Nitroglycerin-Induced S-nitrosylation and Desensitization of Soluble Guanylyl Cyclase Contribute to Nitrate Tolerance

Nazish Sayed, David D. Kim, Xavier Fioramonti, Toru Iwahashi, Walter N. Durán, Annie Beuve

Abstract—Nitrates such as nitroglycerin (GTN) and nitric oxide donors such as S-nitrosothiols are clinically vasoactive through stimulation of soluble guanylyl cyclase (sGC), which produces the second messenger cGMP. Development of nitrate tolerance, after exposure to GTN for several hours, is a major drawback to a widely used cardiovascular therapy. We recently showed that exposure to nitric oxide and to S-nitrosothiols causes S-nitrosylation of sGC, which directly desensitizes sGC to stimulation by nitric oxide. We tested the hypothesis that desensitization of sGC by S-nitrosylation is a mechanism of nitrate tolerance. Our results established that vascular tolerance to nitrates can be recapitulated in vivo by S-nitrosylation through exposure to cell membrane-permeable S-nitrosothiols and that sGC is S-nitrosylated and desensitized in the tolerant, treated tissues. We next determined that (1) GTN treatment of primary aortic smooth muscle cells induces S-nitrosylation of sGC and its desensitization as a function of GTN concentration; (2) S-nitrosylation and desensitization are prevented by treatment with N-acetyl-cysteine, a precursor of glutathione, used clinically to prevent development of nitrate tolerance; and (3) S-nitrosylation and desensitization are reversed by cessation of GTN treatment. Finally, we demonstrated that in vivo development of nitrate tolerance and crosstolerance by 3-day chronic GTN treatment correlates with S-nitrosylation and desensitization of sGC in tolerant tissues. These results suggest that in vivo nitrate tolerance is mediated, in part, by desensitization of sGC through GTN-dependent S-nitrosylation. (Circ Res. 2008;103:606-614.)

Key Words: cGMP ■ nitric oxide ■ nitrosation ■ S-nitrosothiols ■ vascular tolerance

Since the 19th century, nitroglycerin (glyceryl trinitrate [GTN]) has been used in clinical medicine to treat angina pectoris and, more recently, congestive heart failure, acute myocardial infarction, and other cardiovascular diseases.1 It is used because of its excellent therapeutic profile, although it induces nitrate tolerance. Nitrate tolerance is defined as the attenuation or loss of vascular responsiveness to nitrate (eg, GTN) after continuous exposure to GTN or other organic nitrates.2 Crosstolerance corresponds to the lack of vascular responsiveness to nitric oxide (NO) and NO donors (eg, nitrates) and is characterized by an increase in reactive oxygen species.3 NO and nitrates exert their vasorelaxing effects through stimulation of soluble guanylyl cyclase (sGC), which produces cGMP.4 At the molecular level, tolerance corresponds to an absence of increased vascular cGMP production in response to NO or nitrates, highlighting the central role of sGC activity in vascular tolerance. The mechanism of development of nitrate tolerance remains a mystery; the prevalent actual model is impairment of GTN bioconversion through inhibition of mitochondrial aldehyde dehydrogenase, thereby reducing the release of NO or its derivatives.5,6 Other frequently cited mechanisms are (1) depletion of thiols, which are thought to be required for GTN conversion to NO and its derivatives; (2) increased phosphodiesterase activity, which breaks down cGMP; (3) reduced NO bioavailability by affecting the endogenous NO production; and (4) desensitization of sGC.10

Using S-nitrosothiols, we recently demonstrated that S-nitrosylation of sGC is a mechanism of desensitization to NO in primary aortic smooth muscle cells.11 Interestingly, it was shown that in vivo GTN treatment induces an increase in erythrocyte S-nitrosothiol content.12 Earlier, GTN treatment was proposed to increase intracellular concentration of S-nitrosothiols such as S-nitrosothiols (GSNO) and S-nitrosothiols (CSNO).7,13 and, in fact, these S-nitrosothiols were thought to mediate GTN vasorelaxing effects through sGC activation. However, we now know that GSNO and CSNO are also capable of S-nitrosylation, probably through transnitrosylation.14 Together, these findings led us to hypothesize that GTN treatment could S-nitrosylate sGC, leading to its desensitization, which in turn would participate in development of nitrate tolerance. If this hypothesis is correct, then (1) inducing S-nitrosylation in vivo should mimic development of tolerance; (2) GTN treatment should induce S-nitrosylation and desensitization of sGC; and (3) crosstolerance to NO donors that bypass GTN conversion or endothelial dysfunction by acting directly on sGC should be observed. We provide evidence that desensitization of sGC...
through S-nitrosylation could be an additional mechanism for GTN-induced nitrate tolerance.

Materials and Methods

Materials and Reagents

Fetal bovine serum was from Gibco (Carlsbad, Calif). All other cell culture reagents were from ATCC (Manassas, Va). Other materials are described in the “Supplemental Material and Methods.” L-cysteine and CSNO solutions were prepared as previously described.11

Hamster Cheek Pouch Preparation and Arteriolar Diameter Measurements

The hamster left cheek pouch was prepared for intravital microscopy as described.15 Under acute conditions, CSNO or GTN was applied to the cheek pouch through a side port into the suffusate bicarbonate buffer line. For 3-day treatment, GTN or solvent (propylene glycol) was infused through an osmotic pump at a rate of 10 μg/min · kg. GTN or solvent was then topically applied to the cheek pouch to measure arterial vasodilation (see “Supplemental Methods”). All procedures were approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School.

Soluble Guanylyl Cyclase Activity Assay

gc activity was determined by formation of [α-32P]GTP from [α-32P]GTP, as described.16 Forty micrograms of cell cytosol or 50 μg of tissues were used in each assay reaction.

Measurement of cGMP Production

cGMP production was measured by radioimmunoassay in the presence of 1 mmol/L IBMX (see “Supplemental Methods”).

Immunoprecipitation With Anti-S-nitrosocysteine

and Biotin Switch Assay to Detect S-nitrosylation

Immunoprecipitation was detailed in the “Supplemental Materials and Methods.” Biotin switch assay was performed using the NitroGlo Kit from Perkin Elmer17 on the cytosols of cells or tissues (300 μg).11 Statistical analysis is described in the “Supplemental Material and Methods.” Preparation of cytosols from tissues and cells is described in the Supplemental Material and Methods.

Results

S-nitrosocysteine Treatment Induces Vascular Tolerance In Vivo

To show that S-nitrosylation could induce vascular tolerance, we used the hamster cheek pouch preparation in which vasodilation of arterioles is measured by intravital microscopy in response to topical application of vasoactive agents18 (see “Supplemental Methods”). S-nitrosocysteine (CSNO) was used because it (1) induces vasorelaxation (probably through release of NO); (2) is a S-nitrosylating agent that causes S-nitrosylation and desensitization of sGC in cells; and (3) accumulates during GTN treatment.13 L-Cysteine (L-Cys), as control, and CSNO at 10 μmol/L were applied for 5 minutes, vasodilation was measured, and after 10 minutes washout, 10 μmol/L of the NO donor SNAP was applied for 5 minutes and vasodilation assessed. As shown in Figure 1A, following initial application of 10 μmol/L L-Cys, SNAP produced immediate vasodilation of the arteriole. Application of 10 μmol/L CSNO induced vasodilation (as expected for an NO donor), yet after return toward the baseline value, arterioles failed to vasodilate in response to SNAP (Figure 1B). This result shows that the S-nitrosothiol CSNO causes vascular tolerance in an in vivo system under acute conditions.

S-nitrosocysteine-Induced Tolerance Is Associated With S-nitrosylation and Desensitization of Soluble Guanylyl Cyclase

After 5 minutes of 10 μmol/L CSNO or L-Cys treatment (with verification that CSNO induced vasodilation as described previously), the treated pouches and the untreated contralateral pouch (naïve control cysteine or CSNO) were collected, instead of being treated with SNAP, to determine sGC S-nitrosylation and activity. S-nitrosylation was assayed by 3 methods: immunoprecipitation (IP) with anti-SNO antibodies (Figure 1C), the biotin switch assay followed by avidin purification,21 and IP with anti-sGC followed by biotin/avidin switch assay (Figure 1D). Western blots with anti-sGC showed that sGC was strongly S-nitrosylated in the CSNO-treated tissues that exhibited tolerance in comparison to L-Cys-treated pouches or in the contralateral pouches (Figure 1C, D). The same cytosols were assayed for sGC activity. Figure 1E showed that the basal sGC activity was similar between the L-Cys, CSNO-treated tissues and their naive controls, but the sGC in the CSNO-treated pouches lost significant sensitivity to SNAP in comparison to controls. These results indicate that the CSNO-treated pouches that exhibit tolerance, ie, lack of relaxation of their arterioles in response to NO stimulation, contain high levels of S-nitrosylated sGC and displayed a significant decrease in NO-stimulated sGC activity.

Treatment With S-nitrosoglutathione Does Not Induce Tolerance and Does Not Lead to Detectable S-nitrosylation

In vivo treatment with more commonly used S-nitrosothiols such as GSNO or SNAP does not lead to tolerance.22,23 Interestingly, we and others have observed that GSNO does not readily S-nitrosylate intact cells or tissues unlike CSNO.20,24 To investigate further the correlation between S-nitrosylation of sGC and vascular tolerance, the hamster cheek pouch was treated with GSNO under the same conditions as previously described (5 minutes, 10 μmol/L GSNO) and after washout, 10 μmol/L SNAP was applied, as in Figure 1. Figure 2A is a control showing vasorelaxation in response to SNAP. As shown in Figure 2B, GSNO application induces vasorelaxation, as expected for an NO donor, but in contrast to CSNO, there was complete vasorelaxation in response to a subsequent SNAP application indicating that GSNO did not lead to tolerance. Moreover, no S-nitrosylation could be detected in the treated tissues (Figure 2C).

Nitroglycerin Induces S-nitrosylation of Soluble Guanylyl Cyclase and Desensitization in Primary Rat Aortic Smooth Muscle Cells in a Concentration-Dependent Manner

To determine whether GTN treatment leads to S-nitrosylation and desensitization of sGC, we exposed primary smooth muscle cell (SMC) to 100 μmol/L of GTN for 1 hour. As shown in Figure 3A, both biotin–avidin and IP show that GTN induces S-nitrosylation of sGC. No S-nitrosylated sGC could be detected when SMC was treated with methanol (vehicle). We next assayed whether GTN also induces desensitization of sGC by measuring by radioimmunoassay the
cGMP produced in response to SNAP after treatment with various concentrations of GTN. Figure 3B shows that increasing concentrations of GTN (100 to 500 \( \mu \text{mol/L} \)) significantly decreased production of NO-stimulated cGMP by SMC. In parallel, biotin switch assay on the cytosols of GTN-treated SMC indicated that S-nitrosylated sGC levels increased with increasing concentration of GTN (Figure 3B, inset). These results provide evidence that GTN treatment leads to S-nitrosylation of endogenous sGC thiols and induces its desensitization.

Nitroglycerin-Induced S-nitrosylation and Desensitization of Soluble Guanylyl Cyclase in Smooth Muscle Cell Are Prevented by N-acetyl-cysteine Treatment and Are Reversible

S-nitrosylation is a reversible posttranslational modification dependent on the redox state. Therefore, N-acetyl-cysteine (NAC), as a precursor of glutathione synthesis, can prevent S-nitrosylation. To confirm that GTN induces desensitization by S-nitrosylation of sGC, SMC were treated for 2 hours with 2.5 \( \mu \text{mol/L} \) NAC before exposure to GTN (100 \( \mu \text{mol/L} \)) for 5 minutes; n=4. Ten \( \mu \text{mol/L} \) CSNO was used because we did not observe vasodilation in the presence of 1 \( \mu \text{mol/L} \) CSNO and observed persistent vasodilation if 1 mmol/L CSNO was applied. C, Western blots (WB) with anti-sGC after IP with anti-SNO show that S-nitrosylated sGC is pulled down from the CSNO-treated pouches but not from L-Cys-treated pouches or naïve controls. Inputs represent 30% of the precleared cytosols and indicate similar levels of sGC in the various samples. No sGC was pulled down with IgG indicating specificity of the anti-SNO antibodies. D, WB with anti-sGC of biotin–avidin assay before (middle panel) and after IP with anti-sGC (right panel) indicates that 1 \( \mu \text{mol/L} \) CSNO did not have any detectable S-nitrosylated sGC (not shown). The sGC contains \( \alpha 1 \) and \( \beta 1 \) subunits with a molecular weight (MW) of 80 and 72 kDa, indicated by arrows. E, Basal and NO-stimulated activity of the same cytosols showed that sGC from CSNO-treated tissues did not respond to NO stimulation compared with L-Cys and naïve controls. SNAP was used at 1 mmol/L. Experiments were repeated 4 times with each measurement done in duplicate and expressed in pmol/min \( \cdot mg^{-1} \pm SE \). *P<0.05 CSNO treated versus naïve or L-Cys.
induces marked relaxation in the cheek pouch. B, Similar relaxation with SNAP was observed after GSNO treatment (10 μmol/L, 5 minutes). There was no significant difference in the arteriole luminal diameter in response to SNAP in control versus GSNO; n = 3. C, Biotin–avidin assay followed by Western Blot with anti-sGC of 2 cheek pouches treated with GSNO and SNAP did not show S-nitrosylation. sGC was expressed in the cheek pouches (input). Positive control of the assay indicates S-nitrosylation of purified wild-type after treatment with GSNO.

**Mutations of α1C243 and β1C122 Significantly Reduce the Desensitization Observed in the Wild-Type Treated With Nitroglycerin**

We tested whether α1C243 and β1C122, whose S-nitrosylation is involved in desensitization of sGC by CSNO, are also responsible for GTN-dependent desensitization. COS-7 cells, which do not have detectable sGC, were transfected for 48 hours with wild-type, α1C243A/β1 or α1/β1C122A mutants then treated for 1 hour with 100 μmol/L GTN or vehicle; NO-stimulated sGC activity of the various cytosols was assayed. As shown in Figure 5, wild-type lost more than 50% of the response to SNAP after GTN treatment (50.7% ± 1.4% desensitization). Mutants C243A and C122A had a 30.1% ± 4.6% and 33.6% ± 4.5% decrease in NO-stimulated activity, respectively, which corresponds to elimination of approximately 40% of the desensitization seen in the wild-type. These results indicate a causal relationship between S-nitrosylation by GTN and desensitization of sGC in cells. We next tested if this causal relationship could explain nitrate tolerance.

**Nitroglycerin-Induced Nitrate Tolerance Correlates With S-nitrosylation and Desensitization of Soluble Guanylyl Cyclase In Vivo**

Using a similar setup as with CSNO, we first established nitrate tolerance in the hamster cheek pouch preparation under acute conditions (50 minutes topical application of 1 μmol/L GTN). As shown in Supplemental Figure I, after initial vasodilation, the arterioles failed to relax in response to a dose challenge of 10 μmol/L GTN; the nitrate-tolerant tissues contain S-nitrosylated sGC (Supplemental Figure IB), which was desensitized by GTN as shown by the significant decreased in cGMP production in response to 10 μmol/L GTN, 100 μmol/L GTN, and 1 mmol/L SNAP (Supplemental Figure IC).

To study the sGC properties under conditions that mimic the development of nitrate tolerance in clinical settings, we used osmotic pumps to infuse continuously for 3 days low doses GTN (10 μg/min·kg) or solvent (propylene glycol), as previously described. There was vasodilation in response to 10 μmol/L GTN in animals treated with the solvent (1.37 ± 0.13-fold increase) and no vasodilation in the 3-day
GTN-treated animals confirming nitrate tolerance development (Figure 6A). After the 3-day infusion, the lungs and cheek pouches were collected. Both biotin–avidin assay (Figure 6B) and IP with anti-SNO (Figure 6C) show that sGC is S-nitrosylated after chronic exposure to GTN in the pouches and lungs and that no or little S-nitrosylated sGC was detected in the animals infused with vehicle. Specificity of sGC S-nitrosylation was verified by pretreatment with NAC. Cells treated with MeOH or MeOH + NAC did not contain detectable S-nitrosylated sGC. GTN was used at 100 μmol/L. Inputs indicate a similar amount of sGC in the precleared cytosols of the various treatments. B. The cytosols pretreated with NAC are more responsive to NO stimulation compared with the ones treated with GTN only. SNAP: 100 μmol/L. The basal sGC activity was similar for the various treatments. NAC had no effect on NO-stimulated activity for samples treated with MeOH. C. WB of biotin–avidin assay indicating that washout of GTN for 1 hour decreases S-nitrosylation of sGC to a level close to control. Conversely, washout resulted in a drop of desensitization (to 5%) in response to 100 μmol/L SNAP. Measurements of sGC activity were done in 3 independent experiments and expressed as mean ± SE.

Figure 4. GTN-induced S-nitrosylation and desensitization of sGC are prevented by NAC treatment in SMC. A. Western blot (WB) with anti-sGC shows that the amount of sGC pulled down with anti-SNO is greatly reduced in the NAC+GTN cytosols compared with cells not pretreated with NAC. Cells treated with MeOH or MeOH + NAC did not contain detectable S-nitrosylated sGC. GTN was used at 100 μmol/L. Inputs indicate a similar amount of sGC in the precleared cytosols of the various treatments. B. The cytosols pretreated with NAC are more responsive to NO stimulation compared with the ones treated with GTN only. SNAP: 100 μmol/L. The basal sGC activity was similar for the various treatments. NAC had no effect on NO-stimulated activity for samples treated with MeOH. C. WB of biotin–avidin assay indicating that washout of GTN for 1 hour decreases S-nitrosylation of sGC to a level close to control. Conversely, washout resulted in a drop of desensitization (to 5%) in response to 100 μmol/L SNAP. Measurements of sGC activity were done in 3 independent experiments and expressed as mean ± SE.

*P < 0.05 GTN + NAC versus GTN.

We next assayed desensitization of sGC. In the animals chronically treated with GTN, the cGMP production in response to 10 and 100 μmol/L GTN was greatly reduced in comparison to the sham-treated animals in lungs (Figure 6D) and in cheek pouches at 100 μmol/L GTN (Figure 6D, inset). Similarly, sGC desensitization was remarkable in response to 1 mmol/L SNAP, because chronic GTN treatment induced a 59% and 85% NO-dependent desensitization of sGC in lungs and pouches, respectively. The levels of cGMP produced in the lungs were also much higher than in the pouches (not shown), which could be due to the high levels of sGC present in the lungs (Figure 6B, input).

Importantly, we showed that there was crosstolerance; relaxation in response to 10 μmol/L SNAP was significantly reduced in the animals treated with GTN in comparison to the propylene glycol-treated animals (Figure 6E). This decrease in SNAP-dependent relaxation, which was associated with decreased SNAP-dependent cGMP levels (Figure 6D), sug-

Figure 5. Mutations α1C243A and β1C122A significantly alter GTN-dependent desensitization. A. COS-7 cells expressing wild-type (WT) and mutants were treated for 1 hour with GTN 100 μmol/L or methanol (MeOH). NO-stimulated sGC activity was measured in the cytosols with 100 μmol/L SNAP. These experiments were repeated 4 times with 3 transfections; results are expressed as the percentage of NO-stimulated activity with GTN treatment versus vehicle. *P < 0.05. B. Western blot with anti-sGC indicates that expression of sGC and mutants is not altered by GTN.

% desensitization: 51.4 ± 8.7 5.0 ± 14.3

Figure 6. A B C D

A

B

C

D

% desensitization: 51.4 ± 8.7 5.0 ± 14.3

% of NO-stimulated sGC activity

0 20 40 60 80 100

WT (α1/β1) α1C243A/β1 α1/β1C122A

α β

α1 β1

α1 β1

α1 β1
suggests that sGC desensitization per se is, in part, responsible for the observed tolerance.

Discussion

In these in vitro and in vivo studies, we provide evidence that nitroglycerin treatment, whether at low therapeutic doses in animals or at higher doses in cells, is associated with S-nitrosylation of sGC and its desensitization, which correlate with the development of nitrate tolerance and crosstolerance in an in vivo model of hamster cheek pouch. This link between S-nitrosylation of sGC and vascular tolerance was supported by the observations that the S-nitrosylating compound CSNO induced vascular tolerance in vivo and that the tolerant tissues contain S-nitrosylated sGC, which is desensitized. This CSNO-induced tolerance, which we define as the lack of relaxation of arterioles in the hamster cheek pouch preparation in response to SNAP, is intriguing because to date, organic nitrates such as GTN were the only established tolerance inducers. Tolerance is not readily observed after treatment with an NO donor such as sodium nitroprusside and S-nitrosothiols such as SNAP or GSNO. These NO donors, unlike CSNO, remain in the extracellular compartment or in the cell membranes and release NO extracellularly. Interestingly, we have observed

Vascular Tolerance Is Obtained With the Cell-Permeable S-nitrosocysteine, Which Induces S-nitrosylation and Desensitization of Soluble Guanylyl Cyclase

We showed that topical application of an S-nitrosylating agent, CSNO, is sufficient to induce vascular tolerance in vivo and that the tolerant tissues contain S-nitrosylated sGC, which is desensitized. This CSNO-induced tolerance, which we define as the lack of relaxation of arterioles in the hamster cheek pouch preparation in response to SNAP, is intriguing because to date, organic nitrates such as GTN were the only established tolerance inducers. Tolerance is not readily observed after treatment with an NO donor such as sodium nitroprusside and S-nitrosothiols such as SNAP or GSNO. These NO donors, unlike CSNO, remain in the extracellular compartment or in the cell membranes and release NO extracellularly. Interestingly, we have observed
that CSNO strongly S-nitrosylated sGC in intact cells, but not the other S-nitrosothiols GSNO or SNAP (not shown). These observations were in support of results showing that S-nitrosylation and modification of intracellular thiols depend on the ability of CSNO to readily enter cells through L-Cys transporters and do not depend on the release of NO. To strengthen the potential link between S-nitrosylation of sGC and tolerance, we repeated the topical application with GSNO under the same conditions as with CSNO and showed that the relaxation of arterioles in response to SNAP was maintained, indicating no development of tolerance. Moreover, the GSNO-treated tissues did not have detectable S-nitrosylated sGC, unlike the CSNO-treated ones. Yet, we cannot rule out that part of this phenotype is due to reversion by GSNO reductase. These results suggest that CSNO induces tolerance because it S-nitrosylates sGC potentially through transnitrosylation after its transport into cells. It should be mentioned that in our study, the concentrations of CSNO (and GSNO) required to induce arterioles vasorelaxation are higher than in other reports. One potential explanation for the decreased response to these NO donors is the fact that the hamster cheek pouch preparation is different from conventional ex vivo models such as isolated aortic rings that are precontracted with norepinephrine.

**Does Nitroglycerin Treatment Induce S-nitrosylation of Soluble Guanylyl Cyclase and Thereby Its Desensitization Thus Contributing to Tolerance?**

This hypothesis is based on our result showing that vascular tolerance could be caused by CSNO-induced S-nitrosylation, hence desensitization of sGC, and the recent report that GTN treatment leads to increased S-nitrosylation of tissues as measured by RSNO formation. GTN and other nitrates vasorelaxing effects are mediated by stimulation of sGC, and many communications reported on desensitization of sGC by GTN treatment and its relationship to nitrate tolerance but without providing a mechanism of desensitization. We showed that GTN treatment, whether at low therapeutic doses in vivo or at various doses in SMC, lead to dose-dependent S-nitrosylation of sGC. We previously showed in a purified sGC system using GSNO and by mutational analysis of the S-nitrosylated cysteine αC243 and βC122 that S-nitrosylation directly causes desensitization. Thus, it was not surprising to observe that GTN-induced S-nitrosylation correlated with desensitization of sGC in cells. Moreover, replacement of αC243 and βC122 led to partial elimination of GTN-dependent desensitization, supporting a causal relationship between GTN treatment and sGC desensitization. Elimination of desensitization was not complete suggesting that other cysteine could be involved in GTN-dependent desensitization of sGC. We observed the same association in our in vivo model of nitrate tolerance. We first use an “acute” model (50 minutes GTN infusion in the cheek pouch) because the same tissue is assayed for tolerance (measurement of arteriole relaxation), sGC S-nitrosylation, and desensitization (Supplemental Figure I). However, nitrate tolerance develops clinically under prolonged exposure to low doses of GTN. Thus, to mimic development of nitrate tolerance in patients, we used osmotic pumps to infuse clinically relevant doses of GTN and showed that tolerant tissues contained S-nitrosylated sGC that was desensitized to GTN and to SNAP (crosstolerance). As reported by others, we detected an increase in total sGC amount in 3-day GTN-treated animals; yet, this increase was accompanied by an increase in the S-nitrosylated form of sGC, hence desensitized sGC, thus explaining the apparent discrepancy between higher sGC expression and decreased cGMP production.

**Thiols Depletion and the Soluble Guanylyl Cyclase S-nitrosylation/Desensitization Model**

The reversion of both S-nitrosylation and desensitization after 1 hour washout after GTN treatment and the fact that NAC prevents S-nitrosylation and desensitization of sGC in SMC fit well with the pharmacodynamics of nitrate tolerance. Reversibility of S-nitrosylation and desensitization could explain why nitrate sensitivity is progressively restored (4 to 12 hours) in patients after cessation of GTN treatment; this characteristic is used clinically in “intermittent nitrate therapy” to avoid development of nitrate tolerance. Likewise, NAC, a precursor of glutathione synthesis, is used to prevent the development of nitrate tolerance in patients treated with nitroglycerin based on the early Needleman’s model suggesting that nitrate tolerance is due to free thiol depletion (yet others have not observed thiol depletion). In this model, GTN produced nitrosothiols through reactions with free thiols, which in turn activate sGC; therefore, prolonged treatment leads to depletion of the thiols and cessation of activation. However, we observed that GTN-dependent reduced vasorelaxation and desensitization of sGC is seen in response to the NO donor SNAP, which does not require free thiols to activate sGC. Thus, we speculate that NAC facilitates the reversion of S-nitrosylation due to thiol depletion, which has been associated with increased S-nitrosylation of protein thiols. It is known that superoxide, generated by GTN treatment, is responsible for the production of reactive nitrogen species, in particular N2O3, which could directly S-nitrosylate thiol of proteins or convert glutathione or L-Cys to form GSNO and CSNO, respectively. This latter reaction not only will deplete the cells of free thiols, but also generate S-nitrosothiols, known to be increased by GTN treatment, which could S-nitrosylate proteins through transnitrosylation. We speculate that continuous GTN treatment by affecting the equilibrium between the reactive oxygen species and reactive nitrogen species produced and intracellular glutathione levels could favor S-nitrosylation.

**How Does Soluble Guanylyl Cyclase Desensitization by S-nitrosylation Fit in the Mechanism-Based, Classical Tolerance?**

The development of nitrate tolerance is multifactorial and the cGMP-dependent loss of vasorelaxation is not well understood. Chronic GTN treatment leads to generation of superoxide and this reactive oxygen species production could induce thiol oxidation. In this study, we measured specifically S-nitrosylation and therefore we cannot rule out that the sGC could be desensitized by other thiol modifications, including disulfide bond formation or glutathionylation. Upstream of
sGC, the high production of reactive oxygen species was shown to lead to endothelial dysfunction, which is proposed to be a mechanism of nitrate tolerance; for example, chronic GTN treatment resulted in a loss of acetylcholine-induced relaxation. Consequently, this reduced NO availability should decrease sGC activation. Recently, inhibition of mitochondrial aldehyde dehydrogenase, which is involved in GTN bioconversion, has been identified as a major mechanism of nitrate tolerance. This impairment in GTN bioconversion is reflected by a decreased stimulation of sGC because of reduced NO or derivatives. Downstream of sGC, the high production of reactive oxygen species was shown to contribute to nitrate tolerance; for example, chronic nitroglycerin treatment (or application of the S-nitrosylating agent CSNO) leads to a partial loss of arterioles relaxation and decreased cGMP production in response to SNAP, suggesting crosstalk. This result indicates that sGC activity per se is affected by the nitroglycerin/CSNO treatment as SNAP spontaneously released NO, bypassing GTN bioconversion or endothelial NO availability, at least in the vascular system of the hamster cheek pouch. Nonetheless, the response to SNAP was not as blunted as the response to GTN suggesting that desensitization of sGC is another site affected by nitroglycerin in addition to the main upstream site (mitochondrial aldehyde dehydrogenase inhibition) for establishment of nitrate tolerance. Thus, nitroglycerin-induced tolerance could be the result of a double setback for sGC; impairment of GTN bioconversion deprived sGC from activation by NO derivatives and S-nitrosylation of sGC prevents any further activation.

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Disclosures
None.

References


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Expanded Materials and Methods:

**Reagents:** Polyclonal antibodies against the \( \alpha_1 \) and \( \beta_1 \) subunit of sGC were from Cayman Chemicals (Ann Arbor, MI) and anti-SNO-cysteine antibody was from Alpha Diagnostics international (San Antonio, TX). SNAP was from Calbiochem (San Diego, CA). L-cysteine and sodium nitrite were from Sigma (St. Louis, MO). Protein-A Sepharose beads were from Amersham Biosciences (Piscataway, NJ) and Streptavidin-agarose beads from Invitrogen (Carlsbad, CA). Biotin-switch assay was performed using NitroGlo™ Kit from Perkin Elmer (Boston, MA). Nitroglycerin (GTN) was from Cerilliant Corporation (Round Rock, TX). Alzet Osmotic pumps are from Durect Corporation (Cupertino, CA). Primary rat aortic smooth muscle cells were obtained from Dr. Andreas Papapetropoulos (University of Patras, Greece). COS-7 cells and RASMC were grown as previously described \(^1\) and treated with concentrations of GTN ranging from 100 to 500\(\mu\)M for 1hr.

**Preparation of cytosols from tissues and cells:** Collected tissues from hamster cheek pouches and lungs (~100mg) were homogenized in 10 vol. of 50mM HEPES pH 8.0 containing 150mM NaCl, 10% glycerol, 1mM EDTA and protease inhibitors. The samples were then centrifuged. Assays were done with the soluble fraction (supernatant). Smooth muscle cells (SMC) cytosols were prepared as previously described \(^1\).

**Measurement of cGMP Production:** cGMP production was measured by RIA in the presence of IBMX a wide spectrum inhibitor of phosphodiesterase (PDE) activity. Briefly, pouches and lungs were collected in physiological buffer (130mM NaCl, 5.6mM KCl, 2mM CaCl\(_2\), 1mM MgCl\(_2\), 11mM glucose and 10mM HEPES pH 7.4). Tissues were treated with vehicle...
(methanol), or 1mM SNAP, 10µM GTN or 100µM GTN for 1h. SNAP-frozen tissues were
pulverized and resuspended in HEPES buffer containing 2.5% perchloroacetic acid. cGMP from
SMC was measured as in 1.

Hamster cheek pouch preparation and osmotic pump implantation: Golden Syrian Hamsters
(male, 80-120g) were anesthetized with sodium pentobarbital (50 mg/kg IP).
-For hamster cheek pouch preparation: Tracheotomy was performed to ensure a clear airway
passage. The right jugular vein was cannulated for administration of supplemental doses of
anaesthetic. The right carotid artery was cannulated for monitoring blood pressure using a
PowerLab Pressure Monitor (AD Instruments, Colorado Springs, CO). The left hamster cheek
pouch was prepared for intravital microscopy as described previously 2.
-For osmotic pump implantation: on a warm pad (37°C) and under anaesthesia, an incision (1
cm) was made on the upper back of the animal to implant the osmotic pump and the skin was
then sutured with sterile 3-0 nylon thread.
At the completion of the experiments, the hamster was sacrificed by an anaesthetic overdose of
sodium pentobarbital (150 mg/kg IV) while under anaesthesia.

Arteriolar diameter measurements by intravital microscopy: The recording system
comprises an Olympus BH microscope, a TEC-470 camera (Optronics, Goleta, CA) and a Sony
monitor. Arterial luminal diameter was measured as the width of the trans-illuminated blood
column using the MetaMorph image system (Universal Imaging Corporation, Downingtown,
PA). The image system was calibrated with a slide micrometer. 3 to 4 arterioles were studied per
animal. Baseline diameters were normalized to a value of 1. For each vessel, the experimental
diameter was expressed as the ratio of baseline diameter (relative luminal diameter). To compare diameters before and after each intervention, diameters were measured at the same place in the arterioles of interest.

**Immunoprecipitation**: For immunoprecipitation, cytosols (300μg) were precleared with Protein A-Sepharose 4B beads, following the supplier's protocol (Amersham Biosciences). Precleared cytosols were incubated with nonimmune serum or rabbit polyclonal anti-SNO or rabbit polyclonal anti-sGC (α1 & β1) overnight at 4 °C. Protein A beads were added to samples for 2 hrs at 4 °C. Beads were pelleted by centrifugation and washed three times with lysis buffer. Proteins were eluted in 1% SDS buffer and resolved on 8% SDS-PAGE and analyzed by immunoblotting with anti-sGC (α1 & β1 subunits) antibodies.

**Statistical analysis**: All data are expressed as means ± SE. For vessel diameter, one-way analysis of variance was performed using the InStat package (GraphPad, San Diego, CA). The Student-Newman-Keuls test was applied to determine which measurement differed significantly from another. Data for cGMP measurement by RIA or sGC activity assay were obtained from 4 to 5 independent batches of cells or tissues for each condition. Comparison of sGC activities or cGMP production between treatment with methanol (vehicle) and GTN or between treatment with GTN and GTN + NAC were made with Student’s *t* test using Sigma plot version 8.0 software (Systat software, San Jose, CA). *P*<0.05 was considered statistically significant.


**Online Figure I:**

Under acute conditions, nitrate tolerant tissues contain S-nitrosylated sGC, which is desensitized to GTN and NO-donor. (A) 1μM GTN induces vasodilation with a plateau at 1.3 ± 0.1 during 50min suffusion. Tolerance was established as the arterioles failed to relax in response to a 10μM GTN dose challenge, following 15 min washout. n=4. (B) Western blot with anti-sGC, following biotin switch assay, indicate that GTN treated-tissues are strongly S-nitrosylated in comparison to naive or sham (bicarbonate buffer-treated) tissues. A low level of S-nitrosylated sGC was detected in the contralateral pouch (naïve) suggesting that some of the applied GTN suffusion might have entered the systemic circulation. Expression of sGC was similar (input, left side). (C) cGMP-dependent production (measured by RIA) in response to 1mM SNAP, 10 and 100μM GTN is significantly reduced in the cytosols of the GTN treated-tissues compared to sham-treated tissues. In the GTN-treated tolerant tissues, SNAP-stimulated cGMP levels were increased only 5.4 ± 1.0 fold compared to 46.7 ± 17.3 fold in the sham-treated, there was no cGMP increase in response to 10μM GTN (vs. 3.9 ± 1.0 fold increase in control) and a 5.9 ± 2.6 fold cGMP increase with 100μM GTN (14.2 ± 4.9 fold in sham-treated tissues). Results were expressed as fold-stimulation over basal values (obtained with methanol). The values were
normalized using cAMP levels measured in the same samples, which are good indicators of tissues amount and independent of GTN or NO stimulation. *, $P<0.05$ sham vs. GTN-treated.
Online Figure II:

The levels of S-nitrosylated sGC in GTN-treated tissues, detected by biotin-avidin assay, are greatly reduced by pre-treatment with 30mM ascorbate. Input: homogenate of GTN-treated lungs.