ELSEVIER

European Journal of Pharmacology 318 (1996) 47–53

ejp

L- and D-*S*-nitroso-β,β-dimethylcysteine differentially increase cGMP in cultured vascular smooth muscle cells

Mark D. Travis [a,c], Lynn L. Stoll [b,c], James N. Bates [b,c], Stephen J. Lewis [a,c,*]

[a] *Department of Pharmacology, 2-272 Bowen Science Building, University of Iowa, Iowa City, IA 52242-1109, USA*
[b] *Department of Anesthesia, University of Iowa, Iowa City, IA 52242, USA*
[c] *Cardiovascular Center, University of Iowa, Iowa City, IA 52242, USA*

Received 22 July 1996; accepted 6 September 1996

Abstract

We examined the effects of the L- and D-isomers of *S*-nitroso-β,β-dimethylcysteine (L- and D-*S*-nitrosopenicillamine, 10^{-7}–10^{-5} M) on the guanosine 3′,5′-cyclic monophosphate (cGMP) content of cultured porcine aortic smooth muscle cells and the decomposition of these stereoisomers to nitric oxide (NO). L-*S*-nitrosopenicillamine was a more potent generator of cGMP than D-*S*-nitrosopenicillamine although both stereoisomers equally decomposed to NO. The 10^{-7} M concentration of L- or D-*S*-nitrosopenicillamine did not generate detectable amounts of NO although 10^{-7} M L-*S*-nitrosopenicillamine but not D-*S*-nitrosopenicillamine generated significant amounts of cGMP. This study shows that the stereoisomeric configuration of *S*-nitrosopenicillamine is an important factor in its biological potency. The data suggest that the extracellular or intracellular generation of NO is not the only mechanism by which this *S*-nitrosothiol generates cGMP in vascular smooth muscle.

Keywords: *S*-nitrosothiol (stereoisomer); Nitric oxide (NO); cGMP; Vascular smooth muscle

1. Introduction

The vasorelaxant properties of *S*-nitrosothiols are generally ascribed to their decomposition to nitric oxide (NO), which subsequently generates guanosine 3′,5′-cyclic monophosphate (cGMP) within vascular smooth muscle cells via the activation of soluble guanylate cyclase (Moncada et al., 1991; Ignarro, 1990; Furchgott and Vanhoutte, 1989). However, there is now considerable evidence that the spontaneous decomposition of *S*-nitrosothiols to NO cannot fully account for the biological actions of these compounds (Mathews and Kerr, 1993; Rosenblum, 1992; Kowaluk and Fung, 1990; Myers et al., 1990). Kowaluk and Fung (1990) have also provided evidence that *S*-nitrosothiols do not necessarily have to enter vascular smooth muscle cells to produce their vasorelaxant effects. This suggests that *S*-nitrosothiols may undergo a denitrosation step upon contact with vascular smooth muscle membranes and that the liberated NO then enters these cells. The denitrosation of these compounds may arise simply by their chemical interaction with reducing entities such as thiols or heme-proteins on the cell surface (Stamler et al., 1992). However, it would seem possible that the site at which the denitrosation step takes place actually represents a specific *S*-nitrosothiol binding site.

We have provided in vivo evidence that the stereoisomeric configuration of *S*-nitroso-β,β-dimethylcysteine (*S*-nitrosopenicillamine) plays an important role in determining the hemodynamic potency of this *S*-nitrosothiol in conscious rats (Travis et al., 1994). More specifically, we found that upon i.v. injection into conscious rats, the L-isomer of *S*-nitrosopenicillamine (L-*S*-nitrosopenicillamine) was a more potent hypotensive and vasodilator agent than the D-isomer (D-*S*-nitrosopenicillamine). Therefore, it would seem possible that the denitrosation of *S*-nitrosothiols takes place at a stereoselective recognition site on vascular smooth muscle membranes. The enhanced degradation of L-*S*-nitrosopenicillamine to NO on vascular membranes would result in greater activation of soluble guanylate cyclase and therefore the production of cGMP in the muscle. Free diffusion of L- and D-*S*-nitrosopenicilla-

* Corresponding author at address a. Tel.: (1-319) 335-7943; Fax: (1-319) 335-7903.

mine into vascular smooth muscle cells would be unlikely since closely related *S*-nitrosothiols such as *S*-nitrosocysteine are highly polar (Kowaluk and Fung, 1990). In order to examine the possibility that the denitrosation of *S*-nitrosopenicillamine to NO and the subsequent production of cGMP involves the interaction of this *S*-nitrosothiol with stereoselective recognition sites, we examined (i) the relative abilities of L- and D-*S*-nitrosopenicillamine (10^{-7}–10^{-5} M) to generate cGMP in cultured porcine aortic smooth muscle cells bathed in serum-free medium, (ii) the relative decomposition of L- and D-*S*-nitrosopenicillamine (10^{-7}–10^{-5} M) to NO upon their direct addition to cultured porcine aortic smooth muscle cells or to serum-free medium bathing cultured porcine aortic smooth muscle cells. Serum-free medium does not contain thiols which can be nitrosated by L- and D-*S*-nitrosopenicillamine (Dulbecco and Freeman, 1959). This is important since D-*S*-nitrosopenicillamine would be able to produce an L-*S*-nitrosothiol by the nitrosylation of sulphur-containing L-amino acids which could potentially confound the interpretation of the data.

2. Materials and methods

2.1. Measurement of cGMP

Guanosine 3′,5′-cyclic monophosphate (cGMP) levels were measured by radioimmunoassay, using Amersham ^{125}I-labeled cGMP kits (Stoll and Spector, 1993). Cultured porcine aortic smooth muscle cells (10–12 passages) were grown at 37°C in a humidified atmosphere containing 5% CO_2 to confluency on 12-well plates (Corning Glass Works, Corning, NY, USA) as described previously (Stoll and Spector, 1993). Cells underwent one change of complete Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO, USA), MEM vitamin solution (Gibco), 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 25 μM gentamicin (Shering, Kenilworth, NJ, USA) and 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes; Sigma). Prior to incubation of the cells with the *S*-nitrosothiol, the growth medium was removed and the cells were washed once with serum-free DMEM (serum-free medium). They were then reincubated at 37°C in serum-free medium and allowed to equilibrate for 30 min to minimize interference from the effects of handling. The *S*-nitrosothiols were prepared immediately prior to use (see below).

Porcine aortic smooth muscle cells were placed on a slide warmer (Model 77, Fisher Scientific, Pittsburgh, PA, USA) at 37°C. The serum-free medium was removed and 1 ml of incubation media was applied to the porcine aortic smooth muscle cells. The incubation medium consisted of serum-free medium, 100 μM 3-isobutyl-1-methyl xanthine (IBMX) to prevent the enzymatic degradation of cGMP, and the test compounds. The test compounds were L- and D-*S*-nitrosopenicillamine (10^{-7}–10^{-5} M) and sodium nitroprusside (10^{-7}–10^{-5} M). After 30 s or 1 min, the media was removed and 500 μl of ice-cold 5% trichloroacetic acid was added to stop the reaction. Cells were placed in the refrigerator at 4°C for 1 h before obtaining samples. The supernatant containing cGMP was gently mixed with a pasteur pipet, and without disturbing the coagulated protein in the bottom of the well, a 400 μl aliquot was transferred to a borosilicate test tube. Samples were extracted four times with 800 μl of diethyl ether, evaporated to dryness, and dissolved in 0.5 M sodium acetate buffer (pH 5.8). Samples from the extraction procedure were acetylated immediately before beginning the assay to increase sensitivity of the radioimmunoassay. Reagent blanks were carried throughout the entire extraction and assay procedure. The remainder of the assay was performed according to the procedure accompanying the kit. A complete standard cGMP curve was run with each assay and cGMP levels (fmol/well) were determined utilizing this curve.

2.2. Measurement of NO

NO was measured using an NO analyzer (Dasibi, Model 2108, Glendale, CA, USA) as described previously (Myers et al., 1990). The analyzer was initially calibrated using known concentrations of NO. Cultured porcine aortic smooth muscle cells were grown to confluency on 12-well plates as described above. 24 h prior to experimentation, the DMEM medium was removed, cells were washed one time with serum-free medium and allowed to equilibrate overnight. Porcine aortic smooth muscle cells were then placed on a slide warmer at 37°C and the serum-free medium was removed and replaced by a fresh 1 ml volume of serum-free medium. L- or D-*S*-nitrosopenicillamine (100–10 000 pmol) was added to the serum-free medium bathing the porcine aortic smooth muscle cells (10^{-7}–10^{-5} M, initial concentration of L- or D-*S*-nitrosopenicillamine). In the other experiments, the medium was removed from each well and the cells in the sealed well were purged of oxygen by a stream of nitrogen gas. L- and D-SNPEN (5, 10, 50, 100, 250 nmol) were injected into the sealed chamber onto the porcine aortic smooth muscle cell membranes (total volume 100 μl). The released NO was carried in a stream of nitrogen gas under vacuum to the chemiluminescence NO analyzer.

2.3. Drugs

The isomers of β,β-dimethylcysteine (penicillamine) were obtained from Sigma. Stock solutions of the stereoisomers of *S*-nitrosopenicillamine were prepared fresh just prior to use by reacting 1 ml solutions of 0.2 M sodium nitrite (containing 100 μl of 1 M HCl) and 0.2 M L- or D-penicillamine which resulted in a stable (pH ≈ 3)

Table 1
A summary of the effects of L- or D-SNPEN on the cGMP contents of cultured porcine aortic smooth muscle cells

Experiment	L-SNPEN (M)			D-SNPEN (M)		
	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
1	1176 ± 35 [a]	4049 ± 303 [a]	4841 ± 256	158 ± 6	1410 ± 313	4049 ± 320
2	n.d.	1133 ± 167 [a]	n.d.	n.d.	339 ± 101	n.d.
3	6162 ± 160 [a]	6108 ± 407 [a]	4270 ± 345	4926 ± 78	4988 ± 232	4630 ± 323

L-SNPEN = L-*S*-nitrosopenicillamine; D-SNPEN = D-*S*-nitrosopenicillamine; n.d. = not determined. The values represent the mean ± S.E.M. of the arithmetic increases in cGMP (fmol/well) produced over control values by the 30 s exposure of L- or D-SNPEN to cells in four separate experiments. [a] $P < 0.05$, L-SNPEN vs. D-SNPEN.

0.1 M stock solution of the respective isomer of *S*-nitrosopenicillamine. Sodium nitroprusside was obtained from Abbott Laboratories (North Chicago, IL, USA).

2.4. Statistical analysis

The data are represented as the mean ± S.E.M. The S.E.M. terms were determined by the formula $(EMS/n)^{1/2}$ where EMS is the error mean square term from the analysis of variance (ANOVA) and n is the number of samples per group. The data were analyzed by repeated-measures ANOVA (Winer, 1971) followed by Student's modified *t*-test with the Bonferroni correction for multiple comparisons between means (Wallenstein et al., 1980) using the error mean square terms from the ANOVA (Winer, 1971).

Table 2
A summary of the effects of SNP on the cGMP contents of cultured porcine aortic smooth muscle cells

Experiment	*n*	Control	SNP (M)		
			10^{-7}	10^{-6}	10^{-5}
1	4	223 ± 9	1168 ± 94	1670 ± 153	6053 ± 326
2	4	408 ± 33	351 ± 32	245 ± 40	355 ± 30
3	3	23 ± 2	350 ± 12	836 ± 103	9078 ± 501
4	3	12 ± 1	28 ± 3	418 ± 52	13618 ± 751
	13	188 ± 46	465 ± 119 [a]	750 ± 163 [a]	6740 ± 1451 [a]

SNP = sodium nitroprusside. The values represent the mean ± S.E.M. of cGMP (fmol/well) produced by a 60 s exposure of cells to either serum-free medium alone (control) or SNP (in serum-free medium) in four separate experiments (n = number of replicates). [a] $P < 0.05$, SNP vs. saline.

3. Results

3.1. Effects of L- and D-S-nitrosopenicillamine on cGMP levels

In our preliminary experiments, we examined the cGMP levels in porcine aortic smooth muscle cells that had been exposed to L- or D-*S*-nitrosopenicillamine for 30 s. Control values were derived from the cells treated with serum-free medium alone. The results of three such experiments are shown in Table 1. The control values for experiments 1–3 were 123 ± 28, 3 ± 47 and 94 ± 28 fmol/well, respectively ($n = 4$ replicates). As can be seen, the 10^{-7} and 10^{-6} M concentrations of L-*S*-nitrosopenicillamine produced significantly greater increases in cGMP levels than these concentrations of D-*S*-nitrosopenicillamine. However, it was evident that the actual amounts of cGMP produced by the isomers varied markedly. This variability may have arisen because the cells were not exposed to L- or D-*S*-nitrosopenicillamine for a sufficient amount of time. In order to determine if this variability could be overcome by increasing the time of exposure to L- and D-*S*-nitrosopenicillamine, we examined the cGMP levels in porcine aortic smooth muscle cells that had been treated for 1 min with L- or D-*S*-nitrosopenicillamine (10^{-7}–10^{-5} M) or sodium nitroprusside (10^{-7}–10^{-5} M). Again, control values were derived from the cells treated with serum-free medium alone. The results of four such experiments are summarized in Tables 2 and 3. The control and sodium nitroprusside values for experiments 1–4 in Table 2 and the *S*-nitrosopenicillamine values in Table 3 were derived from

Table 3
A summary of the effects of L- or D-SNPEN on the cGMP contents of cultured porcine aortic smooth muscle cells

Experiment	*n*	L-SNPEN (M)			D-SNPEN (M)		
		10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
1	4	3407 ± 253	3319 ± 206	7233 ± 183	586 ± 37	2033 ± 134	5105 ± 185
2	4	3345 ± 416	8099 ± 496	10183 ± 423	333 ± 20	2319 ± 359	8098 ± 608
3	4	4246 ± 594	8120 ± 671	6050 ± 247	141 ± 23	4320 ± 998	5854 ± 602
4	3	4878 ± 523	11322 ± 743	7490 ± 1170	82 ± 4	5150 ± 971	8257 ± 1043
	14	3767 ± 235 [a]	7306 ± 822 [a]	7912 ± 485	315 ± 55	3083 ± 405	6721 ± 491

L-SNPEN = L-*S*-nitrosopenicillamine; D-SNPEN = D-*S*-nitrosopenicillamine. The values represent the mean ± S.E.M. of cGMP (fmol/well) produced by the 60 s exposure of L- or D-SNPEN to cells in four separate experiments (n = number of replicates). [a] $P < 0.05$, L-SNPEN vs. D-SNPEN.

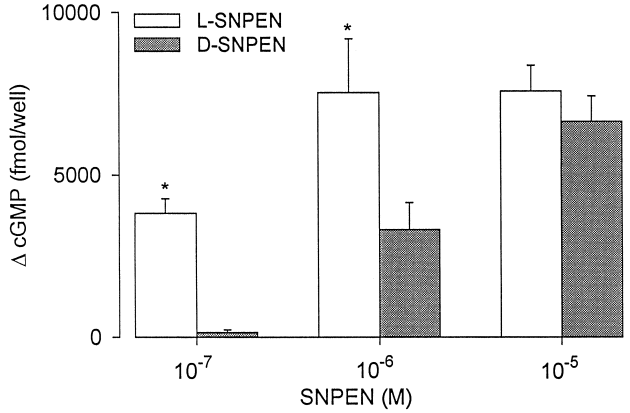

Fig. 1. The changes in cGMP contents (fmol/well, arithmetic change over control values) in porcine aortic smooth muscle cells cells 60 s after the addition of L- or D-*S*-nitrosopenicillamine (L- or D-SNPEN, 10^{-7}–10^{-5} M). The values represent the mean ± S.E.M. of the four individual experiments described in Table 2 and Table 3. * $P < 0.05$, L-SNPEN vs. D-SNPEN.

the same four experiments. Sodium nitroprusside produced concentration-dependent increases in cGMP levels except in experiment 2 (see Table 2). L- and D-*S*-nitrosopenicillamine produced concentration-dependent increases in cGMP levels (Table 3). However, L-*S*-nitrosopenicillamine produced more cGMP than D-*S*-nitrosopenicillamine at 10^{-7} M and 10^{-6} M concentrations in each of the experiments. The 10^{-5} M concentrations of L- or D-SNPEN produced equivalent increases in cGMP. The changes in cGMP (arithmetic increase over control values) produced by L- and D-*S*-nitrosopenicillamine are summarized in Fig. 1. The values are the means ± S.E.M. of the mean values from the four individual experiments described in Tables 2 and 3. The 10^{-7} M and 10^{-6} M concentrations of L-*S*-nitrosopenicillamine produced greater amounts of cGMP than these concentrations of D-*S*-nitrosopenicillamine. The 10^{-5} M concentrations of L- and D-*S*-nitrosopenicillamine produced similar increases in cGMP levels.

In three other experiments, the differences between L- and D-*S*-nitrosopenicillamine were not as obvious as those described above (see Table 4). In the first of these experiments, the 10^{-5} M concentrations of L- and D-*S*-nitrosopenicillamine produced significantly smaller increases in cGMP than either the 10^{-7} M or 10^{-6} M concentrations, although the 10^{-7} M concentration of L-*S*-nitrosopenicillamine generated more cGMP than this concentration of D-*S*-nitrosopenicillamine. In the second of these experiments, the amounts of cGMP generated by L- and D-*S*-nitrosopenicillamine were considerably less than those described above although 10^{-6} M L-*S*-nitrosopenicillamine generated more cGMP than this concentration of D-*S*-nitrosopenicillamine. In the third experiment, all concentrations of L- and D-*S*-nitrosopenicillamine and sodium nitroprusside generated very little cGMP.

3.2. Liberation of NO from L- and D-S-nitrosopenicillamine

The addition of 10, 100 and 1000 pmol of authentic NO ($n = 8$) to the chamber in the absence of serum-free medium and porcine aortic smooth muscle cells yielded 9 ± 2, 97 ± 5 and 976 ± 68 pmol of NO, respectively. The addition of 10 pmol (10^{-8} M, $n = 4$) and 100 pmol of authentic NO (10^{-7} M, $n = 4$) to serum-free medium bathing porcine aortic smooth muscle cells yielded 9 ± 2 and 96 ± 3 pmol of NO, respectively, over 1 min. The total amounts of NO (pmol) measured over 1 min following the addition of L- and D-*S*-nitrosopenicillamine (10^{-7} M and 10^{-5} M) to serum-free medium are shown in Fig. 2. NO was not detected following the addition of 100 pmol (10^{-7} M) of L- or D-*S*-nitrosopenicillamine. Detectable amounts of NO were observed following the addition of 1000 pmol (10^{-6} M) and 10 000 pmol (10^{-5} M) of L- or D-*S*-nitrosopenicillamine to serum-free medium. Each stereoisomer decomposed to similar amounts of NO. The decomposition of the stereoisomers to NO at 30 s was also similar ($P < 0.05$ for all comparisons). The recovery of NO suggested that approximately 10–15% of the higher concentrations of the stereoisomers had decomposed to the free radical over 1 min.

Table 4
A summary of the effects of SNP or L-or D-SNPEN on the cGMP contents of cultured porcine aortic smooth muscle cells

Experiment	*n*	Control	SNP (M)		
			10^{-7}	10^{-6}	10^{-5}
1	4	217 ± 15	1 301 ± 71 [a]	1 837 ± 112 [a]	5 504 ± 388 [a]
2	4	24 ± 1	438 ± 4 [a]	1 714 ± 130 [a]	329 ± 364 [a]
3	4	142 ± 17	196 ± 18	248 ± 24 [a]	336 ± 28 [a]

Experiment	*n*	L-SNPEN (M)			D-SNPEN (M)		
		10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
1	4	5 632 ± 413 [b]	6 078 ± 457	2 057 ± 361	4 014 ± 176	5 058 ± 323	2 850 ± 323
2	4	31 ± 4	2 666 ± 164 [b]	10 740 ± 626	31 ± 7	636 ± 29	11 921 ± 145
3	4	36 ± 7	136 ± 12	268 ± 25 [b]	79 ± 11	109 ± 18	142 ± 27

SNP = sodium nitroprusside; L-SNPEN = L-*S*-nitrosopenicillamine; D-SNPEN = D-*S*-nitrosopenicillamine. The values represent the mean ± S.E.M. of cGMP (fmol/well) produced by a 60 s exposure of cells to either serum-free medium alone (control), SNP (in serum-free medium) or L- or D-SNPEN (in serum-free medium) in three separate experiments (n = number of replicates). [a] $P < 0.05$, SNP vs. saline. [b] $P < 0.05$, L-SNPEN vs. D-SNPEN.

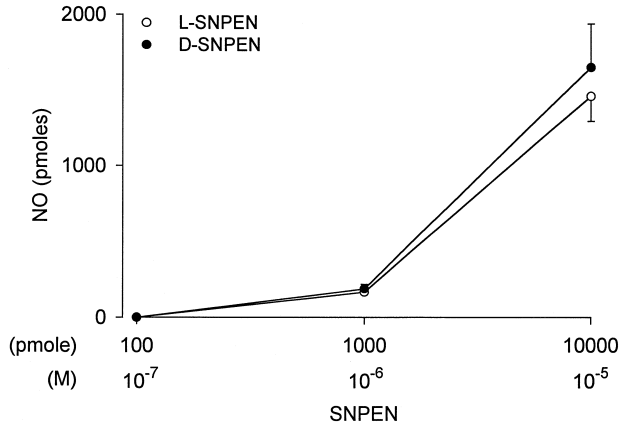

Fig. 2. The total amounts of NO (pmol) measured over 60 s following the application of 100 μl of L- or D-*S*-nitrosopenicillamine (L- or D-SNPEN, 100–10 000 pmol) to 1 ml volume of serum-free medium bathing porcine aortic smooth muscle cells. Each value represents the mean ± S.E.M. of 4–5 replicates.

The total amounts of NO (pmol) detected following the addition of 100 μl amounts of L- and D-*S*-nitrosopenicillamine (10^{-7} M and 10^{-5} M in acidified saline) to cultured porcine aortic smooth muscle cells are shown in Fig. 3. The total amounts of NO detected were less than in Fig. 2 because smaller amounts of L- and D-*S*-nitrosopenicillamine were added to the preparation. The total amounts of NO detected increased with increasing concentrations of L- and D-*S*-nitrosopenicillamine. Again, each stereoisomer decomposed equally to NO. However, it was evident that the percentage amounts of NO recovered in the head space *decreased* with increasing concentrations of L- and D-*S*-nitrosopenicillamine (Table 5). Approximately 80% of the 10 pmol amounts of L- and D-*S*-nitrosopenicillamine was recovered as NO in the head space. In contrast, approximately 14% of the 1000 pmol amounts of L- and D-*S*-nitrosopenicillamine were recovered as NO in the head space. This suggests that the denitrosation capacity of the membranes is rapidly saturated by L- and D-*S*-nitrosopenicillamine.

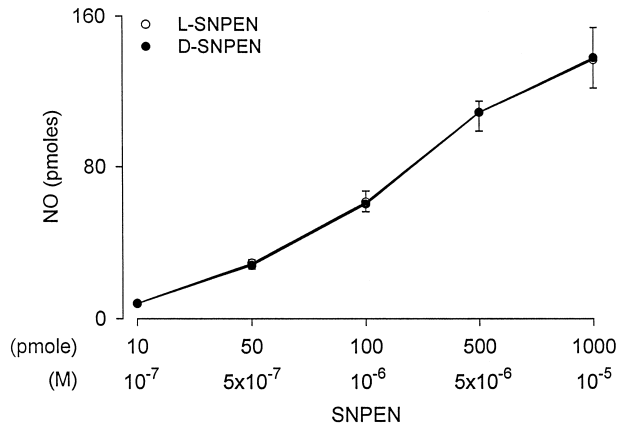

Fig. 3. The total amounts of NO (pmol) measured over 60 s following the addition of 10–1000 pmol of L- or D-*S*-nitrosopenicillamine (L- or D-SNPEN in 100 μl of acidified saline) to cultured porcine aortic smooth muscle cells. Each value represents the mean ± S.E.M. of 4–5 replicates.

Table 5
A summary of the total amounts of NO recovered after the addition of L- or D-SNPEN to porcine aortic smooth muscle cells

Concentration (M)	pmol added	L-SNPEN		D-SNPEN	
		NO (pmol)	NO (%)	NO (pmol)	NO (%)
1×10^{-7}	10	8 ± 1	78.0 ± 5.8	8 ± 1	80.8 ± 5.0
5×10^{-7}	50	29 ± 2	58.2 ± 4.1	28 ± 2	57.6 ± 4.3
1×10^{-6}	100	61 ± 6	61.0 ± 6.3	60 ± 4	60.3 ± 4.4
5×10^{-6}	500	109 ± 6	21.9 ± 1.3	109 ± 10	21.8 ± 2.0
1×10^{-5}	1000	137 ± 15	13.7 ± 1.5	138 ± 16	13.8 ± 1.6

L-SNPEN = L-*S*-nitrosopenicillamine; D-SNPEN = D-*S*-nitrosopenicillamine. Each value represents the mean ± S.E.M. of the NO recovered 1 min after the addition of L- or D-SNPEN (10–1000 pmol in 100 μl of acidified saline) to porcine aortic smooth muscle cells ($n = 4$). The percentage decomposition of L- and D-SNPEN to NO (%NO) as predicted by NO measurement in the headspace is also shown.

4. Discussion

There is evidence that the spontaneous decomposition of *S*-nitrosothiols to NO in biological solutions is not responsible for the vasorelaxant activities of these compounds (Kowaluk and Fung, 1990; Mathews and Kerr, 1993). In addition, these compounds do not necessarily have to enter vascular smooth muscle cells to exert their vasorelaxant effects (Kowaluk and Fung, 1990; Mathews and Kerr, 1993). *S*-nitrosothiols such as *S*-nitrosocysteine and *S*-nitroso-*N*-acetylpenicillamine are highly polar (Kowaluk and Fung, 1990). This would suggest that *S*-nitrosopenicillamine would also be poorly lipophilic and therefore not readily enter porcine aortic smooth muscle cells. Our original thought was that the enhanced vasodilator effects of L-*S*-nitrosopenicillamine as compared to D-*S*-nitrosopenicillamine in vivo may be due to the relatively greater or more rapid denitrosation of the L-isomer at vascular smooth muscle membranes. Such evidence would suggest that the denitrosation of *S*-nitrosothiols may occur at a stereoselective recognition site on these membranes. However, we found that L- and D-*S*-nitrosopenicillamine produced identical amounts of NO upon application to serum-free medium bathing porcine aortic smooth muscle cells or upon direct application to the cells. This clearly suggests that the denitrosation of *S*-nitrosopenicillamine does not depend upon the stereoisomeric configuration of this *S*-nitrosothiol. The greater potency of L-*S*-nitrosopenicillamine as compared to D-*S*-nitrosopenicillamine in generating cGMP must therefore be due to a mechanism other than the decomposition of the stereoisomers to NO.

The present study demonstrates that the L- and D-isomers *S*-nitrosopenicillamine generate concentration-dependent increases in cGMP in cultured porcine aortic smooth muscle cells bathed in serum-free medium. In most of the

experiments it was clearly evident that L-*S*-nitrosopenicillamine was a more potent generator of cGMP than D-*S*-nitrosopenicillamine. To our knowledge this is the first biochemical evidence that the stereoisomeric configuration of an *S*-nitrosothiol is an important factor in determining its biological potency. This study also shows that the marked difference in the potencies of L- and D-*S*-nitrosopenicillamine were not as evident in some of the experiments. At present, we have no explanation for these findings since we kept our experimental conditions as constant as possible. In addition, the cells used in these studies were used between passages 8–10. The lack of marked differences in the potencies of L- and D-*S*-nitrosopenicillamine in some of the experiments was not related to the passage number of the cells. At present, we can only conjecture that the lack of a clear difference in the potency of the L- and D-isomers was because there may have been a down-regulation of the putative *S*-nitrosothiol recognition sites or their signal transduction mechanisms in these particular cells. We chose to do these studies in serum-free medium because this particular medium does not contain thiols which could be *S*-nitrosated by *S*-nitrosothiols (Dulbecco and Freeman, 1959). Serum-free medium contains methionine but the sulfur in this molecule is not available for nitrosation because it is a thioether instead of a sulfhydryl. We also found that L- and D-*S*-nitrosopenicillamine decompose equally to NO upon addition to serum-free medium. NO could not be detected from the 10^{-7} M concentrations of L- and D-*S*-nitrosopenicillamine. It is possible that the NO was trapped by reactive compounds and that we underestimated the production of this free radical. However, it may be unlikely that this is due to trapping or degradation of NO to nitrate/nitrite since the addition of relevant amounts of authentic NO to serum-free medium resulted in the virtually complete recovery of NO in the head space. Consequently, the ability of the 10^{-7} M concentrations of L-*S*-nitrosopenicillamine to generate relatively large amounts of cGMP cannot be readily explained by its decomposition to NO in serum-free medium.

The possible lack of decomposition of 10^{-7} M L- and D-*S*-nitrosopenicillamine to NO in the serum-free medium argues that the relatively greater potency of the L-*S*-nitrosopenicillamine to generate cGMP in porcine aortic smooth muscle cells must involve some mechanism other than the generation of the free radical. These findings support established evidence that lower vasorelaxant concentrations of *S*-nitrosothiols exert their effects by mechanisms other than their decomposition to NO. This evidence includes (i) the vasodilator potencies of *S*-nitrosothiols are not obviously related to their decomposition to NO as some *S*-nitrosothiols were biologically active despite their not decomposing to NO (Kowaluk and Fung, 1990; Mathews and Kerr, 1993) and (ii) extracellular NO cannot be detected from lower vasorelaxant concentrations of *S*-nitrosocysteine by either chemiluminescence (Myers et al., 1990), agarose-hemoglobin trapping or by electron paramagnetic spectroscopy (Rubanyi et al., 1991). The 10^{-6} M concentrations of L-*S*-nitrosopenicillamine generated more cGMP than this concentration of D-*S*-nitrosopenicillamine. The 10^{-5} M concentrations of L- and D-*S*-nitrosopenicillamine produced equivalent increases in cGMP. The 10^{-6} and 10^{-5} M concentrations of L- and D-*S*-nitrosopenicillamine generated detectable amounts of NO upon addition to serum-free medium or porcine aortic smooth muscle cells. Since the decomposition to NO was equivalent for both stereoisomers it is likely that the NO generated by L- and D-*S*-nitrosopenicillamine contributes equally to the production of cGMP.

Taken together, these results do not support the concept that the decomposition of *S*-nitrosopenicillamine to NO or the entry of *S*-nitrosopenicillamine into porcine aortic smooth muscle cells is solely responsible for the observed increases in cGMP. Moreover, since the 10^{-7} M concentrations of L-*S*-nitrosopenicillamine significantly elevated cGMP levels without decomposing to NO, it appears that the NO-mediated activation of soluble guanylate cyclase is not involved in the increase in cGMP. In theory, our results could be explained by the interaction of *S*-nitrosopenicillamine with a stereoselective recognition site. The interaction of *S*-nitrosopenicillamine with this site may then initiate the stimulation of cGMP production in the porcine aortic smooth muscle cells. The capacity of the recognition site may be saturable, such that 10^{-6} M L-*S*-nitrosopenicillamine fully saturates the recognition site and this is why 10^{-5} M L-*S*-nitrosopenicillamine had no further effect on cGMP levels. Moreover, D-*S*-nitrosopenicillamine may have less affinity for, or potency at, these recognition sites. The 10^{-7} M concentration of D-*S*-nitrosopenicillamine may not interact with the stereoselective recognition site sufficiently enough to generate the amounts of cGMP produced by this concentration of L-*S*-nitrosopenicillamine. Increasingly larger concentrations of D-*S*-nitrosopenicillamine may interact sufficiently enough with the recognition site such that the 10^{-5} M concentration of the D-isomer generates similar amounts of cGMP as this concentration of the L-isomer.

An unresolved question is how the interaction of *S*-nitrosopenicillamine with a stereoselective recognition site on vascular smooth muscle membranes would result in increases in cGMP with the vascular smooth muscle cell. One possibility is that the putative *S*-nitrosothiol recognition site represents a membrane bound *S*-nitrosothiol receptor which is linked to particulate guanylate cyclase. The second possibility is that L- and D-*S*-nitrosopenicillamine differentially interact with atrial naturetic peptide receptors which are particulate guanylate cyclases (Wong and Garbers, 1992). We are currently examining which of these mechanisms may be responsible for the *S*-nitrosopenicillamine-induced increases in cGMP. In summary, the present study provides evidence that the steroisomeric configuration of *S*-nitrosopenicillamine is a critical factor in the ability of this *S*-nitrosothiol to generate cGMP in cultured

porcine aortic smooth muscle cells. The relatively greater potency of L-*S*-nitrosopenicillamine is not due to the enhanced degradation of this stereoisomer to NO.

References

Dulbecco, R. and G. Freeman, 1959, Tissue Culture Standards, In Vitro 8, 396.

Furchgott, R.F. and P.M. Vanhoutte, 1989, Endothelium-derived relaxing and contracting factors, FASEB J. 3, 2007.

Ignarro, L.J., 1990, Nitric oxide: A novel signal transduction mechanism for transcellular communication, Hypertension 16, 477.

Kowaluk, E.A. and H. Fung, 1990, Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by *S*-nitrosothiols, J. Pharmacol. Exp. Ther. 25, 1256.

Mathews R.W. and S.W. Kerr, 1993, Biological activity of *S*-nitrosothiols: The role of nitric oxide, J. Pharmacol. Exp. Ther. 267, 1529.

Moncada S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology and pharmacology, Pharmacol. Rev. 43, 109.

Myers P.R., R.L. Minor, Jr., R. Guerra, Jr., J.N. Bates and D.G. Harrison, 1990, Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide, Nature 345, 161.

Rosenblum W.I., 1992, Endothelium-derived relaxing factor in brain blood vessels is not nitric oxide, Stroke 23, 1527.

Rubanyi G.M., A. John, D. Wilcox, J.N. Bates and D.G. Harrison, 1991, Evidence that a *S*-nitrosothiol, but not nitric oxide, may be identical with endothelium-derived relaxing factor, J. Cardiovasc. Pharmacol. 17, 541.

Stamler J.S., D.J. Singel and J. Loscalzo, 1992, Biochemistry of nitric oxide and its redox-activated forms, Science 258, 1898.

Stoll L.L. and A.A. Spector, 1993, Lysophosphatidylcholine causes cGMP-dependent verapamil-sensitive Ca^{2+} influx in vascular smooth muscle cells, Am. J. Physiol. 264, C885.

Travis M.D., R.L. Davisson, L.L. Stoll, J.N. Bates and S.J. Lewis, 1994, Evidence that *S*-nitrosopenicillamine may active stereoselective *S*-nitrosothiol receptors on vascular membranes, Hypertension 24, 390.

Wallenstein S., C.L. Zucker and J.L. Fleiss, 1980, Some statistical methods useful in circulation research, Circ. Res. 47, 1.

Winer, B.J., 1971, Statistical Principles of Experimental Design (McGraw-Hill, New York, NY) p. 752–809.

Wong S.K.-F. and D.L. Garbers, 1992, Receptor guanylyl cyclases, J. Clin. Invest. 90, 229.