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BIOCHEMICAL AND PHARMACOLOGICAL INTERACTIONS BETWEEN NITROGLYCERIN AND THIOLS

EFFECTS OF THIOL STRUCTURE ON NITRIC OXIDE GENERATION AND TOLERANCE REVERSAL

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Abstract—Co-administration of *N*-acetylcysteine (NAC) with nitroglycerin (NTG) has been shown to partially reverse nitrate tolerance and to potentiate the hypotensive effect of NTG in humans. However, a high clinical dose of NAC was required for this pharmacologic interaction resulting in the production of unwanted side-effects. Therefore, sulfhydryl compounds more active than NAC need to be identified if this interaction is to be exploited clinically. We previously suggested that the effect of sulfhydryl compounds on NTG may be mediated by the formation of *S*-nitrosothiol or nitric oxide (NO) extracellularly to the vascular smooth muscle cell (e.g. in plasma) (Fung *et al.*, *J Pharmacol Exp Ther* **245**: 524–530, 1988). In an attempt to understand the structural features which govern this thiol-catalyzed NO generation from NTG, nineteen different aliphatic and ten aromatic sulfhydryl compounds were examined with respect to their catalytic activity to generate NO from NTG in plasma. Significantly enhanced production of NO was observed with most sulfhydryl compounds examined when compared to buffer control. Among the aliphatic thiols, only mercaptosuccinic acid was more potent than NAC (2×), whereas among the aromatic thiols, both thiosalicylic acid (TSA, 10×) and TSA-methyl ester (3×) were more potent than NAC. Comparative *in vitro* relaxation studies were carried out using isolated (and nitrate-tolerant) rat aortic rings with NTG/TSA and NTG/NAC, in the presence of 0.5% (v/v) plasma. Under these conditions, partial reversal of NTG tolerance could be achieved with TSA, but not with NAC. These data are consistent with the view that extracellular production of NO or *S*-nitrosothiol serves as a tolerance-reversing mechanism of thiols on NTG. TSA appears to be a more potent sulfhydryl compound than NAC in this biochemical and pharmacologic interaction.

Continuous administration of nitroglycerin (NTG†) via the intravenous or transdermal route is commonly associated with the development of tolerance (i.e. attenuation of vasodilating effect) in patients with either chronic stable angina [1, 2] or congestive heart failure [3, 4]. Current hypotheses of organic nitrate action propose that organic nitrates must undergo metabolic activation [i.e. production of nitric oxide (NO) via denitration] before inducing vascular smooth muscle relaxation [5]. During this metabolic activation process, free sulfhydryl groups in the vascular smooth muscle have been suggested as a necessary cofactor. Depletion of this intracellular sulfhydryl pool during prolonged exposure to organic nitrates has been suggested to be the underlying mechanism for tolerance development [6]. Recently, however, *in vitro* [7–9] and *in vivo* [10–12] evidence has appeared in the literature which questioned the determinant role of the sulfhydryl pool in governing nitrate tolerance. A summary addressing the positive and negative evidence regarding the "sulfhydryl depletion hypothesis" has appeared [13]. The conflicting results have not been successfully reconciled since the studies reported involved different drugs, doses, preparations (e.g. species and vascular tissues), as well as conditions of study.

Nevertheless, co-administration of the sulfhydryl donor, *N*-acetylcysteine (NAC), has been demonstrated to partially reverse nitrate tolerance in patients [10, 14], and to potentiate the hypotensive effect of NTG in humans [15] and in conscious rats [16]. We have examined the mechanism of this thiol–nitrate interaction and have shown that NTG degradation in the presence of NAC resulted in the production of a pharmacologically active *S*-nitrosothiol (and possibly nitric oxide) in plasma [16]. Extracellular production of *S*-nitrosothiol or NO, therefore, was postulated as a possible mechanism for the beneficial pharmacologic interaction between NAC and NTG. If the interaction between NTG and sulfhydryl compounds in the extracellular space (e.g. plasma) plays an important role in reversing nitrate tolerance, then thiols with a higher catalytic activity than NAC might be better suited to improve nitrate therapy. In searching for such thiols, it is desirable to understand the structural

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† Abbreviations: CYST, L-cysteine; GDN, glyceryl dinitrate; LFER, linear free energy relationship; MSA, mercaptosuccinic acid; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; NTG, nitroglycerin; PE, phenylephrine; RCD, redox-chemiluminescence detector; RSH, reduced sulfhydryl compounds; SOD, superoxide dismutase; TM, thiosalicylic acid-methyl ester; and TSA, thiosalicylic acid.

features which govern the thiol-catalyzed NO generation from NTG.

The primary objective of this report, therefore, was to examine the catalytic activity of various aliphatic and aromatic sulfhydryl compounds toward NTG degradation and NO production in plasma. The thiols chosen were primarily structural analogs of either NAC or thiosalicylic acid, both of which have shown substantial interaction with NTG. The extent of NO generation from such an interaction can be viewed as a potential index of the ability of the thiol to reverse nitrate tolerance. *In vitro* pharmacologic validation of this hypothesis was carried out with two selected thiols.

MATERIALS AND METHODS

Materials. NTG aqueous solution [Perlinganit®, 1.04 mg/mL in 5% (w/v) dextrose solution] was obtained from Pharma-Schwarz GmbH (Monheim, West Germany). Human plasma was obtained from the American Red Cross (Buffalo, NY). Superoxide dismutase (SOD), *N*-acetyl-L-cysteine (NAC), L- and D-cysteine, methyl- and ethyl-esters of cysteine, glutathione, dithiothreitol, cysteamine, dimethylcysteamine, penicillamine, *N*-acetyl-DL-penicillamine, thioglycolic acid, mercaptoethanol, homocysteine, mercaptopropionic acid, mercaptosuccinic acid and dimercaptosuccinic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). Thiosalicylic acid (TSA), 2-aminothiophenol, thiophenol, 4-hydroxythiophenol, *o*-thiocresol, 2-bromothiophenol, 2-methoxybenzenethiol, benzylmercaptan and 1,2-benzenedithiol were obtained from the Aldrich Chemical Co. (Milwaukee, WI). *S,S*- and *R,S*-Captopril were gifts from E. R. Squibb & Sons, Inc. (Princeton, NJ). Methionine, oxidized glutathione, *N*-acetylserine and salicylic acid (as controls with no free thiol group), and phenylephrine hydrochloride were also purchased from the Sigma Chemical Co. TSA-methyl ester was synthesized by heating a TSA/methanol mixture with a few drops of concentrated sulfuric acid at 30° overnight. The mixture was then evaporated to dryness at room temperature under reduced pressure and the residue re-dissolved in ethyl acetate. After washing with a saturated solution of sodium bicarbonate, the organic layer was separated and evaporated under reduced pressure. The UV (254 nm) absorbing fraction was separated using a silica-gel column and the structure of TSA-methyl ester was confirmed by NMR and i.r. [^{14}C]NTG (courtesy of G. D. Searle & Co., Chicago, IL; sp. act. 47.5 mCi/mmol) was purified (99.5%) using a thin-layer chromatographic procedure described previously [17]. All glassware used in this experiment was acid-washed and silanized to prevent NTG adsorption [18].

Quantitation of nitric oxide generation. NO generated from the interaction between NTG and sulfhydryl agents in human plasma was quantified via direct injection of the headspace sample into a Redox Chemiluminescence Detector (RCD) specific for NO (model 207B, Sievers Research, Boulder, CO) [19]. The detector response was calibrated by injecting various volumes of a NO standard (10.5 ppm in nitrogen, Linde Specialty Gases, Hamburg, NY) directly into the detector via a 10-mm teflon-faced microseptum (Supelco Inc., Bellefonte, PA). The amount of NO injected into the RCD was calculated using the ideal gas law and ranged from 4.32 to 21.61 pmol/injection during the calibration procedure. The RCD response (expressed in terms of peak area) was linearly correlated ($r > 0.99$) with the injected amount of NO. The lowest detection limit under the condition employed was about 1.0 pmol NO/injection. Typically, the intra-sample variation was less than 10% over the range of the calibration curve.

Nitric oxide generation from nitroglycerin and sulfhydryl compounds in various incubation media. The effects of various aliphatic and aromatic sulfhydryl compounds on the generation of NO from NTG degradation were examined in human and rat plasma, and also in phosphate buffer (pH 7.4, 0.1 M). Aliquots of 2.0 mL of incubation medium were transferred into air-tight incubation vials (total volume of 5.8 mL) and preincubated for 10 min at 37°. Aliquots of stock solutions of superoxide dismutase (SOD, 100 μL), thiol (50 μL) and NTG (200 μL) were then added to the incubation vial. Phosphate buffer (pH 7.4, 0.1 M) was used to prepare the stock solutions of SOD and aliphatic thiols. Due to the inadequate aqueous solubility of aromatic thiols, ethanol was used to prepare the stock solution of aromatic thiols. Preliminary experiments showed that the presence of a small volume of ethanol (50 μL) in the incubation medium did not affect the production of NO from NTG when NAC was used as a thiol source (data not shown). The presence of SOD was essential since no significant amount of NO could be detected (due to rapid NO degradation) in the headspace of the incubation vial when SOD was not present. The final concentrations of SOD, thiol and NTG in the incubation mixture were 102 units/mL, 996 μM and 196 μM, respectively. These concentrations were selected based on preliminary experiments, and they represented convenient concentrations at which the differential effects of various thiols could be clearly detected. These mixtures were then incubated, and mixed by means of a teflon stirring bar, at 37° for 2 hr; headspace samples were withdrawn periodically for the quantitation of NO.

Effect of heat pretreatment on the production of nitric oxide in human plasma. To examine whether the catalysis of NO production from NTG and thiols was enzymatically mediated by plasma proteins, the above experiments were repeated with human plasma which was previously heated at 65° for 60 min. A representative aliphatic (NAC) thiol and aromatic (TSA) thiol were chosen in this examination.

Effects of various sulfhydryl compounds on in vitro *nitroglycerin degradation in human plasma.* The effects of NAC, L-cysteine, TSA, TSA-methyl ester and mercaptosuccinic acid on the rate of NTG degradation and the formation of the dinitrate metabolites (GDN) were examined in human plasma. The incubation mixture, with respect to composition and concentration, was identical to that used in the NO generation experiments except that ^{14}C-labeled NTG was also added to the medium. The final concentration of ^{14}C-labeled NTG in the incubation

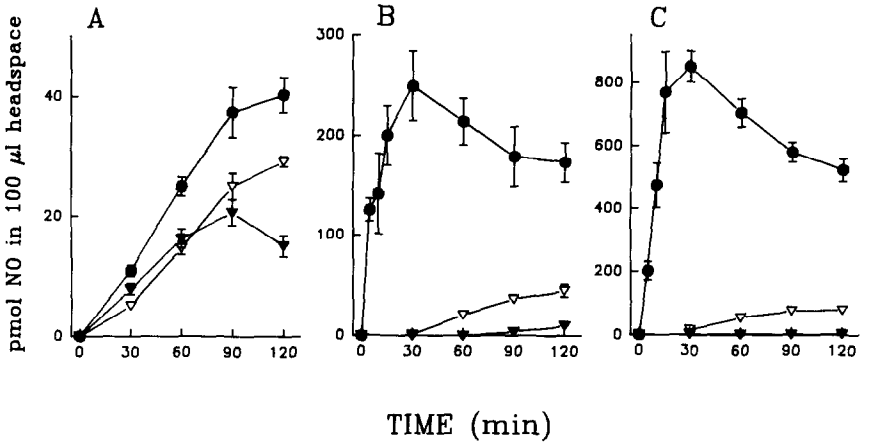

Fig. 1. Production of NO from thiol-NTG interaction in (A) phosphate buffer (0.1 M, pH 7.4), (B) human plasma and (C) rat plasma. Key: (●) TSA, (∇) NAC, and (▼) L-cysteine. Apparent headspace/solution distribution ratios of NO in plasma and phosphate buffer were approximately 0.05 and 4, respectively. Values are means ± SD (N = 3).

mixture was 0.22 μM. The mixture was incubated at 37° for 2 hr, and aliquots (200 μL) of plasma sample were withdrawn periodically using a gas-tight syringe through a teflon-faced septum. Each plasma sample was immediately treated with a 3-mL aliquot of cold methanol and 50 μL of 0.1 M silver nitrate. After collecting the supernatant, the residue was further extracted twice with 2-mL aliquots of methanol. The pooled methanol extracts were stored at −20° until assayed. The concentrations of NTG and the metabolites were determined after thin-layer chromatographic separation and liquid scintillation counting [17].

Effects of sulfhydryl compounds on NTG tolerance in the presence of rat plasma. The relaxation experiments were conducted according to Fung *et al.* [16] Segments of abdominal aorta were obtained from male Sprague–Dawley rats weighing 300–325 g. After equilibration, NTG was added to the tissue baths (to a final concentration of 0.22 mM), and incubated for 1 hr. Control ring segments were incubated in the bathing medium with vehicle alone for the same duration. At the end of incubation period, aortic rings were washed 8–10 times over 30 min with Krebs buffer. Ring segments were then contracted with phenylephrine (PE, 2 10^{-6} M at which approximately 80% of the maximum contraction was observed), and when the contraction plateaued, 100 μL of a mixture containing NTG and thiol (in a vehicle of equal volumes of rat plasma and 5% dextrose, w/v) was added to the bathing medium. The final NTG and thiol (either TSA or NAC) concentrations were 8×10^{-6} and 8×10^{-5} M, respectively. Results are reported in percentage of relaxation of phenylephrine-induced tone.

Data analysis. The area under the headspace NO versus time curve (AUC) was calculated from 0 to 120 min by the linear trapezoidal method. $P < 0.05$ was accepted as denoting statistical significance. Data are expressed as means ± SD, unless otherwise indicated.

RESULTS

Nitric oxide generation from nitroglycerin and sulfhydryl compounds. The biochemical interaction between NTG and various aliphatic and aromatic thiols led to the production of NO which could be detected from the headspace of incubation vials. Shown in Fig. 1 are the time-dependent NO-generating profiles of three selected thiol–NTG systems (thiol = TSA, NAC or L-cysteine). Comparison between results in panel A versus those in panels B and C shows that the catalytic activities of these thiols were much greater in human and rat plasma than those in phosphate buffer (note the differences in the y-scales). For the most active thiol, viz. TSA, the peak NO concentration observed was about 40, 250, and 850 pmol/100 μL of headspace in buffer, human and rat plasma, respectively.

Tables 1 and 2 show, respectively, the NO-generating activities of nineteen aliphatic thiols and ten aromatic thiols when each was individually incubated with NTG in human plasma. Several non-thiol compounds were included for comparison. Data in Table 1 suggested that both L- and D-cysteine were considerably less active (0.1×) than NAC, while the L-cysteine esters were more catalytic than their parent amino acids, but only about half as potent as NAC. Among the aliphatic thiols examined, only mercaptosuccinic acid showed a higher (2×) catalytic activity than NAC. Compounds that contained no free thiol group, viz. methionine, oxidized gluthathione, and *N*-acetylserine, were unable to generate detectable NO *in vitro*. Results in Table 2 show that both TSA (10×) and TSA-methyl ester (3×) exhibited higher potency than NAC in catalyzing NO production from plasma. Again, non-thiol related compounds, such as salicylic acid, did not generate detectable NO from NTG.

A linear free energy relationship (LFER) was used to correlate the NO-generating activity from various aromatic thiols. The AUC values reported

Table 1. NO generation from NTG and aliphatic thiols in human plasma*

Thiol added (N)†	$AUC^{0-120\,min}$‡ (pmol min)	Relative area (NAC = 1)
None (3)	ND§	
S,S-Captopril (7)	526 ± 65‖	0.21
R,S-Captopril (3)	560 ± 38	0.22
Cysteamine (4)	115 ± 16	0.05
Dimethylcysteamine (3)	702 ± 55	0.28
L-Cysteine (9)	274 ± 102	0.11
D-Cysteine (3)	329 ± 60	0.13
L-Cysteine methyl ester HCl (3)	1,455 ± 195	0.58
L-Cysteine ethyl ester HCl (3)	1,182 ± 225	0.47
N-Acetyl-L-cysteine (14)	2,508 ± 265	1.00
Homocysteine (3)	1,000 ± 149	0.40
Penicillamine (3)	ND	
N-Acetyl-DL-penicillamine (3)	658 ± 90	0.26
Dithiothreitol (4)	2,369 ± 461	0.94
Thioglycolic acid (3)	317 ± 33	0.13
Mercaptoethanol (3)	100 ± 17	0.04
Mercaptopropionic acid (3)	433 ± 17	0.17
Mercaptosuccinic acid (3)	5,786 ± 341	2.31
Dimercaptosuccinic acid (3)	ND	
Reduced glutathione (4)	615 ± 34	0.25
Oxidized glutathione (4)	ND	
Methionine (4)	ND	
N-Acetylserine (3)	ND	

* The initial concentrations of NTG and thiol were 196 and 996 μM, respectively.
† N = number of runs.
‡ $AUC^{0-120\,min}$ values of all aliphatic thiols tested, except for dithiothreitol, were statistically different compared to that of NAC ($P < 0.001$).
§ ND = not detected.
‖ Values are means ± SD.

Table 2. NO generation from NTG and aromatic thiols in human plasma*

Thiol added (N)†	$AUC^{0-120min}$‡ (pmol min)	Relative area (NAC = 1)
Thiophenol (3)	555 ± 70§	0.22
2-Bromothiophenol (3)	1,056 ± 30	0.42
o-Thiocresol (3)	690 ± 33	0.28
2-Methoxybenzenethiol (3)	96 ± 11	0.04
2-Aminothiophenol (3)	ND‖	
Thiosalicylic acid (8)	23,102 ± 2,970	9.21
Methylthiosalicylic acid (3)	7,734 ± 190	3.08
4-Hydroxythiophenol (3)	35 ± 20	0.01
Benzylmercaptan (3)	413 ± 46	0.16
1,2-Benzenedithiol (3)	ND	
Salicylic acid (3)	ND	

* The initial concentrations of NTG and thiol were 196 and 996 μM, respectively.
† N = number of runs.
‡ $AUC^{0-120min}$ values of all aromatic thiols tested were statistically different compared to that of NAC ($P < 0.001$).
§ Values are means ± SD.
‖ ND = not detected.

in Table 2 were correlated with the Hammett's sigma (σ) and the fractional partititon coefficient (π) parameters [20]. The best correlation found: AUC = 11713 σ − 4579 π + 2528 was statistically significant ($N = 7$, $r^2 = 0.97$ and $P < 0.001$).

Effect of heat pretreatment on the production of nitric oxide in human plasma. Figure 2 shows that heat pretreatment substantially reduced the NO-generating activities of both TSA and NAC. The apparent reduction was much more pronounced with TSA (73% reduction) than with NAC (43% reduction). However, even after heat pretreatment,

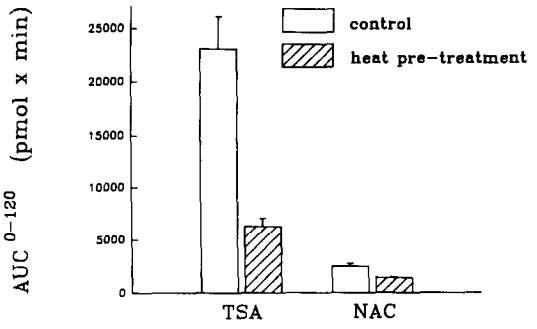

Fig. 2. Effect of heat pretreatment on the production of NO from the thiol–NTG interaction in human plasma. Values are means ± SD (N = 3). Statistical significance: $P < 0.05$ control vs heat pretreatment.

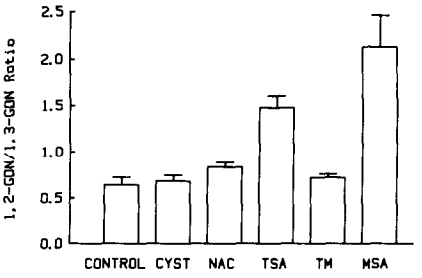

Fig. 4. Metabolite ratio (1,2-GDN/1,3-GDN) obtained from various thiol–NTG interaction studies in human plasma. Key: CYST, L-cysteine; NAC, *N*-acetylcysteine; TSA, thiosalicylic acid; TM, TSA-methyl ester; MSA, mercaptosuccinic acid. Values are means ± SD (N = 6–10).

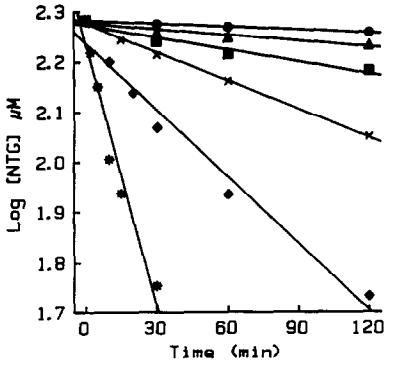

Fig. 3. Degradation of NTG in the presence of thiols in human plasma. Experimental data points were average values of duplicate experiments. The solid lines represent linear-regressed lines. Key: (*) TSA, (◆) TSA-methyl ester, (×) mercaptosuccinic acid, (■) NAC, (▲) L-cysteine, and (●) control, no RSH.

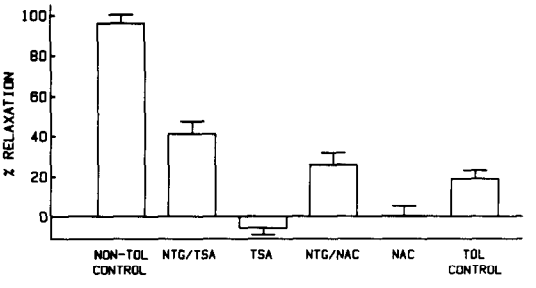

Fig. 5. *In vitro* relaxation of rat abdominal aortas subjected to various treatments of thiol alone (TSA and NAC) and thiol–NTG combinations (NTG/TSA and NTG/NAC). Data are means ± SEM (N = 6–12).

TSA was still more potent (4) than NAC in catalyzing NO production from NTG in plasma.

Kinetics of in vitro *nitroglycerin degradation and dinitrate metabolites formation in human plasma in the presence of sulfhydryl compounds.* Figure 3 shows the kinetic profiles of *in vitro* NTG degradation in human plasma in the presence of various thiols. The rate process in all cases could be described by apparent first-order kinetics. Consistent with the observation that TSA showed the greatest NO-generating potency, NTG degradation in the presence of TSA in human plasma was substantially faster ($T_{\frac{1}{2}} = 13$ min) than that of control (no thiol, $T_{\frac{1}{2}} = 22$ hr) and other thiols (e.g. TSA-methyl ester, $T_{\frac{1}{2}} = 1.1$ hr; mercaptosuccinic acid, $T_{\frac{1}{2}} = 2.7$ hr; NAC, $T_{\frac{1}{2}} = 6.2$ hr; L-cysteine, $T_{\frac{1}{2}} = 12.9$ hr). It is also interesting to note that the metabolite ratio of 1,2- to 1,3-GDN generated during the catalysis was different among the different thiol catalysts added (Fig. 4). Generally, the 1,2-GDN/1,3-GDN ratio increased in the presence of thiol; however, the magnitude of the increase was not related to the catalytic potency of the thiols.

Effects of sulfhydryl compounds on nitroglycerin tolerance. Figure 5 shows that aortic rings made tolerant with 0.22 mM NTG at pH 7.4 for 1 hr (tolerant control) were much less responsive ($P < 0.01$) to NTG challenge (at 8×10^{-6} M) than rings incubated with vehicle alone (non-tolerant control), indicating that vascular tolerance to NTG was achieved with the incubation conditions described. NAC alone (i.e. without NTG challenge) did not alter the PE-induced tone of tolerant vessels, while TSA alone (i.e. without NTG challenge) caused a small contraction (6%, $P < 0.05$ compared to the baseline). Thus, thiol addition alone produced little or no effect on the relaxation of our preparation. NTG challenge in combination with TSA (NTG/TSA) produced a statistically greater ($P < 0.05$) relaxation than NTG challenge alone, while NTG/NAC did not ($P > 0.05$), although there was a trend of partial reversal of tolerance. Analysis of variance also indicated that the relaxation achieved by NTG/TSA was statistically larger than that by NTG/NAC ($P < 0.05$). Based on these statistical results, the NTG/TSA combination caused a greater degree of partial reversal of NTG tolerance than NTG/NAC.

DISCUSSION

In this study, we showed that NO production from

NTG in human plasma was enhanced significantly by various aliphatic and aromatic thiols. With the aliphatic thiols, the stereochemistry of the molecule did not appear to play an important role in this catalytic reaction since stereoisomers of both cysteine and captopril produced similar amounts of headspace NO. It is also interesting to note that the NO-generating activity could be enhanced by attaching an acetyl moiety on the amine group (thus increasing the lipophilicity) of both cysteine and penicillamine (Table 1).

Amongst all the thiols examined, TSA appeared to be the best catalyst in promoting NO-generation from NTG in plasma. It is apparent from panels B and C of Fig. 1 that the amount of headspace NO detected in the presence of TSA reached a plateau at around 30 min, and then declined. NO degrades via a second-order reaction (i.e. the higher the concentration in the headspace, the faster it degrades) [21, 22], and the kinetic profile observed with TSA in the headspace of human plasma is consistent with this rate process. Interestingly, the activity of TSA-methyl ester was substantially lower than that of TSA (Table 2). Thus, esterification of the carboxylate end of the thiol appeared to increase the catalytic activity of aliphatic thiols, but decrease that of aromatic thiols. The reason for this dichotomy is not presently known.

All other aromatic thiols examined were analogs of TSA, the carboxylic acid moiety of which was replaced by different functional groups (R) such that, except for 4-hydroxythiophenol, the sulfhydryl and R groups were ortho to each other. This array of compounds allowed the possibility of constructing a LFER, although it was recognized that the number of data points was quite limited ($N = 7$). This quantitative structure–activity analysis suggested that catalytic activity in aromatic thiols was increased in compounds with higher Hammett's σ values (i.e. more electron withdrawing) and/or with lower π values (i.e. more polar). However, acetylation of the amino group in either cysteine or penicillamine enhanced the NO-generating activity.

The inability of non-thiol containing compounds (e.g. *N*-acetylserine and salicylic acid) to catalyze NO production from NTG is consistent with current hypotheses of nitrate action in which free sulfhydryl compounds are required as cosubstrates. *N*-Acetylserine and salicylic acid are identical to NAC and TSA, respectively, in chemical structure except that they contain a hydroxy in place of a sulfhydryl functional group. The inability of *N*-acetylserine to catalyze generation of detectable amounts of headspace NO is consistent with our previous report [16] which showed *N*-acetylserine to have no effect on the *in vivo* hypotensive potency of NTG.

Results in Fig. 1A indicated that TSA, NAC and L-cysteine were about equipotent in catalyzing NO generation from NTG in phosphate buffer, consistent with the results of Feelisch and Noack [23] who showed that the chemical reactivities of these thiols toward NTG were similar. However, in the presence of human or rat plasma, the NO-generating potency of these thiols increased and differed quite significantly. In particular, the potency of TSA to catalyze NO production in rat plasma was approximately two orders of magnitude higher than that shown in buffer.

It is apparent from Fig. 2 that the thiol-catalyzed NO generation from NTG was mediated, in part, by some heat-labile components in plasma since heat pretreatment substantially reduced NO generation from both NTG/NAC and NTG/TSA systems. Previously, we have shown that several plasma proteins (viz. human serum albumin, γ-globulin and α-1-acid glycoprotein) were unlikely to be involved in producing *S*-nitrosothiol from NTG and NAC [24]. Thus, the heat-labile component(s) in plasma is unlikely to be any of these plasma proteins. The data also suggested that a heat-stable component of the reaction also existed; this component was contributed, at least in part, by the chemical reaction in buffer (see Fig. 1A).

We have reported previously that NAC significantly potentiated the hypotensive effect of NTG in conscious rats, and that the potentiation may be mediated by enhanced formation of *S*-nitrosothiol (or NO) in the systemic circulation [16]. A subsequent report by Munzel *et al.* [25] also suggested the possibility of extracellular interaction between NTG and NAC. Consistent with these reports in the literature, the present study showed that NTG interacted biochemically with thiols in plasma to produce NO, the active vasodilator messenger for nitrates. Using this biochemical index, TSA appeared to be the best catalyst among the thiols tested in promoting NO production from NTG in plasma. In support of this observation, it is shown (Fig. 5) that NTG/TSA produced a stronger partial reversal of NTG tolerance than NTG/NAC. These results are consistent with the observation that NTG/TSA was more potent in activating rat liver guanylate cyclase (i.e. accumulation of cGMP) than NTG/NAC [26].

In conclusion, we have found that most sulfhydryl compounds examined can catalyze NO production from NTG in plasma and this catalysis appears to be mediated by a heat-labile component(s) in plasma. TSA was the most potent sulfhydryl agent that we tested, in terms of its ability to promote biochemical generation of NO from NTG in plasma. TSA was also found to be more potent in reversing *in vitro* NTG tolerance in the presence of plasma than NAC. These results are consistent with the hypothesis that the generation of extracellular NO or *S*-nitrosothiol may be a contributory mechanism for the interaction between NTG and sulfhydryl compounds. The question of whether TSA will be a more potent *in vivo* interactant than NAC to reverse nitrate tolerance remains to be answered, since other factors, such as the aqueous solubility, pharmacokinetics and toxicity of the thiols, will also determine their utility.

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