Evidence that the Nitrergic Neurotransmitter and Endothelium-Derived Relaxing Factor Might be S-Nitrosothiols in the Mouse Corpus Cavernosum

Kansu BÜYÜKAFŞAR[a]*, Cemil GÖÇMEN[b], Ata SEÇILMIŞ[b], Yusuf KARATAŞ[b], Sinem GÖKTÜRK[b] and Nuri Ihsan KALYONCU[c]

[a]Department of Pharmacology, Medical Faculty, Mersin University, Campus Yenişehir 33160 Mersin, TR-Turkey, [b]Department of Pharmacology, Medical Faculty, Çukurova University, 01330 Adana, TR-Turkey and [c]Department of Pharmacology, Medical Faculty, Karadeniz Technical University, 61080 Trabzon, TR-Turkey

The effects of thimerosal, a sulfhydryl oxidizing agent on nitrergic, endothelium-dependent and -independent relaxations were investigated to examine the possibility that the nitrergic neurotransmitter and endothelium-derived relaxing factor (EDRF) could be S-nitrosothiol or free nitric oxide (NO) in the isolated mouse corpus cavernosum. Thimerosal ($5 \times 10^{-6} - 2 \times 10^{-5}$ M) inhibited or almost abolished electrical field stimulation-(EFS, 30 V, 0.5 ms, 15 sec, 1, 2, 4, 8, 16 Hz), acetylcholine-(ACh, $5 \times 10^{-8} - 1.25 \times 10^{-6}$ M), glyceryl trinitrate-(GTN, $3 \times 10^{-7} - 3 \times 10^{-6}$ M), and S-nitrosoglutathione-(GSNO, $5 \times 10^{-6} - 1.25 \times 10^{-4}$ M) induced relaxations. Thiomerosal inhibition seems to be specific to L-arginine NO pathways since it had no effect on acidified sodium nitrite-($10^{-4} - 5 \times 10^{-4}$ M), photoactivated sodium nitrite-(2×10^{-4} M), isoprenaline-(10^{-6} M), or papaverine-(10^{-4} M) elicited relaxations. Moreover, the inhibitory effect of thimerosal on the nitrergic, ACh- or GTN-induced relaxations were partly reversed by sulfhydryl-containing compounds, L-cysteine (10^{-3} M), dithiothreitol (10^{-3} M), or glutathione (10^{-3} M). However L-methionine (10^{-3} M), which contains a methyl group on the sulphur atom, failed to restore the thimerosal inhibition. Thimerosal did not change the contraction produced by 10^{-4} M N^G-nitro-L-arginine methyl ester. These findings indicate that the nitrergic neurotransmitter as well as EDRF may not be free NO but NO-transferring molecules, probably S-nitrosothiols, in the mouse corpus cavernosum.

Key words: nitric oxide, endothelium-derived relaxing factor, nitrergic neurotransmitter, thimerosal, corpus cavernosum, S-nitrosothiols

Although it has been well established that the L-arginine nitric oxide (NO) pathway mediates the phenomenon of endothelium-dependent relaxation of blood vessels and nitrergic relaxation in many tissues of different species (1, 2) there is controversy over the actual identity of the inhibitory neurotransmitter and of the endothelium-derived relaxing factor (EDRF). This has arisen from the findings that superoxide anion-generating drugs, namely LY 83583, hydroquinone and pyrogallol, were effective in inhibiting the relaxant effect of exogenous NO, but not that of non-adrenergic non-cholinergic (NANC) nerve stimulation in the mouse anococcygeus muscle, guinea-pig trachea, the rat gastric fundus, and bovine retractor penis muscle (BRP) (3–6). Some authors suggest that the nitrergic neurotransmitter is not S-nitrosothiol but simply NO in rat gastric fundus and ileocolonic junction (7–10). Some others, however, propose that it seems to be a NO-carrying, superoxide anion-resistant molecule probably an S-nitrosothiol in the BRP (11), mouse anococcygeus (5), mouse corpus cavernosum (12) and rat gastric fundus (13, 14). The precise nature of EDRF in blood vessels is still being debated as well. It has been suggested that NO release accounts for the biological activity of EDRF (15). Feelish *et al.* (16) also proposed that EDRF, as described by Furchgott and Zawadzki (17), is NO. However, Myers *et al.* (18) have demonstrated

*To whom correspondence should be addressed.

that EDRF more closely resembles S-nitrosocysteine than free NO.

Therefore, the objective of the present study was to investigate the possibility that the nitrergic neurotransmitter and EDRF could be S-nitrosothiols or authentic NO by use of a hydrophilic SH-group-oxidizing substance, thimerosal (19-21).

Materials and Methods

Male albino mice weighing 30-40 g were killed by cervical dislocation. Penises were removed and placed in a Petri dish containing Krebs solution (composition mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, $NaHPO_4$ 1.2, glucose 11). The glans penis and urethra were excised and adherent tissues were carefully removed keeping the tunica albuginea intact. Preparations of corpus cavernosum were mounted in an organ bath (15 ml) filled with Krebs solution bubbled with 95 % O_2 and 5 % CO_2 under 0.2 g tension. Tissues were allowed to equilibrate for 1h. During this time they were washed with Krebs solution every 15 min. Tissue responses were recorded by isotonic transducers (Ugo Basile, 7006, Varese, Italy) on a polygraph paper (Ugo Basile, 7070). Following the equilibration, the tissue was precontracted by 5×10^{-6} M L-phenylephrine. After achieving a steady state contraction, electrical field stimulation (EFS, 30 V, 0.5 ms) was delivered for 15 sec at frequencies of 1, 2, 4, 8 and 16 Hz at 2 min intervals via 2 platinum wire electrodes connected to a Grass S 88 stimulator (Grass Instruments, Quincy, MA, USA).

In another groups of experiments, following contraction by phenylephrine, acetylcholine (ACh, 5×10^{-8} M, 2.5×10^{-7} M, 1.25×10^{-6} M), glyceryl trinitrate (GTN, 3×10^{-7} M, 10^{-6} M, 3×10^{-6} M), S-nitrosoglutathione (GSNO, 5×10^{-6} M, 2.5×10^{-5} M, 1.25×10^{-4} M) or acidified $NaNO_2$ (10^{-4} M, 5×10^{-4} M) was added to the bathing medium. 0.05 ml of a solution of acidified $NaNO_2$ was added to the 15 ml incubation chamber without significantly changing the pH value of the bathing solution. After all drug applications, recording was stopped and the tissue was washed out with fresh Krebs solution to relax back to the baseline. Fifteen minutes latter, phenylephrine was added to the incubation chamber and recording was restarted when the tone of the tissue had nearly reached its previous contraction level. This procedure was repeated for every concentration of chemicals, thus yielding the first series of responses. After performing EFS- and chemical-induced relaxations in the first series, the tissue was rinsed with fresh Krebs solution and allowed to rest for 30 min. Thereafter, the second series of responses were recorded as a control in the same manner. In some groups of experiments, after the first series (control) were obtained, the tissue was incubated with thimerosal (5×10^{-6} M $- 2 \times 10^{-5}$ M) for 30 min, and the second series were performed. In the other series of experiments, in which the reversing effects of L-cysteine, glutathione, dithiothreitol and L-methionine on thimerosal inhibition were investigated, the first (control) and the second (thimerosal) series were obtained. Thereafter, L-cysteine (10^{-3} M), glutathione (10^{-3} M), dithiothreitol (10^{-3} M) or L-methionine (10^{-3} M) was co-incubated with thimerosal (10^{-5} M). Subsequently, the third series was performed. In some experiments, after the EFS-induced responses were obtained in the presence of 2×10^{-5} M thimerosal, contractile response to N^G-nitro-L-arginine (L-NAME, 10^{-4} M) was assessed. A matched control group series was also performed. In a separate group of experiments, NO was also generated in the incubation chamber by ultraviolet light (366 nm). For this purpose, the tissue was incubated with 2×10^{-4} M $NaNO_2$ throughout experiments and, irradiated for 15 sec. Ultraviolet lamp (6 W, Vilber Lourmat VL 6 LC, Cedex, France) was placed next to outer wall of glass incubation chamber. The distance between the chamber and lamp was about 2-3 cm. The concentration of thimerosal was chosen after preliminary experiments.

Drugs used. L-Phenylephrine, acetylcholine chloride, L-cysteine, DL-dithiothreitol, glutathione, thimerosal, L-NAME, isoprenaline, papaverine and GSNO (all from Sigma Chemicals Co., St. Louis, MO, USA) were dissolved in distilled water. GTN (Merck Co., Darmstadt, Germany) solution of 1 % was diluted with distilled water to desired concentration. NO was generated from photoactivation or acidification (pH = 2) of $NaNO_2$ (Merck Co.). Acidified $NaNO_2$ was stored at -4 °C between successive applications. It was prepared on the day of experiments. Application of acidified $NaNO_2$ did not significantly alter the pH value of the medium.

Statistical analysis Relaxation was expressed as a percentage reduction of the phenylephrine-induced contraction. Data are given as mean $\pm$ S.E.M and n represents the number of preparations. Statistical analysis of data was performed with the paired or unpaired Student's t test using Instat statistical package program

(R. Chambers, Tulane University, Med. Ctr., USA). P values of less than 0.05 were considered to be significant.

Results

Effects of EFS, ACh, GSNO, acidified $NaNO_2$, photoactivated $NaNO_2$, GTN, papaverine and isoprenaline. Electrical field stimulation (EFS, 30 V, 0.5 ms, 15 s, 1, 2, 4, 8, 16 Hz) produced frequency-dependent and reproducible relaxation (Fig. 1, Table 1). Sodium nitrite ($NaNO_2$) incubation did not alter EFS-induced relaxations. In the presence of $NaNO_2$, the corpus cavernosum relaxations in response to EFS were 4.9 ± 0.7, 14.6 ± 1.8, 28.7 ± 2.4, 40.7 ± 2.5, and 42.9 ± 3.1 % at 1, 2, 4, 8 and 16 Hz respectively (n = 22, not different from those obtained in the absence of $NaNO_2$). Glyceryl trinitrate (GTN, $3 \times 10^{-7} - 3 \times 10^{-6}$ M; Table 2), S-nitrosoglutathione (GSNO, $5 \times 10^{-6} - 1.25\ 10^{-4}$ M; Table 2), acidified $NaNO_2$ ($10^{-4} - 5 \times 10^{-4}$ M; Table 2), photoactivated $NaNO_2$ (2×10^{-4} M; Fig. 1, Table 2) isoprenaline (10^{-6} M; Table 2), papaverine (10^{-4} M; Table 2), and acetylcholine (ACh, $5 \times 10^{-8} - 1.25 \times 10^{-6}$ M; Fig. 2) relax-

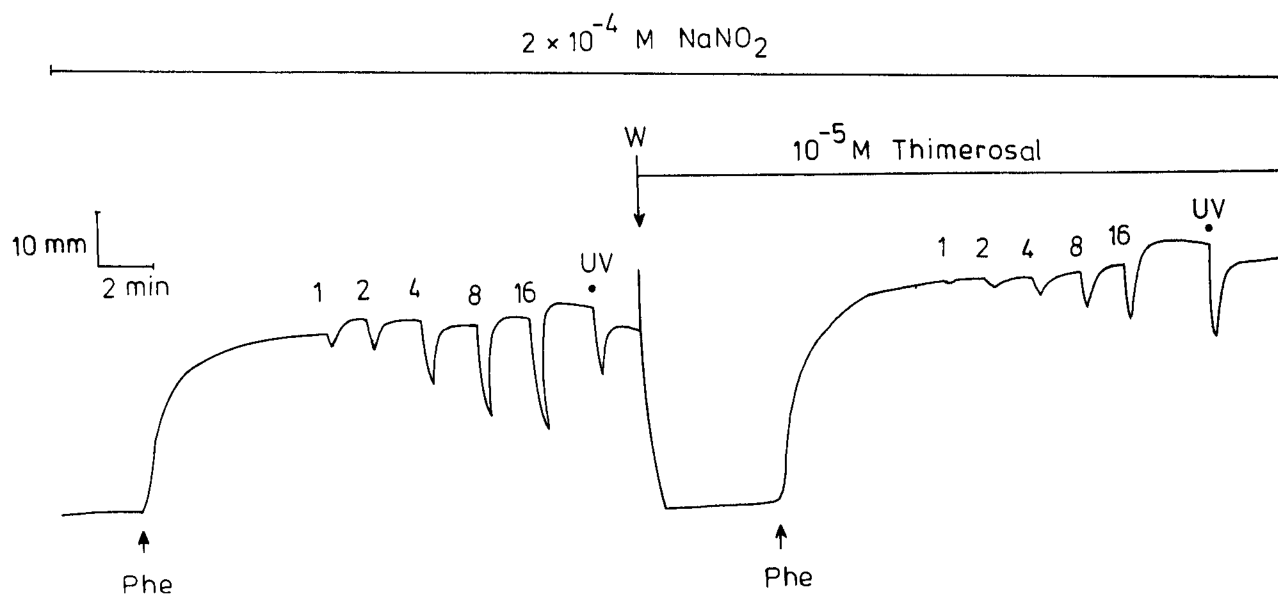

Fig 1 Representative tracings showing the effect of thimerosal (10^{-5} M) on electrical field stimulation (EFS, 30 V, 0.5 ms, 15 sec, 1, 2, 4, 8, 16 Hz)- and photoactivated sodium nitrite ($NaNO_2$). In order tq produce NO, the incubation chamber containing 2×10^{-4} M $NaNO_2$ is exposed to long wave ultraviolet light (366 nm) for 15 sec. Phe: Phenylephrine; W: Washing.

Table 1 Effects of some sulfur containing compounds (all 10^{-3} M) on the inhibition of electrical field stimulation by 10^{-5} M thimerosal. These agents were co-incubated with thimerosal in the third series (see text for details). Relaxation is expressed as percentage of phenylephrine-induced contraction.

	1	2	4	8	16 Hz
Control (first series, n = 33)	5.1 ± 0.9	14.8 ± 1.7	32.9 ± 2.7	44.7 ± 2.9	49.1 ± 2.9
Thimerosal (second series, n = 24)*	1.0 ± 0.5	1.8 ± 0.5	4.6 ± 1.0	9.4 ± 1.2	13.0 ± 2.0
Thimerosal (third series, n = 10)*	0 ± 0	0 ± 0	2.2 ± 0.4	3.1 ± 0.6	4.9 ± 1.1
Thimerosal + Cysteine (n = 9)**	2.5 ± 0.5	8.9 ± 1.3	23.1 ± 3.2	34.2 ± 3.8	35.7 ± 3.9
Thimerosal + Glutathione (n = 5)**	2.5 ± 0.7	8.2 ± 1.4	24.0 ± 3.5	34.4 ± 4.1	34.8 ± 4.2
Thimerosal + Dithiothreitol (n = 4)**	2.1 ± 0.4	6.5 ± 0.9	18.1 ± 2.1	27.7 ± 2.8	29.9 ± 3.2
Thimerosal + Methionine (n = 7)*	0 ± 0	0 ± 0	1.9 ± 0.4	2.8 ± 0.8	4.2 ± 1.5

Data are given as mean ± S.E.M. n indicates numbers of preparations of mouse corpus cavernosum. *$P < 0.001$, different from control; **$P < 0.001$, different from thimerosal (third series) group.

ed the corpus cavernosum in a concentration dependent manner. Tissue was exposed to the relaxant substances until a peak level of relaxation was achieved.

Effects of thimerosal on EFS, ACh, GTN, GSNO, papaverine, isoprenaline, acidified $NaNO_2$ ***and photoactivated*** $NaNO_2$***-evoked relaxation.*** 10^{-5} M thimerosal inhibited relaxation by EFS (1-16 Hz; Fig. 1), ACh ($5 \times 10^{-8} - 1.25 \times 10^{-6}$ M; Fig. 2), GTN ($3 \times 10^{-7} - 3 \times 10^{-6}$ M; Table 2) and GSNO ($5 \times 10^{-6} - 1.25 \times 10^{-4}$ M; Table 2). However, photoactivated $NaNO_2$-(2×10^{-4} M; Fig. 1, Table 2), acidified $NaNO_2$-($10^{-4} - 5 \times 10^{-4}$ M; Table

Table 2 Effect of thimerosal (10^{-5} M) on the relaxation induced by glyceryl trinitrate, S-nitrosoglutathione, acidified $NaNO_2$, photoactivated $NaNO_2$, isoprenaline and papaverine. Relaxation is expressed as percentage of phenylephrine-induced contraction.

		Control	Thimerosal	
Glyceryl trinitrate	3×10^{-7} M	18.7 ± 3.1	0.8 ± 0.5**	
	10^{-6} M	30.3 ± 5.2	2.1 ± 1.1**	(n = 6)
	3×10^{-6} M	41.1 ± 5.6	5.4 ± 2.6**	
S-nitrosoglutathione	5×10^{-6} M	21.5 ± 1.8	12.1 ± 1.8*	
	2.5×10^{-5} M	38.4 ± 4.9	19.8 ± 3.7*	(n = 5)
	1.25×10^{-4} M	64.6 ± 2.5	33.9 ± 3.3*	
Acidified $NaNO_2$	10^{-4} M	29.2 ± 6.3	32.0 ± 6.6	
	5×10^{-4} M	53.7 ± 7.1	51.1 ± 6.0	(n = 5)
Photoactivated $NaNO_2$	2×10^{-4} M	35.5 ± 2.4	39.4 ± 2.1	(n = 23)
Isoprenaline	10^{-6} M	76.0 ± 3.5	75.4 ± 4.5	(n = 8)
Papaverine	10^{-4} M	98.5 ± 4.2	105.7 ± 4.6	(n = 6)

Data are given as mean ± S.E.M. n indicates numbers of preparations of mouse corpus cavernosum. *$P < 0.01$, **$P < 0.001$, different from control.

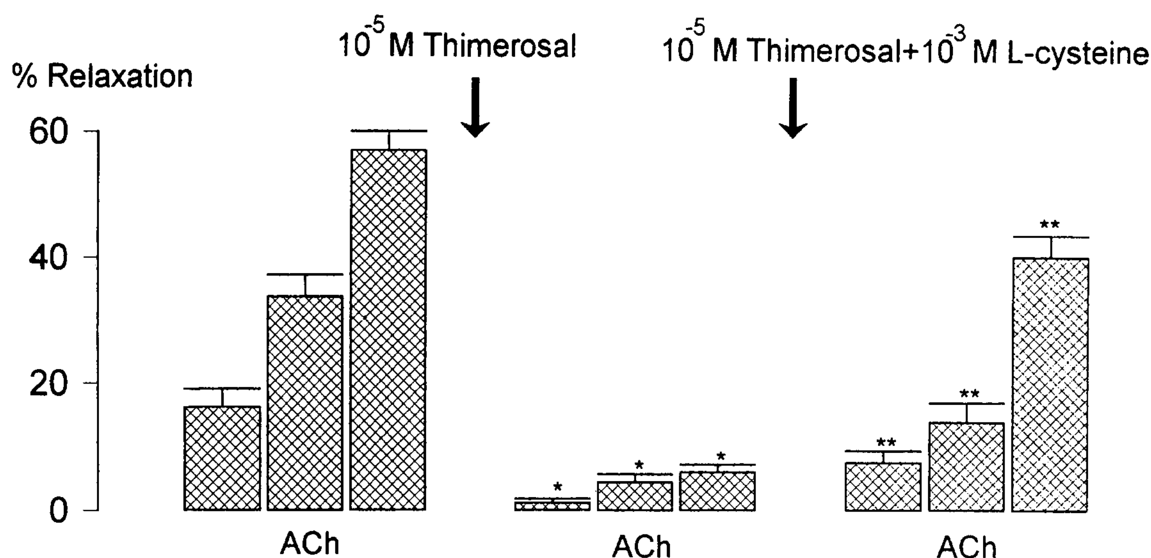

Fig 2 The effects of acetylcholine (ACh, 5×10^{-8} M, 2.5×10^{-7} M and 1.25×10^{-6} M, respectively) in control and in the presence of 10^{-5} M thimerosal and, the reversing effect of 10^{-3} M L-cysteine on thimerosal inhibition. ACh-induced relaxation was expressed as percentages of phenylephrine induced tone. * $P < 0.005$, significantly different from control; ** $P < 0.01$, significantly different from thimerosal group. Data represent mean ± S.E.M. of 7-21 observations.

2), isoprenaline-(10^{-6} M; Table 2), and papaverine-(10^{-4} M; Table 2) induced relaxation was unaffected by thimerosal (10^{-5} M).

Effects of L-cysteine, glutathione, dithiothreitol and L-methionine on the inhibitory effect of thimerosal. 10^{-3} M L-cysteine partially reversed the inhibitory effect of 10^{-5} M thimerosal on GTN- ($3 \times 10^{-7} - 3 \times 10^{-6}$ M) induced relaxation. It produced 41.8 % and 48.3 % restoration in the relaxation induced by 10^{-6} M and 3×10^{-6} M GTN, respectively. It also reversed in part the inhibition induced by thimerosal on ACh- ($5 \times 10^{-8} - 1.25 \times 10^{-6}$ M) elicited relaxation (Fig. 2). The reversing effects of glutathione, cysteine and dithiothreitol on thimerosal inhibition are summarized in Table 1. On the other hand, 10^{-3} M L-methionine failed to reverse the inhibitory effect of 10^{-5} M thimerosal on EFS-(1-16 Hz) induced relaxation (Table 1).

Effect of thimerosal on the contraction by L-NAME. There were no significant differences between contraction produced by 10^{-4} M L-NAME in the presence or absence of 2×10^{-5} M thimerosal (Fig. 3). Percent contractions obtained after phenylephrine-induced tone in the absence or presence of thimerosal were 33.1 ± 2.6 % (n = 11) and 31.3 ± 2.3 % (n = 8), respectively.

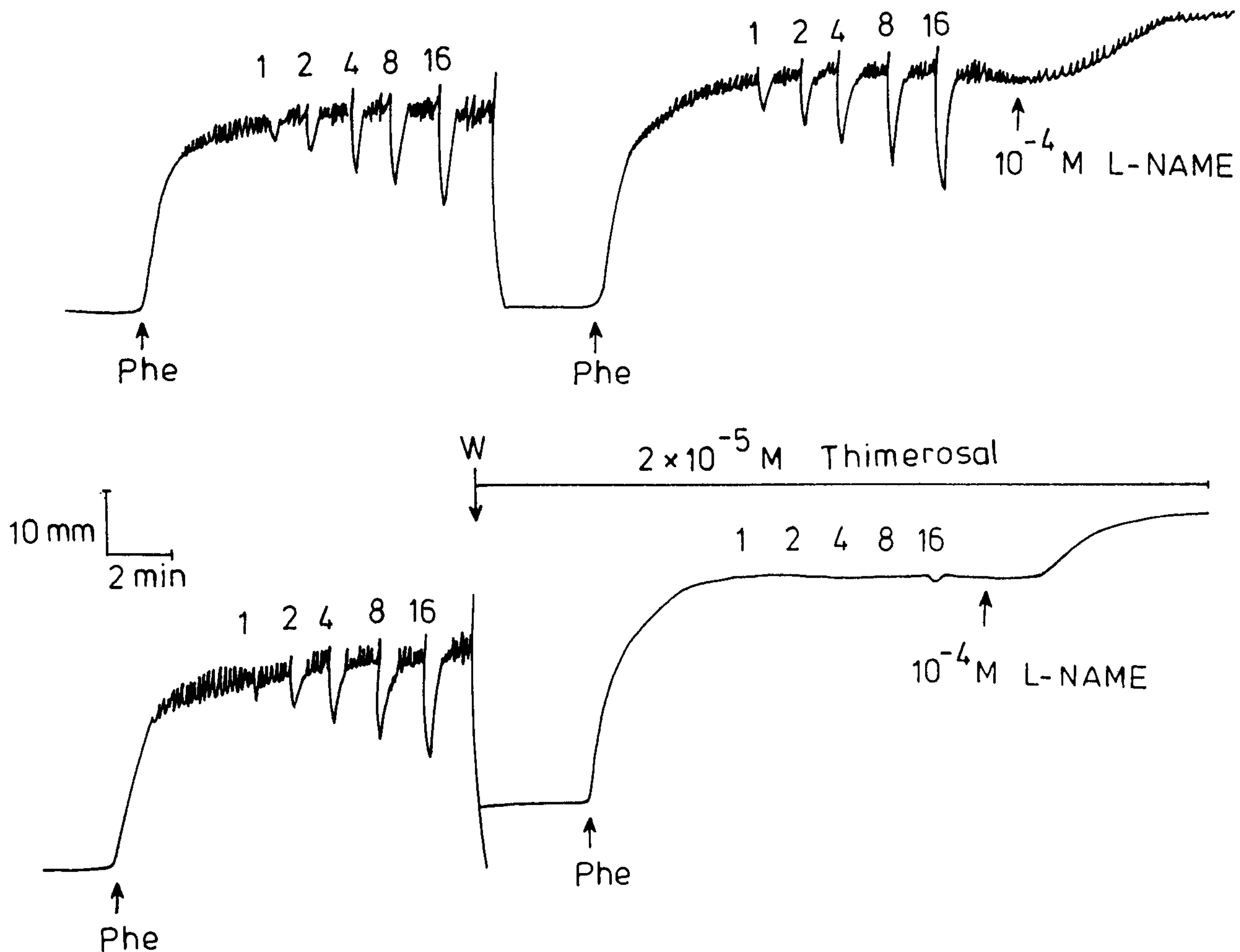

Fig 3 Representative tracings showing N^G-nitro-L-arginine (L-NAME)-induced contractions in control (upper panel) and in the presence of 2×10^{-5} M thimerosal (lower panel). Note the disapperence of electrical field stimulation-induced relaxation but the persistence of L-NAME-elicited contracture over the phenylephrine-induced tone. Phe: Phenylephrine, W: Washing.

Discussion

In the present study we have examined the possibility, which has recently been the subject of much debate, that both EDRF and the nitrergic neurotransmitter could be S-nitrosothiols or free NO in the isolated mouse corpus cavernosum by employing thimerosal, a sulfhydryl-oxidizing agent (19-21).

Several decades ago it was demonstrated that cellular thiols were required to liberate NO from organic nitrates (22). Therefore, we chose GTN as a reference substance for the understanding of the involvement of sulfhydryl groups in thimerosal action. This thiol oxidizing agent dramatically blocked GTN-induced relaxation. The inhibition was partly reversed by L-cysteine, pointing out a sulfhydryl contribution. Liu *et al.* (11) tried sulfhydryl inactivating agents, diamide and N-ethylmaleimide. Although those compounds inhibited NANC relaxation, this effect seems to be non-specific, as authentic NO and isoprenaline induced relaxation was also inhibited. In preliminary experiments, we also employed N-ethylmaleimide. However, since it almost abolished phenylephrine-induced contraction it was avoided to use. In the present study, however, the action of thimerosal, at least at these concentrations and for this exposure duration, on L-arginine NO pathway seems to be specific as neither isoprenaline- nor papaverine-elicited relaxation could be inhibited in the presence of this substance. Furthermore, thimerosal had no effect on the relaxation induced by acidified $NaNO_2$ and photoactivated $NaNO_2$ indicating that free NO apparently does not need reduced thiols to exert its relaxing effect. The fact that cysteine, glutathione and dithiothreitol, all of which contain reduced thiol groups, reversed in part the inhibitory effect of thimerosal on EFS- and ACh- elicited relaxation confirms the specificity of thimerosal on thiols. However, methionine, which has an unreduced moiety, failed to restore the inhibitory effect of thimerosal, indicating that the only the reduced form of thiols can reverse the inhibition. Interestingly enough, thimerosal also inhibited GSNO-induced relaxation. This may show that S-nitrosothiols require reduced thiols at the smooth muscle membrane to transport NO inside to activate soluble guanylate cyclase (23). Likewise, it has been demonstrated that the denitrosation of S-nitrosothiols is not spontaneous, and those substances might be catalyzed at the external vascular membrane of rat aorta (24).

It has been demonstrated that ultraviolet light (366 nm) can cause the release of NO from nitrite ions (NO^-_2) (25). In the present study, since the incubation chamber contained $NaNO_2$, the generation of NO could take place very near and/or inside the tissue. Following this kind of NO application, the failure of thimerosal to inhibit free NO-mediated relaxation is interesting, although it markedly inhibited EFS- and ACh-induced relaxation, both of which stimulate nitric oxide synthase (NOS). In this case, the possibility that thimerosal might inhibit NOS can be put forward as an explanation. But after the thimerosal inhibition had been established on EFS-elicited relaxation, L-NAME produced further contraction over the phenylephrine-induced tone, which was not different from that in the absence of thimerosal. This finding might exclude the possibility that thimerosal inhibits NOS.

It has been reported that NO synthesis apparently does not require thiols (26). Therefore, it seems unlikely that S-nitrosothiols are produced enzymatically by NOS. Instead, it is possible that they are formed non-enzymatically from NO and cellular thiols (27), which comprise the majority of the mammalian fraction of sulphur that exists as free sulfhydryl (28) and which react readily with NO under physiological conditions (29). However, it is generally believed that because of its highly diffusive nature, NO itself cannot be stored in nerve endings or endothelium but is synthesized on demand and is released to the smooth muscle cell by passive diffusion rather than by vesicular stimulus-secretion coupling (30) triggered by Ca^{2+} ions (31). Indeed, it is hard to believe that authentic NO would be stored, but recently a growing body of evidence has emerged that NO, packaged and stabilized in the forms of S-nitrosothiols, could be released as an inhibitory neurotransmitter or an EDRF from nitrergic nerves and endothelium, respectively (11, 12, 14, 18, 32).

Although it has been reported that thimerosal has some side effects (19, 33), it seems to be a discriminative agent between relaxations elicited by free NO, and the nitrergic neurotransmitter as well as EDRF because it has no effect on free NO-mediated relaxation but a dramatic inhibition on the nitrergic nerve stimulation- and ACh-induced relaxant responses.

In conclusion, it appears that the controversy surrounding the true nature of the nitrergic neurotransmitter and EDRF will go on until an accurate method is found to clearly distinguish between free NO, S-nitrosothiols, EDRF and nitrergic neurotransmitter. Nevertheless, our

findings might indicate that both EDRF and the nitrergic neurotransmitter are not simply NO, but an NO-carrying molecule, probably an S-nitrosothiol in the isolated mouse corpus cavernosum.

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