Chemical Stabilization of a Vasoactive *S*-Nitrosothiol with Cyclodextrins Without Loss of Pharmacologic Activity

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S-Nitrosothiols have been proposed as the endogenous chemical representing the vasoactive endothelium-derived relaxing factor, as well as the active cellular intermediates responsible for the therapeutic action of organic nitrates. The relatively stable analogue *S*-nitroso *N*-acetyl penicillamine (SNAP) is a potent vasodilator producing less pharmacologic tolerance than nitroglycerin upon prolonged administration. The therapeutic potential of this new class of vasodilators, however, may be limited by their chemical instability in solution ($t_{1/2}$ of SNAP is 26 hr in 5% dextrose). We examined the usefulness of several cyclodextrins (CD) to stabilize this polar compound in solution. At cyclodextrin concentrations of 12 m*M*, hydroxypropyl-βCD was most effective at stabilizing SNAP ($t_{1/2}$ = 77 hr) when compared to αCD (41 hr), βCD (69 hr), γCD (36 hr), and βCD–tetradecasulfate (38 hr). Stability constants for the complexation of SNAP with the various cyclodextrins were determined by the classical solubility technique and were found to range from 26 to 435 M^{-1}. Increased complexation brought about better SNAP stability. Complexation of SNAP with cyclodextrins, however, did not decrease the relaxation potency of SNAP as determined in an *in vitro* blood vessel preparation. Cyclodextrin complexation may be a useful approach to stabilize labile and polar compounds, such as *S*-nitrosothiols, without loss of pharmacologic activity.

KEY WORDS: vasodilator; cyclodextrin; stabilization.

INTRODUCTION

Nitrate vasodilators, such as nitroglycerin and isosorbide dinitrate, have been used extensively for many years in the treatment of cardiovascular diseases. The rapid action of these compounds, their safety, their venoselective hemodynamic actions, and the availability of convenient sustained-release dosage forms, such as the transdermal nitroglycerin patches, have made this class of compounds important therapeutic entities in the treatment of angina pectoris and congestive heart failure. However, recent clinical studies have suggested that, despite their initial favorable effects, nitrate vasodilators induce rapid development of pharmacologic tolerance after continuous administration (1), thus limiting their usefulness in the chronic management of cardiovascular diseases.

The mechanism of nitrate-induced pharmacologic tolerance has not yet been clearly defined; a popular hypothesis, however, suggests that this phenomenon is brought about by reduced metabolic activation of these compounds to their cellular intermediate(s), nitric oxide and/or *S*-nitrosothiols (2). Nitric oxide has been identified as the endothelium-derived relaxing factor, which has an important role in regulating vascular tone (3). Interestingly, this simple molecule is produced by the nervous and immune systems as well (4) and is probably responsible for many regulatory functions in the body. Nitric oxide, therefore, is a potent endogenous substance of therapeutic relevance. However, its physicochemical properties (being a gas at room temperature) limits its utility in many experimental and therapeutic settings. *S*-Nitrosothiols are labile compounds which form rapidly when thiols are exposed to nitric oxide (5). Therefore, *S*-nitrosothiols are convenient potential prodrugs of nitric oxide since both the formation and the breakdown of these compounds, from and to nitric oxide, are extremely rapid (6).

We have recently found the most stable *S*-nitrosothiol known in the literature, *S*-nitroso *N*-acetyl penicillamine (SNAP), to be a potent vasodilator both *in vitro* (7) and *in vivo* (8). SNAP provided hemodynamic effects similar to nitroglycerin in an experimental model of heart failure but, unlike nitroglycerin, did not develop any pharmacologic tolerance during continuous intravenous administration (8). These studies therefore suggested that *S*-nitrosothiols, including SNAP, may be potentially useful therapeutic entities themselves. The potential utility of *S*-nitrosothiols, however, is currently limited by their chemical instability in solution. We have shown previously that SNAP has a half-life of only 26 hr in 5% dextrose, thus requiring the replacement of infusion solution every 2 hr to maintain a constant *in vivo* SNAP infusion rate (less than 10% fluctuation) (8). In the present studies, we examined the possibility of improving SNAP stability in solution through the use of cyclodextrin complexation. We have investigated the effects of a variety of cyclodextrins in an attempt to characterize the SNAP–cyclodextrin interaction. We also assessed the effect of cyclodextrin complexation on the *in vitro* pharmacologic activity of this potentially useful vasodilator.

MATERIALS AND METHODS

Materials

SNAP (Fig. 1) was synthesized according to the method of Field *et al.* (9). Elemental analysis showed that the resulting product was greater than 99% pure. SNAP crystals were stored at −20°C in a desiccator prior to use, and they were stable under these conditions (9). α-, β-, and γ-cyclodextrins were purchased from Sigma (St. Louis, MO), whereas hydroxypropyl-β-cyclodextrin (HP-βCD; average degree of substitution, 6.3) was obtained from Research Biochemicals Incorporated (Natick, MA). β-Cyclodextrin tetradecasulfate (β-CD-14S) was a generous gift from Takeda Chemical Industries, Pharmaceutical Group (Osaka, Japan).

Measurement of SNAP Stability

The stability of SNAP in 5% dextrose was determined in the absence or presence of cyclodextrins. In all stability experiments the initial concentration of SNAP in solution was 1 mg/ml (4.5 m*M*), which was the concentration used in our previous *in vivo* experiments (8). SNAP concentrations were measured spectrophotometrically at 590 nm (Hitachi 200

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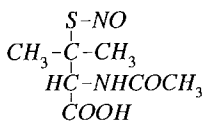

Fig. 1. Structure of *S*-nitroso *N*-acetyl penicillamine (SNAP), molecular weight 220.

spectrophotometer). At this wavelength, SNAP absorbs maximally ($\epsilon = 15.7\ M^{-1}\ cm^{-1}$), while its degradation products and cyclodextrins do not interfere. Solutions (n = 6–12) were stored in sealed polypropylene tubes at room temperature (25 ± 1°C) under constant room light. The SNAP concentration versus time data for each individual run were fitted to a monoexponential equation for the determination of an overall first-order rate constant (k_{obs}), using the computer program PCNONLIN, which was also used to discern the appropriateness of the fit.

Determination of the Complexation Stability Constant, K_c

The complexation of SNAP with cyclodextrins can be viewed simply as

$$\begin{array}{ccc} \text{SNAP} + \text{CD} & \leftrightarrows & \text{SNAP} - \text{CD} \\ \downarrow k_1 & K_c & \downarrow k_2 \end{array}$$

Scheme I

In scheme I, k_1 and k_2 represent the apparent first-order rate constants describing SNAP degradation in the absence of cyclodextrin and SNAP degradation while complexed, respectively. K_c represents the apparent equilibrium (stability) constant describing the SNAP–cyclodextrin complexation.

The classical solubility technique (10) was used to determine K_c for the cyclodextrins examined, assuming 1:1 complexation. Excess SNAP (17 mg) was shaken with varying concentrations of cyclodextrins in 5% dextrose (4 ml). After equilibrium was established, which occurred within 1 hr in all cases, the solution was passed through a 0.45-μm filter (Nylon Acrodisc 13, Gelman Sciences) and the concentration of SNAP in solution was measured. The maximal solubility of SNAP in the presence of cyclodextrin at equilibrium was plotted against cyclodextrin concentration, and K_c was calculated by (10)

$$K_c = \frac{\text{slope}}{\text{intercept } (1 - \text{slope})} \quad (1)$$

Estimation of Rate Constants

According to Scheme I, under the condition of excess cyclodextrin (CD), i.e., when the concentration of CD_{total} is much greater than that of SNAP–CD (11),

$$k_{obs} = k_1 - \frac{CD_{total} \times K_c\ (k_1 - k_2)}{(CD_{total} \times K_c + 1)} \quad (2)$$

where k_{obs} is the experimentally observed first-order degradation rate constant. By rearrangement of Eq. (2), we obtain

$$\frac{(CD_{total} \times K_c)}{(k_1 - k_{obs})} = \frac{(CD_{total} \times K_c)}{(k_1 - k_2)} + \frac{1}{(k_1 - k_2)} \quad (3)$$

Plots of our experimental data for all cyclodextrin solutions according to the Eq. (3) showed that all points fell on the same straight line, indicating that k_2 was similar for all SNAP–cyclodextrin complexes, irrespective of the cyclodextrin used. To provide a more accurate estimate of k_2, all k_{obs} data, where $CD_{total} \gg SNAP_{initial}$, were then fitted to Eq. (2), using PCNONLIN. Since SNAP degradation alone in 5% dextrose (k_1) varied slightly between experimental runs (a range of about 20%), Eqs. (2) and (3) were fitted by using the observed k_1 obtained on the same day.

Measurement of the *in Vitro* Vasodilating Activity of SNAP

The *in vitro* vasodilating effects of SNAP were determined using the isolated rat aorta, as previously described (12). Briefly, male Sprague–Dawley rats (200–250 g; Blue Spruce Farms, Altamont, NY) were sacrificed and aortic ring segments were excised and mounted in 10-ml jacketed tissue baths containing Krebs bicarbonate buffer. The tissue baths were maintained at 37°C and aerated continuously with 95% oxygen/5% carbon dioxide. Ring segment tension was measured using force displacement transducers and displayed on a polygraph. Segments were precontracted with phenylephrine (0.5 μ*M*) and the cumulative concentration–relaxation response curves to SNAP were determined. SNAP solutions were prepared in 5% dextrose and in the presence of 12 m*M* βCD, HP-βCD, or βCD-14S. These solutions were used immediately after preparation. Relaxation was expressed as a percentage of phenylephrine-induced tone. Log molar EC_{50} and E_{max} values were obtained for each individual run by fitting the four-parameter logistic function to the concentration–relaxation data using PCNONLIN (12). Log molar EC_{50} and E_{max} values were statistically compared against the control by Student's t test.

RESULTS

Shown in Fig. 2 are representative plots of SNAP degradation in the absence and presence of cyclodextrins. The degradation data of SNAP were best described by monoexponential decay and apparent first-order half-lives were determined (Table I). All cyclodextrin concentrations tested significantly improved SNAP stability in solution when compared to control. At equimolar concentrations of cyclodextrins, HP-βCD was most effective at stabilizing SNAP. The

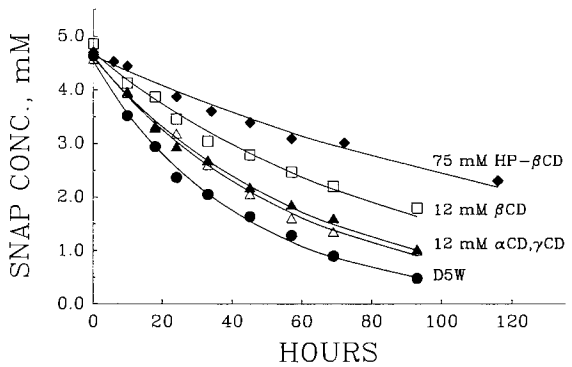

Fig. 2. Representative plots of SNAP degradation in the absence and presence of various cyclodextrins. SNAP solutions were maintained at room temperature with or without cyclodextrins. Lines are computer fitted curves using first-order kinetics.

Table I. Apparent First-Order Half-Lives of SNAP in the Absence or Presence of Cyclodextrins in 5% Dextrose

Cyclodextrin	CD_{total} (m*M*)	pH	Half-life (hr), mean ± SD[a]	Stability enhancement
None	0	2.8	26.1 ± 2.5	1.0
αCD	4	2.8	34.5 ± 2.9	1.29
	12	2.8	40.8 ± 1.4	1.52
βCD	4	2.8	46.1 ± 2.7	1.72
	12	2.9	68.8 ± 3.9	2.40
γCD	4	2.8	30.4 ± 2.6	1.27
	12	2.8	35.5 ± 2.5	1.49
HP-βCD	4	2.9	39.1 ± 2.9	1.40
	12	2.9	76.9 ± 6.1	2.71
	25	3.1	84.9 ± 7.9	3.05
	75	3.4	106 ± 5.7	3.83
βCD-14S	4	2.9	36.6 ± 2.9	1.36
	12	2.9	38.2 ± 1.4	1.42

[a] n = 6–12 runs for each cyclodextrin concentration.

presence of 4 to 12 m*M* cyclodextrins did not affect the pH of the SNAP solutions in 5% dextrose, and the pH did not change throughout the experiments.

Figure 3 shows the time-dependent changes in SNAP solubility in various concentrations of HP-βCD. The increase in solubility was immediate, and equilibrium was rapidly established. All other cyclodextrins behaved similarly.

As expected, all cyclodextrins examined enhanced SNAP solubility. In all cases SNAP solubility was found to be linearly related to cyclodextrin concentration, suggesting a single complexation species (Fig. 4A). Linear saccharides such as maltose and dextran, at equivalent concentrations of sugar units, did not enhance SNAP solubility (Fig. 4B). The linear plots in Fig. 4A were used to determine the apparent stability constants (K_c), by applying Eq. (1), and the results are displayed in Table II. The K_c values ranged from 26 to 435 M^{-1} for the various cyclodextrins and were in the same rank order as observed for the improvement of SNAP stability. We were unable to carry out solubility studies with

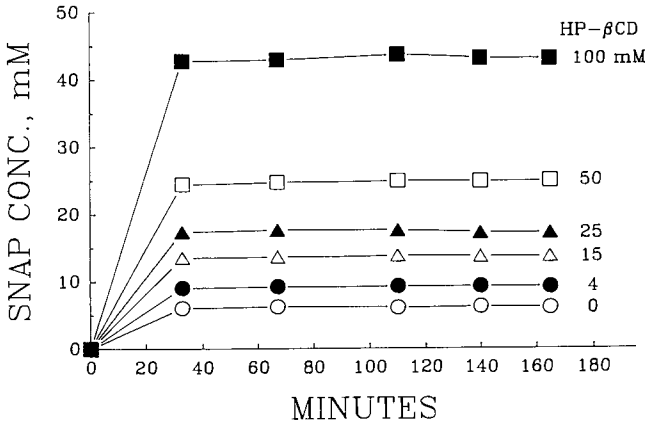

Fig. 3. Rapid solubilization of SNAP in the presence of various concentrations of HP-βCD. Excess SNAP was shaken with cyclodextrin solutions. Apparent maximal solubility of SNAP was achieved in less than 1 hr and was enhanced in the presence of cyclodextrins.

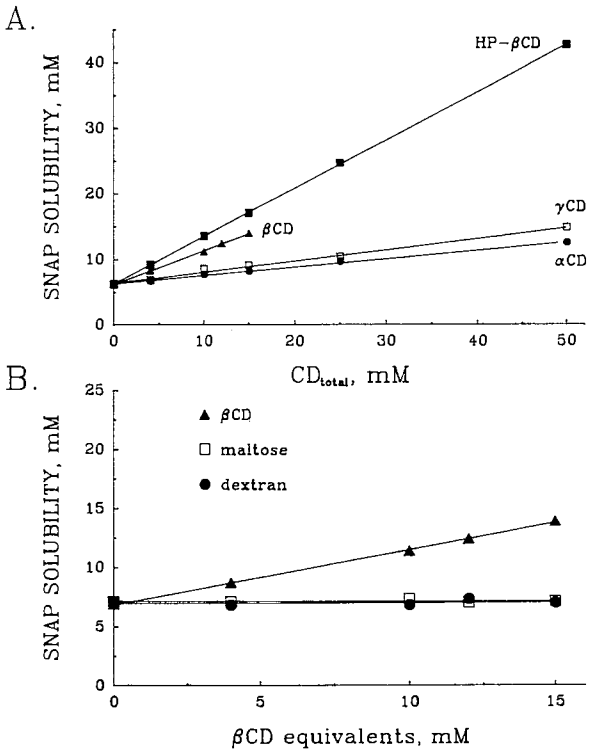

Fig. 4. The effects of cyclodextrins on SNAP solubility. (a) SNAP solubility was a linear function of CD_{total} for all cyclodextrins examined. (B) SNAP solubility was not affected by the presence of maltose or dextran. Data are presented as millimolar equivalents of βCD (corrected for number of glucose residues per mole).

βCD-14S, due to lack of sufficient availability of this new cyclodextrin.

Figure 5 shows the fit of Eq. (2) to the SNAP degradation data when $CD_{total} \gg SNAP_{initial}$ (i.e., cyclodextrin concentrations of 12 m*M* or greater). The K_c values determined previously for each cyclodextrin were used as constants for this fit. Data from βCD-14S were not included since K_c was not determined. Initial data treatment using Eq. (3) showed that all of these data generated from various cyclodextrins fell on a straight line, suggesting an identical value of k_2 for all cyclodextrin complexes (r = 0.994; shown in inset in Fig. 5). Since a reciprocal plot such as Eq. (3) often provides biased estimates due to data transformation and an overemphasis of the large values in the parameter estimation, the

Table II. K_c Values for SNAP–Cyclodextrin Complexation, Cyclodextrin Solubilities, and Cyclodextrin Molecular Weights

Cyclodextrin	K_c (M^{-1})	CD solubility in 5% dextrose (m*M*)	MW[a]
αCD	25.7 ± 1.2	174	973
βCD	167 ± 7.3	15.5	1135
γCD	39.1 ± 1.7	106	1297
HP-βCD	435 ± 6.9	>315	1500
βCD-14S[b]	—	262	1699

[a] Molecular weight.
[b] K_c was not determined for βCD-14S.

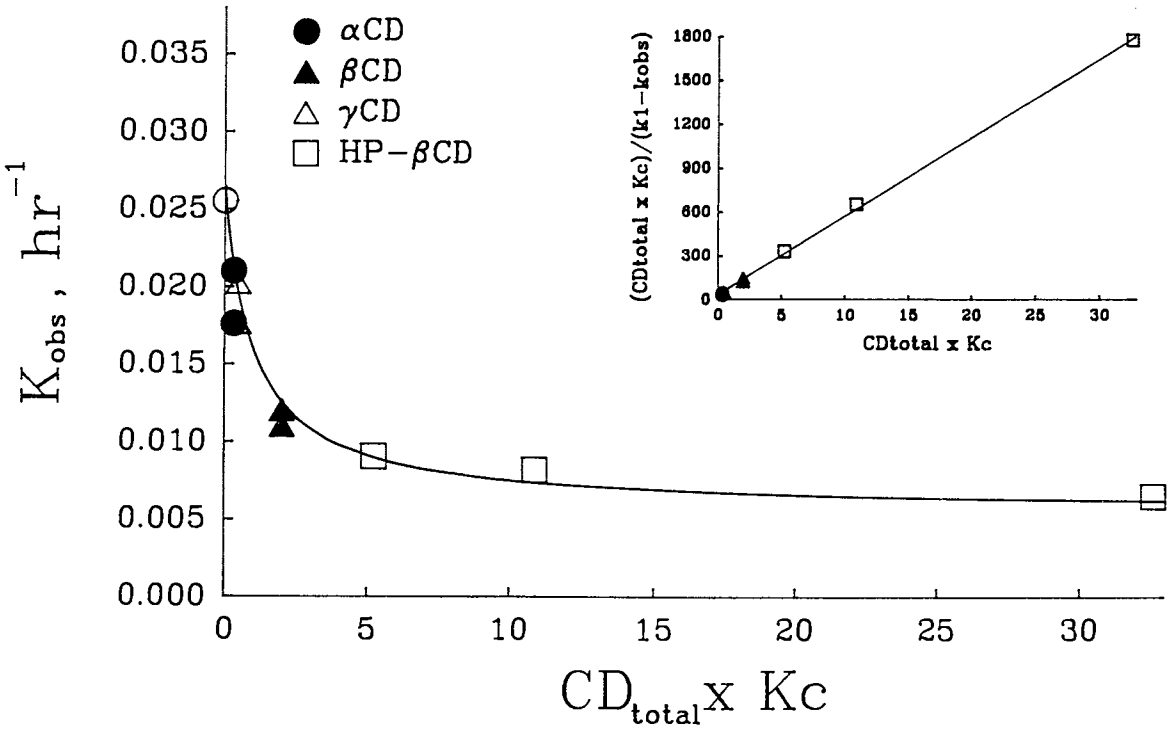

Fig. 5. Experimental determination of k_2. SNAP degradation rate constants were fitted according to Eq. (2) and the half-life of SNAP while complexed was estimated to be approximately 125 hr. The linearized form of Eq. (2) [Eq. (3)] is shown in the inset. Computer-fitted lines are shown.

nonlinear Eq. (2) was used to estimate k_2 (Fig. 5). The PCNONLIN estimate of k_2 was $5.56 \pm 0.62 \times 10^{-3}$ hr^{-1}, corresponding to a $t_{1/2}$ of 125 ± 14 hr, independent of the cyclodextrin used.

The *in vitro* pharmacologic activity of SNAP in the presence of three cyclodextrins was compared to the control (Fig. 6). The effects of cumulative additions of SNAP–cyclodextrin solutions were not significantly different from those obtained from SNAP alone. Log molar EC_{50} values were not different from control for the three cyclodextrins tested (−7.51 ± 0.40 for SNAP, −7.39 ± 0.43 for SNAP + βCD, −7.46 ± 0.39 for SNAP + HP-βCD, −7.62 ± 0.26 for SNAP + βCD-14S). The E_{max} values were also identical. The *in vitro* potency of SNAP–cyclodextrin solutions did not bear any relationship to the relative complexation ability of the various cyclodextrins. βCD-14S appeared to exhibit a slight leftward shift of the concentration vs response curve. This effect, however, was not statistically significant and was probably due to a slight vasorelaxant effect (4–15%) observed with this highly negatively charged cyclodextrin. Cumulative additions of the other cyclodextrins alone had no vascular effects (data not shown).

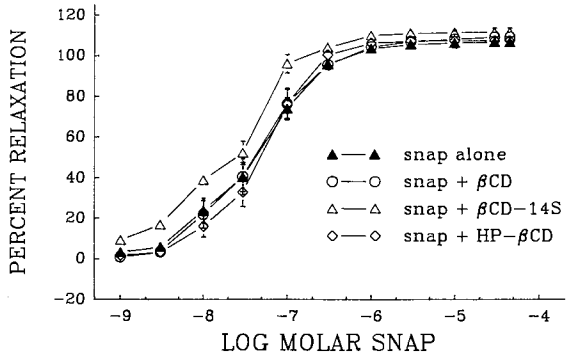

Fig. 6. *In vitro* pharmacologic activity of SNAP in the absence or presence of various cyclodextrins. The presence of cyclodextrins did not alter the vasodilator potency of SNAP.

DISCUSSION

The *S*-nitrosothiols are similar to the organic nitrates in that they produce vascular relaxation via activation of guanylate cyclase within the vascular smooth muscle cell (13), leading to the production of cyclic GMP (14). However, it appears that organic nitrates may require a metabolic activation step which is not required for *S*-nitrosothiol action. We have previously shown that *S*-nitrosothiols are less tolerance producing than nitroglycerin *in vitro* and that there was no cross-tolerance to SNAP in nitroglycerin-tolerant blood vessel segments (12,15). Recently, we have examined the hemodynamic actions and tolerance properties of SNAP, as compared to nitroglycerin, in a rat model of congestive heart failure (8). In this animal model tolerance to nitroglycerin develops rapidly (16), but there was no tolerance observed during continuous SNAP infusion (8). These experimental results suggest that SNAP, or other *S*-nitroso compounds, may be novel vasodilators with important therapeutic potential. However, in general, *S*-nitrosothiols are labile in solution (17), and SNAP is one of the few analogues that can be easily isolated and stored in pure crystal form (9). During our *in vivo* animal experiments we found that SNAP was unstable in our infusion vehicle (5% dex-

trose) (8). This instability led us to examine the possible effects of cyclodextrin complexation on SNAP stability in solution.

Cyclodextrins have been of pharmaceutical interest primarily because of their ability to enhance the aqueous solubility of lipophilic compounds (18). This solubility improvement does not require the use of cosolvents or structural modification of the molecule. Improvements in drug dissolution rates (19), absorption [oral (20) and transdermal (21)], and systemic bioavailability (22,23) have all been observed for selected compounds when incorporated with cyclodextrins. Since inclusion complex formation protects the guest molecule from the surrounding media, its stability is often improved as well (18,24,25). Since the primary degradation pathway of SNAP is the cleavage of the *S*-nitroso bond and the formation of disulfide products (9), inclusion complexation might offer a feasible method to stabilize this compound in solution.

The addition of α-, β-, or γ-cyclodextrins improved SNAP stability in 5% dextrose. Of these three, βCD was the best at stabilizing SNAP and had the highest K_c, suggesting that the interior pore size of this cyclodextrin was optimal for inclusion. Unfortunately, of these three parent cyclodextrins, βCD was also found to be least soluble in 5% dextrose (Table II). The poor solubility of βCD in aqueous media has been observed by others (26) and may in part be responsible for its renal toxicity (27). In contrast, the amorphous derivative HP-βCD is extremely water soluble, and even large doses are well tolerated in animals (28). At equimolar concentrations, we found HP-βCD to be even better at stabilizing SNAP than the parent βCD. Corresponding to this stability enhancement, K_c was nearly threefold higher for HP-βCD than for βCD. The reason for this difference is not clear, since the cavity size is identical for these two cyclodextrins. Some contributing factors might be due to differences in hydrogen bonding, shielding of SNAP molecules by the hydroxypropyl side chains, or other molecular interactions. Muller and Brauns (29) have also shown that the complexation ability of βCD derivatives is highly dependent upon their degree of substitution.

We also examined the effects of straight-chain saccharides on SNAP solubility. Maltose is comprised of 2 glucose units with a linkage identical to those of cyclodextrins, while dextran is a polymer with over 400 glucose units per molecule. The fact that maltose and dextran did not affect the solubility of SNAP suggests that the interaction of SNAP with cyclodextrins is a specific interaction as a result of inclusion complexation. Although we did not attempt to study the structure of the SNAP–cyclodextrin complex, it is possible that some part of the *S*-nitroso moiety might be encapsulated in the interior of the host cyclodextrin. It is interesting that significant complexation occurred despite the relative polar nature of the SNAP molecule (log octanol/5% dextrose partition coefficient was found to be 0.534).

In the presence of cyclodextrins, SNAP stability was a function of both cyclodextrin concentration and K_c. Analysis of our data showed that all SNAP–cyclodextrin complexes examined had a similar value of k_2. This suggests that the degradation of SNAP in cyclodextrin complexes is independent of the "host" cyclodextrin molecule and that the mechanism of this degradation is similar in all complexes. By fitting our data to Eq. (2), we estimated the half-life of SNAP while complexed with cyclodextrins (k_2) to be 125 hr. This theoretical maximum of SNAP stability in the presence of cyclodextrin (i.e., complete complexation) provides an approximate fivefold improvement in stability.

Despite extensive complexation with SNAP, cyclodextrins did not affect the vasodilating activity of SNAP *in vitro*. This is consistent with the findings of Pitha *et al.* (18), who found that HP-βCD significantly improved the solubility and stability of pindolol but did not alter its affinity for β-adrenoreceptors in rat lung homogenates. It is possible that in our system the SNAP–cyclodextrin complex rapidly dissociates upon dilution, since the formation equilibrium seems to be rapid. Previous reports have shown that βCD-14S associates with vascular endothelial cells, due to its high net negative charge (30). Our data, however, did not suggest that SNAP was "directed" to the vessel by this cyclodextrin since we did not observe any significant enhancement of SNAP activity in the presence of this cyclodextrin. This finding may again be due to the rapid dissociation of the complex.

In conclusion, we have found that cyclodextrin complexation can enhance the stability of a potent vasoactive *S*-nitrosothiol. Of the cyclodextrins examined, HP-βCD had the highest affinity for SNAP and was most capable at reducing SNAP degradation. Using a kinetic method to analyze the degradation of different SNAP–cyclodextrin complexes, we found that the type of cyclodextrin used did not affect the degradation rate constant of the complex. Under "idealized" conditions (i.e., complete complexation), cyclodextrins can provide an approximately fivefold improvement of SNAP stability in 5% dextrose. Despite this complex formation, no change in the *in vitro* pharmacologic activity of SNAP was observed in the presence of cyclodextrin. Cyclodextrin complexation may be a potentially useful approach for the stabilization of labile and polar molecules without alteration of pharmacologic activity.

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REFERENCES

1. U. Elkayam, D. Kiluck, N. McIntosh, A. Roth, W. Hsueh, and R. H. Rahimtoola. Incidence of early tolerance to hemodynamic effects of continuous infusion of nitroglycerin in patients with coronary artery disease and heart failure. *Circulation* 76:577–584 (1987).
2. L. J. Ignarro, H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz, and C. A. Gruetter. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: Evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.* 218:739–749 (1981).
3. R. M. J. Palmer, A. G. Ferrige, and S. Moncada. Nitric oxide release accounts for the biologic activity of endothelium-derived relaxing factor. *Nature* 327:524–526 (1987).

4. M. A. Marletta. Nitric oxide: Biosynthesis and biological significance. *Trends Biochem. Sci.* 14:488–492 (1989).
5. H.-L. Fung, S. Chong, E. Kowaluk, K. Hough, and M. Kakemi. Mechanisms for the pharmacologic interaction of organic nitrates with thiols. Existence of an extracellular pathway for the reversal of nitrate vascular tolerance by N-acetylcysteine. *J. Pharmacol. Exp. Ther.* 245:524–530 (1988).
6. E. A. Kowaluk and H.-L. Fung. Spontaneous liberation of nitric oxide cannot account for *in vitro* vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther.* 255:1256–1264 (1990).
7. E. A. Kowaluk and H.-L. Fung. Dissociation of nitrovasodilator-induced relaxation from cyclic GMP levels during *in vitro* nitrate tolerance. *Eur. J. Pharmacol.* 176:91–95 (1990).
8. J. A. Bauer and H.-L. Fung. Differential hemodynamic effects and tolerance properties of nitroglycerin and an S-nitrosothiol in experimental heart failure. *J. Pharmacol. Exp. Ther.* 256:249–254 (1991).
9. L. Field, R. V. Dilts, R. Ravichandran, P. G. Lenhert, and G. E. Carnahan. An unusually stable thionitrite from N-acetyl-D,L-penicillamine; X-ray crystal and molecular structure of 2-(acetylamino)-2-carboxy-1,1-dimethyl thionitrite. *J.C.S. Chem. Comm.* 249–250 (1978).
10. T. Higuchi and K. A. Connors. Phase solubility techniques. *Adv. Anal. Chem. Instr.* 4:117–212 (1965).
11. A. K. Colter, S. S. Wang, G. H. Megerle, and P. S. Ossip. Chemical behavior of charge-transfer complexes. II. Phenanthrene catalysis in acetolysis of 2,4,7-trinitro-9-fluoroenyl *p*-toluenesulfonate. *J. Am. Chem. Soc.* 86:3106–3113 (1964).
12. E. A. Kowaluk, R. Polisczuk, and H.-L. Fung. Tolerance to relaxation in rat aorta: Comparison of an S-nitrosothiol with nitroglycerin. *Eur. J. Pharmacol.* 144:379–383 (1987).
13. S. A. Waldman, R. M. Rapaport, R. Ginsburg, and F. Murad. Desensitization to nitroglycerin in vascular smooth muscle from rat and human. *Biochem. Pharmacol.* 35:3525–3531 (1986).
14. R. A. Keith, A. M. Burkman, T. D. Sokoloski, and R. H. Fertel. Vascular tolerance to nitroglycerin and cyclic GMP generation in rat aortic smooth muscle. *J. Pharmacol. Exp. Ther.* 221:525–531 (1982).
15. E. A. Kowaluk and H.-L. Fung. Dissociation of nitrovasodilator-induced relaxation from cyclic GMP levels during *in vitro* nitrate tolerance. *Eur. J. Pharmacol.* 176:91–95 (1990).
16. J. A. Bauer and H.-L. Fung. Effects of chronic glyceryl trinitrate on left ventricular haemodynamics in a rat model of congestive heart failure: Demonstration of a simple animal model for the study of *in vivo* nitrate tolerance. *Cardiovasc. Res.* 24:198–203 (1990).
17. L. J. Ignarro, B. K. Barry, D. Y. Gruetter, J. C. Edwards, E. H. Ohlstein, C. A. Gruetter, and W. H. Baricos. Guanylate cyclase activation by nitroprusside and nitrosoguanidine is related to formation of S-nitrosothiol intermediates. *Biochem. Biophys. Res. Commun.* 94:93–100 (1980).
18. J. Pitha, T. Irie, P. B. Sklar, and J. S. Nye. Drug solubilizers to aid pharmacologists: Amorphous cyclodextrin derivatives. *Life Sci.* 43:493–502 (1988).
19. H. Arima, T. Irie, and K. Uekama. Differences in the enhancing effects of water soluble β-cyclodextrins on the release of ethyl 4-biphenyl acetate, an anti-inflammatory agent from an oleaginous suppository base. *Int. J. Pharm.* 57:107–115 (1989).
20. J. Pitha, S. M. Harman, and M. E. Michel. Hydrophilic cyclodextrin derivatives enable effective oral administration of steroidal hormones. *J. Pharm. Sci.* 75:165–167 (1986).
21. H. Okamoto, H. Komatsu, M. Hashida, and H. Sezaki. Effects of β-cyclodextrin and di-O-methyl-β-cyclodextrin on the percutaneous absorption of butylparaben, indomethacin, and sulfanilic acid. *Int. J. Pharm.* 30:35–45 (1986).
22. A. Yoshida, M. Yamamoto, T. Itoh, T. Irie, F. Hirayama, and K. Uekama. Utility of 2-hydroxypropyl-β-cyclodextrin in an intramuscular injectable preparation of nimodipine. *Chem. Pharm. Bull.* 38:176–179 (1990).
23. H. W. Frijlink, A. C. Eissens, A. J. M. Schoonen, and C. F. Lerk. The effects of cyclodextrins on drug absorption II. In vivo observations. *Int. J. Pharm.* 64:195–205 (1990).
24. T. Backensfeld, B. W. Muller, M. Wiese, and J. K. Seydel. Effect of cyclodextrin derivatives on indomethacin stability in aqueous solution. *Pharm. Res.* 7:484–490 (1990).
25. F. Glomot, L. Benkerrour, D. Duchene, and M.-C. Poelman. Improvement in availability and stability of a dermacorticoid by inclusion in β-cyclodextrin. *Int. J. Pharm.* 46:49–55 (1988).
26. D. Duchene and D. Wouessidjewe. Physicochemical characteristics and pharmaceutical uses of cyclodextrin derivatives. *Pharm. Tech.* 14:26–34 (1990).
27. D. Frank, J. Gray, and R. Weaver. Cyclodextrin nephrosis in the rat. *Am. J. Pathol.* 83:367–382 (1976).
28. M. E. Brewster, K. S. Estes, and N. Bodor. An intravenous toxicity study of 2-hydroxypropyl-β-cyclodextrin, a useful drug solubilizer, in rats and monkeys. *Int. J. Pharm.* 59:231–243 (1990).
29. B. W. Muller and U. Brauns. Hydroxypropyl-β-cyclodextrin derivatives: Influence of average degree of substitution on complexing ability and surface activity. *J. Pharm. Sci.* 75:571–572 (1986).
30. J. Folkman, P. B. Weisz, M. M. Joullie, W. W. Li, and W. R. Ewing. Control of angiogenesis with synthetic heparin substitutes. *Science* 243:1490–1493 (1989).