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European Journal of Pharmacology 462 (2003) 161–168
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Inhibition by ebselen on nitric oxide mediated relaxations in the rat anococcygeus muscle

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Received 31 December 2002; accepted 7 January 2003

Abstract

The effect of 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (ebselen) on nitric oxide (NO) mediated responses and NO generation from NO donors were studied in vitro. In precontracted rat isolated anococcygeus muscles, relaxations induced by NO donors, electrical field stimulation and 5-[1-(phenylmethyl)-1*H*-indazole-3-yl]-2-furanmethanol (YC-1) were significantly inhibited by ebselen (100 μM), whereas responses elicited by papaverine and theophylline were not affected; those by 8-bromo-cyclic-guanosine-monophosphate (8-Br-cGMP) were slightly enhanced. NO generation from NO gas aqueous solution or acidified nitrite was not affected, but that from *S*-nitroso-*N*-acetyl-penicillamine (SNAP) was attenuated by ebselen, and the attenuation was reserved by glutathione. Both glutathione and cupric sulphate altered the ultraviolet spectrum of ebselen. These findings suggest that ebselen at high concentrations nonselectively inhibited NO-mediated responses, possibly through inhibiting soluble guanylate cyclase. Ebselen does not appear to directly interact with NO, but it may inhibit NO release from nitrosothiols by a thiol- and/or copper-dependent mechanism.

Keywords: Ebselen; Nitric oxide (NO) donor; Nitrergic transmission; Nitric oxide (NO) synthase; Guanylate cyclase, soluble; Anococcygeus muscle, rat

1. Introduction

2-Phenyl-1, 2-benzisoselenazol-3(2*H*)-one (ebselen), a seleno-organic compound, is highly reactive towards thiols and thiol-containing proteins (Schewe, 1995). Accordingly, ebselen reacts with glutathione to form selenenylsulphides, which are in turn converted to ebselen selenol and diselenide (Cotgreave et al., 1992). In addition, it reacts with the sulfhydryl group(s) in proteins leading to the inactivation of a range of enzymes, such as NADPH cytochrome P_{450} reductase (Wendel et al., 1986), lipoxygenases (Schewe et al., 1994), glutathione S-transferase (Nikawa et al., 1994), protein kinase C (Cotgreave et al., 1989) as well as nitric oxide synthase (NOS) isoenzymes (Hattori et al., 1994; Hofmann and Schmidt, 1995; Zembowicz et al., 1993).

Soluble guanylate cyclase is a thiol-containing enzyme. As a target enzyme of nitric oxide (NO), soluble guanylate cyclase has been shown to be widely involved in NO mediated responses in different systems (Lewicki et al., 1982; Denninger and Marletta, 1999). Thus it is reasonable to speculate that ebselen may inhibit the activity of soluble guanylate cyclase, and attenuate the responses mediated by NO. Yet previous studies have revealed inconsistent results. Studies in rabbit and rat aorta, as well as bovine coronary arteries found that ebselen in a concentration ranged between 5 and 100 μM did not affect NO-mediated relaxations (Zembowicz et al., 1993; Kim et al., 2001; Mohazzab et al., 1999). In contrast, we found in a preliminary study that relatively high doses of ebselen ($\geq$ 30 μM) markedly reduced responses of sodium nitroprusside, NO aqueous solution and nitrosocysteine in the rat anococcygeus muscle, a smooth muscle preparation widely used in the study of NO pathway in peripheral tissues (Rand and Li, 1994).

Therefore, the aim of the present study was to characterize further the actions of ebselen on responses elicited by various NO donors and a novel activator of soluble guanylate cyclase 5-[1-(phenylmethyl)-1*H*-indazole-3-yl]-2-furanmethanol (YC-1) in the rat anococcygeus muscle. In addition, we studied the possible interactions between ebselen, NO and nitrosothiol by employing NO selective sensor and spectrophotometer techniques. The primary

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doi:10.1016/S0014-2999(03)01304-9

findings of this study have been communicated to a conference of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (Che et al., 2001).

2. Materials and methods

2.1. Organ bath experiment

Male Sprague–Dawley rats (250–350 g) were humanely killed by carbon dioxide asphyxiation followed by decapitation. The anococcygeus muscle and aorta were isolated and set up in 6 ml organ baths filled with physiological salt solution (PSS), maintained at 37 °C with continuously gassed with 5% CO_2/95% O_2. Anococcygeus muscle was equilibrated for 30 min under a resting tension of 1 g, whereas the aorta was equilibrated for 60 min under a tension of 2 g. The change of muscle tension was measured isometrically with a Grass FT03 force-displacement transducer and recorded on a MacLab data acquisition system.

After the equilibration period, the tone of anococcygeus muscle was raised with guanethidine (10 μM) and clonidine (0.1 μM), as previously described (Li and Rand, 1989). Relaxations to various agents including NO gas aqueous solution (0.3 μM), sodium nitroprusside (30 nM), *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 3 μM), Angeli's salt (3 μM), YC-1 (100 μM), cyclic guanosine monophosphate (cGMP) analogue 8-bromo-cGMP (8-Br-cGMP, 100 μM), phosphodiesterase inhibitors papaverine (10 μM) and theophylline (1 mM) as well as to electric field stimulation (15 V, 1 ms, 2 Hz for 10 s, every 2 min) were then obtained in the absence or presence of different concentrations of ebselen (3–100 μM) or its vehicle dimethyl sulfoxide (DMSO). In general, after the initial control response to different stimuli was obtained, ebselen or vehicle was added to the incubation media and remained for 10–15 min before the subsequent response to NO donors/stimulation was conducted. As sustained relaxations were produced by YC-1, 8-Br-cGMP, papaverine and theophylline, the effect of ebselen was tested in a parallel tissue and only a single dose (100 μM) was investigated; whereas ebselen was added cumulatively into the same organ bath in the study of its effect on those transient relaxations produced by NO donors and electric field stimulation.

The endothelium-denuded aortic rings were prepared as previously described (Ellis et al., 2000). The success of removing the endothelium was confirmed by lack of relaxations to acetylcholine (10 μM) in phenylephrine (0.3 μM) precontracted preparations. The effect of ebselen (0.3–30 μM) on the relaxations induced by sodium nitroprusside (0.1 μM) was then tested in phenylephrine (0.3 μM) precontracted preparations.

2.2. NO measurement

An amiNO-700 sensor electrode connected with an inNO measurement system (Innovative Instruments, USA) was employed to quantify the concentration of NO generated from stocks of NO gas aqueous solution (167, 333 and 667 nM), acidified sodium nitrite solution ($NaNO_2$; 50, 100 and 200 nM) and SNAP (50–500 nM), as previously described (Ellis et al., 2001). The amiNO-700 sensor was calibrated before each experiment by adding known concentrations of $NaNO_2$ standard solution (50–500 nM) into potassium iodide buffer (KI; 0.1 M) in the presence of sulphuric acid (H_2SO_4; 0.1 M). After intensive wash, the sensor was immersed and stabilized in PSS for 10 min before detecting NO release from various agents in the presence and absence of ebselen.

2.3. Ultraviolet (UV) spectrum of ebselen

The absorbance of ebselen (100 μM) was scanned from 220 to 350 nm with a UV spectrophotometer (UV-1601, SHIMADZU, Japan) in the absence and presence of SNAP (500 nM), glutathione (100 μM) and cupric sulphate ($CuSO_4$; 1–100 μM).

2.4. NOS activity assay

Rat cerebellum was used as the source for neuronal nitric oxide synthase (nNOS) (Lincoln et al., 1997). The tissues were homogenized in buffer containing 50 mM HEPES, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, 2 mM leupeptin and 1 μM pepstatin A. Tissue homogenate was centrifuged at 14,000 × *g* for 15 min at 4 °C and the supernatant was collected.

The activity of NO synthase was assayed as the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. The reaction mixture contained 100 μl of the supernatant, 0.15 μCi [^{3}H]L-arginine (Sigma), 0.75 mM $CaCl_2$ and 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a total volume of 117.6 μl. The mixture was incubated for 15 min at 37 °C with or without ebselen (1–1000 μM), and the reaction was terminated by the addition of 750 μl of ice-cold stop buffer (pH 5.5) containing HEPES 20 mM, ethylenediaminetetraacetic acid disodium salt (EDTA) 4 mM, and L-arginine 5 mM. The sample was then applied to a 2-ml column of the Na^+ form of Dowex AG50WX-8 (H^+ form from Sigma) followed by 3×1 ml of distilled water, and the [^{3}H]L-citrulline in the effluent was measured by liquid scintillation spectroscopy (TriCarb 2500TR Series, Packard, USA).

2.5. Drugs and reagents used

The following drugs were used: ebselen, guanethidine monosulfate, sodium nitroprusside, glutathione reduced form, 8-Br-cGMP sodium salt, papaverine hydrochloride, theophylline anhydrous, phenylephrine hydrochloride, KI, $CuSO_4$, L-arginine, N^G-nitro-L-arginine methyl ester (L-NAME), dithiothreitol, EDTA, EGTA, HEPES, NADPH, leupeptin, pepstatin A and phenylmethyl sulfonyl fluoride were from Sigma-Aldrich, NSW, Australia. Clonidine hydro-

chloride was from Boehringer Ingelheim, Germany. Angeli's salt, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and SNAP were from Sapphire Bioscience, NSW, Australia. $NaNO_2$ standard solution (0.01 M) was purchased from Innovative Instruments. All drugs were dissolved in Millipore water except for ebselen, which was dissolved in DMSO (Sigma); Angeli's salt in 0.01 M NaOH (Sigma) and SNAP in 10% ethanol (Sigma).

The composition of PSS was as follows (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, EDTA 0.067. However, EDTA was omitted while testing SNAP in both organ bath and NO sensor settings, as copper is essential for SNAP to release NO (see Results).

Saturated NO gas aqueous solution (2 mM) was prepared on the day of experiment as previously described (Rajanayagam et al., 1993). Briefly, vials of deionised water, deoxygenated by bubbling with argon gas for 1 h, were purged with NO gas for 5 min. Compressed argon and NO gas were obtained from Commonwealth Industrial Gases (Australia).

2.6. Statistical analysis of results

Changes in tension were measured in gram or relaxation (% precontraction). The concentration of NO as detected with the amiNO-700 sensor was measured in nM. Data are expressed as means ± standard error of means (S.E.M.); *n* indicates the number of experiments. The significance of differences between means was determined by Student's *t*-test or one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered statistically significant.

2.7. Ethics

The experiments were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to guidelines laid down by the National Health and Medical Research Council of Australia.

3. Results

3.1. Effect of ebselen in the anococcygeus muscle

The average tone developed by guanethidine (10 μM) plus clonidine (0.1 μM) was 9.5 ± 0.4 g ($n = 32$) in the rat anococcygeus muscle. Low concentrations of ebselen (< 30 μM) had no significant effect on the muscle tone (results not shown). However, higher concentrations (30 μM) of ebselen slightly reduced the muscle tone by 12.0 ± 0.1% ($n = 28$, $P < 0.05$, Student's paired *t*-test), as shown in Fig. 1A. Ebselen at 100 μM produced a transient relaxation, which reduced the tone to 32.9 ± 0.6% ($n = 30$), and followed by gradually recovering, which stabilized after about 10 min, resulting in a tone reduction by 17.4 ± 0.1%, compared with the level in its absence (Fig. 1A) ($n = 14$, $P < 0.05$, Student's paired *t*-test). The slight relaxations to ebselen were unaltered in the presence of NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) and an inhibitor of soluble guanylate cyclase 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 1 μM) (data not shown). The vehicle DMSO (0.1%, v/v) did not affect tissue tension (data not shown).

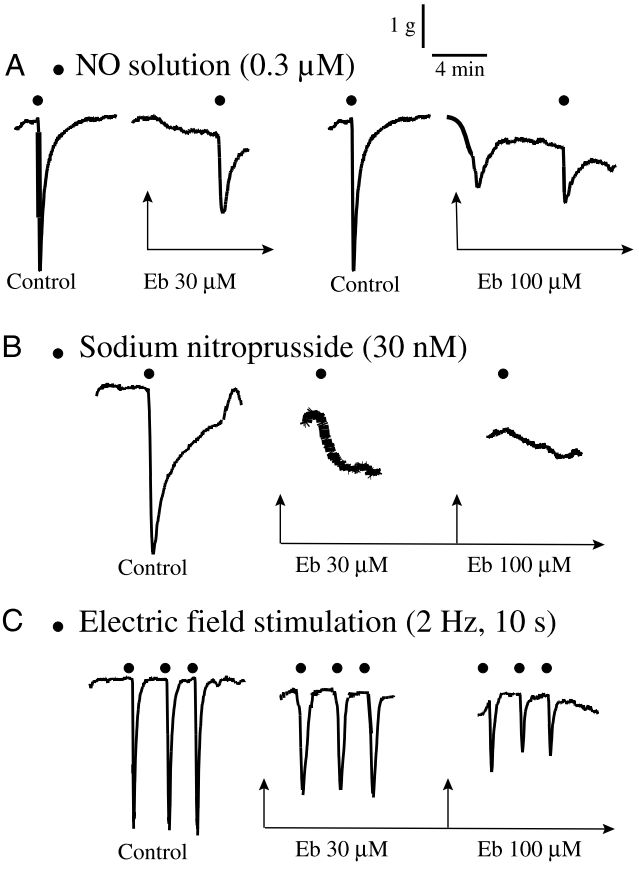

Fig. 1. Typical traces illustrating the relaxant actions induced by ebselen (30 and 100 μM) in the precontracted rat anococcygeus muscle (A), as well as the inhibitory effect of ebselen (30 and 100 μM) on the relaxations induced by NO gas aqueous solution (0.3 μM, A), sodium nitroprusside (30 nM, B) and electric field stimulation (2 Hz, 10 s, C), respectively.

The effects of ebselen (3–100 μM) on the relaxations produced by exogenous NO aqueous solution (0.3 μM), Angeli's salt (3 μM), sodium nitroprusside (30 nM) and SNAP (3 μM), respectively, as well as to electric field stimulation (2 Hz for 10 s) were summarized in Table 1. In general, ebselen at lower concentrations (3–30 μM) had no significant effect on these responses, except that it inhibited sodium nitroprusside-induced relaxation at 10 and 30 μM. However, at 100 μM, ebselen significantly inhibited relaxations to NO donors as well as electric field stimulation ($n = 6$, $P < 0.05$, Student's paired *t*-test); typical traces were illustrated in Fig. 1B and C. DMSO did not affect these relaxations (data not shown).

In separate experiments, slight reduction of the tissue tension by papaverine (1 μM) to a similar magnitude did not significantly affect the relaxations induced by sodium nitroprusside or electric field stimulation (results not shown).

Table 1
Effects of ebselen (3–100 μM) on the relaxations elicited by nitric oxide donors and electric field stimulation in the rat anococcygeus muscle

Stimuli	Control	Ebselen (μM)			
		3	10	30	100
NO solution (0.3 μM)	47 ± 5	44 ± 5	53 ± 8	43 ± 3	24 ± 1[a]
Angeli's salt (3 μM)	69 ± 8	64 ± 8	64 ± 8	63 ± 7	35 ± 5[a]
Sodium nitroprusside (30 nM)	46 ± 5	49 ± 4	30 ± 2[a]	21 ± 2[a]	6 ± 2[a]
SNAP (3 μM)	82 ± 4	81 ± 4	86 ± 6	85 ± 6	68 ± 7[a]
Electric field stimulation (2 Hz, 10 s)	46 ± 5	50 ± 4	46 ± 3	47 ± 2	25 ± 4[a]

Experiments were conducted in tissues precontracted with guanethidine (10 μM) and clonidine (0.1 μM). Results are expressed as percentage of precontraction and are shown as means ± standard error of the mean (S.E.M.), for six individual experiments per group.

[a] $P < 0.05$: significantly different from control value by paired Student's *t*-test.

In the anococcygeus muscle, YC-1 (100 μM) produced a sustained relaxation, which was significantly attenuated by ebselen (100 μM; $n = 4$, $P < 0.05$, Student's unpaired *t*-test), as shown in Fig. 2A. In contrast, ebselen (100 μM) significantly enhanced the relaxations to 8-Br-cGMP (100 μM, $n = 3$, $P < 0.05$, Student's unpaired *t*-test, Fig. 2B). In addition, responses of papaverine (10 μM) and theophylline (1 mM) remained unchanged in the presence of ebselen (Fig. 2C and D).

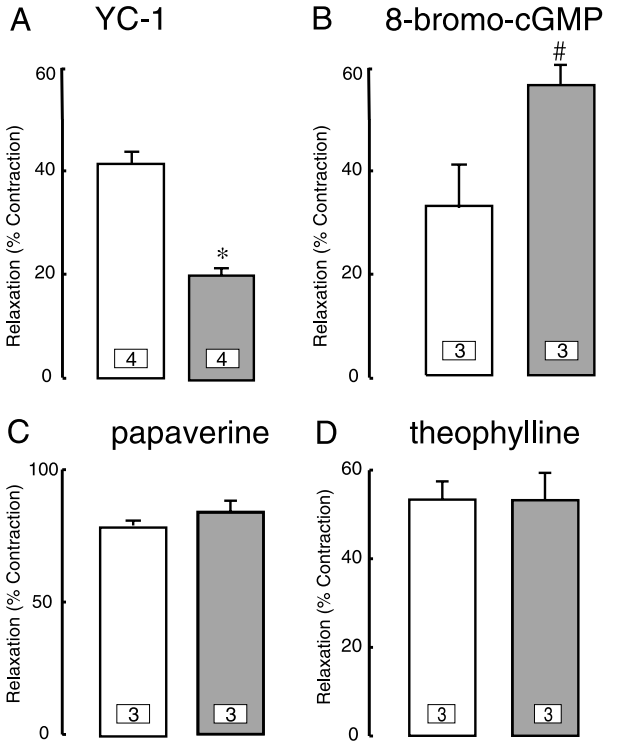

Fig. 2. Effect of ebselen on the relaxations by YC-1 (100 μM, A), 8-bromo-cGMP (100 μM, B), papaverine (10 μM, C) and theophylline (1 mM, D). Results are expressed as percentage of precontraction and shown as means ± standard error of the mean (S.E.M.). Open columns represent the controls, and filled columns are in the presence of ebselen (100 μM). *n* indicates the number of tissue and *, # indicate $P < 0.05$ when compared with the respective control (Student's unpaired *t*-test).

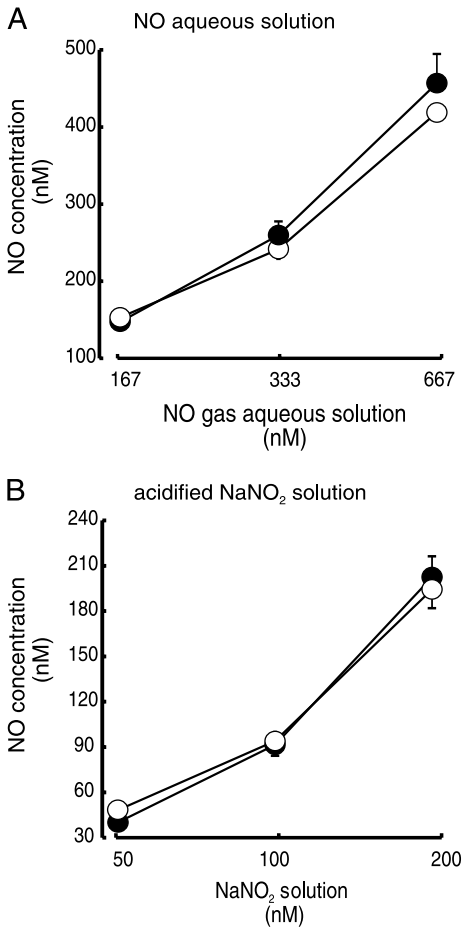

Fig. 3. Effect of ebselen on the NO released from NO gas aqueous solution (A) and sodium nitrite (B) as detected by a selective NO sensor. In both graphs, open circles (○) represent control and closed circles (●) indicate in the presence of ebselen. Symbols represent mean and T-bars indicate the S.E.M.; $n = 4$.

3.2. Effect of ebselen in the endothelium-denuded aorta

In the endothelium-denuded preparations, low concentration of ebselen (≤ 10 μM) had no significant effect on the relaxations to sodium nitroprusside (0.1 μM) (result not shown). However, in the presence of higher concentration of

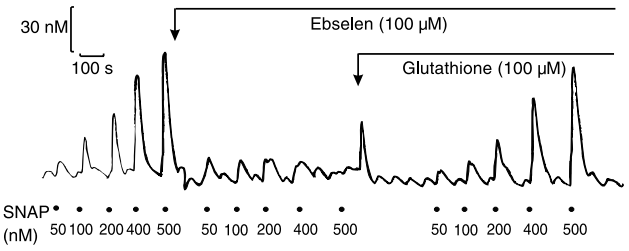

Fig. 4. Traces illustrating the NO release from SNAP (50–500 nM) in the absence or presence of ebselen (100 μM) and ebselen (100 μM) plus glutathione (100 μM), as detected by the amiNO-700 NO sensor.

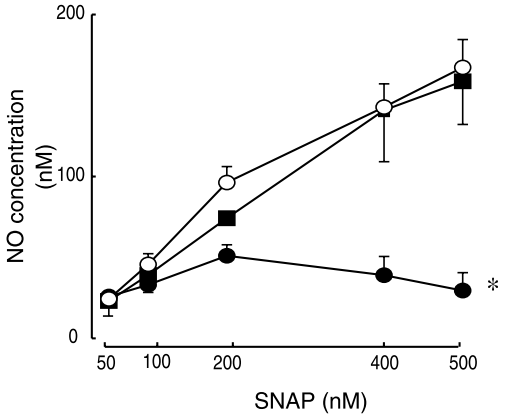

Fig. 5. Effect of ebselen (●) and ebselen plus glutathione (■) on the NO release from SNAP (○). Results are expressed as the absolute concentration of NO as detected by a selective NO sensor and are shown as means ± standard error of the mean (S.E.M.). Symbols represent means, and T-bars indicate the S.E.M.; $n=6$. * indicates $P<0.05$ when compared with SNAP curve without ebselen (one-way ANOVA).

ebselen (30 μM), the relaxation induced by sodium nitroprusside (0.1 μM) was reduced to 12.2 ± 2.5% ($n=3$) from 88.5 ± 2.5% ($n=3$), the level without ebselen ($P<0.05$, Student's paired *t*-test).

3.3. Effect of ebselen on NO release

Bolus addition of NO gas aqueous solution (167, 333 and 667 nM) to the incubation media (PSS) in the organ bath produced transient signals as detected by the amiNO-700 NO sensor; this signal was not affected by ebselen (100 μM), as shown in Fig. 3A. The NO release from acidified $NaNO_2$ solution (50, 100, 200 nM) was also unchanged in the presence of ebselen (100 μM) (Fig. 3B).

Bolus additions of SNAP (50, 100, 200, 400 and 500 nM) did not produce any detectable NO signal if EDTA (0.067 mM) was included in the organ bath. However, when EDTA was excluded from the system, SNAP triggered a concentration-dependent NO release. Ebselen (100 μM) significantly inhibited SNAP-induced NO release, and the effect was more pronounced towards higher concentrations (> 200 nM) of SNAP ($n=6$, $P<0.05$, one-way ANOVA). The inhibitory effect of ebselen (100 μM) on SNAP-induced NO release was reversed by glutathione (100 μM), as shown in Figs. 4 and 5. Neither DMSO nor glutathione (100 μM) had any effect on the NO release from SNAP (data not shown).

3.4. Effect of SNAP, Cu^{2+} and glutathione on the UV spectrum of ebselen

The UV spectrum of ebselen (238–350 nM) was not changed in the presence of SNAP (500 nM, Fig. 6A), but it was significantly altered by $CuSO_4$ (10 and 100 μM; Fig. 6B) and glutathione (100 μM, Fig. 6C). Both glutathione and $CuSO_4$ per se had very weak absorbance within the tested wavelength.

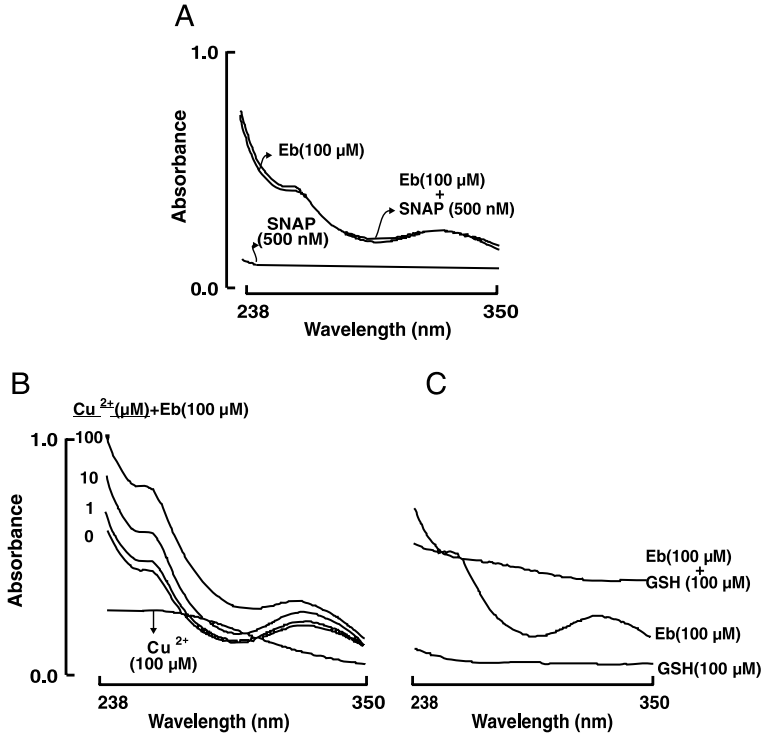

Fig. 6. Effects of SNAP (500 nM, A), $CuSO_4$ (Cu^{2+}, 1–100 μM, B) and glutathione (GSH, 100 μM, C) on the UV spectrum of ebselen (Eb, 100 μM) after incubation at 37 °C for 10 min.

3.5. Effect of ebselen on NOS activity

The formation of [^{3}H]L-citrulline in rat cerebellum homogenate was not affected by 1–100 μM ebselen (data not shown) but it was partially inhibited by 1 mM ebselen (29.5 ± 5.4%, $n=4$, $P<0.05$, Student's paired t-test). By contrast, L-NAME (100 μM) inhibited the NOS activity by 93.1 ± 0.4% ($n=4$). The vehicles (DMSO and water) had no effect on the NOS activity.

4. Discussion

The present study has intended to investigate the possible mechanism involved in the actions of ebselen on NO-mediated responses in the rat anococcygeus muscle, a nitrergically innervated tissue, with a primary aim to determine whether a cGMP-dependent mechanism is involved in actions of relative high concentrations of ebselen. The finding that low concentrations of ebselen (1–30 μM) had no significant effect on NO-mediated responses in the rat anococcygeus is consistent with the previous report in the rabbit aorta (Zembowicz et al., 1993). However, the finding that ebselen (10 and 30 μM) inhibited sodium nitroprusside-induced relaxations in the present study differs from that reported in the rat aorta (Kim et al., 2001). In addition, high concentration of ebselen (100 μM) inhibited responses to all NO donors tested, which is in contrast to the report by Mohazzab et al. (1999) who used the same concentration of ebselen but observed no significant effect on SNAP-induced responses in the bovine coronary artery. The reasons underlying these discrepancies are unclear but could be related to tissue differences.

The NO donors tested in the present study differ in their chemical structures and modes of actions. Accordingly, NO gas aqueous solution is generally regarded as the source of free radical NO ($NO^{\bullet}$); Angeli's salt is a nitroxyl anion (NO^{-}) donor which relaxes anococcygeus muscle by conversion to free radical NO (Li et al., 1999; Nelli et al., 2000); sodium nitroprusside induced NO generation is catalyzed by a membrane-bound enzyme (Feelisch, 1998), whereas SNAP "spontaneously" liberates NO species through a reaction catalyzed by copper ion and other reducing agents (Feelisch, 1998). Thus the inhibitions by ebselen on responses to these NO donors indicate that a common mechanism may be involved.

There are several possibilities that may explain the inhibition by ebselen on relaxations to NO donors. Firstly, the observed effect of ebselen may not be specific to NO-related mechanisms but rather related to a slight reduction in the tissue tone. However, this appears unlikely since the similar reduction of tone by another relaxant agent papaverine did not produce similar inhibition to NO-mediated relaxations, and ebselen also had no effect on relaxations to papaverine and theophylline. Secondly, ebselen may directly scavenge free radical NO, but this is not supported by the finding that ebselen did not affect NO generation/release by NO aqueous solution or $NaNO_2$ reduction. Previous study also suggested that ebselen is a poor scavenger of free radicals (Schewe, 1995). The third and most likely explanation is that ebselen may directly inhibit soluble guanylate cyclase, for it has been well established that relaxations of the rat anococcygeus muscle to NO donors are mediated by a soluble guanylate cyclase/cGMP-dependent mechanism (Rand, 1992). The findings that ebselen markedly attenuated relaxant responses induced by YC-1, a NO-independent activator of soluble guanylate cyclase (Hwang et al., 1999) without inhibiting those to papaverine and theophylline, further support this hypothesis. Furthermore, the results obtained in a separated tissue (rat aorta) further support this possibility as the responses to NO donor sodium nitroprusside were also inhibited by high concentrations of ebselen.

Mammalian soluble guanylate cyclase is a heterodimeric enzyme containing an α subunit and a heme-containing β subunit; NO binds with high affinity to the heme iron, resulting in a change in heme geometry that confers enzyme activation (Ignarro, 1992; Rodgers, 1999). Studies indicate that soluble guanylate cyclase contains fairly amount of sulfhydryl groups which may be critical to the NO activation, since 15 conserved cysteine residues were point-mutated using site-directed mutagenesis, in which mutation of Cys-78 and Cys-214 of β subunit caused a heme deficient and consequently a lose of responsiveness to NO (Friebe et al., 1997). Since ebselen possesses high affinity towards sulfhydryl groups, it is reasonable to predict that a redox reaction between ebselen and cysteine residue(s) in soluble guanylate cyclase may result in a suppression of soluble guanylate cyclase activity and/or a blunted responsiveness to NO. In the present study, ebselen inhibited both YC-1 and NO-mediated responses, indicating it may directly interfere with the cysteine residues of soluble guanylate cyclase in the rat anococcygeus muscle. However, the exact mechanism of ebselen on soluble guanylate cyclase requires further investigation. In this respect, studies on the activity of soluble guanylate cyclase in crude tissue homogenate or ideally the purified enzyme may provide more accurate information including possible interference of endogenous thiols with the enzyme (Friebe et al., 1997). In addition, the exact action of YC-1 on soluble guanylate cyclase also needs further investigation, as it has been suggested to act as an allosteric effector and slow down the rate of dissociation of NO from the heme iron (Friebe and Koesling, 1998).

The interaction between glutathione and ebselen on SNAP-induced NO release is interesting. It is known that NO release from nitrosothiols such as SNAP is greatly accelerated by copper ions, which normally exist in trace concentrations in the incubation buffer (De Man et al., 1996), thus the inhibition of NO release from SNAP by ebselen may involve a copper-chelating action. This is supported by spectra studies that confirmed ebselen directly interfered with copper ion. The reversing action of gluta-

thione on ebselen-induced inhibition of NO release is likely due to a direct interaction with ebselen, possibly by forming an adduct, resulting in the release of free copper ion from the ebselen–copper complex. Alternatively, a thiol-dependent mechanism which is unrelated to copper ions but is sensitive to ebselen may be involved in SNAP-induced NO release.

In the present study, responses to sodium nitroprusside were slightly inhibited by relatively low concentrations of ebselen, compared to other NO donors. The generation of NO from sodium nitroprusside is known to involve certain cellular factors such as thiols and microsomal enzymes (Bates et al., 1991; Kowaluk et al., 1992; Feelisch, 1998), thus there is a possibility that ebselen may also interfere with the NO generation system for sodium nitroprusside, apart from inhibition of soluble guanylate cyclase, resulting in an additional inhibition of sodium nitroprusside-mediated responses. Further studies are necessary to elucidate the exact mechanisms involved. On the other hand, the enhancement of response to 8-Br-cGMP by ebselen is unexpected. It is unclear whether ebselen may modify the intracellular uptake of 8-Br-cGMP, or modulate subsequent cellular events elicited by cGMP. Ebselen has been previously suggested to reduce intracellular calcium (Brüne et al., 1991; Dimmeler et al., 1991), thus it may have a synergic action on reducing intracellular calcium level with cGMP. If it is true, then the magnitude of inhibitory effect of ebselen on NO-mediated responses may be partly underestimated given that soluble guanylate cyclase is involved in relaxations to all NO donors studied (Li et al., 1999).

The mechanism of transient relaxation elicited by high concentrations of ebselen in the anococcygeus muscle is not clear. Similar findings have been previously reported in other tissues such as the rabbit aorta (Zembowicz et al., 1993), guinea-pig lung strips (Leurs et al., 1990) and the rabbit basilar artery (Takase et al., 1999). Since this relaxant effect of ebselen was not affected by NOS inhibitor L-NAME and the soluble guanylate cyclase inhibitor ODQ, it is unlikely the NO/soluble guanylate cyclase pathway is involved. Leurs et al. (1990) suggested that protein thiol alkylation might account for the attenuation of smooth muscle tone by ebselen, based on their observation that *N*-ethylmaleimide, a nonselective thiol alkylator, expressed a similar pattern as those of ebselen and related compounds. On the other hand, Takase et al. (1999) suggested that ebselen might activate protein kinase C to attenuate the smooth muscle tone in rabbit basilar arteries. Furthermore, ebselen might directly modulate calcium homeostasis by a blockade of 1,4,5-inositol-tris-phosphate (IP_3) and consequently reduced calcium release from intracellular store (Brüne et al., 1991; Dimmeler et al., 1991); however, this was disputed by Gogvadze et al. (2000), who demonstrated an induction of Ca^{2+} release by ebselen in mitochondria.

The inhibition of nitrergic relaxations to electric field stimulation in the anococcygeus muscle by ebselen may also involve the inhibition of neuronal NOS, apart from inhibiting soluble guanylate cyclase-dependent mechanism, as suggested by an earlier finding that ebselen can inhibit the activity of purified nNOS (IC_{50} value as low as 0.7 μM, Hofmann and Schmidt, 1995). However, our NOS assay data in rat cerebellum homogenate (containing high concentrations of nNOS) showed that ebselen only partly inhibited NOS activity at very high concentration (1 mM). It should be noted, however, there are different conditions in tissue preparations compared with that of pure enzyme system. We have used millimolar concentration of dithiothreitol in our assay to prevent the formation of disulphide bonds and sustain enzyme activity (Lincoln et al., 1997), but in Hofmann and Schmidt's study, no free thiols were included. It is likely that the ability of ebselen to inhibit NOS activity in the present study was underestimated since the inhibition of nNOS by ebselen can be overcome by glutathione (Hofmann and Schmidt, 1995). Nevertheless, the contribution of ebselen-induced NOS inhibition towards its action on nitrergic relaxations is likely to be small considering the high concentration of thiols found in nitrergic nerves (Rand and Li, 1995).

In conclusion, we have demonstrated that high concentrations of ebselen inhibited relaxations to NO donors, nitrergic nerve stimulation as well as soluble guanylate cyclase activator YC-1 in the rat anococcygeus muscle. Although the exact mechanism for this inhibition requires further investigation, these findings indicate that a direct inhibition of soluble guanylate cyclase by ebselen may be involved. Furthermore, ebselen does not seem to directly interact with NO itself but it can inhibit NO release from nitrosothiols through an unknown mechanism possibly involving thiol or copper ions.

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