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Influence of antioxidant depletion on nitrergic relaxation in the pig gastric fundus

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1 The hypothesis that endogenous tissue antioxidants might explain the inability of the superoxide generators 6-anilino-5,8-quinolinedione (LY83583) and hydroquinone (HQ) and of the NO-scavengers hydroxocobalamin (HC) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) to affect nitrergic neurotransmission in the porcine gastric fundus was tested by selective pharmacological depletion of respectively Cu/Zn superoxide dismutase (Cu/Zn SOD) and reduced glutathione (GSH) in circular smooth muscle preparations.

2 Diethyldithiocarbamate (DETCA; 3×10^{-3} M), which almost completely abolished tissue Cu/Zn SOD activity, had no effect *per se* on nitrergic relaxations induced by either electrical field stimulation (EFS; 4 Hz, 10 s) or exogenous nitric oxide (NO; 10^{-5} M). In these DETCA-treated tissues however, electrically-induced nitrergic relaxations became sensitive to inhibition by LY83583 (10^{-5} M) or HC (10^{-4} M), but not by HQ (10^{-4} M) or c-PTIO (10^{-4} M); only for the combination of DETCA plus LY83583, this inhibition was partially reversed by exogenous Cu/Zn SOD (1000 u ml^{-1}).

3 Immunohistochemical analysis of porcine gastric fundus revealed a 100% colocalization of Cu/Zn SOD and neuronal nitric oxide synthase (nNOS) in the intrinsic neurons of the myenteric plexus.

4 Buthionine sulphoximine (BSO; 10^{-3} M) in the absence or presence of LY83583 (10^{-5} M) or HC (10^{-4} M) did not alter nitrergic relaxations, although it reduced *per se* the tissue GSH content to 62% of control.

5 Pharmacological depletion studies, corroborated by immunohistochemical data, thus suggest a role for Cu/Zn SOD but not for GSH in nitrergic neurotransmission in the porcine gastric fundus. *British Journal of Pharmacology* (2002) **135,** 917–926

Keywords: Porcine gastric fundus; superoxide dismutase; diethyldithiocarbamate; glutathione; buthionine sulphoximine; carmustine; ethacrynic acid; immunohistochemistry; depletion study

Abbreviations: BSO, buthionine sulphoximine; CARM, carmustine; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DETCA, diethyldithiocarbamate; DMSO, dimethylsulphoxide; EA, ethacrynic acid; EFS, electrical field stimulation; GSE, glutathione ethyl ester; HC, hydroxocobalamin; HQ, hydroquinone; 5-HT, 5-hydroxytryptamine; LY83583, 6-anilino-5,8-quinolinedione; NAC, N-acetyl-cysteine; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; NO^+, nitrosonium cation; NO^-, nitroxyl anion; PBS, phosphate-buffered saline; PGP 9.5, protein-gene product 9.5; SNP, sodium nitroprusside; SOD, superoxide dismutase; UA, uric acid; VIP, vasoactive intestinal polypeptide

Introduction

Non-adrenergic non-cholinergic (NANC) inhibitory neurons serve a major role in the enteric nervous system: they provide the main neurotransmitters for regulation of several gastrointestinal motility patterns such as lower oesophagus and pyloric sphincter relaxation (Allescher *et al*., 1992; Preiksaitis *et al*., 1994), gastric adaptive and receptive relaxation (Abrahamsson, 1986) and intestinal accommodation processes (Waterman *et al*., 1994). At the level of the gastric fundus, nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) are generally recognized as the predominant inhibitory NANC neurotransmitters eliciting smooth muscle relaxation (Li & Rand, 1990; Lefebvre *et al*., 1995).

Although there is compelling biochemical and immunohistochemical evidence that neuronal nitric oxide synthase (nNOS) represents the essential enzyme in peripheral nerves for the production of the nitrergic neurotransmitter (Stuehr, 1997), there still is doubt about the exact identity of the latter. The pharmacological observation that a number of drugs (both superoxide generators and NO-scavengers) are potent inhibitors of relaxations induced by exogenous NO but have no or little effect on relaxation due to nitrergic nerve stimulation, is crucial for this controversy (Hobbs *et al*., 1991; Barbier & Lefebvre, 1992; La & Rand, 1999). Three possibilities were considered to explain this paradox, each concentrating on a distinct biochemical form of NO: (1) nitrosothiols (Barbier & Lefebvre, 1994; De Man *et al*., 1995); (2) alternative redox activated states of NO [nitrosonium cation (NO^+) or nitroxyl anion (NO^-)] (Gibson *et al*., 1995; Li *et al*., 1999); (3) the electrically neutral free radical $NO^\bullet$ itself, which should be protected by tissue antioxidants from superoxide attack and NO-scavenger inactivation (Lilley & Gibson, 1996; Colpaert & Lefebvre, 2000).

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Several lines of evidence support a role for antioxidants in the nitrergic neurotransmission process, and thus favour the latter hypothesis: (1) irreversible inhibition of the antioxidant enzyme Cu/Zn superoxide dismutase (Cu/Zn SOD) with the Cu-chelator diethyldithiocarbamate (DETCA) rendered the nitrergic neurotransmitter, released by electrical nerve stimulation, sensitive to superoxide generated by hypoxanthine/xanthine oxidase and pyrogallol in the bovine retractor penis muscle (Martin *et al*., 1994), by duroquinone in the mouse anococcygeus (Lilley & Gibson, 1995) and by LY83583 in the rat gastric fundus (Lefebvre, 1996); (2) colocalization of respectively Cu/Zn SOD and haeme oxygenase-2, the rate limiting enzyme in the production of the potent antioxidant and free radical scavenger bilirubin, with nNOS has been demonstrated in nitrergically-innervated gastrointestinal tissues (Liu *et al*., 1997; Zakhary *et al*., 1997); (3) reduced glutathione, ascorbate and α-tocopherol preserved the bioavailability of exogenously added NO in the mouse anococcygeus from some or all of the tested superoxide generators and direct NO-scavengers (Lilley & Gibson, 1996); (4) significant amounts of the antioxidant ascorbate were released from the rat anococcygeus upon nerve depolarization (Lilley & Gibson, 1997).

We have previously shown that exogenous Cu/Zn SOD and reduced glutathione (GSH) exhibit the broadest pattern of protection of exogenous NO against inactivation by superoxide generators and a NO-scavenger in the porcine gastric fundus (Colpaert & Lefebvre, 2000). The aim of the present study was to investigate the potential need for these antioxidants to protect the endogenous nitrergic neurotransmitter in the porcine gastric fundus by depleting the tissues of these antioxidants.

Methods

Tissue preparation and general methodology

Experiments were carried out on isolated circular smooth muscle strips of the porcine gastric fundus. The stomach was removed from healthy 6 months old male castrated pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution. After the mucosa was removed, strips (15×3 mm) were cut from the fundus in the direction of the circular muscle layer. All tissues were used immediately. Strips were mounted vertically between two platinum plate electrodes under a load of 2 g in 20 ml organ baths, containing physiological salt solution at 37°C and gassed with 95% O_2/5% CO_2. The composition of the physiological salt solution was (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 124.1, HCO_3^- 25, $H_2PO_4^-$ 1.2 and glucose 11.5 (Mandrek & Milenov, 1991). To obtain NANC conditions, atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were continuously present in the medium. In one set of experiments, atropine was omitted from the medium in order to study cholinergic contractions of the preparations. The mechanical activity of the preparations was recorded *via* isotonic transducers (T_3, Palmer Bioscience, U.S.A.) on a recorder (FWR 3701 Graphtec Linearcorder or MC6625 Graphtec Multicorder, Japan). Electrical field stimulation (EFS, 40 V, 0.1 and 0.5 ms, 4 Hz, 10 s) was applied by means of a stimulator (S88 Grass, U.S.A.). The tissues were allowed to equilibrate for 90 min with rinsing every 15 min before starting the experiment.

Protocols for organ bath experiments

After the equilibration period, all strips were first contracted with 3×10^{-7} M 5-hydroxytryptamine (5-HT) and subsequently relaxed by 10^{-5} M sodium nitroprusside (SNP). Following an interval of 1 h with regular rinsing, the experiment was then continued.

In a first series of investigations, we studied the influence of tissue antioxidant depletion on relaxations induced by nitrergic nerve stimulation or exogenous NO. Tone was raised with 3×10^{-7} M 5-HT; it induced stable plateau contractions [in concentration-response curves for 5-HT, obtained in previous experiments (Lefebvre *et al*., 1995), the contractile response to 10^{-7} and 10^{-6} M 5-HT was $59 \pm 7\%$ and $93 \pm 3\%$ of maximum in circular muscle strips]. When a stable plateau contraction was obtained, two relaxant stimuli were consecutively studied with an 8 min interval in between: EFS (40 V, 0.1 ms) at 4 Hz for 10 s and a bolus of exogenous NO (10^{-5} M). The parameter values for these nitrergic relaxations were chosen so that the amplitude of relaxation was at least 50% of the relaxation induced by 10^{-5} M SNP at the beginning of the experiment. Tissues were repetitively rinsed and after 10 min the antioxidant depletors were administered and left in contact with the tissue for respectively 30 min [ethacrynic acid (EA)], 1 h [diethyldithiocarbamate (DETCA) and carmustine (CARM)] or 2 h [buthionine sulphoximine (BSO)]; these compounds were then washed from the tissue baths. Subsequently LY83583, hydroquinone, hydroxocobalamin or c-PTIO was then added, and 15 min later contraction was again induced with 5-HT and the two relaxant stimuli were repeated. LY83583 and hydroquinone were used as superoxide generators as previous work in porcine gastric fundus established their superoxide generating capacity in this tissue (Colpaert & Lefebvre, 2000). Hydroxocobalamin and c-PTIO are NO-scavengers (Ellis *et al*., 2001). Preparations from the same animal, receiving only the antioxidant depletor, one of the superoxide generators or NO-scavengers or the solvents (time control) were always run in parallel.

In an additional set of experiments, we determined the influence of antioxidant replenishment; the antioxidants [Cu/Zn superoxide dismutase (Cu/Zn SOD), the glutathione precursor N-acetyl-cysteine (NAC), the cell permeable ethyl ester of glutathione (GSE) and uric acid (UA)] were added either just before the superoxide generators or NO-scavengers (series with DETCA) or just before the administration of the antioxidant depletor (series with ethacrynic acid).

In separate experiments, the effects of ethacrynic acid (EA) and of its co-incubation with NAC or GSE on contractions elicited by cholinergic nerve stimulation were also assessed. After 1 h of repetitive rinsing, EFS (40 V, 0.5 ms, 4 Hz, 10 s) was initiated with regular intervals of 5 min. Once a reproducible contraction amplitude of the strips in response to three consecutive stimulations was reached, EA or its solvent or the combination of EA with NAC or GSE was incubated, and 5 min later electrical stimulations were continued for another 20 times respecting a 5 min interval in between.

Depletion strategies

Depletion of Cu/Zn superoxide dismutase (Cu/Zn SOD) was obtained *via* the Cu-chelator diethyldithiocarbamate (DETCA), which irreversibly inhibits both extra- and intracellular Cu/Zn SOD (Kelner *et al*., 1989).

Attempts to influence the endogenous levels of reduced glutathione (GSH) were made by (1) inhibiting γ-glutamylcysteine synthetase, the rate limiting enzyme in the biosynthesis of GSH *via* buthionine sulphoximine (BSO) (Meister, 1988) or (2) inhibiting glutathione reductase, so preventing the regeneration of GSH from glutathione disulphide *via* carmustine (CARM) (Padgett & Whorton, 1997) or (3) complexation of GSH and formation of an adduct with ethacrynic acid (EA) (Tirona *et al*., 1999).

Assay of Cu/Zn SOD activity

Total Cu/Zn SOD activity in fundic supernatants was measured by the use of a commercial kit for SOD activity determination (Oxis Health Products Inc.; Portland, Oregon, U.S.A.). The SOD-525 assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzofluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Porcine gastric fundus strips were incubated in physiological salt solution, as described above for the organ bath experiments, with DETCA (3×10^{-3} M) for 1 h. Parallel preparations from the same animal, without DETCA treatment, served as controls. Tissues were subsequently blotted dry on a piece of filter paper, weighed, minced and homogenized in 0.25 M sucrose homogenization buffer in five volumes (v w^{-1}) at 4°C using a glass pestle homogenizer. Homogenates were then centrifuged at $9000 \times g$ for 10 min at 4°C. Supernatants were immediately assayed spectrophotometrically, according to the kit manufacturer guidelines. Before assay, 250 μl of supernatants were treated with 400 μl of ice-cold ethanol/chloroform 62.5/37.5 (v v^{-1}) to remove interfering compounds, centrifuged at $3000 \times g$ for 10 min at 4°C and the upper aqueous layer collected for the assay. SOD activity is expressed in SOD-525 units (as defined by Oxis) per mg protein. The protein content was measured by the method of Bradford (1976).

GSH determination

Measurements of GSH were performed using the Bioxytech GSH-400 kit from Oxis. Briefly, porcine gastric fundus strips were incubated in physiological salt solution with either ethacrynic acid (10^{-4} M; 30 min), or carmustine (2×10^{-4} M; 1 h) or buthionine sulphoximine (10^{-3} M; 2 h); control strips from the same animal were incubated in parallel. The strips were subsequently blot-dried on filter paper, weighed, frozen in liquid nitrogen and homogenized with a micro-dismembrator (B. Braun A.G., Melsungen, Germany) for 60 s. 1.5 ml of 5% metaphosphoric acid was then added and the material was then homogenized on ice with an ultrasonic probe (B. Braun A.G. 300 s, Melsungen, Germany) four times for 7 s at 15 s intervals. The homogenate was then centrifuged at $3000 \times g$ for 10 min at 4°C. The upper clear aqueous layer was collected and assayed spectrophotometrically following the kit manufacturer instructions. The protein content of the pellet was determined by the method of Bradford (1976).

Immunohistochemistry

For immunohistochemistry, tissue of the gastric fundus was obtained from 6-week-old domestic pigs that were euthanized by an overdose of pentobarbitone (50 mg kg^{-1}) intravenously. A fundic area ($\pm$ 4 cm^2) was dissected out along the major curvature and fixed for 2 h at room temperature in freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The tissues were then rinsed thoroughly in PBS, immersed overnight at 4°C in 30% sucrose in PBS, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN, U.S.A.) and cut into 12 μm thick cryostat sections which were mounted on chrome alum-gelatine-coated glass slides. For double immunostaining for Cu/Zn SOD and nNOS or protein-gene product 9.5 (PGP 9.5), the cryosections were preincubated with a blocking mixture containing 0.25% Triton X-100, 1% bovine serum albumin and 10% normal goat serum in PBS (30 min, room temperature). They were then simultaneously incubated in mouse monoclonal Cu/Zn SOD antiserum (raised against recombinant human Cu/Zn SOD; diluted 1 : 300; Sigma) and a rabbit polyclonal nNOS (raised against a synthetic peptide from the C-terminal of the cloned rat cerebellar NOS; diluted 1 : 1000; Euro-Diagnostica) or rabbit polyclonal PGP 9.5 (diluted 1 : 1000; Biogenesis) antiserum overnight at room temperature in a humid chamber. Visualization of the primary antisera was achieved by immersion in biotinylated sheep anti-mouse antibody (diluted 1 : 200; Jackson Immunoresearch Laboratories Inc.) and CY3-labelled goat anti-rabbit antibody (diluted 1 : 1000; Amersham) for 2 h followed by a 2 h incubation period with FITC-conjugated streptavidin (diluted 1 : 500; Jackson Immunoresearch Laboratories Inc.). Preparations were coverslipped with Vectashield (Vector Labs) and examined under a Zeiss Axiophot fluorescence microscope equipped with the appropriate filter set.

Drugs used

The following drugs were used (supplied by Sigma unless stated otherwise): 6-anilino-5,8-quinolinedione (LY83583; Calbiochem), atropine sulphate, bovine serum albumin, *L*-buthionine-[S,R]-sulphoximine, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; Calbiochem), carmustine, diethyldithiocarbamic acid sodium salt, ethacrynic acid, glutathione ethyl ester, guanethidine sulphate, hydroquinone, hydroxocobalamin acetate, 5-hydroxytryptamine creatinine monosulphate (Janssen Chimica), N-acetyl-*L*-cysteine, normal goat serum, paraformaldehyde, sodium nitroprusside, Cu/Zn superoxide dismutase (from bovine erythrocytes), Triton X-100, uric acid. Drugs were dissolved in deionized water except LY83583, that was dissolved in 100% ethanol, and ethacrynic acid, that was dissolved in dimethylsulphoxide (DMSO). Solvents themselves were without significant effect at the concentrations used in the experiments. Stock solutions were made of LY83583 (10^{-2} M) and Cu/Zn SOD (100,000 u ml^{-1}); other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm & Schrader

(1990), yielding a vial containing NO in a concentration taken to be 2×10^{-3} M.

Data analysis

Relaxations elicited by EFS and NO are expressed as percentage of the relaxation induced by 10^{-5} M sodium nitroprusside at the beginning of the experiment. Responses in the presence of interfering drugs are related to those obtained before administration of these drugs. Experimental data are expressed as means ± s.e.mean and n refers to the number of strips from different animals. Results between tissues are compared by an unpaired t-test. When more than two groups have to be compared, one-way analysis of variance (ANOVA) is performed; if statistical significance is reached ($P<0.05$), comparison per two groups is performed by a t-test, corrected for multiple comparisons (Bonferroni procedure). A difference is considered statistically significant at $P<0.05$.

Results

Effect of NO-inhibitors per se on nitrergic relaxation

The superoxide anion generators LY83583 (10^{-5} M) and hydroquinone (HQ, 10^{-4} M), and the NO-scavengers hydroxocobalamin (HC, 10^{-4} M) and c-PTIO (10^{-4} M) did not exert an inhibitory influence on nitrergic relaxations induced by EFS (4 Hz, 10 s) (Figure 1a–d). In contrast, all these agents reduced the relaxant response to a bolus of exogenous NO (10^{-5} M) (Figure 1a–d).

Influence of DETCA treatment on Cu/Zn SOD activity and on the effect of LY83583, HQ, HC and c-PTIO versus nitrergic relaxation

The total Cu/Zn SOD activity measured in supernatants of control preparations of porcine gastric fundus was 3.07 ± 0.68 SOD-525 units mg^{-1} protein ($n=6$). After 1 h of incubation with DETCA (3×10^{-3} M), the Cu/Zn SOD activity was nearly abolished to 0.11 ± 0.11 SOD-525 units mg^{-1} protein ($n=6$; $P<0.01$) confirming the effectiveness of the DETCA depletion method.

Incubation with the Cu-chelator DETCA (3×10^{-3} M) resulted in a firm decrease in basal tone in the porcine gastric fundus preparations, but did not alter the amplitude of contraction induced by 3×10^{-7} M 5-HT. DETCA had no influence *per se* on nitrergic relaxation elicited by EFS (4 Hz, 10 s) or exogenous NO (10^{-5} M) (Figure 1a–d). After DETCA-pretreatment, the superoxide anion generator LY83583 (10^{-5} M) and the NO-scavenger HC (10^{-4} M) significantly reduced the relaxant response to EFS [to respectively 37.6 ± 4.8% ($n=8$; $P<0.001$; Figure 1a) and 43.7 ± 8.2% ($n=6$; $P<0.05$; Figure 1c) of the response before administration of any drug]; the inhibitory effect of HC on the relaxation induced by exogenous NO was also increased [Figure 1c; ($P<0.05$)]. DETCA-pretreatment did not alter the influence of HQ (10^{-4} M) and c-PTIO (10^{-4} M) upon electrically- and NO-induced responses (Figure 1b,d).

We have previously shown that exogenous Cu/Zn SOD did not influence the relaxations induced by exogenous NO or EFS in the porcine gastric fundus (Colpaert & Lefebvre, 2000). We now tested whether it was able to interfere with the DETCA-induced effects. Addition of exogenous Cu/Zn SOD (1000 u ml^{-1}) partially reversed the inhibitory effect of the combination DETCA plus LY83583 on the relaxation to EFS (Figure 2a). However, the inhibitory action of DETCA plus HC on both electrically- and NO-elicited relaxations was not influenced by exogenous Cu/Zn SOD (Figure 2d). Reduced glutathione (GSH) and uric acid (UA) were previously shown to influence the relaxation by exogenous NO in the porcine gastric fundus, while the nitrergic relaxation induced by EFS was not influenced (Colpaert & Lefebvre, 2000). GSH changed the response to exogenous NO from monophasic to biphasic and the amplitude of the second phase was potentiated when compared to the response induced by exogenous NO in the absence of GSH. Uric acid also potentiated the response to exogenous NO, that either stayed monophasic or became biphasic. We therefore assessed whether GSH [by use of glutathione ethyl ester (GSE), that has a similar effect as GSH] or uric acid were able to influence the DETCA-induced effects of LY83583 and HC. When glutathione ethyl ester (GSE, 3×10^{-3} M) or uric acid (UA, 4×10^{-4} M) were added after DETCA, DETCA no longer potentiated the inhibition of the NO-induced relaxation by HC (Figure 2e,f); for the combination DETCA plus GSE plus LY83583, the amplitude of the NO-induced relaxation was restored to that in the absence of any drug (Figure 2b). Neither GSE nor UA could prevent the inhibitory action of the combination DETCA plus LY83583 or DETCA plus HC on the electrically evoked relaxations (Figure 2b,c,e,f).

Immunohistochemistry for Cu/Zn SOD, nNOS and PGP 9.5

Immunoreactivity for Cu/Zn SOD was found in neurons of both the submucosal and myenteric plexus, in nerve fibres running through the muscle layers and in endothelial cells lining blood vessels. Double labelling for Cu/Zn SOD and the neuronal marker PGP 9.5 confirmed the presence of Cu/Zn SOD in nerve cell bodies and fibres (data not shown), while double labelling for Cu/Zn SOD and nNOS clearly demonstrated the presence of Cu/Zn SOD in all nitrergic neurons of the myenteric plexus (Figure 3).

Effect of EA, CARM and BSO on the GSH content of porcine gastric fundus smooth muscle strips

Incubation of smooth muscle strips ($n=6$) with the GSH complex-forming agent EA (10^{-4} M) for 30 min or with the GSH reductase inhibitor CARM (2×10^{-4} M) for 1 h did not result in a significant decrease of the GSH level (8.89 ± 1.06 nmol GSH mg^{-1} protein in EA-treated strips and 6.81 ± 1.36 nmol GSH mg^{-1} protein in the CARM-treated preparations) when compared to the respective control preparations (coming from the same animal) which were incubated in parallel in physiological salt solution alone (7.22 ± 1.78 nmol GSH mg^{-1} protein for the EA control strips and 9.2 ± 1.35 nmol GSH mg^{-1} protein for the CARM control preparations). However, strips treated for 2 h with the GSH biosynthesis inhibitor BSO (10^{-3} M) showed a significantly lower GSH content than untreated control

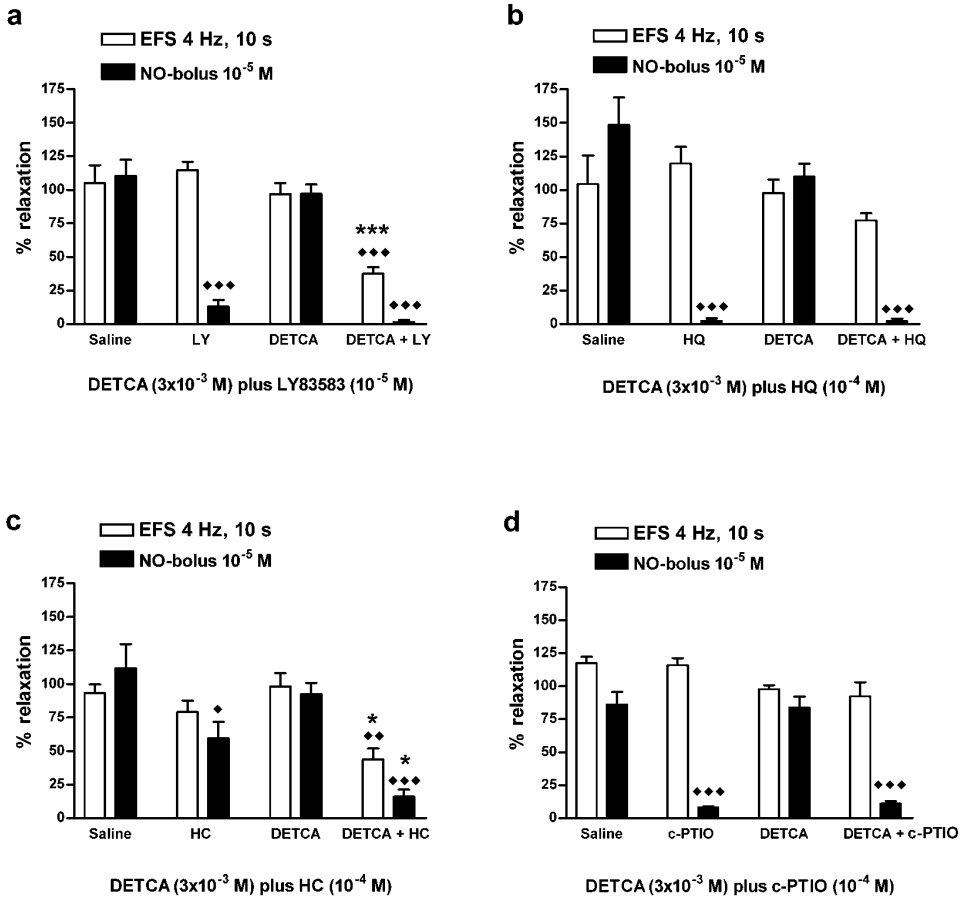

Figure 1 Influence of diethyldithiocarbamate (DETCA, 3×10^{-3} M) *per se* and of DETCA-pretreatment on the effect of LY83583 (10^{-5} M) (a), hydroquinone (HQ, 10^{-4} M) (b), hydroxocobalamin (HC, 10^{-4} M) (c) and carboxy-PTIO (c-PTIO, 10^{-4} M) (d). Relaxant responses induced by electrical field stimulation (4 Hz, 10 s) and exogenous NO (10^{-5} M) are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means$\pm$s.e.mean of $n=5-8$ are shown. ◆$P<0.05$, ◆◆ $P<0.01$, ◆◆◆ $P<0.001$: significantly different from saline; *$P<0.05$, ***$P<0.001$: significantly different from the response in the presence of LY83583 or HC alone (ANOVA followed by Bonferroni multiple comparison *t*-test).

strips: 3.86 ± 0.68 nmol GSH mg^{-1} protein versus 6.25 ± 0.68 nmol GSH mg^{-1} protein ($n=6$; $P<0.05$).

Influence of BSO, CARM and EA on the effect of LY83583 or HC versus nitrergic relaxation

Incubation with BSO (10^{-3} M) did not influence the basal tone of the porcine preparations nor did it alter the amplitude of contraction obtained by 5-HT. BSO had no effect *per se* on nitrergic relaxation evoked by EFS or exogenous NO (Figure 4). The combination of BSO with LY83583 or HC did not affect the influence of LY83583 or HC on nitrergic relaxations induced by EFS or exogenous NO (Figure 4).

CARM (2×10^{-4} M) induced a firm decrease in basal tone, but did not alter the amplitude of contraction induced by 5-HT. CARM had no effect *per se* on nitrergic relaxations and also did not influence the effect of LY83583 or HC thereupon (data not shown).

Treatment with EA (10^{-4} M) did not affect basal tone nor the 5-HT contraction amplitude of our experimental preparations and had also no modulatory effect on their GSH level, but was demonstrated to strongly reduce the nitrergic relaxation by EFS while leaving the NO-induced relaxation unaffected; this strong inhibition of the electrically-induced relaxation by EA was fully prevented by addition of N-acetyl-cysteine (NAC, 3×10^{-3} M) or GSE (3×10^{-3} M) together with EA (Figure 5a). Neither NAC nor GSE exerted an influence *per se* on the nitrergic relaxation induced by EFS (Figure 5a). The combination of EA with LY83583 or HC did not alter the influence of LY83583 or HC on nitrergic relaxations, with the exception of the combination of EA plus HC that enhanced the inhibitory effect of HC on the NO-induced relaxation (data not shown). In an additional set of experiments, we found that EA (10^{-4} M) time-dependently decreased the amplitude of cholinergic contractions induced by EFS (4 Hz, 0.5 ms, 40 V) in the absence of atropine (Figure 5b); this decrease in contraction level to EFS was not influenced by co-incubation of EA with NAC (3×10^{-3} M), while addition of EA together with GSE (3×10^{-3} M) partially prevented the blocking action of EA (Figure 5b).

Discussion

The present study aimed at investigating the influence of pharmacological depletion of the tissue antioxidants Cu/Zn

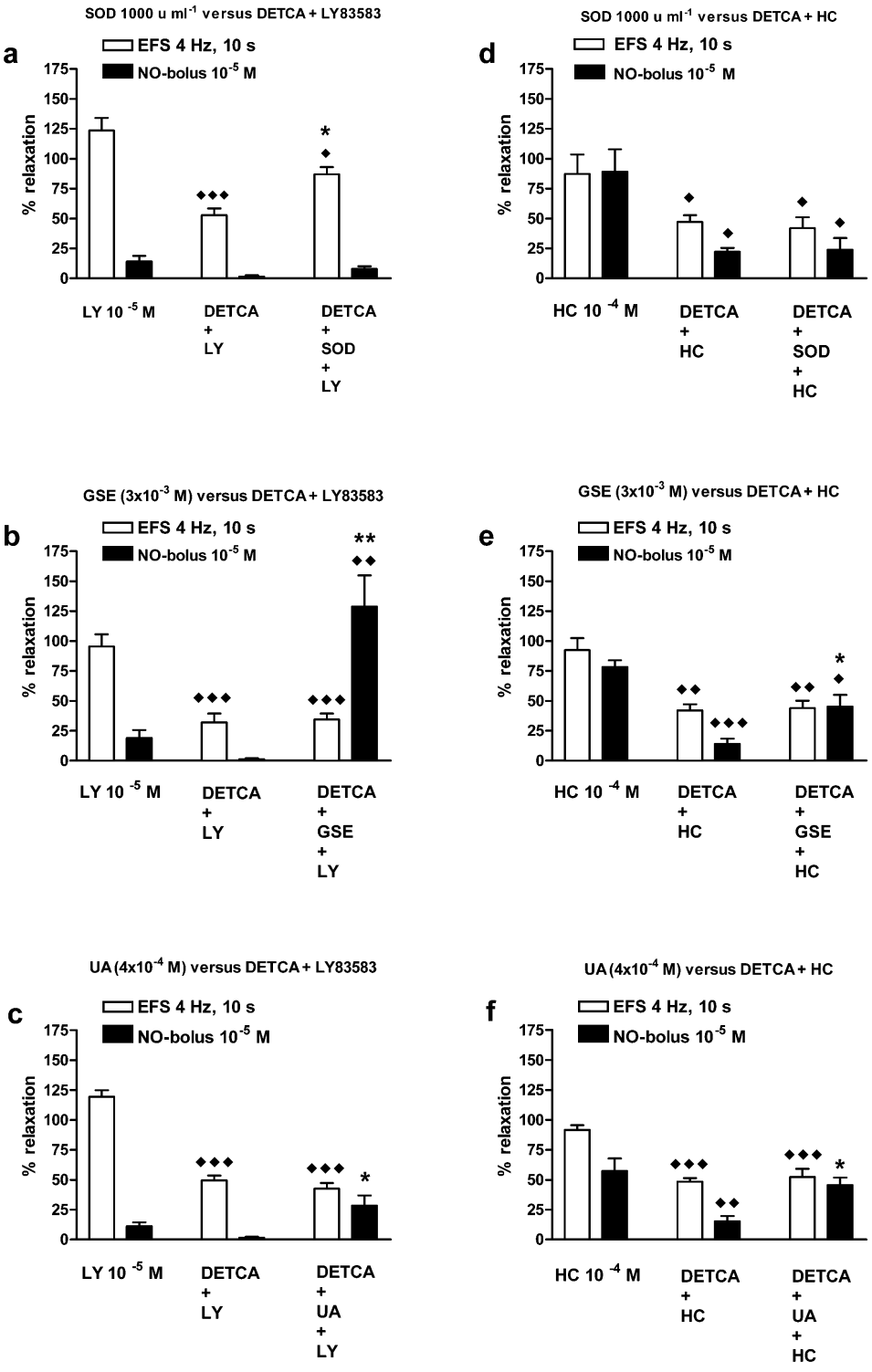

Figure 2 Influence of superoxide dismutase (SOD, 1000 u ml^{-1}) (a,d), glutathione ethyl ester (GSE, 3×10^{-3} M) (b,e) and uric acid (UA, 4×10^{-4} M) (c,f) on the inhibitory effect of LY83583 (10^{-5} M) (a,b,c) or hydroxocobalamin (HC, 10^{-4} M) (d,e,f) versus relaxations induced by electrical field stimulation (4 Hz, 10 s) and exogenous NO (10^{-5} M). Relaxations are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of $n = 4-7$ are shown. ◆$P<0.05$, ◆◆ $P<0.01$, ◆◆◆$P<0.001$: significantly different from LY83583 or HC alone; *$P<0.05$, **$P<0.01$: significantly different from the response in the presence of the combination of DETCA with LY83583 or HC (ANOVA followed by Bonferroni multiple comparison *t*-test).

SOD and GSH on nitrergic relaxation in the porcine gastric fundus. In the depleted porcine gastric fundus preparations, the influence of the superoxide generators LY83583 and HQ and the NO-scavengers HC and c-PTIO was determined

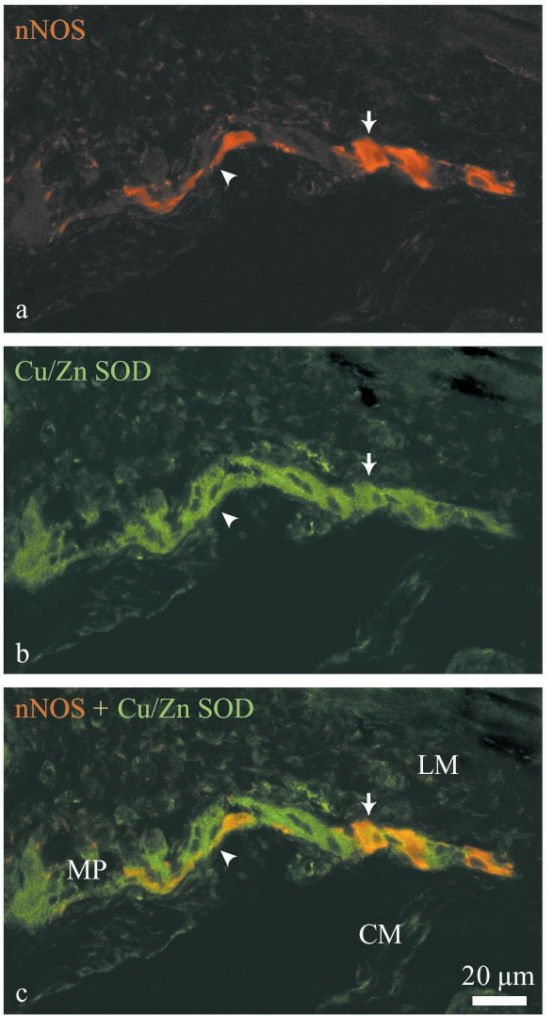

Figure 3 (a) Neuronal nitric oxide synthase (nNOS; red CY3-fluorescence) and (b) Cu/Zn superoxide dismutase (Cu/Zn SOD; green FITC-fluorescence) immunolocalization in neurons of the myenteric plexus (MP) of porcine gastric fundus. (c) Represents the double exposure (=combined fluorescence) photomicrograph. The arrow and arrowhead indicate respectively a myenteric nerve cell body and a nerve fibre in which the colocalization of nNOS and Cu/Zn SOD is clearly visible. LM=longitudinal muscle layer; CM=circular muscle layer.

versus two types of nitrergic relaxations: either induced by electrical field stimulation (EFS; 40 V, 0.1 ms, 4 Hz, 10 s) or by exogenously added NO (10^{-5} M).

Depletion of Cu/Zn SOD

Spectrophotometric analysis of homogenates of tissue pretreated with the irreversible Cu/Zn SOD inhibitor DETCA clearly showed the effectiveness and potency of this depletion method since we observed a 96% inhibition of the specific Cu/Zn SOD activity in the DETCA-incubated strips. The total Cu/Zn SOD activity in control preparations of porcine gastric fundus (3.07 SOD-525 units mg^{-1} protein) is somewhat lower than the control value found in the sheep urethra (9.3 SOD-525 units mg^{-1} protein; Garcia-Pascual *et al*., 2000), which might be indicative for a species and tissue specific quantitative distribution of Cu/Zn SOD, or alternatively might reflect differences in efficiency of the homogenization procedure.

Pretreatment with DETCA, blocking both the extracellular and cytosolic Cu/Zn isoform of the enzyme, did not result in an effect *per se* on nitrergic relaxations. This might be explained by a rather low background of oxidative stress in the porcine gastric fundus or by compensation for the loss of Cu/Zn SOD by other protective antioxidants. When the superoxide generator LY83583 was added to DETCA-treated preparations, the electrically evoked nitrergic relaxation was markedly reduced. Similar results on nitrergic neurotransmission were also obtained with the combination of DETCA plus LY83583 in the bovine retractor penis muscle (Martin *et al*., 1994) and in the rat gastric fundus (Lefebvre, 1996). The observation that repletion with exogenous Cu/Zn SOD only partially restored nitrergic relaxation by EFS when it was reduced by the combination of DETCA plus LY83583 in our strips, points to the involvement of extracellular and intracellular superoxide anions in the interaction of DETCA plus LY83583 with the nitrergic neurotransmitter since exogenous Cu/Zn SOD can not enter cells. The present finding adds further credit to the hypothesis that Cu/Zn SOD preserves the bioactivity of the nitrergic transmitter *via* a mechanism related to its superoxide dismutating activity. In contrast to LY83583, the resistance of electrically-induced nitrergic relaxations to inhibition by hydroquinone, a compound also acting through extracellular and intracellular superoxide production in the pig gastric fundus (Colpaert & Lefebvre, 2000), was not influenced by pretreatment with DETCA. We do not have an explanation for this differential effect.

An important new finding of the present study is that hydroxocobalamin, thought to be a NO-sequestering agent, significantly reduced relaxations induced by nitrergic nerve stimulation when strips were depleted from Cu/Zn SOD by DETCA. This contrasts to the inability of the other NO-scavenger tested in our study, c-PTIO, to produce a similar effect. NO-scavenging agents have yielded variable effects on nitrergic transmission in different experimental tissues: hydroxocobalamin exerted no effect *per se* on relaxations by nitrergic nerve stimulation in the rat and pig gastric fundus (Lefebvre, 1996; Colpaert & Lefebvre, 2000), while a clear inhibition was observed in the bovine retractor penis (Paisley & Martin, 1996); also c-PTIO produced a powerful blockade of nitrergic transmission in the latter tissue, but was ineffective in the sheep urethra (Garcia-Pascual *et al*., 2000) and in our present study. However, even in the bovine retractor penis muscle, the magnitude of the inhibition of the electrically-induced nitrergic relaxation by hydroxocobalamin or carboxy-PTIO was unaffected following treatment with DETCA. In our study, DETCA also markedly potentiated the inhibitory effect of hydroxocobalamin on the relaxation by exogenous NO, an effect which has been previously described in the rat gastric fundus (Lefebvre, 1996). Administration of exogenous SOD however did not reverse this enhancing influence of DETCA, neither did it prevent the inhibitory action of hydroxocobalamin on electrically-induced nitrergic relaxations in the DETCA-pretreated porcine gastric fundus strips. We can thus conclude that extracellular superoxide anions are not implicated in the observed inhibitory mechanism; the pathway through which Cu-

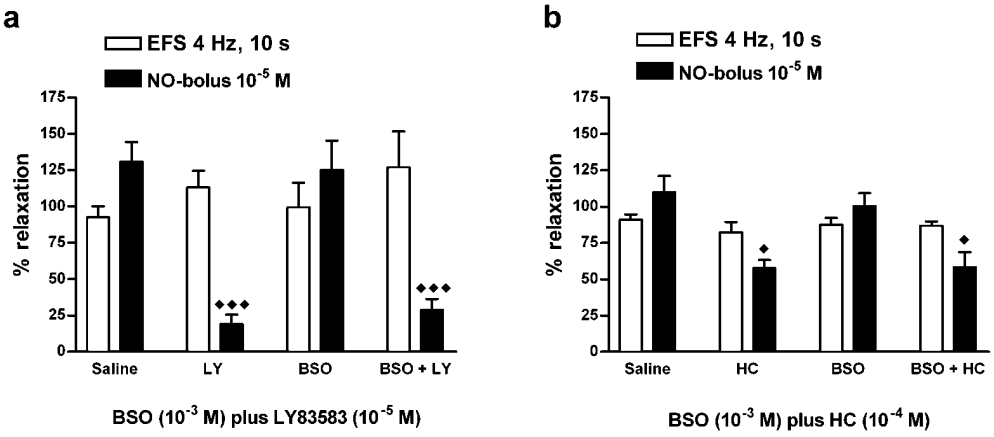

Figure 4 Influence of buthionine sulphoximine (BSO, 10^{-3} M) *per se* and of BSO-pretreatment on the effect of LY83583 (10^{-5} M) (a) and hydroxocobalamin (HC, 10^{-4} M) (b). Relaxant responses induced by electrical field stimulation (4 Hz, 10 s) and exogenous NO (10^{-5} M) are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of $n=6$ are shown. ◆$P<0.05$, ◆◆◆$P<0.001$: significantly different from saline (ANOVA followed by Bonferroni multiple comparison *t*-test).

chelation by DETCA ultimately leads to a potentiation of the sequestering action of hydroxocobalamin versus the endogenous nitrergic neurotransmitter and exogenous NO remains unclear.

To fully substantiate the above conclusion that in porcine gastric fundus Cu/Zn SOD contributes to the protection of the nitrergic neurotransmitter against superoxide anions and NO-scavenging activity, we used an indirect immunohistochemical technique to investigate the topographic relationship between Cu/Zn SOD and nNOS. Our immunofluorescence results establish this intimate connection between Cu/Zn SOD and nNOS since all nitrergic neurons of the myenteric plexus colocalize for Cu/Zn SOD.

Besides Cu/Zn SOD, other antioxidants are also able to protect against oxidative influences; we therefore investigated the possibility that addition of GSH or uric acid might compensate for the depletion of Cu/Zn SOD obtained by DETCA in our experimental preparations. Both glutathione ethyl ester (GSE), a cell permeable GSH derivative, and uric acid counteracted the potentiating effect of DETCA-pretreatment on the inhibitory activity of hydroxocobalamin versus the NO-induced relaxation, but were ineffective against inhibition by either LY83583 or hydroxocobalamin of relaxations induced by nitrergic nerve stimulation in the DETCA-treated strips. The protection afforded by GSH and uric acid on the NO-induced relaxations might be due to nitrosylation reactions resulting in the formation of respectively S-nitrosoglutathione and a nitrated uric acid product (see Colpaert & Lefebvre, 2000). The lack of protection versus the endogenous nitrergic neurotransmitter might then be related to the limited time for potential interaction of the neurotransmitter and GSH or uric acid bearing in mind the short distance the nitrergic neuromuscular transmitter has to travel in comparison to exogenous NO to reach the effector enzyme in the adjacent smooth muscle cells.

Depletion of GSH

Glutathione (*L*-γ-glutamyl-*L*-cysteinylglycine; GSH) is the predominant low molecular thiol in mammalian cells and plays a major role in cellular defenses against oxidative stress (Meister & Anderson, 1983). The possible role for GSH in nitrergic neurotransmission has initiated studies investigating the influence of GSH depletion strategies on functional responses in smooth muscle preparations with nitrergic innervation (De Man *et al*., 1996; Garcia-Pascual *et al*., 2000), but the influence of the employed depletion strategies on the actual GSH content of the tissues was not determined.

Quantitative spectrophotometric analysis of the GSH content of our control preparations yielded values (varying between 6 and 9 nmol GSH mg^{-1} protein) comparable with reported values in other smooth muscle preparations (Megson *et al*., 2000). In the present study, three approaches of GSH depletion have been used with the action of each agent being validated on the porcine gastric fundus smooth muscle tissue. (1) Inhibition of the *de novo* synthesis of GSH with buthionine sulphoximine (BSO) led to a significant reduction of the tissue GSH content of our experimental preparations, yet failed to affect nitrergic relaxations elicited by exogenous NO or nitrergic nerve stimulation. Since DETCA-pretreatment *per se* also exerted no effect on relaxations evoked by nitrergic nerve stimulation yet rendered these relaxations sensitive to inhibition by LY83583 or hydroxocobalamin, BSO might perform likewise provided that GSH plays an essential role in nitrergic neurotransmission. However, BSO-treatment had no effect on the action of LY83583 or hydroxocobalamin on nitrergic relaxations. The observation that BSO could only reduce the tissue GSH content by 38% might clarify this non-effect; however, even reductions of GSH content limited to 15% impaired NO-induced relaxations in normal rabbit thoracic aorta treated with carmustine (Adachi & Cohen, 2000). This indicates that even relatively small reductions of tissue GSH may lead to alterations in nitrergic signalling. The results with BSO do thus not support a major role for GSH in the protection of the nitrergic neurotransmitter in the porcine gastric fundus. (2) Carmustine (CARM), that prevents the reductase mediated regeneration of GSH from glutathione disulphide, also did not influence both types of nitrergic relaxant responses but was proven to be an ineffective GSH depletor

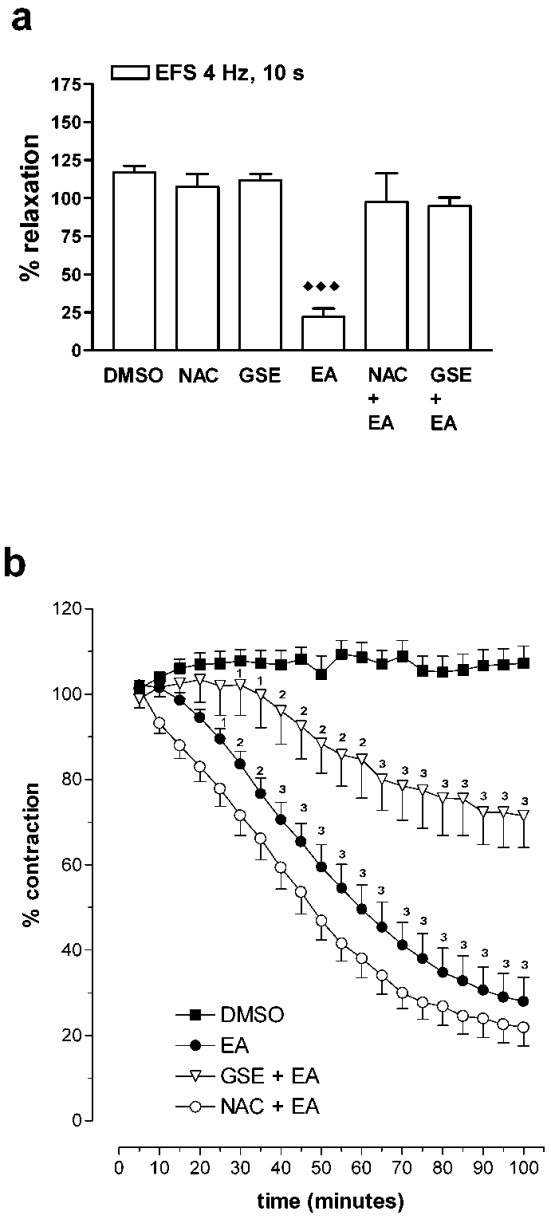

Figure 5 (a) Influence of ethacrynic acid (EA, 10^{-4} M) *per se*, of its solvent dimethylsulphoxide (DMSO), of N-acetyl-cysteine (NAC, 3×10^{-3} M) or glutathione ethyl ester (GSE, 3×10^{-3} M), and of the combination of EA with either NAC (3×10^{-3} M) or GSE (3×10^{-3} M) on nitrergic relaxations induced by electrical field stimulation (EFS; 4 Hz, 10 s). Relaxations are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± s.e.mean of $n=5-8$ are depicted. ◆◆◆$P<0.001$: significantly different from the control response (DMSO) (ANOVA followed by Bonferroni multiple comparison *t*-test). (b) The effect of EA (10^{-4} M) or its solvent DMSO on cholinergic contractions elicited by electrical field stimulation (40 V, 0.5 ms, 4 Hz, 10 s) and the influence of co-incubation with NAC (3×10^{-3} M) or GSE (3×10^{-3} M) are demonstrated. Contractile responses to 20 successive stimulations starting immediately after the administration of drugs are shown, expressed as a percentage of the last contraction before addition of these drugs. Means ± s.e.mean of $n=6$ are shown. $^1P<0.05$, $^2P<0.01$, $^3P<0.001$: significantly different from the response to DMSO (results obtained with EA) or to EA (results obtained with GSE + EA) (ANOVA followed by Bonferroni multiple comparison *t*-test).

in our experimental conditions. (3) Since GSH depletions effected by thiol-reactive oxidants and electrophiles are more rapid than can be achieved with either BSO or CARM (Griffith, 1999), we submitted the porcine gastric fundus preparations to ethacrynic acid pretreatment. Ethacrynic acid (EA) is known to form an adduct with GSH which lacks antioxidant properties. Measurement of the level of GSH after EA pretreatment revealed no difference when compared with the GSH content in the controls. However, EA strongly reduced the relaxation induced by electrical field stimulation while leaving the NO-induced relaxations unaffected. In conformity to our data, Li *et al.* (1994) have found, in the rat anococcygeus muscle, that EA strongly reduced relaxations to nitrergic nerve stimulation but under their experimental conditions, NO-induced relaxations were also almost abolished. In contrast, in the rat gastric fundus (De Man *et al.*, 1996) and in the sheep urethra (Garcia-Pascual *et al.*, 2000) EA was without effect on nitrergic relaxations. It should be noted, however, that ion channel inhibiting activities of EA have been reported in the literature (Miller & Schnellmann, 1993). Our observation that EA also inhibited contractions induced by activation of cholinergic nerves corresponds with this possibility. This neurotoxic effect exerted by EA was not prevented by NAC and only partially by GSE for the cholinergic responses, while a complete prevention of the inhibitory effect both by NAC and GSE was observed for the nitrergic relaxations. This side effect of EA should thus be considered when using it as a GSH depleting agent in functional studies, all the more since it is often promoted as a compound able to firmly decrease the mitochondrial GSH content in addition to the cytosolic GSH.

Summary and conclusion

We have demonstrated that nitrergic nerve mediated relaxations of porcine gastric fundus preparations are inhibited by both a superoxide generator and a NO-scavenger only after irreversible inhibition of Cu/Zn SOD; replenishment with exogenous Cu/Zn SOD partially prevented the inhibitory action of the superoxide generator. Of the GSH modulators tested, only BSO was able to significantly reduce the GSH content of the experimental preparations but did not influence the effect of superoxide generation and NO-scavenging on nitrergic relaxation. In conclusion, pharmacological depletion studies together with immunohistochemical data suggest a role for Cu/Zn SOD, but preclude a similar role for GSH, in the nitrergic neurotransmission process in the porcine gastric fundus *via* protection of free radical NO from superoxide attack and NO-scavenging activity.

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