Acute Effects of Nitroglycerin Depend on Both Plasma and Intracellular Sulfhydryl Compound Levels In Vivo

Effect of Agents With Different Sulfhydryl-Modulating Properties

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Background. Changes in sulfhydryl (SH) compound availability may alter the hemodynamic effect of nitroglycerin (NTG). Data on the relation between NTG effect and thiol levels are, however, limited to in vitro experiments. The present study investigates how intracellular and extracellular changes in SH group concentrations (cysteine and glutathione [GSH]) affect the responsiveness to NTG in vivo.

Methods and Results. GSH and cysteine levels in plasma, vena cava, and aorta were measured after administration of N-acetylserine (placebo, n=6), N-acetylcysteine (NAC, extracellular and intracellular SH donor, n=6), oxothiazolidine (OXYO, intracellular SH donor, n=6), buthionine sulfoximine (BSO, intracellular GSH-depleting agent, n=6), BSO+NAC (n=6), and BSO+OXO (n=6) in chronically catheterized conscious rats. In addition, the effect of 2.5 mg NTG/kg i.v. on mean arterial pressure (MAP) was determined before and after the same treatment. NAC (5 mmol/kg i.v. for 2 hours) significantly (p<0.05) increased extracellular cysteine and GSH levels and potentiated the hypotensive effect of NTG (from 26±3 to 31±4 mm Hg [mean±SEM], p<0.05). OXYO (5 mmol·kg⁻¹·hr⁻¹ i.v. for 2 hours) significantly increased intracellular cysteine and GSH levels but had no effect on NTG responsiveness (p>0.05). BSO (1 g i.p. three times within 24 hours) significantly decreased intracellular GSH levels (p<0.05) and attenuated the effect of NTG (from 28±3 to 16±2 mm Hg).

Conclusions. The results suggest that the acute hypotensive effect of NTG in vivo is 1) increased by high extracellular GSH and/or cysteine levels (NAC), 2) decreased by low intracellular GSH levels (BSO), and 3) unaffected by high intracellular levels of cysteine and GSH (OXO). (Circulation 1993;87:547–553)

Key Words • nitrates • nitroglycerin • cysteine • glutathione

Previous studies have suggested that activation of soluble guanylate cyclase by organic nitrates and/or their biotransformation to vasoactive S-nitrosothiols and nitric oxide (NO) requires the presence of sulfhydryl-containing compounds (e.g., cysteine and glutathione [GSH]).¹ ² In accordance with this, administration of the sulfhydryl donor N-acetylcysteine (NAC) potentiates the acute effect of nitroglycerin (NTG),³–⁶ whereas sulfhydryl oxidizing and alkylating agents (in vitro) appear to reduce NTG vasorelaxation.⁷ ⁸ These findings led to the hypothesis that decreased and increased intracellular thiol levels may diminish and augment the hemodynamic effect of NTG, respectively. However, data on thiol levels are limited to

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in vitro experiments,⁹ ¹⁰ and in these studies, high intracellular cysteine levels⁹ and low GSH levels¹⁰ apparently do not affect NTG-induced vasorelaxation. In vivo data on the relations between sulfhydryl compound levels and vascular nitrate responsiveness have not been reported.

To study these relations, we determined the effect of a specific intracellular cysteine donor and an extracellular and intracellular cysteine donor on plasma and vascular cysteine and GSH concentrations and on the degree of NTG-induced hypotension in conscious rats. In addition, the effect of GSH depletion was investigated in rats treated with a specific GSH synthesis inhibitor. The combined effect of GSH depletion and subsequent cysteine donor administration on sulfhydryl compound levels and NTG responsiveness was also examined.

Methods

Animals

For chronic catheterization, female Wistar rats (weight, 200–260 g) were anesthetized with 1–3% halothane and N₂O/O₂ (2:1). One catheter (medical grade Tygon catheters) was implanted with its tip in the
ascending aorta through the left carotid artery, and two catheters were placed in the superior vena cava via the left jugular vein. Catheters were filled with a solution of 50% glucose and 500 IU/ml heparin and plugged with a nylon pin. Each catheter was externalized through the back in the neck region. After catheter implantation, the rats were housed individually and exposed to a 12-hour light–12-hour dark cycle and with free access to a standard rat chow and tap water. Postoperatively, rats were allowed to recover from surgery until they had regained their preoperative weights and appeared healthy (6–8 days). Details regarding this rat model with chronically catheterized conscious rats have been described previously.\textsuperscript{11}

**Drugs**

NTG (100 mg/ml) was dissolved in 5% glucose. N-Acetyl-l-cysteine (NAC), N-acetylsorine (NAS), and L-buthionine-[S,R]-sulfoximine (BSO) were purchased from Sigma Chemical Co., St. Louis, Mo. L-2-Oxothiazolidine-4-carboxylic acid (OXO) was obtained from Bachem AG, Bubendorf, Switzerland. All solutions were adjusted to pH 7.4 with NaOH and prepared in 0.9% NaCl.

NAC. NAS is identical to NAC except that it contains a hydroxy group in place of a sulphydryl group, and any differences in response to NAC and NAS are probably due to this difference.

NAC. GSH levels may be increased by several methods. Cysteine is normally the limiting amino acid in GSH synthesis, but its administration is toxic. Administered NAC leads to increased cysteine levels after deacetylation (primarily in the kidney) and thus increased GSH levels.\textsuperscript{12,13}

OXO. OXO is a nonthiol, nontoxic cysteine delivery drug and is readily transported into cells and converted into cysteine by 5-oxyprolinease, a widely distributed intracellular enzyme.\textsuperscript{14} OXO is a specific intracellular sulphydryl donor (whereas NAC has both intracellular and extracellular sulphydryl donor properties) and is more effective in increasing intracellular cysteine and GSH content than NAC\textsuperscript{15} (Figure 1).

BSO. GSH is synthesized intracellularly by the consecutive actions of gamma-glutamylcysteine synthetase and GSH synthetase.\textsuperscript{16} Gamma-glutamylcysteine synthetase is the rate-liming step in GSH synthesis and is feedback inhibited by the end product, GSH.\textsuperscript{17} Availability of substrates, especially cysteine, may also regulate cellular GSH levels. Cellular GSH is normally transported out of cells.\textsuperscript{18} Cellular GSH levels are lowered by the administration of BSO, a specific inhibitor of gamma-glutamylcysteine synthetase\textsuperscript{19} (Figure 1).

**Experimental Protocol**

Conscious, unrestrained, chronically catheterized rats were divided into six different groups. In all groups, the blood pressure–lowering effect of intravenous bolus doses of NTG (0 mg/ml [NTG vehicle, placebo]) and 2.5 mg/kg) was determined immediately before the beginning of the experiments. The total volume of each bolus dose was 0.4 ml, and each bolus injection was separated by a 30-minute interval. Blood pressure during baseline infusion conditions (before NTG bolus administration) and blood pressure alterations during NTG bolus challenges were recorded continuously by Trantec pressure transducers (model 60–800) connected to the left carotid catheter. Tracings were displayed on a Watanabe linear recorder (Watanabe Instruments Corp.).

**Sulfhydryl compound supplementation.** The effects of NAC (n=7), OXO (n=7), and NAS (control, n=8) infusion on the blood pressure response to NTG were studied in equimolar amounts of 5 mmol·kg\textsuperscript{-1}·hr\textsuperscript{-1} in a volume of 1.5 ml/hr. The infusions lasted for 3 hours, and after 2 hours, the preinfusion NTG bolus challenge doses were repeated, and each animal served as its own control.

**Sulfhydryl compound depletion.** After the baseline NTG bolus testing, eight rats were given BSO (1 g i.p. 3 times within 24 hours (8 hours, 16 hours, and 24 hours). Two hours after the last BSO dose, the NTG bolus injections were repeated, and the blood pressure changes were monitored.

**Sulfhydryl compound administration in GSH-depleted rats.** Two additional groups of rats were treated with BSO as described. Immediately after the last BSO dose, NAC (n=8) or OXO (n=8) was infused at a dose of 5 mmol·kg\textsuperscript{-1}·hr\textsuperscript{-1} for 3 hours. NTG bolus injections were repeated after 2 hours of infusion.

**Sulfhydryl compound levels.** In another series of experiments, the rats were prepared and divided into the six treatment groups described above (n=6 in each group). At the end of the treatment period, arterial blood was sampled during halothane and N\textsubscript{2}O/O\textsubscript{2} anesthesia for determination of plasma cysteine and GSH levels, and the abdominal aorta and inferior vena cava

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**Figure 1.** Chart demonstrating metabolism of glutathione (GSH) and thiol-modulating agents. BSO, buthionine sulfoximine.
were removed to determine vascular (intracellular) cysteine and GSH levels.

Measurement of Cysteine and GSH Levels

Reduced glutathione was determined by a modification of the method described by Fahey and Newton. Astre. Arterial blood was placed in prechilled tubes together with serine/borate at a final concentration of 20 mM, spun in a fast accelerating centrifuge, and 100 μl of plasma was obtained within 2 minutes for derivatization with monobromobimane (Calbiochem). Vascular tissue was immediately frozen in liquid nitrogen, pulverized in liquid nitrogen, treated with serine/borate for immediate derivatization with monobromobimane, and deproteinized.17

Ten microliters of the samples was injected into a Merck/Hitachi high-performance liquid chromatography gradient system consisting of a model L-6200 pump, a model L-6000 pump, a model AS-2000 autosampler, and a model F-1050 fluorescent detector set at 415-nm excitation and 490-nm emission. The system was connected to a PC-based high-performance liquid chromatography manager (Hitachi model d-6000, Chromatography Data Station Software) for automation and integration. Gradient elution was performed with two solvents. Solvent A was prepared from 2.5 ml acetic acid, 50 ml acetonitrile, 7.03 g sodium perchlorate, and water to a total volume of 1 l; the pH was adjusted to 3.4. Solvent B was prepared from 2.5 ml acetic acid, 7.03 g sodium perchlorate, and acetonitrile to a volume of 1 l. The gradient schedule was started with 98% A and 2% B, and after 5 minutes, linear gradients were introduced: at 5 minutes, 87% A and 13% B; at 14 minutes, 87% A and 13% B; at 16 minutes, 0% A and 100% B. From 20 to 35 minutes, the initial conditions were resumed: 98% A and 2% B. The flow was constant through a Beckmann Ultrasphere ODS 5 μ 4.6×25-cm reverse-phase C4 column. Distinct peaks of cysteine (4.32 minutes), GSH (7.16 minutes), and NAC (9.73 minutes) were obtained. Recovery of the three sulfhydryl compounds was greater than 80%. The coefficient of variation of repeated measurements of the three sulfhydryl compounds was below 3.6%. Initially, internal standards consisting of D-pencilamine were included, but the results were so reproducible that this step could be omitted.

Statistical Analysis

Mean arterial blood pressure (MAP) was estimated as diastolic pressure + (systolic pressure – diastolic pressure) / 3 in mm Hg. Presented alterations in MAP to NTG represent the difference between the pre-NTG bolus value and the nadir on the blood pressure curve after NTG. All data are presented as mean ± SEM. Differences between pretreatment and posttreatment means were determined by Student’s paired t test. Comparisons between treatment groups were performed by ANOVA. Statistical significance was assumed at a value of p < 0.05.

Results

Baseline (control) levels of cysteine and GSH in plasma, aorta, and vena cava are shown in Table 1. Intracellular GSH is present in high concentrations compared with cysteine; the intracellular/extracellular concentration ratio is ~100:1. Sulfhydryl compound levels are similar in arterial and venous tissues.

Effect of Sulfhydryl Compound Supplementation on Plasma and Intracellular Cysteine and GSH Content

Compared with controls, infusion of NAC has no effect on intracellular GSH levels and induces only a small increase in intracellular cysteine. NAC treatment increases extracellular cysteine and GSH levels about 18 times (Table 1). In contrast, OXO infusion induces a marked increase in vascular (intracellular) cysteine and GSH levels while having relatively little effect on extracellular (plasma) cysteine and GSH levels as compared with NAC treatment (Table 1). Thus, the results confirm previous observations that show that under physiological conditions (normal intracellular thiol levels before drug administration), OXO primarily acts as an intracellular sulfhydryl donor.

Differential Effect of Sulfhydryl Compound Donors on NTG Pharmacodynamics

Baseline MAP and heart rate were similar in all treatment groups and not affected by treatment regimens (data not shown). Administration of NTG placebo (NTG vehicle) caused no significant changes in MAP in any of the experiments.

NAC infusion significantly increases the hypotensive effect of NTG (pretreatment, 26±3 mm Hg versus

| Table 1. CYS and GSH Levels in Plasma and Vascular Tissue in Normal Control Animals and After Treatment With NAC, OXO, BSO, BSO+NAC, and BSO+O XO |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Control         | NAC             | OXO             | BSO             | BSO+NAC         | BSO+O XO        |
| CYS             |                 |                 |                 |                 |                 |                 |
| Plasma (μM)     | 11±2           | 201±34          | 32±3            | 2±0.5           | 578±92          | 47±2           |
| Aorta (nmol/g)  | 37±11          | 96±21           | 296±78          | 53±19           | 345±150         | 138±77         |
| Vena cava (nmol/g) | 30±14         | 36±15           | 211±200         | ……              | 334±162         | 132±15         |
| GSH             |                 |                 |                 |                 |                 |                 |
| Plasma (μM)     | 7±1            | 132±26          | 21±1            | 21±1            | 303±43          | 11±1           |
| Aorta (nmol/g)  | 729±123        | 911±167         | 1,559±221       | 173±34          | 186±43          | 318±29         |
| Vena cava (nmol/g) | 972±300       | 1,014±286       | 1,355±99        | 82±31           | 179±46          | 291±13         |

Values are mean ± SEM; n = 6 in each group. NAC, N-acetylcysteine; OXO, oxathiolodione; BSO, buthionine sulfoximine; CYS, cysteine; GSH, glutathione.

*p < 0.05 compared with control; †p < 0.05 compared with BSO; ‡p < 0.05 compared with NAC; §p < 0.05 compared with aorta.
posttreatment, 31 ± 4 mm Hg; p < 0.05) (Figure 2). Neither OXO nor the nonthiol NAS influenced the pharmacodynamic response to NTG (Figure 2). Therefore, intracellular thiol levels above the normal range (OXO) do not alter NTG responsiveness. Thus, the increased hypotensive effect of NTG (approximately 19%) after NAC infusion probably depends on the marked increase in plasma cysteine and GSH produced by NAC rather than by the NAC-induced changes in intracellular cysteine and GSH levels.

**Effect of BSO Treatment on Cysteine and GSH Content and NTG Responsiveness**

The major source of plasma GSH is the liver, and the observed small increase in plasma GSH suggests that liver gamma-glutamylcysteine synthetase was not fully inhibited by BSO (despite a rigorous dose regimen) in this experiment. However, compared with control levels, BSO significantly lowered intracellular GSH content (Table 1). GSH levels in vena cava were significantly lower than in aorta, suggesting a likely difference in susceptibility to BSO-induced GSH depletion in the two vascular beds. Cysteine levels in aorta were not significantly altered. As shown in Figures 3 and 4, GSH depletion induced by giving BSO significantly decreased the hypotensive effect of NTG (43%).

**Effect of Sulfhydryl Compound Supplementation in GSH-Depleted (BSO-Treated) Animals**

As expected, NAC or OXO infusion in BSO-pre-treated animals did not normalize the low intracellular GSH levels (Table 1). However, slightly increased intracellular GSH levels after BSO+NAC and BSO+OXO compared with BSO treatment suggests that not all vascular gamma-glutamylcysteine synthetase was inhibited by BSO. Intracellular cysteine and plasma levels of cysteine were significantly increased because cysteine cannot be used for GSH synthesis.

The potentiated NTG response seen in animals treated with NAC alone (Figure 2) is abolished in the BSO+NAC group of animals with low intracellular GSH levels (Figure 4), further suggesting that low intracellular GSH levels attenuate NTG responsiveness in this animal model. However, because the pharmacodynamic effect of NTG is less inhibited in the BSO+NAC and BSO+OXO group compared with the BSO group (Figure 4) (vascular GSH levels are comparably low), it is likely that the very high levels of intracellular cysteine and/or plasma cysteine and GSH may compensate some of the effects of intracellular GSH depletion.

**Discussion**

In the current study, the pharmacodynamic response to NTG during treatment with different sulfhydryl-modulating drugs has been correlated with actual plasma and tissue thiol levels, thus providing new information about nitrate–thiol interactions in vivo. The hypotensive effect of NTG in vivo was altered either by depletion of intracellular (vascular) GSH content (which inhibits the response to NTG) or by increasing the extracellular (plasma) cysteine and/or GSH levels (which potentiates the effect of NTG). Clearly, supraphysiologically high intracellular levels of cysteine and GSH are not associated with changes in NTG responsiveness in normal animals (Figure 5).

The mechanism by which NTG is metabolized to vasoactive substances in vascular smooth muscle remains a subject of some controversy. At present, hypotheses involve interaction of NTG with intracellular thiols at one or more sites during its biotransformation to vasoactive nitrosothiols. In vitro, cysteine appears to be required for nitrate-induced activation of guanylate cyclase, and cysteine and GSH may accelerate the degradation of NTG in plasma. Recently, a direct “one step” conversion of NTG to a nitrosothiol in the presence of cysteine has been proposed. Thus, nitrosothiols and/or nitrosothiolamine may be intermediates in the biotransformation of NTG and may directly or indirectly (via release of nitric oxide) activate guanylate cyclase and mediate smooth muscle relaxation.
Although diverging results exist, clinical studies have shown that NAC may augment the acute hemodynamic effects of NTG. Similarly, sulphydryl compound–depleting agents and sulphydryl compound donors have generally been shown to reduce and augment vasoreactivity to NTG in vitro, respectively. The results in this study on the effects of NAC and sulphydryl compound depletion (which has not been investigated previously in vivo) are consistent with and extend these in vitro observations.

At present, it is not clear whether this interaction takes place within and/or outside the vascular smooth muscle cell. The present study provides information about the relative contribution of extracellular/intracellular metabolic pathways in vivo.

We find that 1) animals with very high plasma levels of cysteine and GSH and physiological intracellular sulphydryl levels (after NAC treatment) showed an augmented response to NTG, suggesting that there is an important extracellular NTG–thiol interaction. In vitro studies in which plasma enzyme activity may augment NTG-induced activation of soluble guanylate cyclase in the presence of NAC appear to support this observation. 2) Supraphysiological high intracellular cysteine and GSH and relatively normal extracellular levels (OVO treatment) do not affect the hypotensive effect of NTG. This implies that intracellular sulphydryl levels in normal animals are not rate limiting for the conversion of NTG to nitric oxide, an observation in line with findings that elevation of intracellular cysteine levels does not affect NTG-induced relaxation in vitro. 3) BSO-induced inhibition of intracellular GSH synthesis significantly lowers intracellular GSH levels in vascular tissue and reduces the hypotensive effect of NTG. This result suggests that a critical amount of intracellular GSH is required for optimal conversion of NTG in vivo. Subsequent treatment with NAC (BSO+NAC group) does not reverse the low GSH levels but provides high levels of intracellular cysteine and high levels of extracellular cysteine and GSH. Although these changes tended to normalize the pharmacodynamic effect of NTG, the NTG-potentiating effect of NAC was abolished. Together, these findings further emphasize that intracellular GSH plays a critical role in the metabolism of NTG.

GSH (synthesized from cysteine, glutamate, and glycine within the cells) is found almost exclusively intracellularly and constitutes the major cellular nonprotein sulphydryl pool. Cysteine is rapidly metabolized, and GSH may be regarded as a storage and transport form of cysteine; cysteine levels in most tissues are present in much lower concentrations (10–100 μM) than those of GSH (500–10,000 μM). A continuous and irreversible transport of GSH out of the cell is the source of extracellular GSH and may augment extracellular sulphydryl availability by extracellular or membranous metabolism of GSH. Thus, synthesis and degradation of GSH includes intracellular and extracellular reactions and involves the immediate environment on both sides of the cell membrane (Figure 1).

Although the levels of GSH and cysteine in plasma are normally very low, it is probable that their concentration in interstitial fluid in proximity to the cell membrane are greater than plasma levels as a consequence of the cellular GSH export process. Recently, it has been reported that enzymes involved in the metabolic activation of NTG are located mainly in the cell membrane fragment of vascular smooth muscle cells. One may speculate that the metabolism of NTG may take place at or in near proximity to the cell membrane of the smooth muscle cell and may require an enzyme located on the external surface of the cell membrane and that such metabolism requires continuous export of intracellular GSH (and perhaps membranous degradation of GSH to cysteine). This hypothesis is consistent with most of the published observations, including extracellular formation of nitrosothiols in the presence of high extracellular sulphydryl compound levels and the in vitro effects of different sulphydryl compound–oxidizing and alkylating agents. It may also...
account for the differential effect of NAC (in providing high extracellular GSH and cysteine levels) and OXO (which produces only slight changes in extracellular sulfhydryl compound levels) on NTG responsiveness. Similarly, normal intracellular sulfhydryl compound levels may be required for membranous transport and delivery of GSH (and cysteine sulfhydryl).

Qualitative changes in sulfhydryl compound levels after systemic treatment with NAC, OXO, and BSO are similar in all tissues investigated,\textsuperscript{9,27} and the measured changes in sulfhydryl levels are therefore probably not specific for changes in other vascular beds. Large vessels show a sulfhydryl-dependent response to NTG in vitro,\textsuperscript{5} and sulfhydryl compound supplementation potentiates the effect of NTG in the peripheral arterial system,\textsuperscript{4} the venous system,\textsuperscript{29} and the coronary circulation\textsuperscript{3} in vivo. However, in most studies, no attempt has been made to differentiate between sulfhydryl-dependent effects in small and large vessels. In this context, it is interesting that small coronary resistance vessels (<100 \mu m) are not sensitive to NTG\textsuperscript{29} and appear to respond to NTG in the presence of exogenous cysteine.\textsuperscript{8,21} The validity of this finding has not been confirmed in the systemic circulation, but it is likely that NAC, besides having an NTG-potentiating effect in large arteries and the venous system, also facilitates vasodilation of small arterioles not normally responsive to NTG. This mechanism may thus contribute to the observed NAC-induced changes in blood pressure.

The amount of specific sulfhydryl compounds in these small vessels is uncertain, but it has been suggested that they lack specific pools of either intracellular and/or membrane-associated sulfhydryl groups necessary for the biotransformation of NTG.\textsuperscript{5,21} This study does not allow a clear differentiation between these possibilities. NAC and OXO have, however, different effects on intracellular and extracellular sulfhydryl levels, and elevation of intracellular sulfhydryl levels (OXO) was not associated with a potentiated effect of NTG. Thus, assuming that some of the NTG-potentiating effect of NAC is elicited by a sulfhydryl-dependent, small vessel dilatation, an effect on membrane-bound sulfhydryl groups is most compatible with the present results.

Development of tolerance to organic nitrates has been associated with a depletion of intracellular sulfhydryl compounds.\textsuperscript{19} Interestingly, the decreased hemodynamic response to NTG in the present BSO experiments mimics the attenuated hemodynamic effect of NTG during nitrate tolerance.\textsuperscript{30--33} and, as in nitrate tolerance, a decreased response during BSO treatment may be reversed by NAC administration. At present, it is unknown whether this effect of NAC is mediated by replenishment of intracellular sulfhydryl stores or results from a tolerance-independent NTG potentiating effect, as in the present study. However, the results from this and other studies\textsuperscript{13} suggest that OXO, which is well tolerated in humans,\textsuperscript{34} is a better precursor of intracellular cysteine and GSH than NAC. Thus, it is possible that OXO may be more effective than NAC in prevention and/or reversal of clinical tolerance to organic nitrates. Recent in vitro data appear to support this view.\textsuperscript{35}

**Summary**

The present research is the first report on the relations in vivo between pharmacodynamic response to NTG and plasma and vascular cysteine and GSH. The results show that the hypotensive effect of NTG in vivo is inhibited by depletion of intracellular (vascular) GSH content and that it is potentiated by an increase in extracellular but not intracellular cysteine and/or GSH levels. We conclude that two different mechanisms operate in vivo; one is related to intracellular GSH content and the other is related to plasma sulfhydryl compound levels. Thus, in vivo NTG responsiveness depends on normal intracellular sulfhydryl compound levels and may be modulated by both intracellular and extracellular changes in sulfhydryl group availability.

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