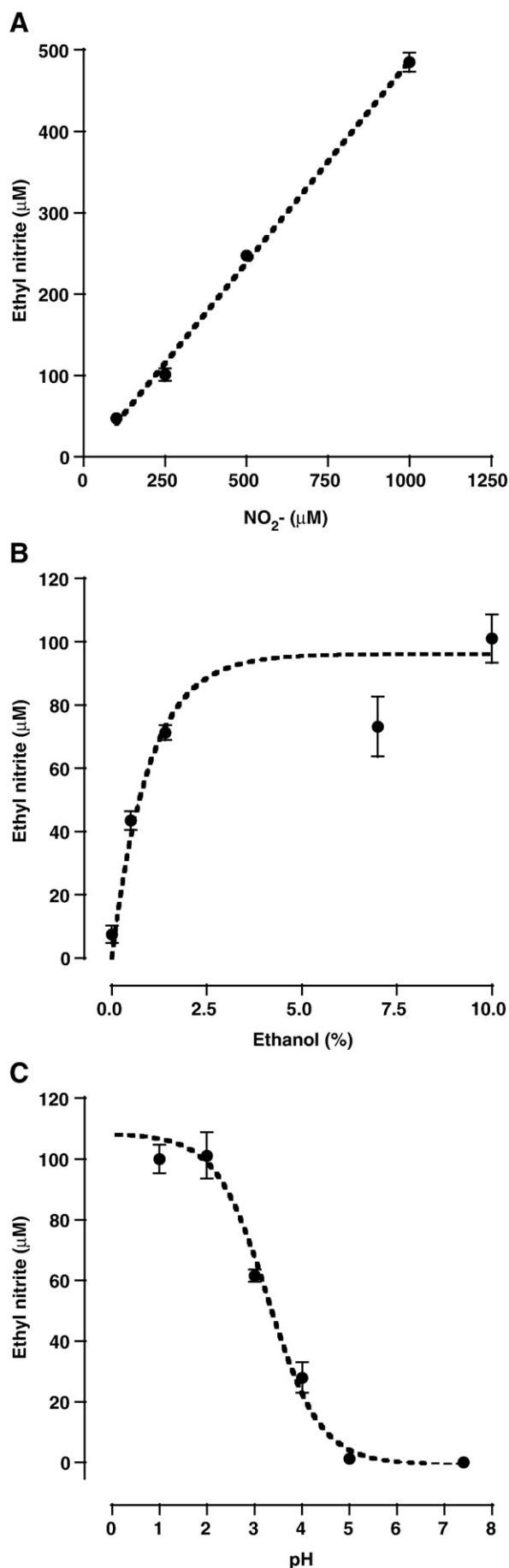


Fig. 3. Ethyl nitrite production from 250 μM nitrite in alcoholic drinks at pH 2.0. Wine and brandy were diluted to reach the concentration of 7% ethanol and the pH fixed to 2.0. Values are mean±S.E.M. \*\* $p < 0.01$  relative to control, ## $p < 0.01$  relative to brandy.

#### Ethyl nitrite production under simulated gastric conditions

The production of ethyl nitrite was studied as a function of pH as well as of nitrite and ethanol concentration (Fig. 2). Concentrations in a range compatible with those found *in vivo* following food intake were used, comprising 100 to 1000 μM for nitrite, 0.015 (2.5 mM) to 10% (1.7 M) for ethanol, and a pH range between 1.0 and 7.4. The mixture of 250 μM nitrite with 10% ethanol at pH 2.0 was chosen as a representative dietary condition. For 10% ethanol at pH 2.0 the production of ethyl nitrite is a linear function of nitrite concentration (Fig. 2A). For 250 μM nitrite the ethyl nitrite production was  $101.0 \pm 33.3$  μM. When varying the concentration of ethanol for a constant concentration of 250 μM nitrite, the formation of ethyl nitrite increases up to 1.4% ethanol (Fig. 2B) but with higher ethanol concentrations no further increase was found, suggesting a saturation beyond this concentration of ethanol.

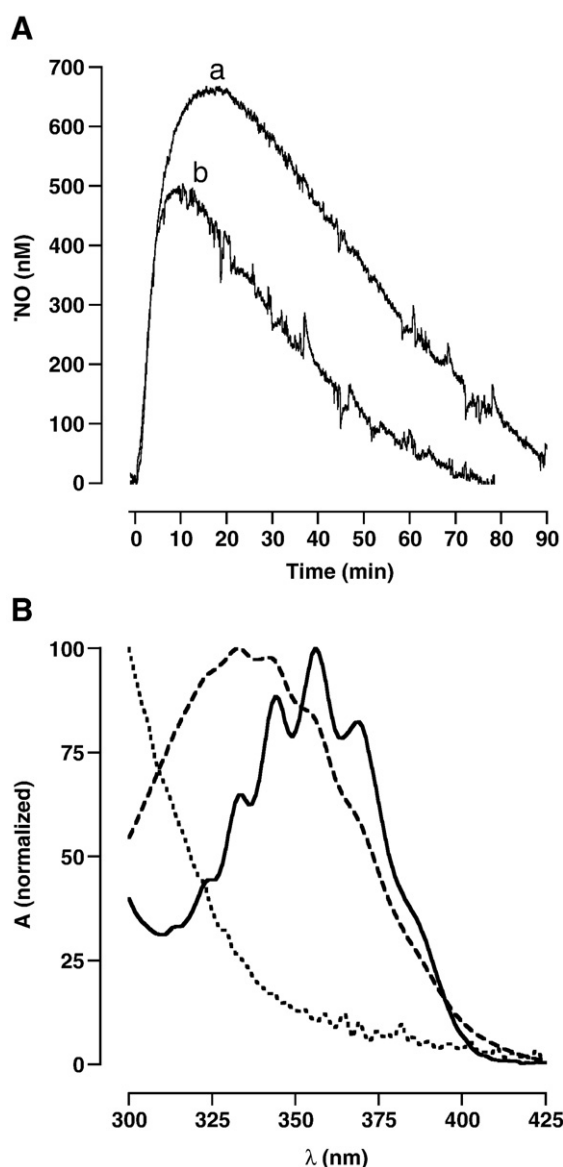
The pH critically affected the formation of ethyl nitrite. As shown in Fig. 2C, the addition of 250 μM nitrite to 10% ethanol generated around 100 μM ethyl nitrite in the pH range between 1.0 and 2.0 (physiological pH of the fasting stomach), dropping sharply at higher pH values. At pH 3.3 (curve extrapolation) the  $pK_a$  of HNO<sub>2</sub>, ethyl nitrite formation was about 50% of maximum. At pH 5.0,  $1.2 \pm 0.8$  μM ethyl nitrite was produced, while at pH 7.4 the levels were below the detection limit.

Two different alcoholic drinks were used as a source of ethanol, red wine (14% v/v) and brandy (40% v/v). These beverages represent two different sources of ethanol, fermentation for red wine and distillation for brandy. In addition they differ in polyphenols content (compounds that may potentially interfere in ethyl nitrite production), which is higher in red wine [32–34]. Finally, a solution of ethanol (p.a.) was used as control. Fig. 3 shows the formation of ethyl nitrite at pH 2.0 when 250 μM nitrite reacted with diluted red wine or brandy (final ethanol concentration of 7%). Under these conditions, the production of ethyl nitrite promoted by red wine, brandy and ethanol solution (control) was  $38 \pm 1.4$ ,  $84 \pm 3.4$  and  $79 \pm 4.0$  μM respectively. There was a significant difference ( $p < 0.01$ ) between red wine and control but not between control and brandy ( $p > 0.05$ ).

Fig. 2. Ethyl nitrite production from nitrite:ethanol mixture. A) 100, 250, 500 and 1000 μM nitrite in 10% ethanol at pH 2.0; B) 250 μM nitrite in 0.015, 0.5, 1.4, 7 and 10% ethanol at pH 2.0; C) 250 μM nitrite in 10% ethanol at pH 1.0, 2.0, 3.0, 4.0, 5.0 and 7.4. Values are mean±S.E.M.

### Nitric oxide release from ethyl nitrite standards and from nitrite:ethanol mixtures: Effect of glutathione

Fig. 4A shows a representative tracing of  $^1\text{NO}$  release from 50  $\mu\text{M}$  ethyl nitrite standard in 50 mM phosphate buffer saline at pH 7.4 alone (Fig. 4A, line a) or in the presence of 5 mM GSH (Fig. 4A, line b). The maximal releases of  $^1\text{NO}$  and the  $t_{80}$  are presented in Table 1. The results show that the presence of GSH in the medium decreases the maximal release of  $^1\text{NO}$  from ethyl nitrite (Fig. 4A, line a versus line b). In separate experiments 10–100  $\mu\text{M}$  DTPA was added to the buffer solution to study the influence of metal traces on  $^1\text{NO}$  release from ethyl nitrite (50  $\mu\text{M}$  ethyl nitrite at pH 7.4). No changes were observed in  $^1\text{NO}$  release in the presence of DTPA (data not shown). At pH 7.4, ethyl nitrite concentration decreases with time, as followed spectrophotometrically at 280 nm (not shown). In the presence of



**Fig. 4.**  $^1\text{NO}$  release from ethyl nitrite at pH 7.4. A) Representative time course of  $^1\text{NO}$  release from, 50  $\mu\text{M}$  ethyl nitrite in the absence (line a) or presence (line b) of 5 mM GSH.  $^1\text{NO}$  detection was made electrochemically, in the dark, at room temperature and under constant stirring; B) GSNO production from GSH and ethyl nitrite at pH 7.4. Spectrum of 5 mM GSH (....), 1 mM ethyl nitrite (—) and from the mixture of 5 mM GSH and 1 mM ethyl nitrite (---). Spectra were taken immediately after the addition of ethyl nitrite, at room temperature, under constant stirring. All assays were made in phosphate buffer saline at pH 7.4 prepared with ultra pure water.

**Table 1**

$^1\text{NO}$  release from 50  $\mu\text{M}$  ethyl nitrite at pH 7.4 in the presence of GSH

	$^1\text{NO}$ Peak (nM)	$t_{80}$ (min)
Ethyl nitrite (n=8)	970 $\pm$ 36	8.7 $\pm$ 0.9
+ 1 mM GSH (n=3)	801 $\pm$ 54*	7.8 $\pm$ 0.1
+ 5 mM GSH (n=3)	610 $\pm$ 31**	3.9 $\pm$ 0.2**

Values are mean $\pm$ S.E.M.; \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to ethyl nitrite alone.

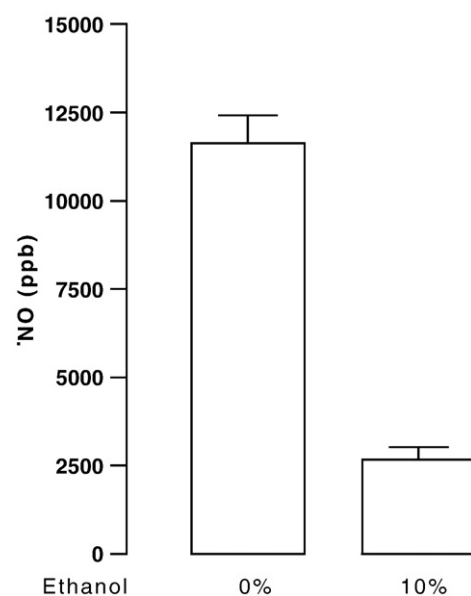
5 mM GSH at pH 7.4, the addition of 1 mM ethyl nitrite promotes the formation of GSNO as detected by the typical absorption maximum of RSNO at 336 nm (Fig. 4B, dashed line). Fig. 4B, dot line and solid line represent the spectra of GSH and ethyl nitrite, respectively.

Fig. 5 shows the release of  $^1\text{NO}$  from the nitrite:ethanol mixture and from nitrite alone. As expected, at this acidic pH, nitrite spontaneously yielded  $^1\text{NO}$ . The presence of ethanol however led to an almost 80% decrease in  $^1\text{NO}$  release most likely due to the formation of ethyl nitrite. Such  $^1\text{NO}$  scavenging effect of ethanol has been described before [35].

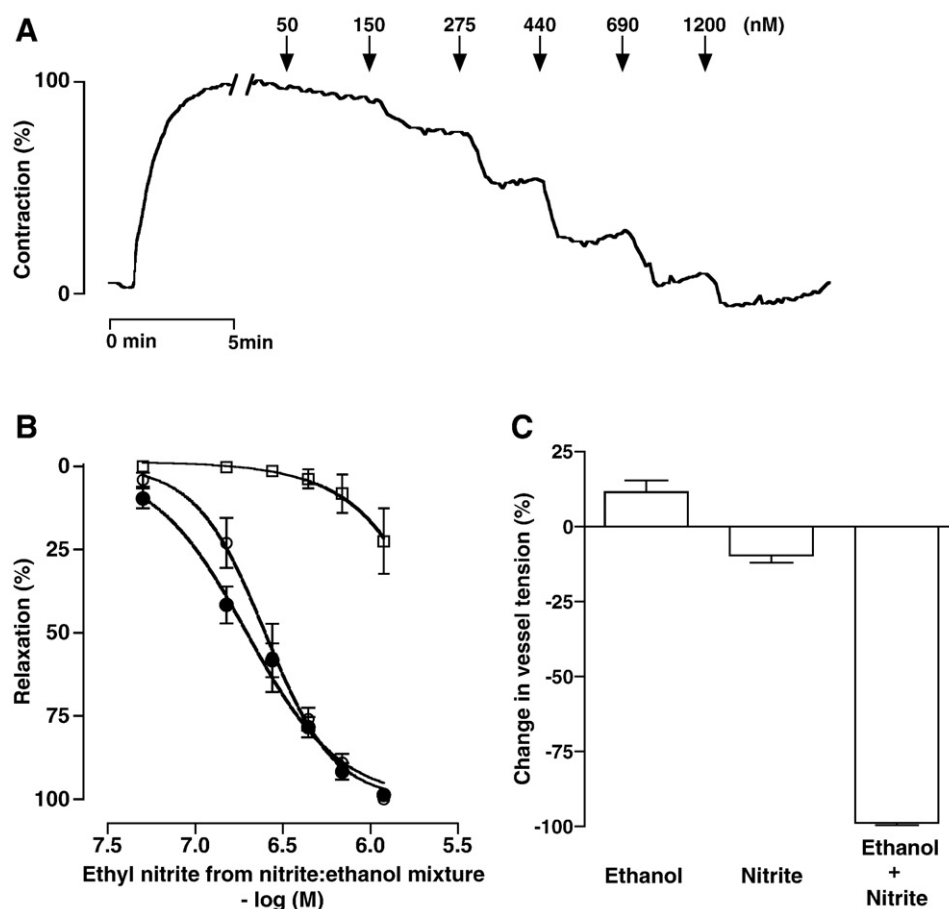
### Rat femoral artery rings and gastric fundus strips: responses to nitrite:ethanol

A dose-dependent vascular relaxation was obtained when the nitrite:ethanol mixtures were added to the femoral artery rings (Fig. 6). For the initial dose of 50 nM ethyl nitrite 10 $\pm$ 3% relaxation was observed. 50% of relaxatory effect ( $\text{EC}_{50}$ ) was accomplished with the cumulative dose of 198 $\pm$ 11 nM ethyl nitrite and a complete relaxation was achieved for the cumulative dose of 1.2  $\mu\text{M}$  ethyl nitrite (Fig. 6B, close circles). The dose-response curve was not significantly changed in the presence of 1 mM L-NAME (Fig. 6B, open circles). In contrast in the presence of 1  $\mu\text{M}$  ODQ, the dose-response curve for ethyl nitrite was strongly rightward shifted (Fig. 6B, open squares). A small but significant relaxation (10 $\pm$ 3%,  $p < 0.05$ ) was produced by nitrite alone (3  $\mu\text{M}$ ) while ethanol (20.6 mM - 0.12%) alone evoked a significant contraction (12 $\pm$ 4%,  $p < 0.05$ , Fig. 6C).

The pre-contracted gastric fundus strips relaxed dose-dependently in response to nitrite:ethanol mixtures (Table 2). In contrast, control



**Fig. 5.**  $^1\text{NO}$  release after addition of 250  $\mu\text{M}$  nitrite to either 0% or 10% ethanol at pH 2.0. Values are mean $\pm$ S.E.M.  $^1\text{NO}$  detection was made by chemiluminescence in the headspace of the reaction chamber. Acidified nitrite yields  $^1\text{NO}$  via disproportionation. With ethanol less free  $^1\text{NO}$  is detected due to rapid formation of ethyl nitrite.



**Fig. 6.** Dose-response of phenylephrine pre-contracted rat femoral artery rings to the mixture of 250  $\mu\text{M}$  nitrite in 10% ethanol at pH 2.0. A) Representative recording of rat femoral artery rings in an organ bath containing Krebs solution pH 7.4, when pre-contracted with phenylephrine (1  $\mu\text{M}$ ) followed by relaxation evoked by an increasing concentration of ethyl nitrite from the nitrite:ethanol mixture achieved by successive dilutions. Concentrations of ethyl nitrite were calculated based on the quantification of the total amount of ethyl nitrite in the original mixture by GC-MS; B) dose-response curves to cumulative concentrations of ethyl nitrite from the nitrite:ethanol mixture (close circles), in the presence of 1 mM L-NAME (open circles) and in the presence of 1  $\mu\text{M}$  ODQ (open squares); C) Response to the total cumulative dose of the nitrite:ethanol mixture (1.2  $\mu\text{M}$  ethyl nitrite), ethanol alone (0.12%) and nitrite alone (3  $\mu\text{M}$ ) at pH 2.0.

strips exposed to the same amounts of nitrite or ethanol alone, showed no significant change in tension (data not shown).

## Discussion

Nitrite is now emerging as an important alternative substrate for  $\text{NO}$  generation in processes that are independent on  $\text{NO}$  synthases. [1–6].  $\text{NO}$  formation from nitrite proceeds via enzymatic as well as non-enzymatic pathways. Nonenzymatic acid-catalyzed reduction of nitrite to  $\text{NO}$  is the predominate pathway in the stomach [6,8,9].

In a previous study we have shown that consumption of red wine increases intragastric  $\text{NO}$  formation following the intake of nitrate and suggested a role for wine polyphenols as catalysts of nitrite reduction to  $\text{NO}$  [23]. Considering the well-known reaction of aliphatic alcohols with  $\text{HNO}_2$  to yield alkyl nitrites [25] by O-nitrosation [24] it became pertinent to assess whether the ethanol component of alcoholic drinks, would react with nitrite, and if the reaction product would be bioactive. Clearly, it is demonstrated here that the ethanol content of red wine and other alcoholic drinks, at dilutions found *in vivo*, reacts with physiological amounts of nitrite under gastric conditions to yield ethyl nitrite (Fig. 3) a compound endowed with vasoactive properties (Fig. 6) via  $\text{NO}$  release (Figs. 4 and 5).

Chemically the formation of ethyl nitrite may encompass reaction 4 and 5, in which alcohols react with  $\text{HNO}_2$  [36,37] and  $\text{N}_2\text{O}_3$

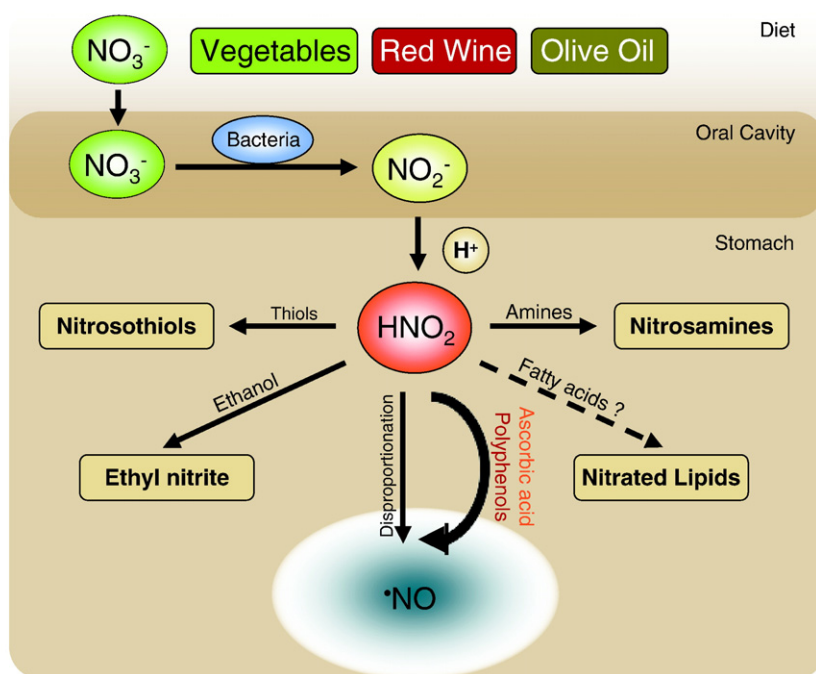
[26,27,38] to generate alkyl nitrites. Given that nitrite is continually produced in human saliva by oral commensal bacteria [13,39,40],  $\text{HNO}_2$  will be present in the gastric juice as long as a low pH is maintained. Via its reduction,  $\text{HNO}_2$  produces high levels of  $\text{NO}$  in the stomach lumen [8,9,20,23] which in turn is in equilibrium with  $\text{N}_2\text{O}_3$  (reaction 3). In this scenario when ethanol reaches the gastric compartment it is likely to react with both,  $\text{HNO}_2$  and  $\text{N}_2\text{O}_3$ , and O-nitrosation reactions are prone to happen. These reactions will depend not only on the rate constants for the individual reactions that govern the complex equilibrium between nitrogen oxides, but also on several

**Table 2**

Relaxation of carbachol pre-contracted rat fundus stomach strips in response to a nitrite:ethanol mixture

Nitrite:ethanol mixture ( $\mu\text{M}$ ethyl nitrite)	% of relaxation
0.5	17 $\pm$ 4
2.5	57 $\pm$ 5***

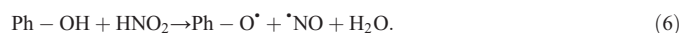
50  $\mu\text{l}$  of nitrite:ethanol mixtures (250 or 1000  $\mu\text{M}$  nitrite in 10% ethanol at pH 2.0) were added to the organ bath (10 ml) and the relaxatory response was determined. Controls with the same amounts of nitrite or ethanol alone evoked no significant changes in tension. Ethyl nitrite values represent the final calculated concentrations in the organ bath as detected by GC-MS in the original nitrite:ethanol mixtures. Values are mean  $\pm$  S.E.M. (n=16); \*\*\* p<0.0001 (unpaired t test).



**Fig. 7.** Simplified scheme showing how dietary nitrate can interact chemically with other nutrients to generate a number of novel bioactive compounds. In this example the focus is on vegetables, olive oil and wine which constitute a central part of the typical Mediterranean diet. Many vegetables including spinach, lettuce and beetroot are very rich in inorganic nitrate ( $\text{NO}_3^-$ ). After ingestion, nitrate rapidly accumulates in saliva where it is reduced by commensal bacteria to form nitrite ( $\text{NO}_2^-$ ). In the acidic stomach nitrite forms nitrous acid ( $\text{HNO}_2$ ) and a variety of reactive nitrogen oxides (RNOs), which in turn can interact with other dietary constituents. It is now clear that, not only potentially carcinogenic nitrosamines are formed from the interaction of RNOs with nutrients. First,  $\text{HNO}_2$  spontaneously yields  $\cdot\text{NO}$  via disproportionation, and this reduction is greatly enhanced in the presence of dietary polyphenols and ascorbic acid. Ethyl nitrite is formed from the reaction of  $\text{HNO}_2$  with ethanol as shown in this study and S-nitrosation reactions give rise to nitrosothiols. Unsaturated fatty acids (e.g. linoleic and oleic acid) from olive oil may undergo nitration following reaction with RNO's (in particular the  $\text{NO}_2$  radical) giving rise to nitrated lipids. The existence of this latter reaction however, is yet to be proven *in vivo*. Nitrite, nitrosothiols, ethyl nitrite and nitrated lipids can all act as transducers of  $\cdot\text{NO}$ -like bioactivity including vasodilation, stimulation of mucus generation, antimicrobial and anti-inflammatory effects. Most of these compounds are fairly stable and readily absorbed which allows also for systemic effects. It is interesting that all nutrients highlighted in this figure may divert the chemistry in the stomach away from nitrosamine formation and instead favor the formation of other chemically related bioactive compounds. Future studies will clarify if the well-known health promoting effects of a Mediterranean diet is related to this alternative chemical fate of dietary nitrate.

variables and conditions present in the stomach lumen. In particular the following may be considered:

(1) The amount of nitrite in saliva will directly influence the levels of  $\text{N}_2\text{O}_3$ ,  $\text{HNO}_2$  and  $\cdot\text{NO}$  in the stomach [20] and thereby also the levels of ethyl nitrite (Fig. 2A); (2) ethanol acts as substrate for nitrosation and small changes in its concentration will influence the production of ethyl nitrite. In a normal situation after ethanol consumption, ethanol will be in large excess compared to  $\text{HNO}_2$ . As shown in Fig. 2B, ethanol (0.015%) and nitrite (250  $\mu\text{M}$ ) yields low amounts of ethyl nitrite but already at 1.4% the levels had reached a plateau and were not statistical different from the ones achieved for 10% ethanol, demonstrating that nitrite is likely the limiting factor; (3) pH influences the equilibrium between all the nitrosative species. An increase in pH from 2.0 (fasting) to 5.0 (post-prandial) results in major changes in the equilibrium between the different nitrogen oxides with reduced  $\text{HNO}_2$  and  $\text{N}_2\text{O}_3$  concentrations. For pH values lower than 3.0, ethyl nitrite production was independent of pH, but when pH increases above the  $\text{pK}_a$  of  $\text{HNO}_2$  (~3.3), a rapid decrease was observed (Fig. 2C). This demonstrates the crucial importance of  $\text{HNO}_2$  for ethanol O-nitrosation in the stomach; (4) the content of  $\text{HNO}_2$ -reducing agents like polyphenols and ascorbic acid, affects reaction 2, shifting it to the right with an increase in  $\cdot\text{NO}$  production [23,41–43]. Ethyl nitrite production was more than 2 times lower for red wine compared with brandy or the control ethanol solution. In contrast to brandy [34], red wine has a high polyphenolic content [32,33]. These polyphenols (Ph-OH) greatly accelerate  $\cdot\text{NO}$  formation from  $\text{HNO}_2$  as shown in recent studies [23,41]:



Taking into consideration reactions 4 and 6, the red wine phenols will compete for  $\text{HNO}_2$  thereby diminishing the nitrosation reaction of ethanol.

In the bioassay studies with rat femoral artery rings, the nitrite: ethanol mixture produced a dose-dependent relaxation with an  $\text{EC}_{50}$  around 200 nM (Fig. 6). In blood the half-lives of alkyl nitrites are in the range of seconds (isobutyl nitrite around 1.4 min) [44], and they can decompose by nonenzymatic hydrolysis as well as by enzymatic cleavage. In the case of ethyl nitrite it is known that it spontaneously releases low fluxes of  $\cdot\text{NO}$  in aqueous solution [45] by homolytic cleavage of the O-NO bond [46]. The inhibition of vascular relaxation in the presence of OEQ and the inability of L-NAME to block the vasodilatory effect, strongly suggest that the relaxation was caused by  $\cdot\text{NO}$  from ethyl nitrite. One might argue that ethanol and nitrite produced these effects independently of ethyl nitrite formation, since vasoactivity has been described for both these compounds [47,48]. However, when applied in high concentrations separately, ethanol and nitrite were devoid of significant vasodilatory activity. In fact, with ethanol we even noted a minor vasoconstriction (Fig. 6C) which is in accordance with recent studies [49]. The small vascular relaxation observed with nitrite alone when prepared at pH 2.0 (Fig. 6C) is likely a result of free dissolved  $\cdot\text{NO}$  being generated by acidic disproportionation (this reaction stops abruptly however, once the solution is mixed with the physiological buffer in the organ baths). With the combined nitrite: ethanol mixture profound vasorelaxation occurred despite less free  $\cdot\text{NO}$  being generated in the solution (Fig. 5), supporting ethyl nitrite as being the active component. Likely, this ethyl nitrite readily diffuses into the smooth muscle where it effectively releases  $\cdot\text{NO}$  in close vicinity of the guanylyl cyclase.

On the other hand, since ethyl nitrite is endowed with nitrosative properties, transnitrosation reactions are prone to occur in the presence of substrates like alcohols, thiols and amines. As shown in Fig. 4 and in Table 1, there is a decrease in maximal release of  $\cdot\text{NO}$  from ethyl

nitrite in the presence of GSH; an observation in accordance with the transnitrosation of the <sup>14</sup>N-NO moiety from ethyl nitrite (Fig. 4B, solid line) to GSH (Fig. 4B, dotted line a), with the final formation of GSNO (Fig. 4B, dashed line). GSNO is a more stable product and <sup>14</sup>N-NO release will be less obvious.

The rapid transnitrosation reaction between ethyl nitrite and GSH will transfer the "NO-like" biological activity of ethyl nitrite to GSNO.

From this study it is also clear that the yield of ethyl nitrite from nitrite:ethanol mixture is rapid and remarkably large under acidic conditions. In fact in the *in vitro* experiments almost 50% of added nitrite instantly formed ethyl nitrite (Fig. 2A). In contrast, under the same conditions but without ethanol the formation of <sup>14</sup>N-NO from nitrite via acidic disproportionation is much slower.

The pharmacological effects of alkyl nitrites have been known for almost a century and ethyl nitrite is being tested in clinical studies as a hemodynamic regulator for treatment of hypertension in the newborn [50] and in animals during laparoscopic procedures [51], and in reversing surgical vasospasm [52]. In the stomach ethyl nitrite will easily reach  $\mu$ M concentration and it is very likely that it will have biological effects. For nitrite such effects have already been shown and they include mucosal vasodilatation, increased mucus generation, destruction of pathogens and protection against gastric ulcer [19,20,22]. While <sup>14</sup>N-NO formation from nitrite and ethyl nitrite can clearly be beneficial in gastroprotection, detrimental effects have also been suggested including formation of carcinogenic nitrosamines from nitrite/<sup>14</sup>N-NO and its reaction products [18]. It is not known how ethyl nitrite will affect these potentially carcinogenic pathways. It will be interesting to study if ethyl nitrite can survive passage to deeper mucosal structures and cause relaxations of the underlying smooth muscle layer. This effect is in fact suggested by the relaxatory effect of the nitrite:ethanol mixture seen in the experiments with rat stomach strips (Table 2) [53]. Such effects if they occur also *in vivo*, could contribute to gastric adaptive responses as well as the cardia relaxation and concomitant gastro-esophageal reflux commonly seen after alcohol ingestion [54–56]. Finally, an intriguing but yet unproven possibility exists that some ethyl nitrite is absorbed intact in the GI tract, escapes liver metabolism to reach the systemic circulation. In this context it is tempting to speculate that some of the described acute effects of ethanol on the cardiovascular system (such as reduction in blood pressure and increases in forearm blood flow) [48,57,58], involve <sup>14</sup>N-NO release from circulating ethyl nitrite.

This study and other recent reports [23,59] highlight the stomach as a bioreactor where bioactive molecules are produced from common dietary components. For dietary nitrate the focus over the past 50 years has been entirely on the formation of potentially carcinogenic nitrosamines via nitrite-mediated N-nitrosation [14,18]. It is now becoming clear however, that a variety of other bioactive compounds are formed from nitrite under acidic gastric conditions (Fig. 7). These include <sup>14</sup>N-NO, S-nitrosothiols [20], as well as O-nitrosation products such as the ethyl nitrite described here. In addition, nitration reactions will likely occur including the formation of nitroalkenes from dietary unsaturated fatty acids. The latter is an interesting possibility, as nitroalkenes have powerful anti-inflammatory activity *in vitro* [60]. In general, it is becoming clear that dietary nitrate and its reaction products are not necessarily a threat to human health. In fact, numerous recent studies now imply beneficial effects of nitrate in the gastrointestinal tract and the cardiovascular system [5]. The nutritional implications of this are potentially intriguing. As an example, the typical Mediterranean diet with a high intake of vegetables, fruits, olive oil and wine, provides a rich supply of nitrate/nitrite, polyphenols, unsaturated fatty acids and ethanol. It remains to be studied if the well-known health promoting effects of this diet in any way is related to the chemical interactions between these dietary components.

We conclude that the potent vasodilator ethyl nitrite is generated under gastric conditions from physiological amounts of nitrite and ethanol in red wine or brandy. The physiological and pathophysiological role of gastric ethyl nitrite formation remains to be elucidated.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.04.027.

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