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Nitrate Tolerance In Vivo Is Not Associated With Depletion of Arterial or Venous Thiol Levels

Søren Boesgaard, Jan Aldershivle, Henrik Enghusen Poulsen, Steffen Loft, Mary E. Anderson, Alton Meister

Results from in vitro experiments suggest that development of nitrate tolerance is due to a depletion of vascular thiol compounds (ie, cysteine and glutathione [GSH]) necessary for the bioconversion of organic nitrates. However, it is unknown whether in vivo tolerance development is associated with changes in thiol levels. This study measures plasma and vessel tissue GSH and cysteine levels in nontolerant rats, nitrate-tolerant rats, and rats treated with the two characteristically different thiol donors N-acetyl-L-cysteine and l-2-oxothiazolidine-4-carboxylic acid (OXO). Chronically catheterized conscious rats received an intravenous infusion of either nitroglycerin (NTG, 0.2 mg/h) or matching placebo for 3 days. At day 3, the hypotensive effect of 2.5 mg NTG/kg was decreased by 74±6% (mean±SEM, P<.05) in the NTG-treated group (n=7), indicating the development of tolerance. No change in the hypotensive effect of NTG was seen in the placebo group (n=6, P>.05). Hemodynamic tolerance is not associated with changes in aorta cysteine or GSH levels as compared with the placebo group (cysteine, 77±14 versus 57±11 [mean±SEM] nmol/g; GSH, 414±62 versus 399±89 nmol/g; P>.05). However, the increase in vascular thiol levels seen after OXO treatment in nontolerant rats is completely absent in nitrate-tolerant animals. The results suggest that (1) depletion of vascular cysteine and/or GSH is apparently not the mechanism underlying development of nitrate tolerance in vivo, and (2) the metabolic handling of thiol compounds and/or the activity of enzymes that act on these thiols is altered during the development of nitrate tolerance in vivo. (Circ Res. 1994;74: 115-120.)

Key Words • nitroglycerin • cysteine • glutathione • nitrate tolerance

Although tolerance to organic nitrates has long been known, the mechanisms responsible for vascular tolerance development to organic nitrates is unclear. Previous studies have shown that generation of S-nitrosothiols and/or nitric oxide from organic nitrates depends on interactions with cellular thiol compounds like cysteine and glutathione (GSH).1-4 On the basis of in vitro studies by Needleman et al,1,5 the primary mechanism underlying vascular tolerance development has been attributed to a depletion of the vascular thiol compounds and diminished production of vasoactive intermediates during continuous nitrate exposure. Indirect evidence supporting the “thiol depletion theory” is derived from in vitro and more recent in vivo studies in which the pharmacologic manipulation of cellular thiol compounds either increases (thiol donors) or decreases (eg, thiol-oxidizing compounds) the hemodynamic response to nitroglycerin (NTG).4-8 Although these studies demonstrate significant thiol-nitrate interactions, they do not address the role of thiol levels during continuous nitrate therapy. Currently, there are no in vivo data on thiol levels during continuous nitrate treatment and tolerance development. Thus, it is unknown whether “thiol depletion” occurs during the development of tolerance in vivo.

The present study was carried out to determine whether or not tolerance to NTG is associated with changes in thiol levels in vivo. Plasma and vascular cysteine and GSH levels in normal and nitrate-tolerant rats were compared. In addition, the effects of two potent, but characteristically different, thiol donors on thiol levels and NTG hemodynamics were also investigated.

Material and Methods

GSH and Thiol Metabolism

GSH is found almost exclusively intracellularly, where it constitutes the major nonprotein thiol pool. GSH is synthesized intracellularly from cysteine, glutamate, and glycine by the consecutive actions of γ-glutamylcysteine synthetase and GSH synthetase.9 Cysteine is rapidly metabolized, and GSH serves as a storage and transport form of cysteine.10 γ-Glutamylcysteine synthetase is the rate-limiting step in GSH synthesis and is feedback-inhibited by the product of the pathway, GSH.9,11 Availability of substrates, especially cysteine, may also regulate cellular GSH levels.

Cysteine and GSH levels may be increased by several methods. N-Acetyl-L-cysteine (NAC) leads to increased plasma and cellular cysteine levels after deacetylation.9,12 l-2-Oxothiazolidine-4-carboxylic acid (OXO) is a nonthiol nontoxic cysteine delivery drug that is readily transported into cells and converted into cysteine by 5-oxoprolinase.9,13 Thus, whereas NAC has both intracellular and extracellular thiol-donor properties, OXO is a specific intracellular thiol donor and is more effective in increasing intracellular cysteine and GSH content than is NAC.9

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Animals

Female Wistar rats (210 to 250 g) were anesthetized with 1% to 3% halothane and Na$_2$O$_2$O$_2$ (2:1) during the chronic catheterization procedure. One catheter (medical-grade Tygon tubing) was implanted with its tip in the ascending aorta through the left carotid artery, and three catheters were placed in the superior vena cava via the left (two) and right (one) jugular veins. The catheters were filled with a solution of glucose (500 g/L) and heparin (300 U/mL) and plugged with a nylon pin. Each catheter was externalized through the neck skin. After catheter implantation, rats were housed individually and exposed to a 12 hour–12 hour light-dark cycle and had free access to standard rat chow and tap water. After surgery, rats were allowed to recover until they had regained their preoperative weights (6 to 8 days).

Experimental Protocol

Induction of nitrate tolerance. At the end of the recovery period (day 0), an osmotic minipump (Alza Corp) was placed subcutaneously and connected to one of the intravenous catheters. NTG or placebo (99% ethanol [NTG-vehicle]) was delivered from the minipump at a constant rate of 0.2 mg/h IV (10 µL/h) for 72 hours (day 3). In this model, which previously has been described in detail,14 tolerance to the blood pressure–lowering effect of NTG develops within 24 hours.

Hemodynamic response. The hemodynamic response to NTG was estimated from the blood pressure–lowering effect of intravenous bolus doses of NTG (0 mg [placebo] and 2.5 mg NTG/kg) at day 3 in all treatment groups. The total volume of each bolus dose was 0.4 mL, and each 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 pressure transducers (Baxter Corp, Uden, Holland) connected to the arterial catheter. Tracings were displayed on a Graphtec linear recorder (Watanabe Instruments Corp, Japan).

Treatment Groups

Five different groups of conscious unrestrained chronically catheterized rats were studied. NTG responsiveness in nontolerant and tolerant rats. Three groups of rats were investigated. Seven rats received a long-term infusion of NTG (0.2 mg/h) for 3 days (NTG group). Six rats received placebo NTG infusion (NTG-vehicle) for a similar period of time (placebo group). In addition, another control group of six chronically catheterized, but otherwise nonmedicated, rats was included (untreated group). The hypotensive effect of NTG bolus injections was determined on day 3.

NTG responsiveness in tolerant rats after NAC and OXO administration. On day 3 of the constant NTG infusion, two groups of rats (n=6 in each) additionally received either 3-hour intravenous infusions of NAC (NTG+NAC group) or OXO (NTG+OXO group). These infusions were given in a separate catheter in equimolar amounts of 5 mmol·kg$^{-1}$·h$^{-1}$ (1.5 mL/h). The hypotensive effect of NTG bolus injections was determined immediately before the start of NAC or OXO infusion and repeated after 2 hours of infusion.

Plasma and vascular thiol levels in nontolerant and tolerant rats. In another series of experiments, the rats were prepared and divided into the five treatment groups described above (n=6 in each group). Because of the unexpected findings in the OXO-treated group, the effect of OXO administration was additionally repeated in a group of nontolerant rats [OXO group], confirming previous findings, and then examined in another group of NTG-tolerant rats [n=31]. At the end of the treatment period, arterial blood was sampled during halothane and Na$_2$O$_2$O$_2$ anesthesia for determination of plasma (extracellular) cysteine and GSH levels, and the abdominal aorta and inferior vena cava were removed to determine vascular (intracellular) cysteine and GSH levels.

Measurement of Cysteine and GSH Levels

Arterial blood was placed in prechilled tubes together with serine/borate (to inhibit γ-glutamyltranspeptidase) at a final concentration of 20 mmol/L and spun in a fast-accelerating centrifuge, and 100 µL plasma was obtained within 2 minutes for derivatization with monobromobimane (Calbiochem, Switzerland). Vascular tissue was immediately frozen in liquid nitrogen, pulverized in liquid nitrogen, treated with serine/borate for immediate derivatization with monobromobimane, and deproteinized. Cysteine and GSH (reduced form) were measured on a high-performance liquid chromatography gradient system with fluorescent detection as previously described.4

Drugs

NTG solutions were prepared from a stock solution (100 mg NTG in 1 mL of 99% ethanol). For prolonged infusion, NTG was dissolved in 99% ethanol, and for bolus injections, NTG was diluted with 5% glucose. NAC and OXO were purchased from Sigma Chemical Co, St Louis, Mo. Solutions of NAC and OXO were adjusted to pH 7.4 with NaOH and prepared in 0.9% NaCl.

Calculations and Statistics

Mean arterial blood pressure (MAP) was estimated as follows: diastolic pressure + (systolic pressure – diastolic pressure)/3 (in millimeters of mercury). The reported reduction in MAP after NTG bolus injections represents the difference between the pre–NTG bolus baseline value and the nadir value on the pressure-response curve. Within 10 minutes, blood pressure in all rats recovered to the pre–NTG bolus level. 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 using ANOVA. Statistical significance was assumed at P<.05.

Results

NTG Responsiveness in Nontolerant and Tolerant Rats

Development of nitrate tolerance was confirmed by the finding that the hypotensive effect of bolus injections of NTG (2.5 mg/kg) was significantly reduced in the group infused with NTG for 3 days as compared with the placebo-treated group (NTG group, 8±4 mm Hg; placebo group, 23±3 mm Hg; P<.05) (Fig 1). The response to NTG in the placebo group did not differ from the response in the nonmedicated (untreated) group (P>.05) (Fig 1). Baseline MAP values (before NTG bolus challenges) were similar in all treatment groups (data not shown). Bolus doses of NTG-placebo caused no significant changes in MAP in any of the experiments.

Plasma and Vascular Thiol Levels in Nontolerant and Tolerant Rats

Plasma and vascular cysteine and GSH levels are shown in the Table and Fig 2. There are no differences between the extracellular (plasma) and intracellular (vascular [aorta and vena cava]) levels of cysteine and GSH in tolerant and nontolerant (placebo-treated) rats. There is a trend (Table) to decreased intracellular thiol levels in rats during placebo and NTG infusions compared with levels in the untreated rats, but this trend is not significant (P>.05).

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Effect of NAC Administration on Thiol Levels and NTG Responsiveness in Nitrate-Tolerant Rats

Rats treated for 3 days with NTG are nitrate tolerant, as demonstrated by the weak hypotensive response to a bolus dose of NTG before the start of the 3-hour OXO or NAC infusions (Fig 3). Although the infusion of NAC significantly potentiated the hypotensive effect of NTG (from 10±2 to 17±3 mm Hg, *P<.05) (Fig 3), the response is not as great as that of the placebo-treated nontolerant group (23±3 mm Hg). The hemodynamic response to NAC is accompanied by a marked increase in plasma cysteine levels and a significant increase in intracellular cysteine levels (Table). The increase in plasma GSH levels after NAC treatment of tolerant rats is, however, not as great as that of nontolerant rats.*

Effect of OXO Administration on Thiol Levels and NTG Responsiveness in Nitrate-Tolerant Rats

Several studies have shown that administration of OXO leads to increased intracellular levels of cysteine

Cysteine and Glutathione Levels in Plasma and Vascular Tissue in Untreated Rats, Placebo-Treated Rats, and Groups of Rats Treated With Nitroglycerin Alone, Nitroglycerin and N-Acetyl-L-Cysteine, Nitroglycerin and L-2-Oxothiazolidine-4-Carboxylic Acid, and L-2-Oxothiazolidine-4-Carboxylic Acid Alone

<table>
<thead>
<tr>
<th>Cysteine</th>
<th>Untreated (n=6)</th>
<th>Placebo (n=6)</th>
<th>NTG (n=6)</th>
<th>NTG+NAC (n=6)</th>
<th>NTG+OXO (n=6+3)</th>
<th>OXO (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, μmol/L</td>
<td>15±1</td>
<td>18±3</td>
<td>14±2</td>
<td>244±20*</td>
<td>36±3*</td>
<td>39±3*</td>
</tr>
<tr>
<td>Aorta, nmol/g</td>
<td>83±15</td>
<td>57±11</td>
<td>77±14</td>
<td>187±16*</td>
<td>56±8</td>
<td>151±3*‡</td>
</tr>
<tr>
<td>Vena cava, nmol/g</td>
<td>57±7</td>
<td>30±7</td>
<td>25±7</td>
<td>187±18*</td>
<td>38±6</td>
<td>97±4*‡</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GSH</th>
<th>Untreated (n=6)</th>
<th>Placebo (n=6)</th>
<th>NTG (n=6)</th>
<th>NTG+NAC (n=6)</th>
<th>NTG+OXO (n=6+3)</th>
<th>OXO (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, μmol/L</td>
<td>8±1</td>
<td>11±2</td>
<td>9±3</td>
<td>22±2*</td>
<td>9±8</td>
<td>6±2</td>
</tr>
<tr>
<td>Aorta, nmol/g</td>
<td>432±35</td>
<td>399±89</td>
<td>414±62</td>
<td>392±39</td>
<td>396±24</td>
<td>1037±61*‡</td>
</tr>
<tr>
<td>Vena cava, nmol/g</td>
<td>561±56</td>
<td>457±57</td>
<td>454±86</td>
<td>564±25</td>
<td>404±40†</td>
<td>1127±254*‡</td>
</tr>
</tbody>
</table>

Placebo indicates nitroglycerin-vehicle–treated rats; NTG, rats treated with nitroglycerin at 0.2 mg/h for 3 days; NTG+NAC, rats treated with NTG and N-acetyl-L-cysteine; NTG+OXO, rats treated with NTG and L-2-oxothiazolidine-4-carboxylic acid; OXO, rats treated with L-2-oxothiazolidine-4-carboxylic acid alone; and GSH, glutathione. Values are mean±SEM.

*P<.05 compared with NTG.
†P<.05 compared with NTG+OXO.
and GSH in normal animals. \(^4\) In accordance with these findings, the present study shows that administration of OXO to normal (nontolerant) rats produces increases in vascular thiol levels (OXO group, Table).\(^4\) Although plasma cysteine increases slightly after OXO treatment in tolerant rats, the effect of OXO on vascular thiol levels is completely absent in nitrate-tolerant rats (NTG+OXO group, Table). In view of this surprising result, the studies on the effect of OXO in tolerant rats were repeated in another group of rats (n=3); the same result was obtained. OXO treatment did not affect the hemodynamic response to NTG in nitrate-tolerant rats (9±2 versus 10±3 mm Hg, \(P>0.05\)) (Fig 3).

**Discussion**

A major finding of this in vivo study is that arterial and venous cysteine and GSH levels are similar in NTG-tolerant and nontolerant animals. This result does not support the thiol depletion theory of nitrate tolerance. Rather, the present study strongly suggests that depletion of vascular thiol compounds may not be the "cause" of NTG tolerance in vivo.

The metabolism of organic nitrates involves interaction with cellular thios, such as cysteine and GSH, at one or more steps in the biotransformation to vasoactive \(S\)-nitrosothiols and/or nitric oxide.\(^2,3,15\) The mechanism of nitrate tolerance is probably multifaceted, but a loss of pharmacologic activity due to depletion of vascular thiol compounds has been one of two generally accepted mechanisms for tolerance development; the other mechanism is the activation of neurohormonal counterregulatory mechanisms.\(^16,17\) The thiol depletion hypothesis for NTG tolerance is based on studies\(^1,5\) showing that tolerance, induced in vitro, is associated with depletion of nonspecific vascular thiol groups. More recently, it has been reported that vascular GSH content is depleted after in vitro exposure to NTG.\(^4\) The thiol depletion mechanism is indirectly supported by in vivo studies showing that treatment with NAC potentiates the hemodynamic effect of NTG,\(^19-21\); however, these in vivo studies do not report thiol levels. The in vitro results may not accurately reflect the in vivo situation. Thus, the existence of a causal association between thiol depletion and tolerance development in vivo remains to be established.

Prolonged in vivo exposure to NTG has previously been shown to induce a marked tolerance in all vascular beds investigated.\(^22-24\) Tolerance to the hypotensive effect of NTG and tolerance in aorta develops simultaneously in rats.\(^22\) In the present study, aortic tissue was therefore considered nitrate tolerant in rats showing tolerance to the hypotensive effect of NTG. The finding of normal vascular (aorta and vena cava) thiol levels in nitrate-tolerant animals provides new information that does not support the conclusions drawn from previous in vitro studies showing the depletion of thiol levels in aorta and large coronary arteries during tolerance development.\(^1,18\) The discrepancy may relate to differences between the in vitro setup and the more physiological in vivo situation. Thus, plasma NTG levels in the present rat model are approximately 50 \(\mu\)g/L\(^4\) or 2000 to 4000 times lower than NTG concentrations used in the in vitro incubation media.\(^1,18\) Since ethanol is known to deplete intracellular GSH,\(^25,26\) it seems important to include NTG-vehicle (ethanol) as the placebo drug to distinguish between an NTG-induced thiol depletion and an NTG-unrelated alcohol-induced thiol depletion. This is also in accordance with the apparent trend toward decreased cellular thiol levels in the placebo group compared with the nonmedicated (untreated) group in the present study. The finding that nitrate tolerance was induced without changes in vascular GSH content in an in vitro study using NTG-vehicle as a placebo is relevant to this point and in agreement with the present in vivo findings.\(^27\)

A general limitation of the present study and of in vitro studies measuring intracellular thiol levels relates to the use of vascular homogenates, which include different cell types and interstitial fluid. Thus, intracellular thiol levels reported in these kinds of studies are based on thiol measurements in the vasculature as a whole and do not represent thiol levels in specific cell types (eg, vascular smooth muscle). Similarly, the present study does not rule out the possibility that NTG induces thiol depletion in smaller vessels or other specific vascular beds.

Previous studies have shown that pharmacologic manipulation of thiol levels significantly changes the hemodynamic effect of NTG. In the present study, NAC significantly potentiated the hypotensive effect of NTG and increased intracellular and extracellular cysteine levels. However, supraphysiological intracellular levels of cysteine and GSH do not change the vasoreactive effect of NTG either in vitro\(^18\) or in vivo.\(^4\) High extracellular cysteine levels may accelerate the degradation of NTG in plasma\(^21,23,28\) and/or react enzymatically with NTG at the cell membrane of the vascular smooth muscle cell.\(^9,30\) In addition, recent reports suggest that small coronary resistance vessels not normally sensitive to NTG respond to NTG in the presence of exogenous cysteine.\(^29,31\) By extending this finding to the peripheral circulation, it is possible that a significant proportion of the NTG-potentiating effect of NAC is the result of a dilution of resistance vessels that respond to NTG only in the presence of high extracellular cysteine levels.

All of these proposed nitrate-thiol interactions are compatible with the fact that NAC also potentiates the
acute effects of NTG (in conditions without tolerance). The finding that NAC in this rat model increases the hypotensive effect of NTG to a similar extent in tolerant (present study) and nontolerant rats further emphasizes that the NTG-NAC interaction probably does not demonstrate any specificity for tolerance. Similarly, the results of the vascular thiol measurements do not support the long-held theory that an effect of NAC on tolerance is mediated by a repletion of intracellular thiol stores.

In contrast to NAC, OXO is a specific intracellular cysteine delivery drug and is converted to cysteine by the intracellular enzyme 5-oxoprolinase. The pronounced capability of this drug to increase intracellular cysteine and GSH has previously been described in this model and other animal models and is presently illustrated in the nontolerant rats treated with OXO. Surprisingly, this effect is completely absent in the nitate-tolerant rats. Why OXO fails to increase cellular thiol levels in tolerant animals is not known, but this phenomenon may involve NTG-induced changes in the transmembranous transport of OXO or changes in activity of 5-oxoprolinase or other enzymes. NTG-induced changes in the activity of other enzymes like GSH S-transferases and guanylate cyclase have previously been implicated as a mechanism for tolerance development. Another tolerance-related change is suggested by the fact that we noted a twofold increase in plasma GSH after NAC treatment of tolerant rats, whereas in nontolerant rats, plasma GSH increased 18-fold. It is possible that NTG-tolerant rats have a more rapid turnover of GSH, thus explaining why OXO and NAC apparently fail to increase GSH levels; OXO (and NAC) may be used for GSH synthesis as fast as they are converted to cysteine. Although thiol depletion per se may not be the cause of nitrate tolerance in vivo, it appears that the cellular handling or turnover of thiol compounds may be altered in nitrate-tolerant animals as compared with nontolerant animals.

In summary, the present study is the first in which the effect of nitrate tolerance on vascular thiol levels (ie, cysteine and GSH) was examined in vivo. The results show that, in conscious unrestrained rats, hemodynamic tolerance to NTG occurs without changes in arterial or venous cysteine and GSH levels and that the NTG-potentiating effect of thiol administration (NAC) in nitrate tolerance is mediated by a mechanism other than replenishment of intracellular thiol levels. We conclude that (1) depletion of vascular thiol levels is apparently not the “cause” of nitrate tolerance in vivo and (2) the metabolic handling or turnover of thiol compounds and/or the activities of thiol-related enzymes may be significantly changed during the development of nitrate tolerance in vivo.

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