

## Full-length article

**Role of superoxide dismutase enzymes and ascorbate in protection of nitrenergic relaxation against superoxide anions in mouse duodenum**M Ata SECILMIS<sup>1</sup>, Olcay Ergurhan KIROĞLU<sup>1</sup>, Nuran OGULENER<sup>1,2</sup><sup>1</sup>Department of Pharmacology, Cukurova University, Adana 01330, Turkey**Key words**

mouse duodenum; endogenous superoxide dismutase; manganese superoxide dismutase; superoxide anion generators

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**Abstract**

**Aim:** The aim of this study was to investigate whether superoxide dismutase (SOD) enzymes and ascorbate play a role in the protection of the nitrenergic relaxation against superoxide anion inhibition in the mouse duodenum. **Methods:** The effects of exogenous SOD, *N,N'*-bis(salicylidene) ethylenediamine chloride (EUK-8; a synthetic cell-permeable mimetic of the manganese SOD [Mn SOD]) and ascorbate on relaxant responses induced by nitrenergic nerve stimulation, exogenous nitric oxide (NO), and nitroglycerin were investigated in isolated mouse duodenum tissues. **Results:** Diethyldithiocarbamate (DETCA) inhibited the relaxation to exogenous NO and nitroglycerin, but not relaxation to electrical field stimulation (EFS). SOD and ascorbate partially prevented the inhibitory effect of DETCA on relaxation to NO, but not to nitroglycerin. The DETCA-induced inhibition on nitroglycerin was prevented by EUK-8. Hemoglobin, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolinel-oxyl-3-oxide, and hydroxocobalamin inhibited the relaxation to NO, but not to EFS and nitroglycerin in the presence of DETCA. Pyrogallol and hydroquinone inhibited the relaxation to NO, but not to EFS and nitroglycerin. This inhibition was prevented by exogenous SOD and ascorbate, but was not prevented by EUK-8. Pyrogallol and hydroquinone did not inhibit the EFS-induced relaxation in the presence of DETCA. Duroquinone and 6-anilino-5.8-quinolinedione inhibited the relaxation to EFS, NO, and nitroglycerin, and this inhibition was prevented by EUK-8. **Conclusion:** These results suggest that the nitrenergic neurotransmission in the mouse duodenum is protected by endogenous tissue antioxidants against superoxide anions, and Mn SOD, in addition to copper/zinc SOD, can protect NO from attack from superoxide anion generators intracellularly. Also, the possibility that the endogenous neurotransmitter may not be the free NO but a NO-containing or NO-generating molecule in the mouse duodenum remains open.

**Introduction**

The release of inhibitory neurotransmitters from non-adrenergic non-cholinergic (NANC) neurons mediates many gastrointestinal motility patterns and is responsible for the maintenance of gastrointestinal muscles in a state of inhibition. The activation of inhibitory nerves in the mouse duodenum causes relaxations, which are inhibited by nitric oxide (NO) synthase inhibitors, indicating that this response involves a nitrenergic neurotransmission<sup>[1,2]</sup>. Also, other pep-

tides or neuropeptides may be released by electrical stimulation, stimulating NO production. The importance of NO as a mediator of NANC neurotransmission is now widely accepted. However, there is still doubt on the exact biochemical identity of the peripheral nitrenergic neurotransmitter. It was reported that superoxide anion generators, duroquinone, pyrogallol, and 6-anilino-5.8-quinolinedione (LY83583), and NO-binding substances, hemoglobin, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolinel-oxyl-3-oxide

(carboxy-PTIO), and hydroxocobalamin inhibit the relaxations induced by exogenous NO, but not that induced by electrical field stimulation (EFS) of the NANC nerves in several tissues<sup>[3-9]</sup>. We have previously shown that hemoglobin and pyrogallol did not affect responses to nitrergic nerve stimulation in the mouse duodenum, although responses to exogenous NO in the same tissues were inhibited<sup>[1]</sup>. Also, in the rat duodenum, carboxy-PTIO, hydroxocobalamin, hydroquinone, and pyrogallol inhibited relaxations to exogenous NO, but have no or little effect on the relaxation elicited by EFS of the nitrergic nerves in NANC conditions<sup>[10,11]</sup>. It has been suggested that the endogenous transmitter is not free NO, but NO-linked to a carrier before release into the neuroeffector region. A possible alternative explanation for this differential effect is that the nitrergic neurotransmitter is free NO that is protected from breakdown by tissue antioxidants. In order to fulfill the neurotransmitter function, the nitrergic neurotransmitter has to diffuse from its neuronal site of synthesis to its target enzyme-soluble guanylate cyclase in the neighboring effector smooth muscle cells; it is vulnerable to superoxide attack and NO-scavenging activity during this journey. It has been proposed that endogenous antioxidants protect the nitrergic neurotransmitter from superoxide anions and even NO-scavenging molecules<sup>[12-15]</sup>. Experiments with copper (Cu) chelator, diethyldithiocarbamate (DETCA), irreversibly inhibited extracellular and intracellular Cu/zinc (Zn) superoxide dismutase (SOD), showed an important role for Cu/Zn SOD in the protection of the nitrergic neurotransmitter<sup>[12,16]</sup>. However, Cu/Zn SOD inhibition did not alter the differential action of superoxide generated by hypoxanthine/xanthine oxidase and pyrogallol in the rat gastric fundus<sup>[16]</sup> and anococcygeus muscle, respectively<sup>[17]</sup>. It is suggested that besides Cu/Zn SOD, other antioxidants can protect NO; in addition to Cu/Zn SOD, as De Man *et al*<sup>[18]</sup> pointed out, there is a mitochondrial manganese SOD 6 (Mn SOD) that is not inhibited by DETCA and is present in the rat gastrointestinal tract muscle and nerve cells, where it is colocalized with NADPH diaphorase, a marker for NO synthase<sup>[19,20]</sup>. Also, another endogenous antioxidant, ascorbate, has been shown to protect exogenous NO from attack by superoxide anions<sup>[13]</sup>, and the release of ascorbate with the nitrergic transmitter upon nitrergic nerve stimulation was demonstrated in the rat anococcygeus<sup>[14]</sup>.

The aim of this study was to investigate whether SOD enzymes, Cu/Zn SOD, and mitochondrial Mn SOD, and ascorbate play a role in the protection of the nitrergic relaxation against superoxide anion inhibition in the mouse duodenum. For this purpose, we have studied the effect of antioxidants, exogenous Cu/Zn SOD, *N,N'*-bis(salicylidene) ethylenedi-

amine chloride (EUK-8), a synthetic cell-permeable mimetic of the mitochondrial Mn SOD and ascorbate; Cu/Zn SOD inhibitor DETCA; NO scavengers, hemoglobin, carboxy-PTIO, and hydroxocobalamin; and superoxide anion generators pyrogallol, hydroquinone, duroquinone and LY83583 on relaxant responses induced by nitrergic nerve stimulation, exogenous NO, and nitroglycerin. The influence of DETCA pretreatment on the effects of superoxide anion generators and NO scavengers were also assessed.

## Materials and methods

**Tissue and preparation** Swiss albino mice of either sex, weighing 20–25 g, were used in these experiments. The mice fasted for 24 h with free access to water. They were killed by stunning and cervical dislocation. Duodenal segments were rapidly removed and the proximal portion of the duodenum (approximately 12–15 mm long) was mounted as a tube under 0.2 g tension in a 15 mL organ bath filled with Krebs solution (in mmol/L: NaCl 117.9, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 0.89, NaHCO<sub>3</sub> 25, and glucose 10.1). Throughout the experiments, atropine sulfate (1 μmol/L) and guanethidine sulfate (1 μmol/L) were present in the bathing medium to inhibit cholinergic and adrenergic responses. In our previous experiments, the strips reached stability at 25 °C, therefore the bath medium was maintained at 25 °C and oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>). The tissues were equilibrated for 60 min with rinsing at 15 min intervals. Changes in muscle length were recorded isotonicity via an isotonic transducer (7006, Ugo Basile, Varese, Italy) connected to an ink writer (Gemini 7070, Ugo Basile, Italy). The protocol of this study was approved by the Ethics Committee of the Faculty of Medicine at Cukurova University (Adana, Turkey).

**Experimental protocols** In cumulative concentration–response curves on mouse duodenal strips, 0.1 μmol/L serotonin produced a contraction, which represented 85.9%±3.1% of the maximum attainable contraction with serotonin<sup>[1]</sup>. All strips were contracted with 0.1 μmol/L serotonin. This resulted in an active tone that reached a stable level within 5 min; at the end of this period, 2 series of control relaxant responses were obtained according to experimental protocol. In the experimental protocol, EFS (5 Hz, 25 V, 1 ms, 15 s train), exogenous NO (100 μmol/L; administered as acidified NaNO<sub>2</sub>), nitroglycerin (50 μmol/L), or isoproterenol (10 nmol/L) were applied without rinsing the tissue between each individual application to a single tissue. After the relaxant responses had been obtained, the tissues were rinsed and incubated for 30 min with the drug under study, and the second series of responses were recorded in the same manner.

**Studies with antioxidants and DETCA** The effects of the

antioxidants, exogenous Cu/Zn SOD (200  $\mu\text{mol/L}$ ), incapable of penetrating intracellular, EUK-8 (300  $\mu\text{mol/L}$ ), a synthetic cell-permeable mimetic of the mitochondrial Mn SOD, and ascorbate (500  $\mu\text{mol/L}$ ) were investigated on the relaxant responses to nitrenergic nerve stimulation, exogenous NO, nitroglycerin, and isoproterenol. All antioxidants were added 2 min before relaxant stimulus was applied.

To study the effect of tissue Cu/Zn SOD inhibition on the relaxation of the mouse duodenum to EFS, NO, nitroglycerin, and isoproterenol the irreversible inhibitor of extracellular and intracellular Cu/Zn SOD, DETCA (8 mmol/L) was used as follows. After the first control responses were obtained, DETCA was added to the medium, and relaxant stimuli were applied for the second time. The tissue was incubated with DETCA for 30 min. To study the influence of exogenous Cu/Zn SOD (200 U/mL), EUK-8 (300  $\mu\text{mol/L}$ ), and ascorbate (500  $\mu\text{mol/L}$ ) on the inhibitory effect of DETCA, the antioxidants were administered 2 min before relaxant responses were obtained in the presence of DETCA. The influence of exogenous Cu/Zn SOD, EUK-8, and ascorbate in the presence of DETCA was studied in the tissues of different animals.

**Studies with NO scavengers** In these experiments, the effects of NO scavengers, hemoglobin (20 mmol/L), carboxy-PTIO (100  $\mu\text{mol/L}$ ), and hydroxocobalamin (500  $\mu\text{mol/L}$ ) were investigated on the relaxation of the mouse duodenum in response to EFS, NO, nitroglycerin, and isoproterenol. After the first relaxations were obtained with EFS, NO, nitroglycerin, and isoproterenol, NO scavengers were added, and tissues were incubated for 30 min with NO scavengers. After incubation, EFS, NO, nitroglycerin, and isoproterenol were applied for the second time. The influence of hemoglobin, carboxy-PTIO, and hydroxocobalamin was studied in tissues from different animals.

**Studies with superoxide anion generators** After the first responses were obtained, the mouse duodenum was treated for 30 min with the superoxide anion generators, pyrogallol (50  $\mu\text{mol/L}$ ), hydroquinone (100  $\mu\text{mol/L}$ ), duroquinone (50  $\mu\text{mol/L}$ ), and LY83583 (10  $\mu\text{mol/L}$ ) and then EFS, NO, nitroglycerin, and isoproterenol were applied second time. Furthermore, the influence of the antioxidants, exogenous Cu/Zn SOD (200 U/mL), EUK-8 (300  $\mu\text{mol/L}$ ), and ascorbate (500  $\mu\text{mol/L}$ ), was studied on the inhibitory effects of pyrogallol, hydroquinone, duroquinone, and LY83583. In some experiments, to study the effect of DETCA (8 mmol/L) plus the superoxide anion generators, pyrogallol (50  $\mu\text{mol/L}$ ), hydroquinone (100  $\mu\text{mol/L}$ ), duroquinone (50  $\mu\text{mol/L}$ ), and LY83583 (10  $\mu\text{mol/L}$ ) were added to the medium in the presence of DETCA, and then EFS, exogenous NO, nitroglycerin, and isoproterenol were applied. Further-more, the influence

of antioxidants, exogenous Cu/Zn SOD (200 U/mL), EUK-8 (300  $\mu\text{mol/L}$ ), and ascorbate (500  $\mu\text{mol/L}$ ) were tested versus DETCA plus the superoxide anion generators. None of the drugs used in this investigation influenced the serotonin-induced contraction of the tissue under study.

**Drugs and solutions** Atropine sulfate, guanethidine sulfate, serotonin hydrochloride (5-hydroxytryptamine), acidified sodium nitrite, isoproterenol hydrochloride, human hemoglobin, carboxy-PTIO, hydroxocobalamin, Cu/Zn SOD, hydroquinone, pyrogallol, duroquinone, LY83583, manganese EUK-8, glutathione, and ascorbate were supplied by Sigma (St Louis, MO, USA). Nitroglycerin was provided by Adeka (Adeka Drug, Samsun, Turkey). Acidified sodium nitrite, which was used as an exogenous NO source, obtained by diluting sodium nitrite in de-aerated water acidified to pH 2 with HCl, was stored at  $-4\text{ }^{\circ}\text{C}$ . As with exogenous NO, 0.05 mL stock solution of acidified  $\text{NaNO}_2$  was added to the 15 mL organ bath<sup>[21]</sup>. The application of acidified  $\text{NaNO}_2$  did not alter the pH value of the bathing medium. Stock solutions of acidified sodium nitrite were prepared freshly on the day of experimentation. Drugs were dissolved in de-ionized water, except duroquinone and 6-anilino-5.8-quinolinedione. Duroquinone was dissolved in acetone, and 6-anilino-5.8-quinolinedione was dissolved in 100% ethanol. Stock solutions were made of LY83583 ( $1 \times 10^{-2}$  mol/L); other solutions were prepared on the day of the experiment. The final concentrations of these solvents showed no significant biological differences.

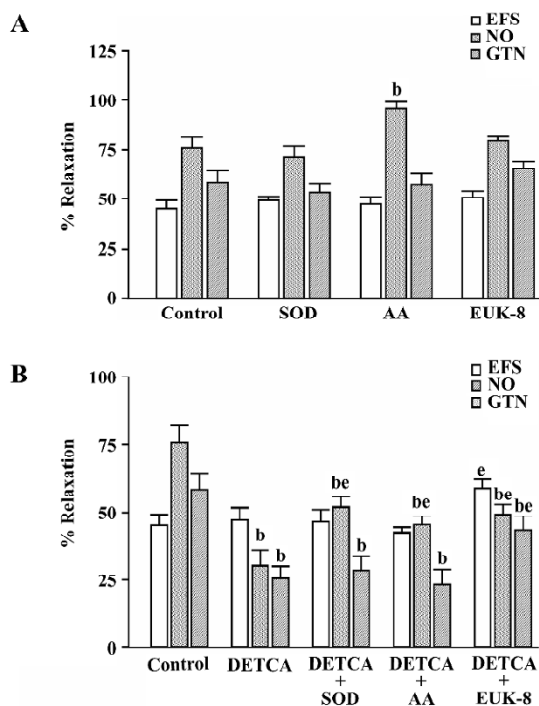
**Presentation of results and statistical analysis** The relaxations were calculated as percentage peak reductions of serotonin contraction. Results were expressed as mean $\pm$ SEM, and *n* referred to the number of animals used for each experiments. Differences in results between tissues were tested by ANOVA and *t*-test corrected for multiple comparisons (*Bonferroni* corrections). *P*-values less than 0.05 were considered to be significant.

## Results

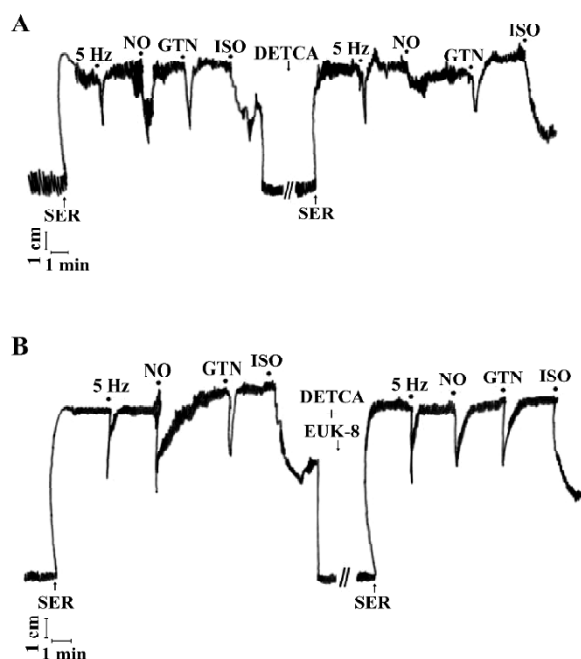
**Relaxant responses to EFS, exogenous NO, nitroglycerin, and isoproterenol** Short-term EFS (5 Hz, 25 V, 1 ms, 15 s train) of nitrenergic nerves elicited a fast and transient relaxation (45.1% $\pm$ 4.2%, *n*=6) in serotonin-contracted tissues treated with atropine and guanethidine. Relaxation was also observed in response to exogenous NO (100  $\mu\text{mol/L}$ ; 75.7% $\pm$ 6.3%, *n*=6) and nitroglycerin (50  $\mu\text{mol/L}$ ; 58.3% $\pm$ 6.1%, *n*=6) that was also characterized by a fast and transient response. In contrast, the relaxation in response to isoproterenol (10 nmol/L; 69.3% $\pm$ 3.1%, *n*=6) was slow in onset and sustained.

**Effect of exogenous Cu/Zn SOD, EUK-8, and ascorbate** Exogenous Cu/Zn SOD (200  $\mu\text{mol/L}$ ) and EUK-8 (300  $\mu\text{mol/L}$ ) did not influence the relaxation to EFS, NO, and nitroglycerin in the mouse duodenum preparations ( $P>0.05$ ; Figure 1A;  $n=6$ ). However, ascorbate (500  $\mu\text{mol/L}$ ) potentiated the relaxation to NO, but did not affect the relaxant response to EFS and nitroglycerin ( $P<0.05$ ; Figure 1A;  $n=6$ ). The isoproterenol-induced relaxation was unaffected by antioxidants (data not shown).

**Effect of Cu/Zn SOD inhibitor DETCA** Treatment of the mouse duodenal tissues with Cu/Zn SOD inhibitor DETCA (8  $\text{mmol/L}$ ) significantly inhibited the relaxation of the duodenum to NO and nitroglycerin, but not that to EFS ( $P<0.05$ ; Figure 1B, 2A;  $n=6$ ). The addition of exogenous Cu/Zn SOD (200  $\mu\text{mol/L}$ ), EUK-8 (300  $\mu\text{mol/L}$ ), and ascorbate (500  $\mu\text{mol/L}$ ) partially prevented the inhibitory effect of DETCA (8  $\text{mmol/L}$ ) on relaxation to NO ( $P<0.05$  of DETCA). The inhibitory effect of DETCA on nitroglycerin was not influenced by exog-



**Figure 1.** (A) effects of SOD (200 U/mL), ascorbic acid (AA; 500  $\mu\text{mol/L}$ ), and EUK-8 (300  $\mu\text{mol/L}$ ), and (B) DETCA (8  $\text{mmol/L}$ ), DETCA (8  $\text{mmol/L}$ ) plus SOD (200 U/mL), DETCA (8  $\text{mmol/L}$ ) plus AA (500  $\mu\text{mol/L}$ ), and DETCA (8  $\text{mmol/L}$ ) plus EUK-8 (300  $\mu\text{mol/L}$ ) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100  $\mu\text{mol/L}$ ), and nitroglycerin (GTN; 50  $\mu\text{mol/L}$ ) in mouse duodenal strips precontracted with 0.1  $\mu\text{mol/L}$  serotonin. All values are mean $\pm$ SEM ( $n=6$ ). <sup>b</sup> $P<0.05$ , significantly different from control; <sup>e</sup> $P<0.05$  significantly different from DETCA. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

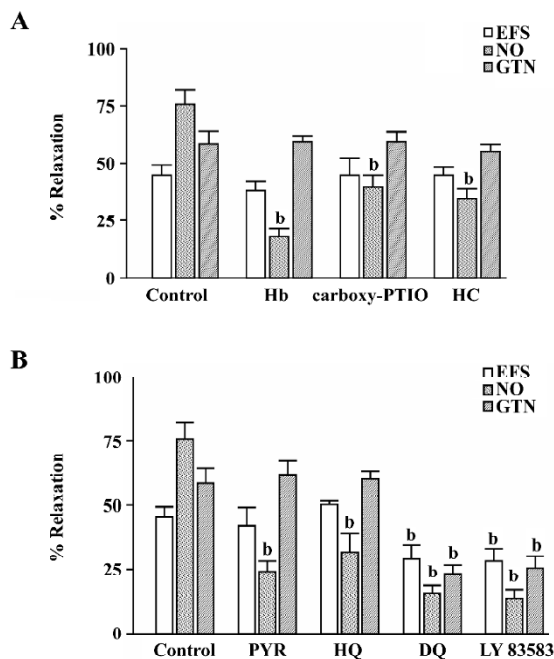


**Figure 2.** Representative traces showing the effects of (A) DETCA (8  $\text{mmol/L}$ ) and (B) DETCA (8  $\text{mmol/L}$ ) plus EUK-8 (300  $\mu\text{mol/L}$ ) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100  $\mu\text{mol/L}$ ), GTN (50  $\mu\text{mol/L}$ ), and isoproterenol (ISO; 10  $\text{nmol/L}$ ) in mouse duodenal strips precontracted with 0.1  $\mu\text{mol/L}$  serotonin.

enous Cu/Zn SOD (200  $\mu\text{mol/L}$ ) and ascorbate (500  $\mu\text{mol/L}$ ), but was partially prevented by EUK-8 (300  $\mu\text{mol/L}$ ;  $P<0.05$  of DETCA; Figure 1B, 2B;  $n=6$ ). Also, the amplitude of the relaxation to EFS was enhanced in the presence of DETCA plus EUK-8. DETCA did not influence the relaxations to isoproterenol ( $P>0.05$ , data not shown).

**Effect of NO scavengers, hemoglobin, carboxy-PTIO, and hydroxocobalamin** Hemoglobin (20  $\text{mmol/L}$ ), carboxy-PTIO (100  $\mu\text{mol/L}$ ), and hydroxocobalamin (500  $\mu\text{mol/L}$ ) significantly inhibited the relaxation of mouse duodenum to exogenous NO (100  $\mu\text{mol/L}$ ;  $P<0.05$ ; Figure 3A;  $n=6$ ). In contrast, all these agents did not exert an inhibitory influence on relaxations induced by EFS (5 Hz, 25 V, 1 ms, 15 s train), nitroglycerin (50  $\mu\text{mol/L}$ ), and isoproterenol (10  $\text{nmol/L}$ ;  $P>0.05$ ; Figure 3A;  $n=6$ ). The NO scavengers did not inhibit the EFS-induced relaxant response even in the presence of DETCA (8  $\text{mmol/L}$ , data not shown).

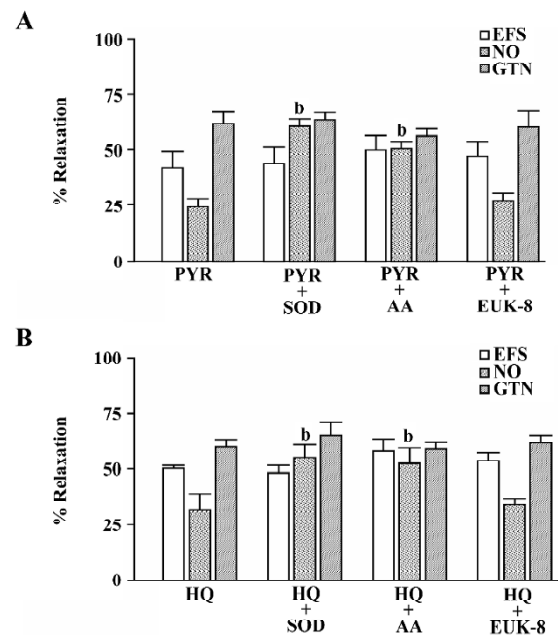
**Effect of superoxide anion generators, pyrogallol, hydroquinone, duroquinone, and LY83583** Pyrogallol (50  $\mu\text{mol/L}$ ) and hydroquinone (100  $\mu\text{mol/L}$ ) significantly inhibited the relaxation of the mouse duodenum to NO, but not the relaxation to EFS and nitroglycerin ( $P<0.05$ ; Figure 3B;  $n=6$ ). The inhibitory effects of pyrogallol and hydro-



**Figure 3.** (A) effects of hemoglobin (Hb; 20 mmol/L), carboxy-PTIO (100 μmol/L), and hydroxocobalamin (HC; 500 μmol/L), and (B) pyrogallol (PYR; 50 μmol/L), hydroquinone (HQ; 100 μmol/L), duroquinone (DQ; 50 μmol/L), and LY83583 (10 μmol/L) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100 μmol/L), and GTN (50 μmol/L) in mouse duodenal strips precontracted with 0.1 μmol/L serotonin. All values are mean±SEM (n=6). <sup>b</sup>P<0.05, significantly different from control. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

quinone on the relaxation in response to NO were reduced by the previous addition of Cu/Zn SOD (200 U/mL; *P*<0.05 of pyrogallol and hydroquinone; Figure 4A,4B; n=6), demonstrating that pyrogallol and hydroquinone are acting by extracellular generation of superoxide anion. Also, ascorbate (500 μmol/L) partially prevented the inhibitory effect of pyrogallol and hydroquinone on the relaxation induced by NO (*P*<0.05 of pyrogallol and hydroquinone, n=6), but EUK-8 (300 μmol/L) did not prevent the inhibition (Figure 4A,4B; n=6).

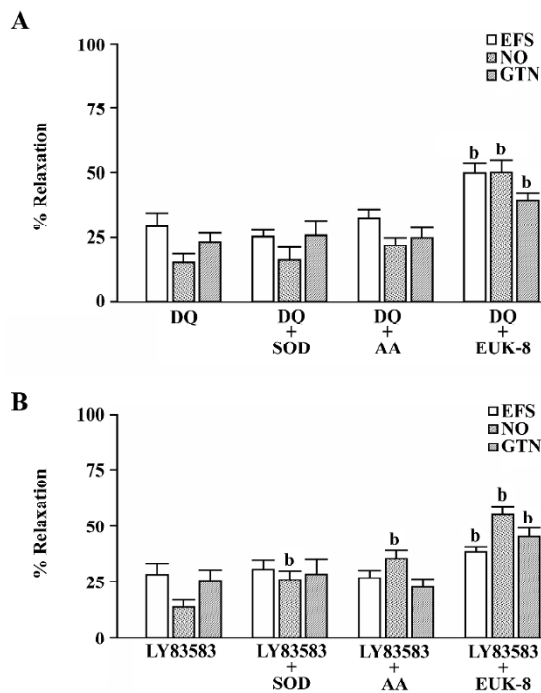
Duroquinone (50 μmol/L) and LY83583 (10 μmol/L) significantly inhibited the relaxation in response to EFS, NO, and nitroglycerin (*P*<0.05; Figure 3B; n=6). The addition of Cu/Zn SOD (200 U/mL) and ascorbate (500 μmol/L) did not prevent the inhibitory effect of duroquinone (50 μmol/L) on the relaxation to EFS, NO, and nitroglycerin, but EUK-8 (300 μmol/L) reduced the inhibition of the relaxation to EFS, NO, and nitroglycerin (*P*<0.05 of duroquinone; Figure 5A; n=6). Also, the inhibitory effect of LY83583 (10 μmol/L) on the relaxation to EFS, NO, and nitroglycerin was partially pre-



**Figure 4.** (A) effects of PYR (50 μmol/L), PYR (50 μmol/L) plus SOD (200 U/mL), PYR (50 μmol/L) plus AA (500 μmol/L), and PYR (50 μmol/L) plus EUK-8 (300 μmol/L), and (B) effects of HQ (100 μmol/L), HQ (100 μmol/L) plus SOD (200 U/mL), HQ (100 μmol/L) plus AA (500 μmol/L), and HQ (100 μmol/L) plus EUK-8 (300 μmol/L) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100 μmol/L), and GTN (50 μmol/L) in mouse duodenal strips precontracted with 0.1 μmol/L serotonin. All values are mean±SEM (n=6). <sup>b</sup>P<0.05, significantly different from PYR and HQ. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

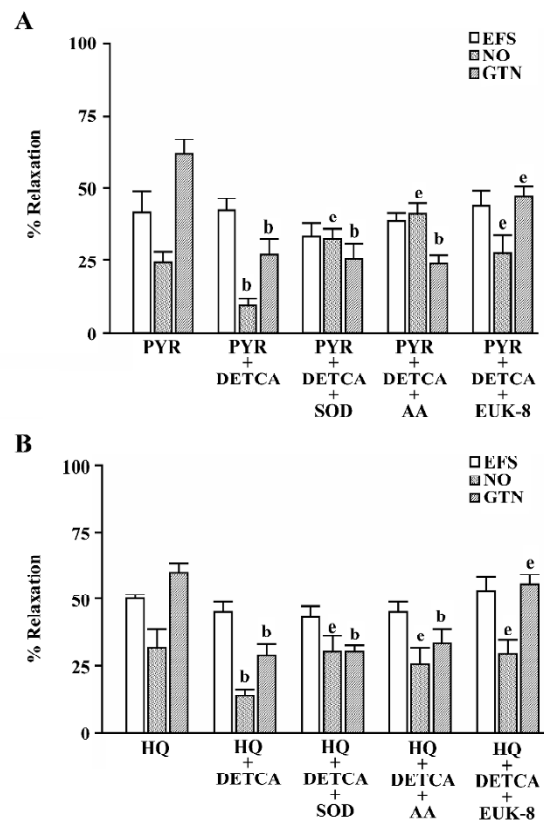
vented by EUK-8 (300 μmol/L). The addition of Cu/Zn SOD (200 U/mL) and ascorbate (500 μmol/L) partially prevented the inhibitory effect of LY83583 on the NO-induced relaxation, but did not influence the inhibition of relaxation to EFS and nitroglycerin (*P*<0.05 of LY83583; Figure 5B; n=6). Superoxide anion generators did not affect the relaxations to isoproterenol (*P*>0.05, data not shown).

**Effect of a combination of DETCA plus superoxide anion generators** To investigate whether the presence of high endogenous levels of Cu/Zn SOD in the neuroeffector junction protects the nitrergic transmitter from inactivation by superoxide anions, preparations were pretreated with Cu/Zn SOD inhibitor DETCA (8 mmol/L). After DETCA pretreatment, the inhibitory effect of pyrogallol (50 μmol/L) and hydroquinone (100 μmol/L) on the NO-mediated relaxation greatly enhanced (*P*<0.05 of pyrogallol and hydroquinone; Figure 6A,6B; n=6); however, pyrogallol (50 μmol/L) and hydroquinone (100 μmol/L) did not inhibit the EFS-induced relaxant response even in the presence of DETCA. Also, the pyrogallol or hydroquinone plus DETCA combination in-



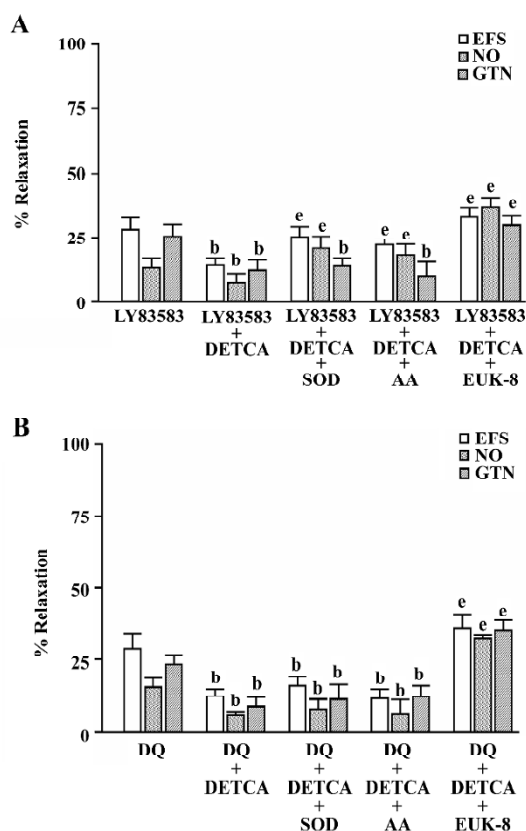
**Figure 5.** (A) effects of DQ (50  $\mu\text{mol/L}$ ), DQ (50  $\mu\text{mol/L}$ ) plus SOD (200 U/mL), DQ (50  $\mu\text{mol/L}$ ) plus AA (500  $\mu\text{mol/L}$ ), and DQ (50  $\mu\text{mol/L}$ ) plus EUK-8 (300  $\mu\text{mol/L}$ ), and (B) effects of LY83583 (10  $\mu\text{mol/L}$ ), LY83583 (10  $\mu\text{mol/L}$ ) plus SOD (200 U/mL), LY83583 (10  $\mu\text{mol/L}$ ) plus AA (500  $\mu\text{mol/L}$ ), and LY83583 (10  $\mu\text{mol/L}$ ) plus EUK-8 (300  $\mu\text{mol/L}$ ) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100  $\mu\text{mol/L}$ ), and GTN (50  $\mu\text{mol/L}$ ) in mouse duodenal strips precontracted with 0.1  $\mu\text{mol/L}$  serotonin. All values are mean $\pm$ SEM ( $n=6$ ). <sup>b</sup> $P<0.05$ , significantly different from duroquinone and LY83583. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

hibited the relaxation to nitroglycerine ( $P<0.05$  of pyrogallol and hydroquinone; Figure 6A and 6B;  $n=6$ ), but this inhibition was not significant from the DETCA-induced inhibition on the relaxation produced by nitroglycerin ( $P>0.05$  of DETCA). The inhibitory effect of the combination of DETCA (8 mmol/L) plus pyrogallol (50  $\mu\text{mol/L}$ ) or hydroquinone (100  $\mu\text{mol/L}$ ) on the relaxation to exogenous NO was partially prevented by the addition of Cu/Zn SOD (200 U/mL), EUK-8 (300  $\mu\text{mol/L}$ ), and ascorbate (500  $\mu\text{mol/L}$ ;  $P<0.05$ ; Figure 6A and 6B;  $n=6$ ). Also, EUK-8 (300  $\mu\text{mol/L}$ ) prevented the inhibitory effect of the combination of DETCA (8 mmol/L) plus pyrogallol (50  $\mu\text{mol/L}$ ) or hydroquinone (100  $\mu\text{mol/L}$ ) on the relaxation to nitroglycerin ( $P<0.05$ ; Figure 6A, 6B;  $n=6$ ). Furthermore, DETCA (8 mmol/L) potentiated the inhibition of the EFS, NO, and nitroglycerine-induced relaxation by duroquinone (50  $\mu\text{mol/L}$ ) or LY83583 (10  $\mu\text{mol/L}$ ;  $P<0.05$ ; Figure 7A and 7B;  $n=6$ ). The addition of Cu/Zn SOD (200 U/mL) and ascorbate (500  $\mu\text{mol/L}$ ) partially prevented the inhibi-



**Figure 6.** (A) effects of PYR (50  $\mu\text{mol/L}$ ), PYR (50  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L), PYR (50  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and SOD (200 U/mL), PYR (50  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and AA (500  $\mu\text{mol/L}$ ), PYR (50  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and EUK-8 (300  $\mu\text{mol/L}$ ), and (B) effects of HQ (100  $\mu\text{mol/L}$ ), HQ (100  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and SOD (200 U/mL), HQ (100  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and AA (500  $\mu\text{mol/L}$ ), HQ (100  $\mu\text{mol/L}$ ) plus DETCA (8 mmol/L) and EUK-8 (300  $\mu\text{mol/L}$ ) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100  $\mu\text{mol/L}$ ) and GTN (50  $\mu\text{mol/L}$ ) in mouse duodenal strips precontracted with 0.1  $\mu\text{mol/L}$  serotonin. All values are mean $\pm$ SEM ( $n=6$ ). <sup>b</sup> $P<0.05$ , significantly different from PYR and HQ, <sup>e</sup> $P<0.05$ , significantly different from PYR plus DETCA or HQ plus DETCA. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

tory action of the combination of DETCA plus LY83583 on the relaxation to EFS and NO, but not nitroglycerin in the mouse duodenum ( $P<0.05$ ; Figure 7A;  $n=6$ ). Also, EUK-8 (300  $\mu\text{mol/L}$ ) partially prevented the inhibition of the EFS, NO, and nitroglycerin-induced relaxation by the combination of DETCA plus LY83583 in the mouse duodenum ( $P<0.05$ ; Figure 7A;  $n=6$ ). The addition of Cu/Zn SOD (200 U/mL) and ascorbate (500  $\mu\text{mol/L}$ ) did not prevent the inhibitory action of the combination of DETCA plus duroquinone on the relaxation to EFS, NO, and nitroglycerin, while EUK-8 (300  $\mu\text{mol/L}$ ) partially prevented the inhibition of the EFS, NO, and nitro-



**Figure 7.** (A) effects of LY83583 (10  $\mu\text{mol/L}$ ), LY83583 (10  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ), LY83583 (10  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and SOD (200  $\text{U/mL}$ ), LY83583 (10  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and AA (500  $\mu\text{mol/L}$ ), LY83583 (10  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and EUK-8 (300  $\mu\text{mol/L}$ ), and (B) effects of DQ (50  $\mu\text{mol/L}$ ), DQ (50  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and SOD (200  $\text{U/mL}$ ), DQ (50  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and AA (500  $\mu\text{mol/L}$ ), and DQ (50  $\mu\text{mol/L}$ ) plus DETCA (8  $\text{mmol/L}$ ) and EUK-8 (300  $\mu\text{mol/L}$ ) on relaxant responses to EFS (5 Hz, 25 V, 1 ms), exogenous NO (100  $\mu\text{mol/L}$ ), and GTN (50  $\mu\text{mol/L}$ ) in mouse duodenal strips precontracted with 0.1  $\mu\text{mol/L}$  serotonin. All values are mean $\pm$ SEM ( $n=6$ ). <sup>b</sup> $P<0.05$ , significantly different from LY83583 and DQ, <sup>e</sup> $P<0.05$ , significantly different from LY83583 plus DETCA or DQ plus DETCA. One-way ANOVA followed by *Bonferroni* multiple comparison *t*-test.

glycerin-induced relaxation with the combination of DETCA plus duroquinone ( $P<0.05$ ; Figure 7B;  $n=6$ )

## Discussion

The aim of this study was to investigate whether SOD enzymes and ascorbate play a role in the protection of the nitrergic relaxation against superoxide anion inhibition in the mouse duodenum. In the present study, we provided evidence that the nitrergic NANC neurotransmitter in the mouse duodenum is protected by endogenous tissue antioxidants against superoxide anions, and also found that Mn SOD, in

addition to Cu/Zn SOD, can protect NO from attack from superoxide anion generators intracellularly. Also, the possibility that the endogenous neurotransmitter is not free NO in the mouse duodenum remains open.

**Influence of the antioxidant** To investigate whether high levels of tissue antioxidants afford protection of the nitrergic transmitter from destruction by SOD, the influence of exogenous Cu/Zn SOD, EUK-8, and ascorbate was determined versus 3 types of nitrergic relaxations. Exogenously-added Cu/Zn SOD, a membrane impermeable antioxidant and EUK-8, a synthetic cell-permeable mimetic of the mitochondrial Mn SOD, did not enhance the relaxations to EFS, NO, and nitroglycerin, suggesting that an endogenous amount of both Cu/Zn SOD and mitochondrial Mn SOD is sufficiently high to protect NO (endogenously released as the nitrergic neurotransmitter or exogenously added) from superoxide-mediated destruction. This is in agreement with previous results obtained in the mouse anococcygeus<sup>[4,13]</sup>, rat anococcygeus<sup>[17]</sup>, rat gastric fundus<sup>[16]</sup>, bovine retractor penis<sup>[22]</sup>, and pig gastric fundus<sup>[15]</sup>. However, ascorbate, which is known to be one of the most important extracellular antioxidants<sup>[23,24]</sup> and is released from nitrergically-innervated smooth muscle<sup>[14]</sup>, significantly enhanced the relaxations to exogenous NO, but did not affect relaxation to EFS and nitroglycerin. It is suggested that ascorbate enhances the activation of soluble guanylate cyclase, and this effect of ascorbate is due to the redox regulation of guanylate cyclase in smooth muscle cells, presumably by maintaining the iron of the metalloporphyrin in a reduced state, thereby facilitating the reaction between NO and the (ferrous) heme cofactor<sup>[25,26]</sup>. However, as ascorbate had no effect on relaxations induced by both EFS and nitroglycerin, this explanation is not suitable in the mouse duodenum. The effect of ascorbate on exogenous NO was not seen with the endogenous nitrergic neurotransmitter released by EFS, suggesting that the effect of ascorbate on exogenous NO might be related to the short distance the endogenous nitrergic neurotransmitter has to travel to reach the smooth muscle cells in comparison to exogenous NO. In contrast to our results, ascorbate did not affect the relaxations to either exogenous NO or nitrergic field stimulation in the mouse anococcygeus and pig gastric fundus in some other studies<sup>[13,15]</sup>. This controversy may have resulted from differences in tissue preparation or in the species used. Also, ascorbate did not affect the relaxations evoked by nitroglycerine. NO generation by nitroglycerine is dependent on intracellular mechanisms, and ascorbate, which acts as an extracellular antioxidant<sup>[14]</sup>, can not affect intracellular NO.

**Depletion of Cu/Zn SOD** In order to further sustain the hypothesis that antioxidants protect the endogenous

nitroergic neurotransmitter, we tried to deplete the endogenous tissue SOD enzyme of duodenal preparations and to determine the sensitivity of the nitroergic transmitter released by nitroergic nerve stimulation, exogenous NO, and nitroglycerin towards superoxide anion generators. Pretreatment with DETCA, which blocked both the extracellular and intracellular cytosolic Cu/Zn SOD isoform of the enzyme<sup>[27,28]</sup>, did not result in an effect on the nitroergic nerve-induced relaxation, while a clear inhibition was observed on the relaxations to exogenous NO and nitroglycerin. The inhibitory effect of DETCA was likely to result from the elevated level of tissue-derived superoxide anion since the addition of exogenous Cu/Zn SOD, EUK-8, and ascorbate partially prevented the DETCA-induced inhibition on relaxation to exogenous NO and nitroglycerin. Our results suggest that besides Cu/Zn SOD, Mn SOD might be involved in protecting the nitroergic relaxation in mouse duodenum. In Mn SOD, a mitochondrial isoenzyme that disposes of superoxide, immunoreactivity is demonstrated in nerve cells and smooth muscle cells in the stomach wall of rats<sup>[20]</sup> and in nerve cells in the myenteric plexus of the opossum esophagus<sup>[29]</sup>. The observation that DETCA had no effect on the EFS-induced relaxation is in agreement with findings of rat gastric fundus<sup>[18]</sup> and also in the rat anococcygeus<sup>[17]</sup>. Also, Colpaert *et al*<sup>[30]</sup> reported that DETCA had no effect on the nitroergic relaxations induced by both EFS and exogenous NO in pig gastric fundus, suggesting a rather low background of oxidative stress in the pig gastric fundus or compensation for the loss of Cu/Zn SOD by other protective antioxidants. Lefebvre<sup>[16]</sup> and Martin *et al*<sup>[12]</sup> demonstrated that DETCA inhibited the relaxations to EFS in the rat gastric fundus and bovine retractor penis, respectively. However, in the mouse duodenum, as DETCA significantly inhibited relaxations to exogenous NO but not relaxation to EFS, it might be suggested that endogenous NO released by nitroergic nerve stimulation may not be free NO but may be a superoxide-resistant, NO-containing, or NO-generating molecule. Further advanced studies are needed to clarify the biochemical identity of the nitroergic neurotransmitter in mouse duodenum.

**Influence of NO scavengers** We previously showed that the relaxant responses to EFS at 5 Hz for 15 s were largely nitroergic in the mouse duodenum preparations, and hemoglobin, pyrogallol, and DETCA did not affect relaxations in response to nitroergic nerve stimulation of the mouse duodenum, suggesting that the nitroergic NANC neurotransmitter is not free NO but a superoxide-resistant, NO-containing molecule and/or free NO which is protected from breakdown by tissue antioxidants<sup>[1]</sup>. In the present study, we also confirmed that NO scavengers, hemoglobin, carboxy-PTIO,

and hydroxocobalamin, significantly inhibited the relaxation to exogenous NO, without affecting those to nitroergic nerve stimulation and nitroglycerin. These findings are consistent with the report of Correia *et al*<sup>[10]</sup> who showed that the nitroergic nerve-mediated relaxation of the rat duodenum were unaffected by carboxy-PTIO and hydroxocobalamin in the presence of DETCA. Also, similar results with NO scavengers were observed in the pig gastric fundus<sup>[15]</sup>, rat gastric fundus<sup>[31]</sup>, mouse anococcygeus<sup>[13]</sup>, and sheep urethra<sup>[32]</sup>. Since tissue SOD might protect the endogenous nitroergic neurotransmitter against NO scavengers, we investigated the effect of NO scavengers on the EFS-induced relaxation in the presence of DETCA. The addition of DETCA did not alter the effect of NO scavengers on the EFS-induced relaxation, suggesting that the other antioxidants (reduced glutathione and ascorbate) may provide protection of neuronally-released NO from attack by scavenging molecules or endogenous NO, which is not free NO in the mouse duodenum. Also, we previously observed that glutathione did not affect the relaxation to nitroergic nerve stimulation and exogenous NO, but its addition prevented the inhibitory effect of DETCA on the exogenous NO- and nitroglycerin-induced relaxation in the mouse duodenum (N OGULENER). In contrast to our findings, in the pig gastric fundus, hydroxocobalamin significantly reduced the relaxations induced by nitroergic nerve stimulation when strips were depleted from Cu/Zn SOD by DETCA, and the administration of exogenous SOD did not prevent the inhibitory action of hydroxocobalamin, suggesting that extracellular superoxide anions are not implicated in the observed inhibitory mechanism<sup>[30]</sup>.

**Influence of superoxide anion generators** Pyrogallol and hydroquinone had no effect on the relaxations to nitroergic nerve stimulation and nitroglycerin, but significantly reduced responses to exogenous NO. The inhibitory effect of pyrogallol and hydroquinone on the exogenous NO-induced relaxation was prevented by the addition of SOD and ascorbate, but not by EUK-8, thus supporting the view that the reduction was due to the superoxide-generating activity of pyrogallol and hydroquinone, and these superoxide anion generators mainly act extracellularly. This finding is consistent with other reports that showed an inhibition of response to exogenous NO, but not to nitroergic nerve stimulation by pyrogallol and hydroquinone in the rat duodenum<sup>[11]</sup>, pig gastric fundus<sup>[15]</sup>, rat gastric fundus<sup>[18]</sup>, and mouse anococcygeus<sup>[13]</sup>. Hydroquinone may act in some tissues as a free radical scavenger<sup>[4,13,17]</sup>, but in our study, since the inhibitory effect of hydroquinone on the relaxations to exogenous NO was prevented by exogenous SOD, it acted as a



superoxide anion generator, similar to those observed in the bovine retractor penis<sup>[9]</sup>.

In contrast to the finding of pyrogallol and hydroquinone, LY83583 and duroquinone significantly inhibited the relaxation in response to EFS in addition to the exogenous NO- and nitroglycerin-induced relaxation in the mouse duodenum. LY83583 was proposed as an inhibitor of soluble guanylate cyclase<sup>[33]</sup>, but it is recognized to produce its actions through the generation of superoxide anions<sup>[5]</sup>. In this study, the addition of SOD and ascorbate resulted in a partial reversal of the inhibitory action of LY83583 on the exogenous NO-induced relaxation, but not those to EFS and nitroglycerin; blockade of inhibition with exogenous SOD provided confirmation that the actions of LY83583 were due to the generation of superoxide oxide. As exogenous SOD can not enter cells and LY83583 can generate superoxide anions extracellularly as well as intracellularly, we studied the effect of EUK-8, a synthetic cell-permeable mimetic of the mitochondrial Mn SOD. When administering EUK-8 before LY83583, we found a significant reversal of the inhibitory action of the superoxide generator LY83583 upon the EFS-, exogenous NO-, and nitroglycerine-induced relaxations. However, Colpaert and Lefebvre<sup>[15]</sup> found that EUK-8 did not prevent the inhibitory effect of LY83583 on the relaxation to exogenous NO in the pig gastric fundus; this may indicate a difference between this tissue and pig gastric fundus. Furthermore, in our study, the inhibitory effect of duroquinone on the relaxation to EFS, exogenous NO, and nitroglycerin was prevented by EUK-8, but not by Cu/Zn SOD and ascorbate, suggesting that duroquinone produces superoxide anions intracellularly, and inhibition takes place mainly in the intracellular compartment. Duroquinone requires conversion to the semiquinone radical via the action of flavoprotein enzymes<sup>[34]</sup>, and therefore, the majority of the superoxide anions will be produced inside the cell. The ability of EUK-8 to protect NO indicates that duroquinone also causes increased superoxide anion concentrations in the intracellular fluid. These findings together with the demonstration that Mn SOD immunoreactivity is demonstrated in nerve cells and smooth muscle cells in the stomach wall of rats, where it is colocalized with NADPH diaphorase, a marker for NO synthase<sup>[20]</sup> and in nerve cells in the myenteric plexus of the opossum esophagus<sup>[29]</sup>, support the theory that Mn SOD might be involved in protecting the nitrenergic neurotransmission process in the duodenum. Also, these findings obtained with LY83583 and duroquinone provide some support for the hypothesis that the nitrenergic transmitter is not free NO, but may be a NO-donating species in the mouse duodenum.

### **Influence of DETCA plus superoxide anion generators**

Superoxide anion concentration in tissues can be controlled by the actions of SOD (either Cu/Zn or manganese) that converts superoxide anions to peroxide, which is then converted via catalase to molecular oxygen. Recent experimental studies clearly demonstrate that, when pre-incubating the smooth muscle preparations with DETCA, the relaxation to the endogenous nitrenergic neurotransmitter, which is released from the nitrenergic nerve endings upon electrical stimulation, becomes strongly sensitive towards superoxide anion-generating compounds pyrogallol, LY83583, and xanthine/xanthine oxidase in the bovine retractor penis<sup>[12]</sup>, to duroquinone in the mouse anococcygeus<sup>[8]</sup>, and to LY83583 in the rat<sup>[16]</sup> and pig<sup>[30,35]</sup> gastric fundus. In the present experiments, the inhibition of endogenous Cu/Zn SOD with DETCA did not significantly alter the effects of pyrogallol and hydroquinone on the EFS-induced relaxations, supporting our hypothesis that endogenous NO may not be free NO in the mouse duodenum. Pretreatment with DETCA markedly potentiated the inhibitory effect of pyrogallol and hydroquinone on the relaxation by exogenous NO in the duodenum, and this inhibitory effect of DETCA plus pyrogallol or hydroquinone on the relaxation to exogenous NO can be only partially overcome with the re-addition of exogenous SOD and ascorbate, indicating that extracellular and intracellular superoxide anions are implicated in the observed inhibitory mechanism. Also, evidence in favor of this was demonstrated in studies that show LY83583 and duroquinone having little effect on responses to nitrenergic field stimulation in concentrations that greatly reduced the relaxations to exogenous NO. After the inhibition of Cu/Zn SOD, nitrenergic relaxations were powerfully reduced by LY83583 and duroquinone in the mouse duodenum. The re-addition of exogenous SOD and ascorbate only partially prevented the inhibitory effect of DETCA plus LY83583, but not that of DETCA plus duroquinone. EUK-8 prevented the inhibitory effect of DETCA plus duroquinone on the nitrenergic relaxations. As EUK-8, a synthetic cell-permeable mimetic of the mitochondrial Mn SOD, can enter cells, the latter observation illustrates that duroquinone can generate superoxide anions intracellularly, and Mn SOD might be involved in protecting the nitrenergic neurotransmission process in the mouse duodenum. To demonstrate whether the antioxidant defense systems, Cu/Zn SOD and ascorbate, specifically protect the nitrenergic NANC neurotransmitter from the extracellular and intracellular superoxide anion-generating system, we studied the effects of antioxidants, superoxide anion generators and NO scavengers, on the relaxation to isoproterenol. These drugs did not affect the relaxation induced by

isoproterenol, which acts by stimulating  $\beta$ -receptors and the activation of adenylate cyclase<sup>[36]</sup>, showing specific effects of the drugs and drug combinations tested on the relaxation induced by NO in the mouse duodenum

Our results in the mouse duodenum demonstrate that antioxidant defense systems, Cu/Zn SOD and ascorbate, can protect the nitrergic NANC neurotransmitter from the extracellular and intracellular superoxide anion-generating system. The important finding of the present study is that Mn SOD, in addition to Cu/Zn SOD, can protect NO from attack from superoxide anion generators intracellularly; the possibility that the endogenous neurotransmitter is not free NO in the mouse duodenum remains open. Further advanced studies are needed to clarify the biochemical identity of the nitrergic neurotransmitter in the mouse duodenum.

### Author contributions

Nuran OGULENER designed research; Olcay Ergurhan KIROĞLU performed research; M Ata SECILMIS, Olcay Ergurhan KIROĞLU contributed new reagents or analytic tools; M Ata SECILMIS, Olcay Ergurhan KIROĞLU analyzed data; Nuran OGULENER wrote the paper.

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