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Influence of polyethylene-glycol-superoxide dismutase and combined depletion and repletion of antioxidants on nitrergic relaxation in the pig gastric fundus

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Abstract

In circular smooth muscle strips of porcine gastric fundus, polyethylene-glycol-superoxide dismutase, a membrane-permeable analogue of endogenous copper/zinc (Cu/Zn) superoxide dismutase, reversed the inhibitory effect of the superoxide anion generator 6-anilino-5,8-quinolinedione (LY83583) on electrically induced nitrergic relaxations of fundic tissues which are depleted of the endogenous antioxidant Cu/Zn superoxide dismutase by diethyldithiocarbamate, to the same extent as exogenously added Cu/Zn superoxide dismutase. Addition of a second antioxidant together with Cu/Zn superoxide dismutase does not result in a higher degree of reversal of the inhibitory effect of LY83583. Depletion of either tissue glutathione or tissue catalase in combination with diethyldithiocarbamate does not increase the inhibitory action of LY83583 or the nitric oxide (NO)-scavenger hydroxocobalamin upon nitrergic relaxations (electrically induced or by exogenous NO) when compared to their action in the presence of diethyldithiocarbamate alone. In conclusion, these results demonstrate that endogenous Cu/Zn superoxide dismutase is the essential antioxidant responsible for safeguarding peripheral nitrergic neurotransmission, whereby extracellular protection of endogenous NO is most important.

Keywords: Polyethylene-glycol-superoxide dismutase; Cu/Zn superoxide dismutase; Diethyldithiocarbamate; 6-Anilino-5,8-quinolinedione; Nitrergic relaxation; Gastric fundus; (Pig)

1. Introduction

Non-adrenergic non-cholinergic (NANC) inhibitory neurones in the myenteric plexus play a major role in the control of gastrointestinal motility. Nitric oxide (NO) is an important mediator of NANC inhibitory neurotransmission during receptive and adaptive gastric relaxation upon food intake. Depending upon the species, NO is involved in initiating or also sustaining proximal gastric relaxations, such as in the rat (Li and Rand, 1990; Boeckxstaens et al., 1991; D'Amato et al., 1992) and pig (Lefebvre et al., 1995), respectively.

In a number of nitrergically innervated tissues, including gastric fundus, compounds which inhibit relaxations induced by administration of exogenous free radical NO, either through superoxide anion generation or via NO-scavenging, are not capable of reducing the relaxations elicited by electrical field stimulation of the nitrergic nerves in NANC conditions (Hobbs et al., 1991; Barbier and Lefebvre, 1992).

The most substantiated hypothesis explaining this discriminatory action of NO-inhibitors on exogenous free radical NO and the endogenous nitrergic neurotransmitter is that tissue antioxidants create a correct redox environment for free radical NO to function as endogenous nitrergic neurotransmitter by fencing off superoxide generator and NO-scavenger attack. On the other hand, exogenously added free radical NO is vulnerable to attack by these substances before reaching the antioxidant protection of the tissue (Gibson and Lilley, 1997). Experiments with the Cu-chelator diethyldithiocarbamate, irreversibly inhibiting extra - and intracellular copper/zinc (Cu/Zn) superoxide dismutase, showed an important role for Cu/Zn superoxide dismutase in the protection of the

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nitrergic neurotransmitter (Martin et al., 1994; Lefebvre, 1996). Besides Cu/Zn superoxide dismutase, other antioxidants also protect against oxidative stress (Halliwell, 1994) and a potential role of antioxidants such as reduced glutathione, α-tocopherol, ascorbate and urate in protection of free radical NO has been suggested (Lilley and Gibson, 1996, 1997).

In our previous studies on the effect of antioxidant depletion on nitrergic neurotransmission in the pig gastric fundus, the superoxide generator 6-anilino-5,8-quinolinedione (LY83583) and the NO-scavenger hydroxocobalamin reduced the nitrergic relaxant response induced by electrical field stimulation by approximately 50%, after nearly complete inhibition of endogenous Cu/Zn superoxide dismutase with diethyldithiocarbamate; still this inhibitory effect is less pronounced than the effect on the relaxation induced by exogenous NO, which is nearly abolished. This inhibitory effect of the combination diethyldithiocarbamate plus LY83583 on the electrically induced nitrergic relaxation is only partially reversed by administration of exogenous Cu/Zn superoxide dismutase. However, the inhibitory action of diethyldithiocarbamate plus hydroxocobalamin on electrically induced relaxations was not influenced by exogenous Cu/Zn superoxide dismutase (Colpaert et al., 2002). The potential role of other antioxidants than Cu/Zn superoxide dismutase was also assessed. In the presence of reduced glutathione, the short-lasting relaxation to exogenous NO in the pig gastric fundus becomes biphasic, potentiated and prolonged (Colpaert and Lefebvre, 2000). Still, reduction of the tissue reduced glutathione content by about 40% using buthionine sulphoximine did not influence nitrergic relaxations in the absence or presence of LY83583 or hydroxocobalamin (Colpaert et al., 2002). Also urate and bilirubin potentiated the relaxant effect of exogenous NO (Colpaert and Lefebvre, 2000).

The aim of the present study in circular smooth muscle strips of pig gastric fundus was therefore to investigate whether: (1) the nitrergic relaxation elicited by electrical field stimulation becomes more sensitive to LY83583 and hydroxocobalamin when combining two antioxidant depleting agents, either diethyldithiocarbamate plus buthionine sulphoximine or diethyldithiocarbamate plus the catalase inhibitor 3-amino-1,2,4-triazole; (2) the reversing capacity of polyethylene-glycol-superoxide dismutase on the inhibitory effect of diethyldithiocarbamate plus LY83583 is more pronounced than with Cu/Zn superoxide dismutase as polyethylene-glycol-superoxide dismutase can penetrate intracellularly, which is not the case for Cu/Zn superoxide dismutase; (3) the addition of a second antioxidant (i.e. uric acid, reduced glutathione, bilirubin, or catalase) to Cu/Zn superoxide dismutase is able to induce a reversion of the inhibitory effect of diethyldithiocarbamate plus hydroxocobalamin, or to induce a more pronounced reversion of the inhibitory effect of diethyldithiocarbamate plus LY83583 than Cu/Zn superoxide dismutase alone.

2. Material and methods

2.1. Tissue preparation

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 of approximately 1.5 cm in length and 0.3 cm in width were cut from the fundus in the direction of the circular muscle layer. All strips were used immediately. Strips were mounted vertically between two platinum plate electrodes (in 20 ml organ baths) or between two wire electrodes (in 2 ml organ baths) under a load of 2 g in physiological salt solution [containing (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 and Milenov, 1991)] maintained at 37 °C and gassed with carbogen (95% O_2/5% CO_2). To obtain NANC conditions, atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were continuously present in the medium. Changes in length were recorded isotonically via Palmer Bioscience T3 transducers on a Graphtec Linearcorder WR 3701 F in the 20 ml organ baths and via Harvard heart/smooth muscle isotonic transducers on a Kipp and Zonen BD112 (Ankersmit, The Netherlands) recorder in the 2 ml organ baths. Electrical field stimulation (EFS; 40 V, 0.1 ms, 4 Hz) was applied by means of a Grass S88 stimulator. The tissues were equilibrated for 90 min with rinsing every 15 min before starting the experiment.

2.2. Experimental protocols

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. Following an interval of 1 h with regular rinsing, the experiment was then continued.

A first series of experiments was performed to study the influence of combinations of tissue antioxidant depleting agents on relaxations induced by nitrergic nerve stimulation or exogenous NO. Tone was raised with 3×10^{-7} M 5-HT; this induced a stable plateau contraction (between 60% and 90% of the maximal response to 5-HT; see Lefebvre et al., 1995). When a stable plateau contraction was obtained, two relaxant stimuli were consecutively studied with a 5-min interval in between: electrical field stimulation (40 V, 0.1 ms) at 4 Hz for 10 s and a bolus of exogenous NO (10^{-5} M). The amplitude of the relaxation induced was at least 50% of the relaxation induced by 10^{-5} M sodium nitroprusside at the beginning of the experiment. Tissues were then repetitively rinsed and after 10 min the antioxidant depletors [diethyldithiocarbamate (3×10^{-3} M), a Cu-chelator irreversibly inhibiting both extra-and intracellular Cu/Zn superoxide dismutase (Kelner et al., 1989); buthionine sulphoximine (10^{-3} M), inhibiting γ-glutamyl-cysteine synthetase, the rate limiting enzyme in the biosynthesis of reduced glutathione

(Meister, 1988); 3-amino-1,2,4-triazole (3×10^{-3} M), a catalase inhibitor] were administered and left in contact with the tissue for respectively120 min (buthionine sulphoximine), 90 min (3-amino-1,2,4-triazole) or 60 min (diethyldithiocarbamate); these compounds were then washed from the tissue baths. Subsequently the superoxide generator LY83583 (10^{-5} M) or NO-scavenger hydroxocobalamin (10^{-4} M) was added, and 15 min later contraction was again induced with 5-HT and the two relaxant stimuli were repeated. Eight preparations from the same animal, receiving either a single one or a combination of the pharmacological compounds under study or only the solvents (control), were run in parallel.

In an additional set of experiments we determined the influence of several protocols of antioxidant replenishment on the inhibitory effect versus nitrergic relaxations of diethyldithiocarbamate plus LY83583 or plus hydroxocobalamin. Polyethylene-glycol-superoxide dismutase (1000 U/ml) or the combination of Cu/Zn superoxide dismutase(1000 U/ml) with the cell permeable ethyl ester of gluthatione (3×10^{-3} M), uric acid(4×10^{-4} M), bilirubin ditaurate (2×10^{-4} M) or catalase (2000 U/ml) was added just before LY83583 or hydroxocobalamin. Polyethylene-glycol-superoxide dismutase (1000 U/ml) was also studied when added 2 h before LY83583.

2.3. Drugs used

The following drugs were used (supplied by Sigma unless stated otherwise): 3-amino-1,2,4-triazole, 6-anilino-5,8-quinolinedione (LY83583 ; Calbiochem), atropine sulphate, bilirubin ditaurate (Calbiochem), L-buthionine-[S,R]-sulphoximine, catalase (from bovine liver), diethyldithiocarbamic acid sodium salt, glutathione ethyl ester, guanethidine sulphate, hydroxocobalamin acetate, 5-hydroxytryptamine creatinine monosulphate (Janssen Chimica), polyethylene-glycol superoxide dismutase (from bovine erythrocytes), sodium nitroprusside, Cu/Zn superoxide dismutase (from bovine erythrocytes), uric acid. Drugs were dissolved in deionised water except LY83583, that was dissolved in 100% ethanol. Solvents themselves were without significant effect at the concentrations used in the experiments. Stock solutions were made of LY83583 (10^{-2} M); other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm and Schrader (1990), yielding a vial containing NO in a concentration taken to be 2×10^{-3} M.

2.4. Data analysis

Relaxations elicited by electrical field stimulation 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.M. and n refers to the number of strips from different animals. Results within tissues are compared by a paired t-test. 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 $P<0.05$.

3. Results

3.1. Effect of diethyldithiocarbamate plus buthionine sulphoximine or diethyldithiocarbamate plus 3-amino-1,2,4-triazole upon nitrergic relaxations elicited by electrical field stimulation or exogenous NO

In control tissues, the relaxant responses to electrical field stimulation at 4 Hz for 10 s and to a bolus of exogenous NO (10^{-5} M) were well maintained, although sometimes we observed a slight decrease in the relaxant response towards electrical field stimulation (Fig. 1A, B, C).

As shown in previous studies, we confirmed that the superoxide anion generator LY83583 (10^{-5} M) and the NO-scavenger hydroxocobalamin (10^{-4} M) per se did not exert an inhibitory influence on nitrergic relaxations induced by electrical field stimulation (4 Hz, 10 s). In contrast, these agents reduced the relaxant response to a bolus of exogenous NO (10^{-5} M) (Fig. 1A, B, C); the effect of hydroxocobalamin versus NO was less pronounced than that of LY83583.

Neither diethyldithiocarbamate (3×10^{-3} M) nor buthionine sulphoximine (10^{-3} M) nor the combination of these two antioxidant depleting agents had an influence per se on nitrergic relaxation evoked by electrical field stimulation (4 Hz, 10 s) or exogenous NO (10^{-5} M) (Fig. 1A, B). After pretreatment with diethyldithiocarbamate (3×10^{-3} M), LY83583 (10^{-5} M) and hydroxocobalamin (10^{-4} M) significantly reduced the relaxant response to electrical field stimulation [to respectively 27.5 ± 5.4% ($n=4$; $P<0.01$; Fig. 1A) and 40.4 ± 3.9% ($n=4$; $P<0.05$; Fig. 1B) of the response before administration of any drug]; the inhibitory effect of hydroxocobalamin on the relaxation induced by exogenous NO was also increased (Fig. 1B). Pretreatment with buthionine sulphoximine (10^{-3} M) did not affect the influence of LY83583 or hydroxocobalamin on nitrergic relaxations induced by electrical field stimulation or exogenous NO (Fig. 1A, B). The combination of diethyldithiocarbamate (3×10^{-3} M) plus buthionine sulphoximine (10^{-3} M) did not result in a more pronounced inhibition of the nitrergic relaxations by LY83583 or hydroxocobalamin when compared to the action of LY83583 or hydroxocobalamin in the presence of diethyldithiocarbamate (3×10^{-3} M) alone (Fig. 1A, B).

Pretreating the experimental tissues with diethyldithiocarbamate (3×10^{-3} M) and 3-amino-1,2,4-triazole (3×10^{-3} M), thus inhibiting both endogenous Cu/Zn su-

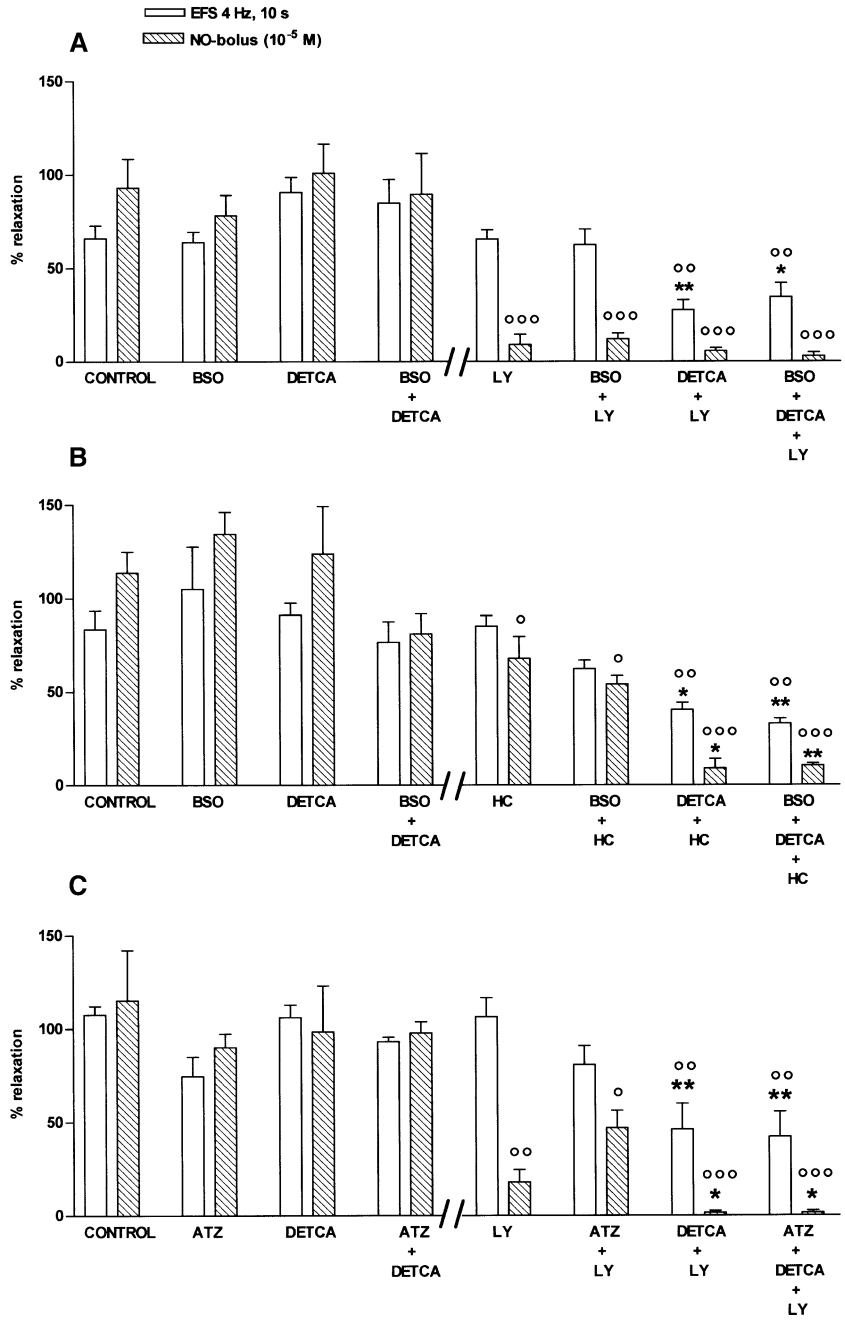

Fig. 1. Relaxant responses to electrical field stimulation (EFS; 40 V, 0.1 ms, 4 Hz, 10 s) and exogenous NO (10^{-5} M) in control tissues (A, B, C) and in tissues pretreated with buthionine sulphoximine (BSO; 10^{-3} M) (A, B), diethyldithiocarbamate (DETCA; 3×10^{-3} M) (A, B, C), 3-amino-1,2,4-triazole (ATZ; 3×10^{-3} M) (C), BSO plus DETCA (A, B) or ATZ plus DETCA (C) (left part of the graphs). In the right part of the graphs, the effect of the same pharmacological compounds was tested but now in the presence of either 10^{-5} M LY83583 (A, C) or 10^{-4} M hydroxocobalamin (HC) (B). Relaxations are expressed as a percentage of the response to the same stimulus before administration of interfering drugs. Means ± S.E.M. of $n = 6-7$ are represented. °$P < 0.05$, °°$P < 0.01$: °°°$P < 0.001$: significantly different from the response in the same tissue before administration of drugs (paired t-test); *$P < 0.05$: **$P < 0.01$: significantly different from pretreatment with either LY83583 or hydroxocobalamin alone (ANOVA followed by Bonferroni multiple comparison t-test).

peroxide dismutase and catalase, had no effect per se on nitrergic relaxations (Fig. 1C). Furthermore, the effect of LY83583 (10^{-5} M) in the presence of the combination of diethyldithiocarbamate (3×10^{-3} M) plus 3-amino-1,2,4-triazole (3×10^{-3} M) was not different from the effect of LY83583 (10^{-5} M) obtained in the presence of diethyldithiocarbamate (3×10^{-3} M) alone (Fig. 1C).

3.2. Influence of polyethylene-glycol-superoxide dismutase on the inhibitory effect of diethyldithiocarbamate plus LY83583 on nitrergic relaxations

An example of the experiments where polyethylene-glycol-superoxide dismutase was compared to Cu/Zn superoxide dismutase is shown in Fig. 2. The reduction of the

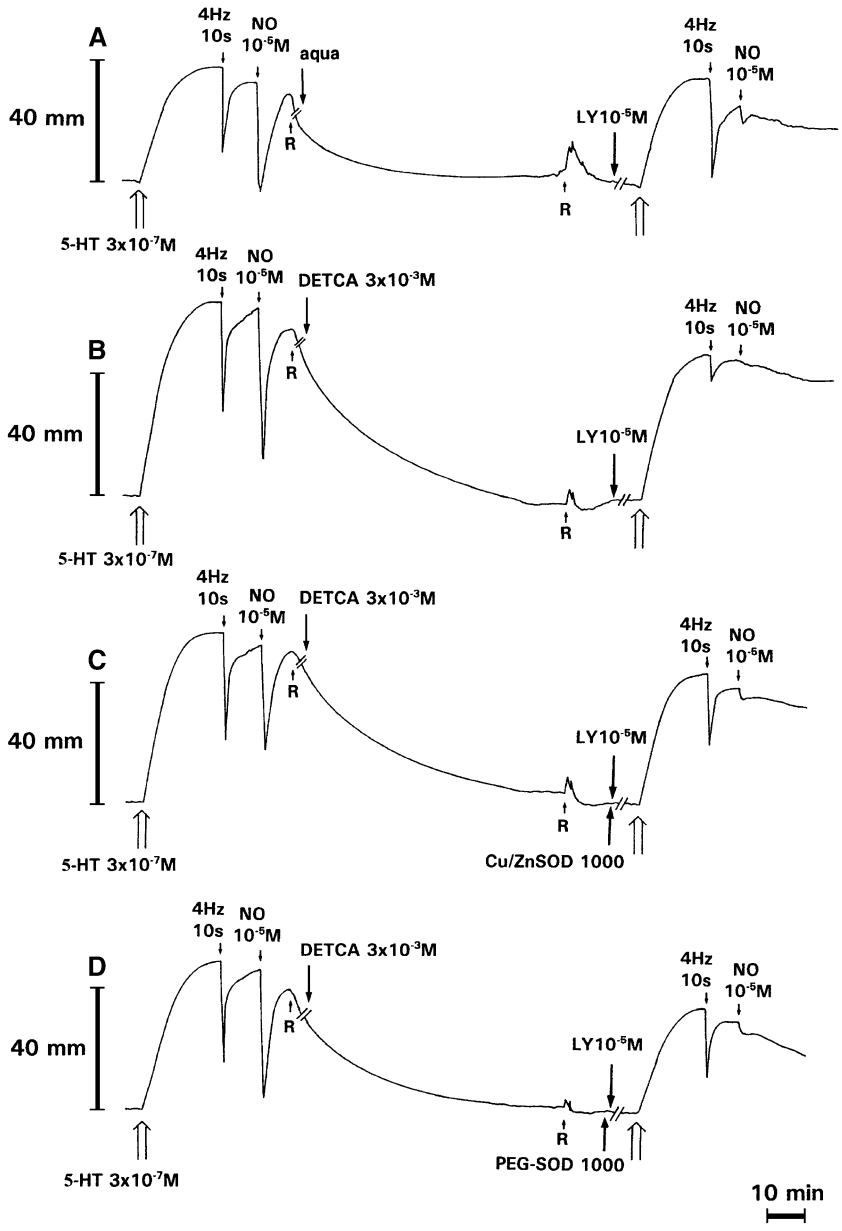

Fig. 2. Representative traces demonstrating: (A) the influence of 10^{-5} M LY83583 on the relaxations induced by electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s) and exogenous NO (10^{-5} M); (B) the inhibitory effect of LY83583 (10^{-5} M) upon the same relaxations when pretreating the tissue with diethyldithiocarbamate (DETCA; 3×10^{-3} M); (C, D) the protective effect of respectively Cu/Zn superoxide dismutase (SOD; 1000 U/ml) (C) and polyethylene-glycol-superoxide dismutase (PEG-SOD; 1000 U/ml) (D) versus these relaxations when tissues are treated with DETCA plus LY83583. During the interval indicated by //–//, the paper speed was reduced fivefold.

electrically induced nitrergic relaxation to $54.2 \pm 9.1\%$ ($n = 4$) by diethyldithiocarbamate (3×10^{-3} M) plus LY83583 (10^{-5} M) was partially reversed (to $90.8 \pm 12.8\%$; $n = 4$; $P < 0.05$ versus diethyldithiocarbamate plus LY83583) by administering polyethylene-glycol-superoxide dismutase (1000 U/ml) to the tissues just before LY83583 (Fig. 3). The degree of reversal was not significantly different from that provided by Cu/Zn superoxide dismutase (1000 U/ml) (to $80.1 \pm 7.8\%$; $n = 4$; $P < 0.05$ versus diethyldithiocarbamate plus LY83583, Fig. 3). Because the uptake of polyethylene-glycol-superoxide dismutase is a slow process (Beckman et al., 1988), polyethylene-glycol-superoxide dismutase (1000 U/ml) was also left in contact with the tissue for 120 min before administering LY83583. Similarly, with this extended incubation time scale, the reversal of the inhibition by diethyldithiocarbamate plus LY83583 with polyethylene-glycol-superoxide dismutase (1000 U/ml) was not significantly different from that with Cu/Zn superoxide dismutase (1000 U/ml). In this series, the electrically induced nitrergic relaxation was $111.8 \pm 8.2\%$ in the presence of LY83583, and $47.5 \pm 9.3\%$ after pretreatment with diethyldithiocarbamate plus LY83583; this reduced response was reversed to $92.2 \pm 5.6\%$ ($P < 0.01$) by administration of polyethyl-

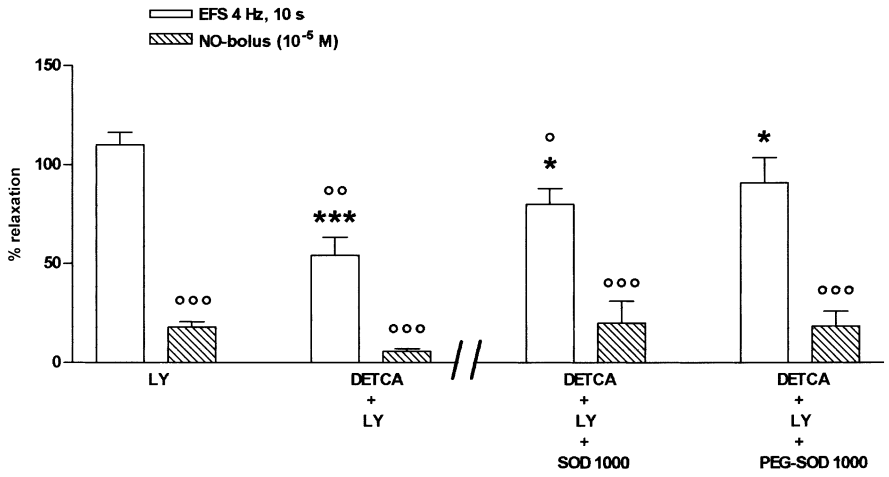

Fig. 3. Influence of incubation with Cu/Zn superoxide dismutase (SOD; 1000 U/ml) or polyethylene-glycol-superoxide dismutase (PEG-SOD; 1000 U/ml) on the inhibitory effect of 3×10^{-3} M DETCA plus 10^{-5} M LY83583 versus relaxations induced by electrical field stimulation (EFS; 40 V, 0.1 ms, 4 Hz, 10 s) and exogenous NO (10^{-5} M). Relaxations are expressed as a percentage of the response to the same stimulus before admistration of interfering drugs. Means ± S.E.M. of $n=4$ are represented. $^{\circ}P<0.05$: $^{\circ\circ}P<0.01$.$^{\circ\circ\circ}P<0.001$: significantly different from the response in the same tissue before administration of drugs (paired t-test); $^{*}P<0.05$: significantly different from pretreatment with DETCA plus LY83583; $^{***}P<0.001$: significantly different from pretreatment with LY83583 alone (ANOVA followed by Bonferroni multiple comparison t-test).

ene-glycol-superoxide dismutase, and to 84.0 ± 2.0% ($P<0.01$) by Cu/Zn superoxide dismutase ($n=4$ for each condition).

Neither Cu/Zn superoxide dismutase (1000 U/ml) nor polyethylene-glycol-superoxide dismutase [1000 U/ml; either given just before LY83583 (Fig. 3) or incubated during

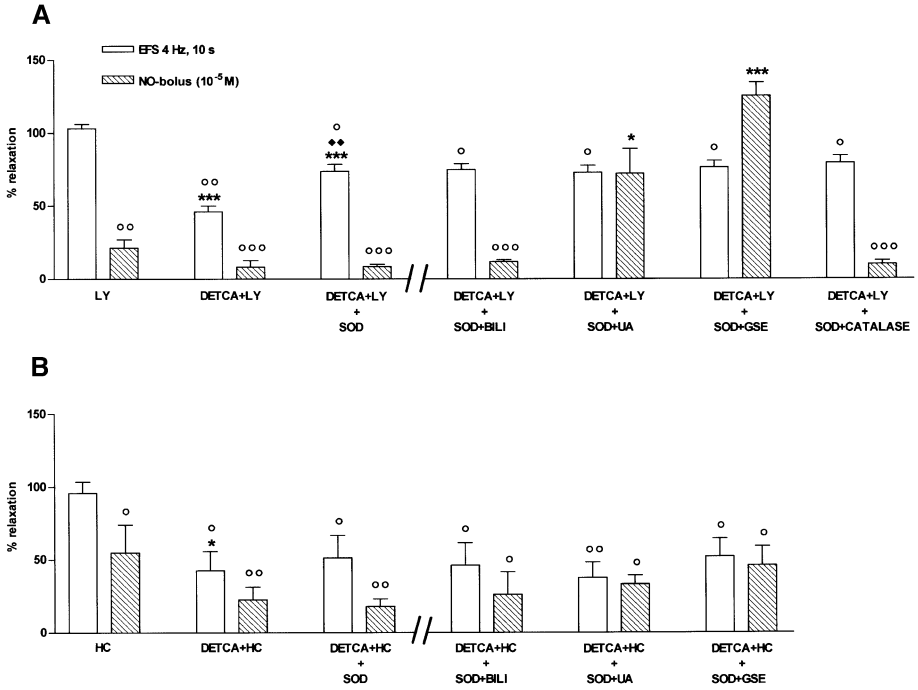

Fig. 4. Influence of Cu/Zn superoxide dismutase (SOD; 1000 U/ml) alone or SOD plus one of the antioxidants indicated [bilirubin (bili; 2×10^{-4} M), uric acid (UA; 4×10^{-4} M), glutathione ethyl ester (GSE; 3×10^{-3} M) and catalase (2000 U/ml)] on the inhibitory effect of 3×10^{-3} M DETCA plus 10^{-5} M LY83583 (A) or 10^{-4} M hydroxocobalamin (HC) (B) versus relaxations induced by electrical field stimulation (EFS; 40 V, 0.1 ms, 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.M. of $n=6$ are shown. $^{\circ}P<0.05$,$^{\circ\circ}P<0.01$, $^{\circ\circ\circ}P<0.001$; significantly different from the response in the same tissue before administration of drugs (paired t-test). Left part of the graphs (–//), $^{*}P<0.05$: $^{***}P<0.001$: significantly different from pretreatment with LY83583 or hydroxocobalamin alone; ♦♦$P<0.01$; significantly different from the response in the presence of the combination of DETCA plus LY83583 (ANOVA followed by Bonferroni multiple comparison t-test). Right part of the graphs (//–): $^{*}P<0.05$: $^{***}P<0.001$: significantly different from pretreatment with DETCA plus LY83583 plus SOD (ANOVA followed by Bonferroni multiple comparison t-test).

a period of 2 h before administering LY83583] altered the effect of LY83583 (10^{-5} M) on the relaxation to exogenous NO (10^{-5} M) in strips which were pretreated with diethyldithiocarbamate (3×10^{-3} M).

3.3. Effect of antioxidant replenishment on the inhibitory effect of LY83583 or hydroxocobalamin on nitrergic relaxations in the presence of diethyldithiocarbamate

As previously shown, we confirmed that the addition of exogenous Cu/Zn superoxide dismutase (1000 U/ml) partially reversed the inhibitory effect of the combination diethyldithiocarbamate (3×10^{-3} M) plus LY83583 (10^{-5} M) on the relaxation to electrical field stimulation [from 46.2 ± 4.7% to 73.7 ± 6.0% ($n = 6$; $P < 0.01$; Fig. 4A)].

We now showed that addition of several antioxidants (bilirubin, uric acid, glutathione ethyl ester or catalase) to Cu/Zn superoxide dismutase did not induce a more pronounced reversal of the inhibitory effect of LY83583 on electrically induced nitrergic relaxations after pretreatment with diethyldithiocarbamate (3×10^{-3} M), than the addition of Cu/Zn superoxide dismutase alone (Fig. 4A).

Combination of Cu/Zn superoxide dismutase (1000 U/ml) with bilirubin (2×10^{-4} M) or with catalase (2000 U/ml) did not result in a reversal of the inhibitory effect of diethyldithiocarbamate plus LY83583 upon the nitrergic relaxation induced by exogenous NO (Fig. 4A). When Cu/Zn superoxide dismutase (1000 U/ml) was combined with uric acid (4×10^{-4} M) or glutathione ethyl ester (3×10^{-3} M), the amplitude of the relaxation to exogenous NO (10^{-5} M) in the presence of diethyldithiocarbamate plus LY83583 highly increased (Fig. 4A). This corresponds to an effect per se of uric acid and glutathione ethyl ester on NO-induced relaxation, reported before (Colpaert and Lefebvre, 2000).

The inhibitory effect of diethyldithiocarbamate (3×10^{-3} M) plus hydroxocobalamin (10^{-4} M) on electrically induced nitrergic relaxations was not influenced by exogenous Cu/Zn superoxide dismutase (1000 U/ml) nor by the combination of Cu/Zn superoxide dismutase with bilirubin (2×10^{-4} M), uric acid (4×10^{-4} M) or glutathione ethyl ester (3×10^{-3} M) (Fig. 4B). Neither Cu/Zn superoxide dismutase nor the combinations of Cu/Zn superoxide dismutase with bilirubin, uric acid or glutathione ethyl ester altered the inhibitory effect of diethyldithiocarbamate plus hydroxocobalamin upon the NO-induced relaxation (Fig. 4B).

4. Discussion

The concept that antioxidants contribute to nitrergic signalling in peripheral organ systems has received growing attention in recent years by several authors (see Martin et al., 1994; Lilley and Gibson, 1996, 1997; Colpaert and Lefebvre, 2000; Colpaert et al., 2002): they have gathered evidence that natural tissue antioxidants, present in the neuroeffector region and/or released by nitrergic nerve terminals, may protect free radical NO against oxyradical attack thereby enabling free radical NO to function as endogenous nitrergic neurotransmitter. Among the antioxidants under study, Cu/Zn superoxide dismutase, reduced glutathione (GSH), uric acid and ascorbic acid were most promising because they provided protection of exogenous NO against several superoxide anion generators and/or NO-scavengers. Although pharmacological depletion and replenishment studies have been performed within nitrergically innervated parts of the gastrointestinal and urogenital tract to further unravel the importance of antioxidants in nitrergic signalling, all studies focused on the depletion or replenishment of merely one antioxidant system (mostly Cu/Zn superoxide dismutase). In the present study, we aimed to further elaborate upon this ‘antioxidant protection theory’ in the pig gastric fundus by combining several antioxidant depletion strategies (in casu Cu/Zn superoxide dismutase together with reduced glutathione or catalase) and by investigating the effect of addition of a second antioxidant in combination with Cu/Zn superoxide dismutase when the endogenous tissue Cu/Zn superoxide dismutase is depleted. Finally, we also compared the effect of administration of polyethylene-glycol-superoxide dismutase, capable of reaching the intracellular milieu, with that of Cu/Zn superoxide dismutase.

Consistent with our previous studies in circular smooth muscle strips of porcine gastric fundus (Colpaert et al., 2002) we confirmed that inhibition of either the tissue antioxidant enzyme Cu/Zn superoxide dismutase (by means of the Cu-chelator diethyldithiocarbamate) or the antioxidant compound reduced glutathione (by use of buthionine sulphoximine, an inhibitor of the biosynthesis of reduced glutathione) did not result per se in an inhibition of nitrergic relaxations (induced by exogenous NO or by electrical field stimulation) of the experimental smooth muscle preparations. Combining both Cu/Zn superoxide dismutase and reduced glutathione depletion strategies also did not affect nitrergic relaxations. When the superoxide generator 6-anilino-5,8-quinolinedione (LY83583) or the NO-scavenger hydroxocobalamin were added to the organ bath, the amplitude of the nitrergic relaxation to electrical field stimulation was reduced to about 50% when experimental tissues were pretreated with diethyldithiocarbamate, but not with buthionine sulphoximine. The combination of diethyldithiocarbamate, buthionine sulphoximine and LY83583 did not induce a more pronounced inhibition of electrically induced nitrergic relaxation than diethyldithiocarbamate plus LY83583. It should of course be kept in mind that treatment of porcine gastric fundus with buthionine sulphoximine only reduces the reduced glutathione content to about 60% of control level (Colpaert et al., 2002). Still, the result implies that weakening of the antioxidant protection, mediated by reduced glutathione, does not induce an additive action with the depletion of Cu/

Zn superoxide dismutase, suggesting a more prominent role for Cu/Zn superoxide dismutase than for reduced glutathione. In another series of experiments, we tested whether the combined depletion of Cu/Zn superoxide dismutase and catalase, the antioxidant enzyme which converts tissue hydrogen peroxide to water and oxygen, renders nitrergic relaxation to electrical field stimulation and exogenous NO more sensitive to inhibition by the superoxide generator LY83583 than Cu/Zn superoxide dismutase depletion alone. It is indeed reported in literature that in mouse gastric fundus catalase may protect the nitrergic neurotransmitter at the prejunctional site against oxyradical attack (De Man et al., 2001). However, administration of 3-amino-1,2,4-triazole, which is an inhibitor of catalase to whose active center it specifically and covalently binds (Ueda et al., 2003) and was used in a concentration as applied by De Man et al. (2001), had no effect per se and did not increase the inhibitory effect of diethyldithiocarbamate plus LY83583 versus nitrergic relaxations. No evidence for a protective effect of catalase was thus obtained in the pig gastric fundus.

The protecting capacity of antioxidants versus oxyradicals is not only determined by their intrinsic chemical reactivity toward radicals, but is equally influenced by important other factors : the concentration of the antioxidant at the microenvironment, the interaction with other antioxidants and the site of action of the antioxidant (Niki et al., 1995). In circular smooth muscle preparations of porcine gastric fundus which were pretreated with diethyldithiocarbamate (i.e. had virtually no Cu/Zn superoxide dismutase enzyme activity), addition of 1000 U/ml of exogenous Cu/Zn superoxide dismutase partially reversed the inhibitory effect of the superoxide generator LY83583 upon the electrically induced nitrergic relaxation. Higher concentrations of exogenous Cu/Zn superoxide dismutase (going to 3000 U/ml; data not shown) did not result in a more pronounced reversal of the inhibitory effect of LY83583, suggesting that the maximal protective activity against the superoxide anions generated by LY83583 was already reached at the lower concentration of 1000 U/ml. We cannot provide a suitable answer for the inability of exogenous Cu/Zn superoxide dismutase to protect the relaxation induced by exogenous NO against the inhibitory action of LY83583 in tissues pretreated with diethyldithiocarbamate, nor for the non-effect of exogenous Cu/Zn superoxide dismutase to protect nitrergic relaxations against the NO-scavenger hydroxocobalamin in tissues treated with diethyldithiocarbamate. Since we showed before that the antioxidants bilirubin, uric acid and reduced glutathione each exhibited protective activity towards the relaxation induced by exogenous NO when confronted with the superoxide generator LY83583 (Colpaert and Lefebvre, 2000), we expected a more pronounced reversal of the inhibitory action of LY83583 upon nitrergic relaxations in tissues pretreated with diethyldithiocarbamate when Cu/Zn superoxide dismutase was added in combination with one of these antioxidants than when added alone. Nevertheless, no combination of Cu/Zn superoxide dismutase with one of the other antioxidants mentioned (including catalase) managed to do so for relaxations elicited by electrical field stimulation. Only for the combination of Cu/Zn superoxide dismutase with uric acid or glutathione ethyl ester (a membrane permeable form of reduced glutathione) did we observe a protective effect towards relaxations induced by exogenous NO in the presence of diethyldithiocarbamate and LY83583. For the combination of Cu/Zn superoxide dismutase with glutathione ethyl ester, the protection was complete, similar to what we have shown before with glutathione ethyl ester alone (see Colpaert et al., 2002). For the combination of Cu/Zn superoxide dismutase with uric acid, the protective effect was higher than when uric acid was given alone (see Colpaert et al., 2002). The addition of Cu/Zn superoxide dismutase together with either bilirubin or uric acid or glutathione ethyl ester was not able to induce any reversal of the inhibitory action of the NO-scavenger hydroxocobalamin on both types of nitrergic relaxations (electrically and NO-induced) in strips which were depleted from tissue Cu/Zn superoxide dismutase by diethyldithiocarbamate. For the action upon the NO-induced relaxations, this contrasts to previous findings in porcine gastric fundus (Colpaert et al., 2002) where both uric acid and glutathione ethyl ester alone were shown to partially protect the NO-induced relaxation against the NO-scavenger hydroxocobalamin in tissues treated with diethyldithiocarbamate. We have no explanation for this observation.

In order to take into account the site (extracellular or intracellular milieu) where an antioxidant can interact with oxyradicals, we also investigated the effect of polyethylene-glycol-superoxide dismutase. In contrast to Cu/Zn superoxide dismutase, which cannot penetrate cell membranes, polyethylene-glycol-superoxide dismutase has also access to the intracellular compartment for interplay with superoxide anions (Liu et al., 1989). Since the Cu-chelator diethyldithiocarbamate irreversibly inhibits both the extracellular and intracellular Cu/Zn superoxide dismutase isoforms (Kelner et al., 1989) in our experimental tissues and as LY83583 produces superoxide anions both intracellularly and extracellularly, addition of exogenous Cu/Zn superoxide dismutase (incapable of penetrating intracellularly) can only protect NO (endogenously released as nitrergic neurotransmitter or exogenously added) against the inactivation by superoxide in the extracellular compartment. This lack of intracellular effect of exogenous Cu/Zn superoxide dismutase might thus explain the incomplete or absent reversal of the inhibitory action of diethyldithiocarbamate plus LY83583 upon nitrergic relaxations. When administering polyethylene-glycol-superoxide dismutase just before LY83583, in a concentration of 1000 U/ml in tissues pretreated with diethyldithiocarbamate, we found a significant reversal of the inhibitory action of the superoxide generator LY83583 upon electri-

cally induced nitrergic relaxations, but not upon NO-induced relaxations. Furthermore, when we compared the reversal induced by 1000 U/ml polyethylene-glycol-superoxide dismutase to that by 1000 U/ml of exogenous Cu/Zn superoxide dismutase, we did not notice a difference in effect. Two feasible explanations can be put forward to explain this observation: firstly, the extracellular interaction between superoxide anions and the endogenous nitrergic neurotransmitter might be far more important than the intracellular interplay so that changes in the intracellular inactivation rate of the nitrergic neurotransmitter do not influence the final result; secondly, polyethylene-glycol-superoxide dismutase experiences difficulties to penetrate through cell membranes in our porcine gastric fundus preparation. Indeed, the latter option merits further attention since in cultured endothelial cells it was already reported that the intracellular penetration of polyethylene-glycol-superoxide dismutase can progress rather slowly (Beckman et al., 1988). We therefore extended the incubation time of polyethylene-glycol-superoxide dismutase to 2 h, but this did not lead to an improvement of the reversal of the electrically induced nitrergic relaxation in diethyldithiocarbamate-treated preparations in the presence of LY83583 when compared to the reversal induced by 1000 U/ml polyethylene-glycol-superoxide dismutase administered just before LY83583. Increasing the concentration of polyethylene-glycol-superoxide dismutase up to 3000 U/ml also did not enhance its effect (data not shown).

In conclusion, in circular smooth muscle strips of porcine gastric fundus, combining reduced glutathione decrease with Cu/Zn superoxide dismutase depletion did not influence the effect of Cu/Zn superoxide dismutase depletion alone on the inhibitory effect of LY83583 and hydroxocobalamin versus nitrergic relaxation. Adding other antioxidants to exogenous Cu/Zn superoxide dismutase did not increase the reversal capacity of Cu/Zn superoxide dismutase versus the inhibitory effect of LY83583 on nitrergic relaxations in tissues depleted of endogenous Cu/Zn superoxide dismutase by diethyldithiocarbamate. These observations point to a crucial role of endogenous tissue Cu/Zn superoxide dismutase in safeguarding peripheral nitrergic neurotransmission. The membrane permeable polyethylene-glycol-superoxide dismutase has no greater reversal capacity than exogenous Cu/Zn superoxide dismutase, suggesting that the extracellular interaction of the endogenous nitrergic neurotransmitter with superoxide radicals is the important one in determining its breakdown.

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