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Blockade of nitric oxide-induced relaxation of rabbit aorta by cysteine and homocysteine

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KEY WORDS aorta; endothelium-derived relaxing factor; cysteine; homocysteine; nitric oxide; phenylephrine; superoxide dismutase; xanthine; xanthine oxidase; cytochrome c

AIM: To examine the inhibition by *L*-cysteine (Cys) and *L*-homocysteine (HoCys) of NO-induced relaxation of aorta. **METHODS**: The tension of rabbit aortic rings in oxygenated Krebs' solution was recorded isometrically. **RESULTS**: Pretreatment of endothelium-denuded rings with Cys or HoCys inhibited the NO-induced increase in cGMP. The inhibitory effects of Cys or HoCys on relaxation responses to subsequent additions of NO 75 nmol·L^{-1} gradually diminished with time, which was consistent with the loss of the sulfhydryl concentration of Cys and HoCys. Superoxide dismutase (SOD) 35 kU·L^{-1} attenuated the inhibition by Cys and HoCys of NO-induced relaxation. Neither boiled SOD nor catalase 100 kU·L^{-1} antagonized the inhibitory effects of Cys. Preaddition of SOD 35 kU·L^{-1} inhibited the reduction of cytochrome C by Cys. Increasing concentrations of SOD from 35 to 350 kU·L^{-1} intensified the cytochrome C reduction. Addition of xanthine 300 μmol·L^{-1} plus xanthine oxidase 1 U·L^{-1} to the mixture of cytochrome C 60 μmol·L^{-1} and Cys 100 μmol·L^{-1} produced an additional augmentation of SOD-inhibitable reduction of cytochrome C. The rate of the reduction of cytochrome C induced by HoCys 100 μmol·L^{-1} was much slower than with Cys. Addition of NO reduced the SH concentrations of both the supernatant of aortic homogenate and Cys in Krebs' solution. **CONCLUSION**: The inhibition by the SH compounds of NO is mediated partly by the superoxide generated by the auto-oxidation of these compounds, and partly by a direct reaction of SH groups with NO.

Endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide (NO)[1,2]. This free radical, released from vascular endothelium, stimulates guanylate cyclase by the formation of a nitrosyl-heme complex at the activator site of the enzyme, and then produces vasorelaxation. Studies of vascular function in patients with homocysteinemia and in animals with a long-term administration of homocysteine (HoCys) demonstrated the essential features of vascular lesion that might be associated with the homocysteine-induced endothelial injury[3,4] with the resultant decrease in production of EDRF[5]. In considering the vascular damage induced by HoCys, sulfhydryl (SH) compounds have a potential for autoxidation accompanied by the generation of some free radicals including O_2^- [6,7] which has been proven to be a powerful inactivator of EDRF and NO[8]. Also, SH compounds which have standard reduction potentials around −0.25 V have a potential to reduce NO with reduction potentials +0.5 V[9]. Therefore, that leaves open the possibility that Cys and HoCys may be a potential antagonist of EDRF or NO under physiological and pathophysiologic conditions. Indeed, the interactions of HoCys with NO and EDRF that influence vascular tone are poorly understood and are a subject requiring further clarification. It was our aim in this study to characterize the effects of Cys and HoCys on vasomoter tone of isolated rabbit aortas in the presence and absence of endothelium, to determine whether the effects of the 2 compounds on NO-induced relaxation paralleled their ability to affect the accumulation of cyclic GMP. Furthermore, we wished to elucidate the mechanism by which Cys and HoCys interfere with NO-induced relaxation.

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METHODS

Preparations of rabbit aortic rings and tension recording The preparation of rabbit aortic rings was similar to that described previously[11]. Some experiments were performed using aortic rings in which the endothelium was removed by rubbing the lumen with a wooden applicator stick for 30 – 60 s. The absence of endothelium in these rings was confirmed by the lack of relaxation to ACh after submaximal contraction to an agonist.

Tension was measured isometrically, using Grass FTO3C transducers, and was displayed on model 7 Grass polygraphs. Rings were allowed to equilibrate for at least 90 min before experiments were begun. Basal tension was maintained at approximately 2 g. Most experiments were carried out on sets of 4 rings from the same aorta. To allow studies on relaxation, each ring was precontracted submaximally (30 % – 70 % of maximum tone) by addition of *L*-phenylephrine (PE) 30 – 100 nmol · L^{-1} to the bathing solution. Results are expressed in % of relaxation of PE-induced tone.

Preparation of NO solutions Solutions of NO were prepared as previously described[10]. Briefly, a 125-mL gas sampling chamber (Kontes K-653100) was flushed for about 10 min with a flow of N_2 gas, and then for about 10 min with a flow of purified NO gas, which first passed through a scrubbing tower containing NaOH pellets to get rid of acid-generating material. Ten mL of nitrogenated anaerobic HCl/KCl solution 50 mmol · L^{-1} (pH 2.0) was injected from a syringe through a self-sealing rubber stopper and distributed with shaking over the inner surface of the chamber for a few minutes. It was then removed by syringe anaerobically through the stopper. Three more similar washes with 10 mL of the same solution was made to eliminate NO_2^-. The NO concentration in the final equilibrated solution was estimated to be close to 1.5 mmol · L^{-1} on the basis of the increment in NO_2^- plus NO_3^- formed from the NO present in the solution when the solution was exposed to oxygen[10].

Measurement of superoxide production Superoxide anion (O_2^-) production was determined by spectrophotometrically measuring the reduction of ferricytochrome C to ferrocytochrome C. The change in absorbance at 550 nm (the peak for ferrocytochrome C) was used to calculate the amount of cytochrome C reduced, using the difference in extinction coefficient at 550 nm of 2.1 m^2 · mol^{-1}, for conversion of the oxidized to the reduced form[11]. The difference in the amount of cytochrome C reduced in the absence and presence of superoxide dismutase (SOD) was taken as a measure of the amount of O_2^- produced[12]. A continuous assay for cytochrome C reduction by Cys and HoCys was carried out to clarify the onset, rate, and extent of ferricytochrome C reduction by these compounds. Twenty mL of ferricytochrome C were added to Krebs' solution in the presence and absence of SOD (35 kU · L^{-1}) at 37 ℃ bubbling with 95 % O_2/5 % CO_2 to give a final concentration of 15 μmol · L^{-1}. Then, Cys or HoCys (10 or 100 μmol · L^{-1}) was added to the above solution in a test tube, followed immediately by vortexing throughout the solution, and placing the solution into a disposable polystyrene cuvette for a continuous scanning in a spectrophotometer at 550 nm. The onset, rate, and extent of change of absorbance at 550 nm of ferricytochrome C on reduction by these SH compounds were continuously monitored in the presence and absence of SOD.

Measurement of SH group Stock standard solutions of Cys and HoCys were freshly prepared on the day of use by dissolving these compounds in distilled water, producing pH 3.5 – 4.5. The color reagent used for determinations of SH group was 2,2'-dithiobis-(5-nitropyridine) (DTNP), which is a sensitive reagent for SH determination over a broad pH range[13]. DTNP was dissolved in acetone (purity >99.5 %). An ethanol-phosphate buffer was prepared by mixing 250 mL of phosphate buffer (100 mmol · L^{-1}, pH 7.0) with 100 mL of distilled water, and then bringing the buffer volume up to 500 mL with absolute ethanol. The final pH of all the mixtures was about 7.5 for the determination. Absorbance was spectrophotometrically measured at 412 nm.

Stock solutions of Cys or HoCys (100 mmol · L^{-1}) were prepared in distilled water, and diluted to 100 μmol · L^{-1} with Krebs' solution, and then incubated in test tubes at 37 ℃. To observe changes in SH content with time under normal condition of O_2 supply or under anaerobic condition, these solutions were bubbled with 95 % O_2/5 % CO_2, or 95 % N_2/5 % CO_2. Aliquots were withdrawn at given intervals, and mixed with ethanol-phosphate buffer and color reagent DTNP for spectrophotometric reading. To compare changes in SH content after addition of NO in the presence and absence of O_2, the test tubes containing 10 mL of Cys (100 μmol · L^{-1}) in Krebs' solution were bubbled with 95 % O_2/5 % CO_2 or 95 % N_2/5 % CO_2. Twenty μL of saturated NO solution were directly added to the Krebs' solution with gentle shaking for 1 min, then 1 mL of the solution was withdrawn and mixed with ethanol-phosphate buffer and DTNP for determination of SH concentrations.

Determination of total SH groups in rabbit aorta A fresh rabbit aorta was cut into rings of 2.5 mm width, and trimmed free of connective tissue in a cooled dish. The rings were homogenized with Krebs' solution (60 g · L^{-1}) in a homogenizer precooled in ice. The ratios of tissue to homogenizing medium were chosen to obtain visible color and an absorbance of 0.1 to 0.8 when total SH groups were estimated. The homogenates were transferred to 2 mL polypropylene tubes and centrifuged at 27 000 × *g* at 4 ℃ for 15 min. Aliquots of 0.2 mL of the supernatant were transferred to 2 mL polypropylene tubes and mixed with 1 mL

of saturated NO (7.5 μmol·L^{-1}) solution, and then 0.6 mL of ethanol-phosphate buffer and 0.2 mL of DTNP 250 μmol·L^{-1} were added to the same tubes, followed by vortexing. A reagent blank (without supernatant) and a sample blank (without DTNP) were prepared. The final mixtures were pipetted into cuvettes, and the absorbance was read at 412 nm. The molar extinction coefficient at 412 nm of 1310 m^2·mol^{-1} was used to calculate SH content[15].

Measurement of cGMP level Endothelium-denuded aortic rings were suspended under 2 g of tension at 37 ℃ in 20-mL capacity water-jacketed chambers. At any time, these chambers could be instantly dropped down, leaving the preparation for immediate freezing in liquid N_2. From preliminary experiments time for freezing tissue and cGMP measurements were selected that corresponded to the period of the peak responses. For example, NO (75 nmol·L^{-1}) was added alone or after SH compounds for 2 min, Cys or HoCys 10 μmol·L^{-1} were added for 3 min. Frozen tissues were homogenized in ice-cold 6% trichloroacetic acid to give an approximately 10 % (wt/vol) homogenate. The homogenates were centrifuged at 2 000 · g at 4 ℃ for 15 min. The supernatant fractions were decanted off and the pellets were washed 4 times with 5 volumes of water-saturated ethyl ether, and the aqueous extracts were saved for assay for cGMP using enzyme-immunoassay kits (Amersham Inc, Amersham, UK), while the pellets were saved for protein determination. After the aqueous extracts were allowed to evaporate, the dried residues were dissolved in 1.2 mL of assay buffer containing 50 mmol·L^{-1} acetate buffer pH 5.8, 0.02 % (wt/vol) bovine serum albumin, and 0.005 % (wt/vol) thimerosal, and assayed for cGMP. A peroxidase-labelled cGMP (100 μL) was used to compete with the acetylated sample cGMP (50 μL) for a limited number of binding sites on the rabbit anti-cGMP serum (100 μL) at 4 ℃ for 1 h. The peroxidase ligand that is bound to the rabbit anti-cGMP was immobilized on polystyrene microtiter wells. After washing out any unbound ligand, the amount of peroxidase labeled cGMP bound to the rabbit anti-cGMP was determined at 450 nm after adding peroxidase substrate tetramethyl-benzidine 210 μL for 30 min and acidifying the reaction mixture with 110 μL of sulfuric acid 1 mol·L^{-1}. Protein precipitated by the trichloroacetic acid was measured[14].

Chemicals Pure NO gas was obtained from Liquid Carbonic Specialty Gas Co (Chicago IL, USA). ACh, *L*-cysteine hydrochloride (Cys), *L*-homocysteine (HoCys), phenylephrine hydrochloride (PE), cytochrome C type Ⅲ from horse heart, xanthine, xanthine oxidase, and superoxide dismutase from bovine erythrocytes were purchased from Sigma Chemical Co (St Louis MO, USA). 2,2′-Dithiobis (5-nitropyridine) was purchased from Aldrich Chemical Co (Milwaukee WI, USA).

Statistical analysis Results are expressed as $\bar{x} \pm s$. Statistical analysis was performed with t test for paired observations (two-tailed).

RESULTS

Inhibitions by Cys and HoCys on relaxations of rabbit aortic rings induced by NO and ACh. On PE-precontracted aortic rings denuded of endothelium, single dose (75 nmol·L^{-1}) of NO induced reproducible relaxations that were of rapid onset and transient duration (Fig 1).

Cumulative addition of Cys and HoCys attenuated relaxation induced by NO. The 2 compounds inhibited both duration of relaxation and peak relaxation responses to NO, with duration usually being inhibited more potently than peak

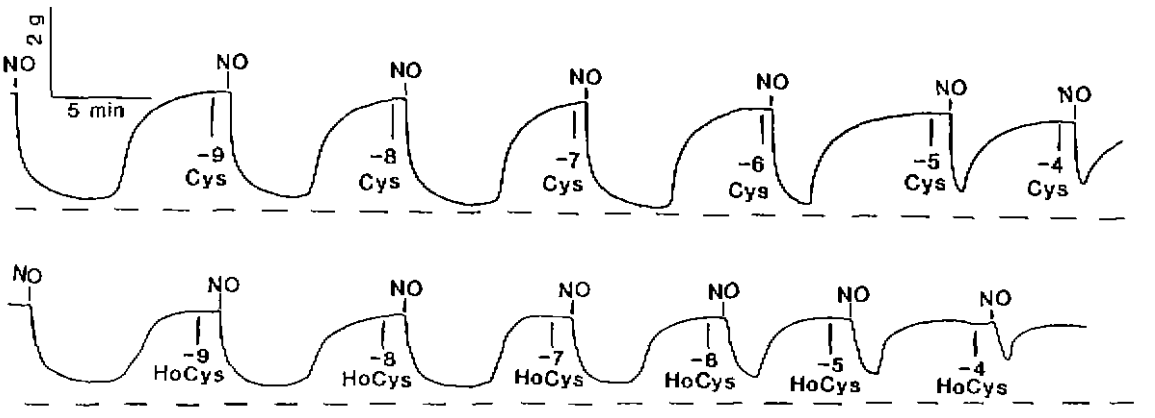

Fig 1. Inhibitory effects of Cys and HoCys on NO-induced relaxation in PE-precontracted rabbit aortic rings without endothelium. Dashed lines show level of basal tone before contraction. Concentrations are expressed as lg mol·L^{-1}. Tracings are typical of 6 experiments.

relaxation (Fig 1).

The concentrations required to shorten by 50 % the duration of relaxation induced by NO 75 nmol·L^{-1} were less for Cys than for HoCys. When the concentrations of Cys and HoCys were increased up to and above 100 μmol·L^{-1}, inhibition of NO-induced relaxation was obscured by the endothelium-independent component of relaxation produced directly by Cys and HoCys on vascular smooth muscle.

Pretreatment of the endothelium-denuded rings with SOD (15 kU·L^{-1}) markedly augmented relaxation induced by NO (75 nmol·L^{-1}), and then considerably attenuated the inhibition of the NO-induced relaxation by Cys (Fig 2), and HoCys (data not shown).

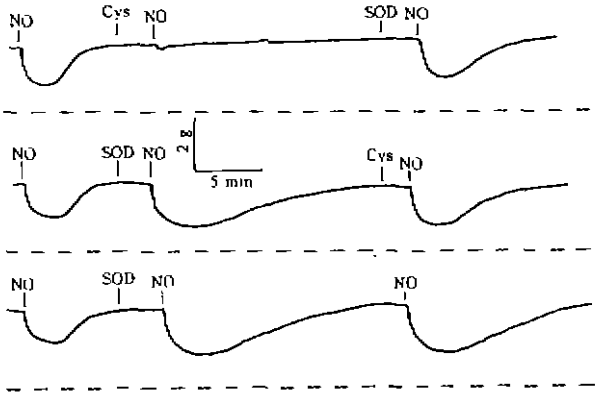

Fig 2. Effects of SOD on NO-induced relaxation. SOD 15 kU·L^{-1} potentiated relaxation induced by NO 15 nmol·L^{-1} (lower tracing). When Cys (10 μmol·L^{-1}) was present, the potentiation was partially curtailed (upper 2 tracings). Dashed lines show level of basal tone. Tracings are typical of 3 experiments.

However, even though SOD was able to reverse the inhibitory effects of Cys and HoCys on NO-induced relaxation, the duration of NO-induced relaxation in the presence of both Cys and SOD was not as prolonged as in the presence of SOD alone (Fig 2).

This inability of SOD to completely abrogate the inhibition of Cys on NO-induced relaxation is also apparent in the inhibition of HoCys on NO-induced relaxation (not shown). Increase in the amount of SOD (up to 35 kU·L^{-1}) did not produce significant change in its effects in this respect. In contrast to SOD, catalase (100 kU·L^{-1}) and boiled SOD did not prolong the relaxation induced by NO, nor antagonize the inhibitory effects of Cys (Fig 3).

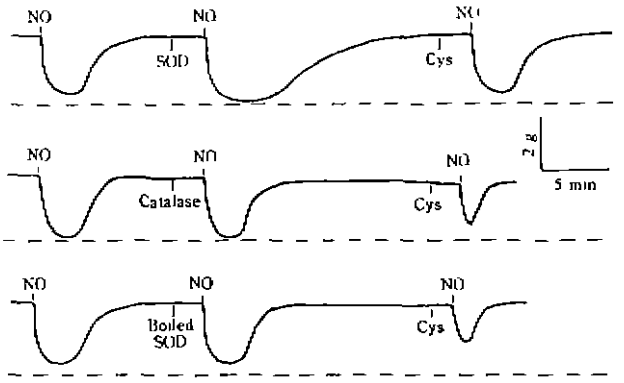

Fig 3. Effects of SOD, catalase, and boiled SOD on relaxation induced by NO 75 nmol·L^{-1} in the presence and absence of Cys (10 μmol·L^{-1}). Only fresh SOD (15 kU·L^{-1}) enhanced the relaxation and antagonized the inhibition by Cys. Catalase (100 kU·L^{-1}) and boiled SOD failed to do so. Dashed lines show level of basal tone. Tracings are typical of 3 experiments.

To evaluate how long the inhibition by Cys and HoCys of NO-induced relaxation lasts after a single addition to an organ chamber, the time-course of the inhibition was observed. With Cys or HoCys, the degree of inhibition of NO-induced relaxation was maintained essentially at the same level for 10 – 15 min after the single addition (Fig 4).

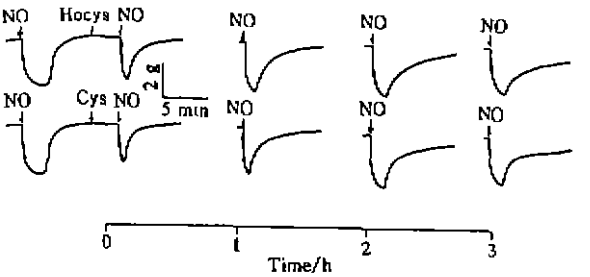

Fig 4. Inhibition by Cys and HoCys of relaxation induced by NO. Tracings of endothelium-denuded rings precontracted above the basal tone (dashed line) with phenylephrine show inhibition by the 2 compounds (10 μmol·L^{-1}) of relaxation induced by NO (75 nmol·L^{-1}) was diminished. The tracings represents 3 experiments.

The inhibitory effects of the 2 compounds were gradually diminished with time when Cys or HoCys was incubated with the aortic rings, which were

relaxed by single addition of NO (75 nmol·L^{-1}) every hour under the physiologic condition.

Incubations of intact rings of rabbit aorta with HoCys 100 μmol·L^{-1} for 4 h resulted in a loss of about half of the relaxation response to ACh as compared to control incubations without HoCys (Fig 5). But these abnormal relaxations to ACh of intact rings of aorta were not significantly shown in the presence of Cys 100 μmol·L^{-1}.

Effects of Cys and HoCys on the NO-induced cGMP content of aortic rings If the effectiveness of NO in relaxing vascular rings parallels its ability to stimulate the activity of guanylate cyclase, then pretreatment with Cys or HoCys (10 μmol·L^{-1}), which partially inhibit relaxation by NO, might affect the cGMP content stimulated by NO. Pretreatment of endothelium-denuded rings with Cys or HoCys (both 10 μmol·L^{-1}) reduced by almost 50 % the increase in the cGMP content stimulated by a 2-min exposure to NO (Tab 1).

Tab 1. NO-induced cGMP content of rabbit aortic rings after pretreatment with Cys and HoCys. $\bar{x} \pm s$. $^{*}P < 0.01$ *vs* NO alone.

Agents	n	Cyclic GMP (pmol·g protein)
Control	10	202 ± 123
NO	10	1 393 ± 158
Cys + NO	9	712 ± 123*
HoCys + NO	8	740 ± 107*

Continuous assay for cytochrome C reduction by Cys and HoCys For determining the time-course of reduction of cytochrome C by the two compounds, absorbance at 550 nm was monitored continuously after mixing the agents in solution in spectrophotometer cuvettes in the presence and absence of SOD. Since ferricytochrome C can be reduced by numerous compounds besides O_2^-, specificity for O_2^- was achieved by measuring not total cytochrome C reduction, but rather the SOD-inhibitable component of that reduction. Cys 100 μmol·L^{-1} reduced cytochrome C (60 μmol·L^{-1}) at higher rate, and the reduction of cytochrome C by Cys reached a steady state (plateau) in about 2 min. Addition of SOD 35 kU·L^{-1} before Cys inhibited the initial rate and the steady state level of cytochrome C reduction by Cys. A mixture of stock solution of SOD 7 000 kU·L^{-1} with Cys 20 mmol·L^{-1} produced cytochrome C reduction similar to the results obtained by respectively adding SOD and Cys to cytochrome C solution. However, the rate of reduction of cytochrome C induced by HoCys was much slower than with Cys, and the extent of the inhibition by SOD of cytochrome C reduction in the presence of HoCys was small (Fig 6). To evaluate the extent of O_2^- involvement in reduction of cytochrome C by Cys, additional experiments were performed. Increasing the concentration of

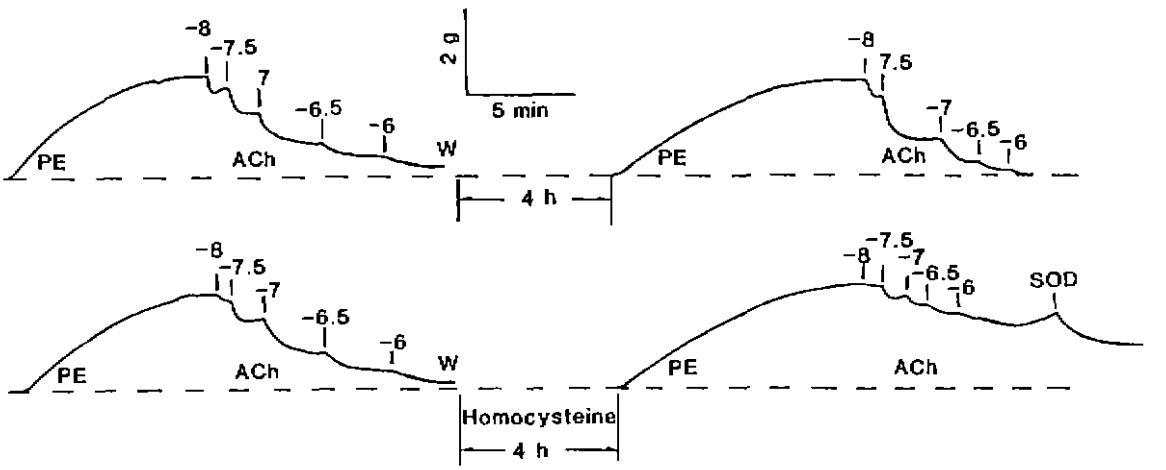

Fig 5. Effect of prolonged incubations of intact rings with HoCys on ACh-induced relaxation. Two rings from the same aorta were incubated in control (upper tracing) or HoCys 100 μmol·L^{-1} (lower tracing) for 4 h. Addition of SOD 35 kU·L^{-1} after incubation partially augmented ACh-induced relaxation of a dysfunctional aorta. Dashed lines show level of basal tone. Concentrations are expressed as lg mol·L^{-1}.

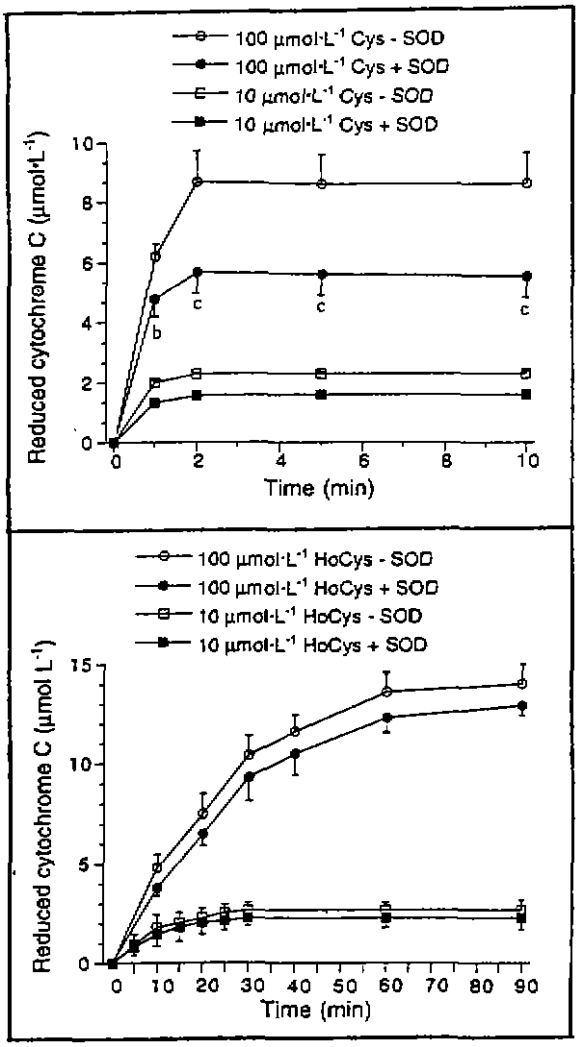

Fig 6. Reduction of cytochrome C (15 μmol·L⁻¹) by Cys or HoCys (10 or 100 μmol·L⁻¹). $\bar{x} \pm s$ of 4 tests. $^bP<0.05$, $^cP<0.01$ vs without SOD.

SOD from 35 to 350 (kU·L⁻¹) intensified the inhibition of cytochrome C reduction induced by Cys 100 μmol·L⁻¹. The SOD dose-dependence of the inhibition of cytochrome C reduction provided additional evidence that O_2^- was involved in cytochrome C reduction by Cys. However, even the very high concentration of SOD could not completely inhibit the cytochrome C reduction, suggesting some direct reduction of ferricytochrome C by Cys. This direct reduction was also proven by the results from experiments of incubation of Cys with cytochrome C bubbling with 95 % N_2/5 % CO_2. Under that condition Cys reduced cytochrome C at a slower rate to the levels close to those obtained under the condition of O_2 supply. The duration (min) from onset of cytochrome C reduction to the plateau was 4.8 ± 0.6 (n = 3), different from 2.2 ± 0.2 (n = 6, $P < 0.05$) obtained under aerobic condition. This result of ferricytochrome C reduction taking place with slower rate under anaerobic conditions was in agreement with observation by Saez's group[7].

To make sure that limiting concentrations of added cytochrome C (15 or 60 μmol·L⁻¹) were not responsible for the rapidly attained plateau of increase in absorbance direct addition of xanthine (300 μmol·L⁻¹) plus xanthine oxidase (1 U·L⁻¹), which are a well-documented combination for generating O_2^-, to the mixture of cytochrome C and Cys was made after attainment of the plateau. Such an addition succeeded in augmenting reduction further only in the absence of SOD (Fig 7).

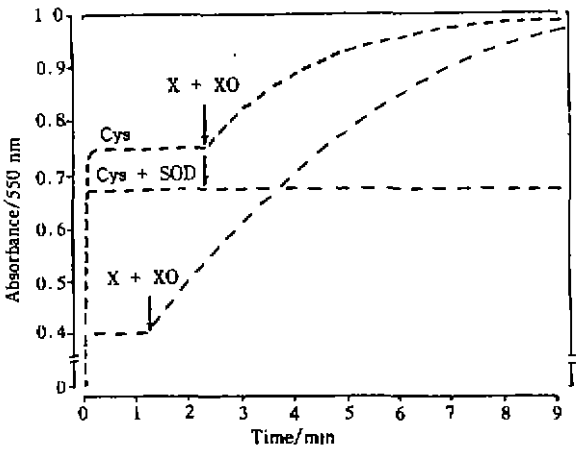

Fig 7. Cytochrome C (60 μmol·L⁻¹) reduction by Cys (100 μmol·L⁻¹) or xanthine (X, 300 μmol·L⁻¹) plus xanthine oxidase (XO, 10 U·L⁻¹) and SOD (35 kU·L⁻¹).

Also, in the absence of Cys, the same amount of xanthine plus xanthine oxidase induced reduction of cytochrome C only in the absence of SOD. In addition, when cytochrome C (60 μmol·L⁻¹) reduction by Cys (10 μmol·L⁻¹) reached the plateau, direct addition of 20 μL of Cys (final concentration 100 μmol·L⁻¹) to the cuvettes containing 1.5 mL of the reaction solution elicited an additional increase in absorbance at 550 nm (not shown). All these results suggest that, compared to xanthine plus xanthine oxidase, Cys has a limited ability to reduce cytochrome C, and that the reduction is only partly the result of O_2^- production.

Effects of NO on content of Cys Using the procedure outlined for preparation of standard curves for SH determination, a linear response between the absorbance reading and the corresponding concentrations of Cys was obtained up to a concentration of 50 $\mu mol \cdot L^{-1}$. When 20 μL of saturated NO (final concentration 3 $\mu mol \cdot L^{-1}$) was added to 10 mL of Krebs' solution containing Cys (final concentration 20 $\mu mol \cdot L^{-1}$) for 1 min under the condition of 95 % O_2/5 % CO_2, aliquots of 1 mL of the solution were withdrawn to determine the changes in SH concentration. The SH concentration of the aliquots was decreased in comparison with control aliquots withdrawn from control Cys solution added with 20 μL of a water medium of NO (Tab 2), regardless of supply with either O_2 or N_2. These results indicate a direct reaction of Cys with NO, which leads to decrease in Cys content.

Tab 2. Cys concentrations after addition of NO. $\bar{x} \pm s$. $^bP < 0.01$ *vs* **Group 1.** $^cP < 0.05$ *vs* **Group 3.**

Group	Cysteine +	n	SH concentration ($\mu mol \cdot L^{-1}$)
1	$O_2 + CO_2$	17	20.3 ± 0.4
2	NO, $O_2 + CO_2$	17	18.2 ± 0.4^b
3	$N_2 + CO_2$	12	20.5 ± 0.7
4	NO, $N_2 + CO_2$	12	17.0 ± 0.7^c

Effects of NO on total tissue SH groups Using the procedure recommended for total tissue SH groups determinations, yellow color was produced immediately after mixture of DTNP with the supernatant obtained from centrifugation of rabbit aortic homogenate. Neither the reagent blank nor sample blank produced visible color. A light milky turbidity developed sometimes, but did not appear to interfere with measurements of color[15]. Control SH content of aortic tissue was 2.42 ± 0.17 $\mu mol \cdot g^{-1}$ ($n = 12$); after addition of NO (2 μL) to the supernatant of aortic homogenate (200 μL), the SH content decreased to 2.20 ± 0.16 $\mu mol \cdot g^{-1}$ ($n = 12$; $P < 0.05$), and 2.21 ± 0.18 $\mu mol \cdot g^{-1}$ ($n = 12$; $P < 0.05$) in the absence and presence of SOD, respectively.

Concentrations of Cys and HoCys in aqueous solution The changes in the concentration of various Cys and HoCys that occurred with time are shown in Fig 8.

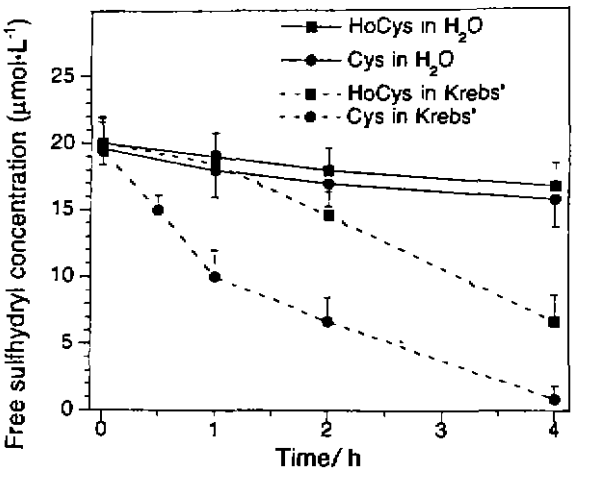

Fig 8. SH concentrations with Cys and HoCys (both 20 $\mu mol \cdot L^{-1}$) were incubated with 95 % O_2/5 % CO_2 in distilled water (pH 4 – 5, solid line), or Krebs' solution (pH 7.4, dashed line). $x \pm s$ **of 4 tests.**

The least change in SH content occurred when these compounds were incubated at 37 ℃ in distilled water, pH 4 – 5. Under these conditions, the concentration of these compounds decreased only 6 % – 12 % during 4 h. However, when these two compounds were incubated in Krebs' solution (pH 7.4), their concentrations decreased more rapidly. After the entire 8-h observation period, a mixture of DTNP with aliquots of Krebs' solution containing 20 $\mu mol \cdot L^{-1}$ of Cys or HoCys did not produce visible color. The slow loss of the SH concentration of the 2 compounds in oxygenated Krebs' solution is consisted with the slow loss of their inhibitory action against NO-induced relaxation of aortic rings over long periods after single additions of the SH compounds in organ chamber experiments (Fig 4).

DISCUSSION

In the present work, we demonstrated that Cys and HoCys partially inhibited the transient relaxation of endothelium-denuded rings of rabbit aortas produced by single additions of NO (75 $nmol \cdot L^{-1}$) to the Krebs' solution. These findings of dose-dependent inhibition of NO-induced relaxation by Cys and HoCys from 0.1 – 100 $\mu mol \cdot L^{-1}$, are consistent with the inhibitory

properties of other low molecular weight thiols, namely glutathione and dithiothreitol[10]. The remarkable similarity in the inhibitory potency among these SH compounds further bespeaks their similar physiochemical properties and a basic mechanism of action due to their common SH group. The degree of inhibition at concentrations of >100 $\mu mol \cdot L^{-1}$ could not be accurately evaluated because of the direct relaxing effect of the SH compounds at high concentrations on the vascular smooth muscle[10].

It is of interest to note that inhibition by these 2 SH-containing amino acids (10 $\mu mol \cdot L^{-1}$) of the NO-induced relaxation of rabbit aortic rings occurs with a concomitant decrease in NO-stimulated cGMP content by about 50 % (Tab 1). These results support the possibility that interaction of the 2 compounds with NO causes a decrease in the concentration of NO, which leads to less stimulation of guanylate cyclase and then less increase in cGMP content in the vascular smooth muscle.

The interaction between NO, vascular endothelium and these SH compounds was further explored in detail. The fact that the inhibition of NO-induced relaxation by Cys and HoCys could be considerablly attenuated by SOD suggests that O_2^- may be involved in this inhibition (Fig 2). In contrast, neither boiled SOD nor catalase (100 $kU \cdot L^{-1}$) could be substituted for SOD in antagonizing the inhibitory effects of Cys 10 $\mu mol \cdot L^{-1}$ on NO-induced relaxation (Fig 3). It might therefore be explained so far that O_2^- generated in the Krebs' solution somehow by the autoxidation of the compounds, and not H_2O_2 formed by dismutation of O_2^-, is partially responsible for their inhibitory action against NO in the absence of SOD.

Nevertheless, the rate of reduction by an equivalent amount of HoCys was not significantly affected by SOD and hence did not significantly proceed via production of O_2^- (Fig 6). It should be emphasized that in all experiments with these 2 compounds, a large part of the reduction of cytochrome C was not inhibitable by SOD, indicating that they were capable of directly reducing cytochrome C. This possibility was confirmed in experiments of cytochrome C reduction by Cys with increasing amounts of SOD. The reduction of cytochrome C was not completely inhibitable even when the amount of SOD was increased to 350 $kU \cdot L^{-1}$ (not shown). In contrast, SOD 35 kU $\cdot L^{-1}$ completely inhibited cytochrome C reduction by the combination of xanthine plus xanthine oxidase (Fig 7), which is a well-documented O_2^- generator. Further evidence supporting SH compounds being able to directly reduce cytochrome C was obtained from experiments involving Cys-induced cytochrome C reduction under anaerobic condition (95 % N_2/5 % CO_2). Under this condition, Cys was found to cause considerable reduction of cytochrome C, although at somewhat slower rate than under aerobic condition (not shown). This finding is in good accord with the report by Saez *et al*[7].

These results suggest a direct reduction of cytochrome C by SH compounds or by the ionized thiol, which would not be inhibited by SOD. In addition, these SH compounds have limiting ability to reduce cytochrome C when compared to xanthine plus xanthine oxidase (Fig 7). The difference between xanthine plus xanthine oxidase and SH compounds in reducing cytochrome C may depend on the difference in the standard reduction potentials of O_2/O_2^- (-0.45 V) as compared with the SH compounds (around -0.25 V).

In addition to O_2^--dependent inhibition of NO-induced relaxation, SH compounds may possess an O_2^--independent component that plays a role, in part, in inhibiting such relaxations. Such an O_2^--independent component would account for the partial reduction by Cys of SOD-potentiated relaxation by NO (Fig 2,3). It would also help explain why the SOD-potentiated relaxation by NO 75 $nmol \cdot L^{-1}$ was partially curtailed by Cys (Fig 2). This O_2^--independent component of inhibition of NO-induced relaxation is likely due to direct reaction between SH groups and NO. This possibility was supported by the results from reactions of NO with Cys (Tab 2), which indicated a decrease in the concentration of SH content after addition of NO under both O_2 and N_2 supply. Pryor *et al*[16] showed that SH are readily oxidized to disulfides by NO or NO_2 at about pH 7 and that the oxidations were inhibited only when pH was reduced to <4. Their observation

appears to rule out direct H atom abstraction from the thiol by NO, which is energetically unfavorable in any case. Furthermore, reaction between NO and Cys to form S-nitrosocysteine under our experiment condition is unlikely to account for the inhibition of the relaxant effect of NO, since S-nitrosocysteine leads to enhanced relaxation of the aortic ring, and this phenomenon of prolonged relaxation due to formation of S-nitrosocysteine was not seen in the present study at all, indicating that S-nitrosocysteine was unlikely to form in effective amounts to produce relaxation when Cys was added up to 100 $\mu mol \cdot L^{-1}$. The additional mechanism by which SH compounds exhibited their O_2^--independent component to inhibit the relaxant effects of NO may be due either to direct trapping of NO or to conversion of the free radical form NO to another reduction state NO^- and N_2O, with accompanying loss of biological activity:

$$2RSH + 2NO \rightarrow 2H^+ - 2NO^- + RSSR$$

$$2H^+ + 2NO^- \rightarrow H_2O + N_2O$$

The chemical stability of SH compounds was affected as the media pH was raised (Fig 8). These results are in agreement with those of Sedlak and Lindsay[15], Misra[6], and Pryor *et al*[16], who found that thiols at pH 7.4 were much more labile than at pH 4.7. Concentrations of Cys and HoCys gradually decreased with time when they were incubated at pH 7.4 (Fig 8). That decrease may account for the gradual diminution of inhibition by the 2 compounds of NO-induced relaxation over an extended period after a single addition of the compounds in organ chamber experiments (Fig 4). A rational explanation for influence of pH on chemical stability of thiol could be related to the ionization of the SH groups under the condition of pH 7.4, and a single electron transfer from the thiol anion (RS^-) to O_2 may result in formation of O_2^- and thiol free radicals, both of which have been found during the oxidation of SH compounds[17], and finally the production of oxidized thiols. Hence, higher pH media could favor the thiol autoxidation, and speed up the decline in concentration of reduced thiols.

Also, it was found that the exposure of endothelium-intact rings of aorta to HoCys for about 4 h inhibited about one-half the relaxation response to ACh as did rings incubated with time controls. Since addition of SOD (Fig 5) or catalase (100 $kU \cdot L^{-1}$, not shown) could only partially attenuate such incompleted relaxation to ACh, and Cys was not as potent as HoCys in this respect, our results suggest the specific damage to vascular endothelium and its product EDRF caused by HoCys. This HoCys-induced dysfunctional endothelium may release inadequate amounts of vasodilators such as EDRF and epoprostenol, thereby enhancing vasoconstraction of the vascular wall. It is interesting to speculate that, based on the biochemical interactions of NO and SH group found in this study, treatment of homocysteinemia patients with NO donors may be a reasonable therapeutic approach[3] which may trans-nitrosylate HoCys, rendering it nontoxic to the cardiovascular system.

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半胱氨酸和高半胱氨酸对一氧化氮诱发兔主动脉舒张的抑制作用

R979.3

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关键词 主动脉；内皮获得性松弛因子；半胱氨酸；高半胱氨酸；一氧化氮；苯福林；超氧化物歧化酶；黄嘌呤；黄嘌呤氧化酶；细胞色素 c

目的：研究半胱氨酸(Cys)和高半胱氨酸(HoCys)对一氧化氮(NO)诱导的动脉舒张的抑制作用的机制. **方法**：用 Cys 或 HoCys 对去内皮细胞血管环预处理，观察张力变化. **结果**：Cys 或 HoCys 抑制 NO 诱导的 cGMP 增加. 加入 Cys 或 HoCys，再加入 NO 75 nmol·L^{-1}，抑制血管舒张的效应随时间逐渐消失，与此二化合物在 Krebs' 液中巯基的丢失一致. 超氧化物歧化酶(SOD) 35 kU·L^{-1} 抑制但不完全阻断 Cys 和 HoCys 对 NO 诱导的血管舒张的抑制作用. 相反，加热失活的 SOD 和过氧化氢酶(100 kU·L^{-1})均不拮抗 Cys 的抑制作用. 在 Krebs' 液中预先加入 SOD 35 kU·L^{-1} 显著抑制 Cys 诱导的细胞色素 C 的还原. SOD 浓度增至 350 kU·L^{-1}，效应虽增强，但不能完全抑制细胞色素 C 的还原. 直接将黄嘌呤 300 μmol·L^{-1} 和黄嘌呤氧化酶 1 U·L^{-1}加入细胞色素 C 60 μmol·L^{-1}和 Cys 100 μmol·L^{-1}的混和物仍可见 SOD 对细胞色素 C 还原的抑制作用，提示 Cys 降低细胞色素 C 能力有限，且部分由于产生超氧化物所致. HoCys 100 μmol·L^{-1}诱导的细胞色素 C 的还原速率比 Cys 慢. 在主动脉匀浆和含 Cys 的 Krebs' 液中加入 NO 均能降低其中的巯基浓度. **结论**：巯基化合物对 NO 的抑制作用部分由这些化合物自氧化产生的超氧化物所介导，部分为巯基与 NO 的直接作用.