Nitroglycerin Fails to Lower Blood Pressure in Redox-Dead Cys42Ser PKG1α Knock-In Mouse

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Background—Although nitroglycerin has remained in clinical use since 1879, the mechanism by which it relaxes blood vessels to lower blood pressure remains incompletely understood. Nitroglycerin undergoes metabolism that generates several reaction products, including oxidants, and this bioactivation process is essential for vasodilation. Protein kinase G (PKG) mediates classic nitric oxide–dependent vasorelaxation, but the 1α isof orm is also independently activated by oxidation that involves interprotein disulfide formation within this homodimeric protein complex. We hypothesized that nitroglycerin-induced vasodilation is mediated by disulfide activation of PKG1α.

Methods and Results—Treating smooth muscle cells or isolated blood vessels with nitroglycerin caused PKG1α disulfide dimerization. PKG1α disulfide formation was increased in wild-type mouse aortas by in vivo nitroglycerin treatment, but this oxidation was lost as tolerance developed. To establish whether kinase oxidation underlies nitroglycerin-induced vasodilation in vivo, we used a Cys42Ser PKG1α knock-in mouse that cannot transduce oxidant signals because it does not contain the vital redox-sensing thiol. This redox-dead knock-in mouse was substantively deficient in hypotensive response to nitroglycerin compared with wild-type litters as measured in vivo by radiotelemetry. Resistance blood vessels from knock-ins were markedly less sensitive to nitroglycerin-induced vasodilation (EC50 = 39.2 ± 10.7 μmol/L) than wild-types (EC50 = 12.1 ± 2.9 μmol/L). Furthermore, after ~24 hours of treatment, wild-type controls stopped vasodilating to nitroglycerin, and the vascular sensitivity to nitroglycerin was decreased, whereas this tolerance phenomenon, which routinely hampers the management of hypertensive patients, was absent in knock-ins.

Conclusions—PKG1α disulfide formation is a significant mediator of nitroglycerin-induced vasodilation, and tolerance to nitroglycerin is associated with loss of kinase oxidation. (Circulation. 2012;126:287-295.)

Key Words: blood pressure ■ nitroglycerin ■ oxidative stress ■ signal transduction ■ vascular response

Nitroglycerin, generally referred to as glyceryl trinitrate (GTN) clinically, remains a treatment for unstable angina and hypertension.1 However, chronic treatment of patients with GTN is substantially limited by the development of tolerance, a condition in which the vasodilatory and blood pressure action of GTN is lost or higher doses of the drug are required.1 GTN is metabolized by smooth muscle cells to yield a molecular form that mediates vasodilation. This bioactivation process yields nitric oxide (NO), S-nitrosothiols, inorganic nitrite, and glycerol-1,2-dinitrate,2 as well as reactive oxygen species.3 GTN is bioactivated principally by mitochondrial aldehyde dehydrogenase (mtALDH)2,4 but also by the cytosolic isoform,5 and this metabolic conversion is essential for its vasodilatory actions. It has been commonly assumed that the NO generated is responsible for GTN-induced vasodilation. In this scenario, NO would bind to and activate soluble guanylate cyclase to stimulate cGMP production, which activates cGMP-dependent protein kinase (PKG). PKG then phosphorylates a number of target proteins, which results in smooth muscle relaxation and vasodilation.1 However, recent studies have provided evidence that NO does not mediate the relaxation of vessels to GTN. For example, GTN relaxes vessels without elevating cellular NO levels,6 which suggests the classic NO-cGMP-PKG pathway was not in operation and that another mechanism or bioactivation product was responsible for the vasodilation.

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We have previously shown that PKG1α can be activated wholly independently of the classic NO-cGMP pathway by thiol oxidants such as hydrogen peroxide (H2O2)7 or the nitrosothiol nitrosocysteine.8 PKG1α is a parallel-aligned homodimer held together by the electrostatic attraction of its N-terminal leucine zipper. This dimerization domain also
contains 2 thiols (from Cys42 on each of the chains) that align directly opposite one another. Oxidants induce an interprotein disulfide between the 2 cysteines, and this activates the kinase by increasing its affinity for substrates, which results in their phosphorylation. Indeed, this oxidative activation of PKG1α is a major molecular mechanism by which oxidants relax blood vessels ex vivo and in vivo.

A logical possibility is that nitrosothiols generated during bioactivation recruits oxidative disulfide activation of PKG1α, and this is a major mechanism underlying GTN-induced vasodilation. This possibility is supported by GTN-inducing protein S-nitrosylation in vivo. S-nitrosylated protein thiols will readily react with a proximal thiol to generate a disulfide, a process we showed indeed occurs in PKG1 when cells are exposed to nitrosothiols. Furthermore, GTN bioactivation also promotes reactive oxygen species formation, which provides another potentially synergistic mechanism that could also drive oxidative activation of PKG1α. Indeed, GTN-induced oxidative stress results in oxidation of ALDH, in which a disulfide bond forms in its active-site cysteine. Oxidized ALDH accumulates because the reducing equivalents required for its cyclic reduction (ie, reduced lipoic acid and NADPH) become depleted as they are consumed during GTN bioactivation. This oxidation inactivates ALDH, which prevents GTN bioactivation and contributes to tolerance.

In the present study, we test the hypothesis that GTN-dependent vasodilation is mediated by oxidative activation of PKG1α. Our studies have been aided significantly by the use of a Cys42Ser PKG-1α knock-in (KI) mouse in which wild-type (WT) kinase has been systemically replaced by a “redox-dead” form in which the cysteinyl thiol has been replaced by a hydroxy group. This mutation prevents oxidative disulfide activation of PKG1α while maintaining classic NO-cGMP–dependent stimulation. We demonstrate using radiotelemetric monitoring of blood pressure in vivo that KI mice are deficient in their hypotensive response to GTN compared with WT mice. Furthermore, comparison of the dose-dependent relaxation of isolated blood vessels showed KIs were intrinsically less sensitive to GTN than WTs. Unlike their WT littermates, KI mice also fail to become tolerant, albeit the interpretation of this observation is complicated by their deficient response to GTN basal.

**Methods**

**Cys42Ser Redox-Dead PKG1α KI Mice**

All procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 in the United Kingdom. Mice constitutively expressing PKG1α Cys42Ser were generated for us on a pure C57BL/6 background by TaconicArtemis (Köl n, Germany). A targeting vector was constructed that involved polymerase chain reaction amplification of the murine Prkg1, which introduced the Cys42Ser mutation into exon 1a (which is specific for the α-isofrom) by site-directed mutagenesis, and insertion of an FRT-flanked neomycin selection marker (to allow for selection of transfected embryonic stem cells) close to the mutation to favor homologous recombination. Next, screening by Southern blot was performed to identify whether homologous recombination had occurred, followed by validation of the positive clones. Embryonic stem cell transfection was then performed, followed by chimera generation. The chimeras were bred directly with an Flp deleter for the in vivo deletion of the selection marker. Because the embryonic stem cells always go germline, chimeras can be bred directly to the deleter to obtain germline transmission and selection marker deletion at the same time.

**Cultured Vessels**

Rat aortic smooth muscle cells (A10) were grown on 12-well plates in an incubator at 37°C with a 95% O2–5% CO2 environment. Once confluent, A10 cells were treated with or without GTN 100 μmol/L for 4 hours. For some samples, additional treatments of GTN 100 μmol/L were added either at 2, 3, or 4 hours.

**Isolated Vessels**

Mice were euthanized by pentobarbital overdose, and vessels were isolated and cleaned from surrounding tissues and fat in an ice-cold Krebs solution. Thoracic aorta vascular rings (5 mm) or third-order mesenteric vessels were incubated with 3 to 300 μmol/L nitroglycerin (GTN; 1 mg/mL from Hameln Pharmaceuticals Ltd) at 37°C with a 95% O2–5% CO2 environment and frozen and homogenized in liquid nitrogen. NADPH fluorescence (340 nm excitation; 450 nm emission) was measured in isolated aortas before and after GTN (1 μmol/L, 5 minutes) treatment with a FLUOstar microplate reader (BMG Labtech GmbH).

**Immunoblotting**

Immunoblotting for PKG1α disulfide dimer was performed as described previously, with maleimide (100 mM) used in preparation buffers to alkylate thiols and prevent thiol disulfide exchange. Antibodies used in these studies included cGKα (E-17; Santa Cruz Biotechnology) or cGKα (ADI-KAP-PKO05; Enzo Life Science), phosphoSer239-VASP (16C2; Millipore), and GAPDH (sc-20357; Santa Cruz Biotechnology). Horseradish peroxidase–linked secondary antibody (Dako) and ECL reagent (GE Healthcare) were used. Digitized immunoblots were analyzed quantitatively with a Gel-Pro Analyzer 3.1. The percentage of PKG1α disulfide dimer was quantified from a total PKG1α protein expression.

**In Vivo Mouse Studies**

Mean arterial pressure (MAP) and heart rate were assessed by telemetry in conscious mice as described previously. Briefly, mice were anesthetized with isoflurane, and a TA11PA-C10 probe catheter (Data Science International) was implanted into the aortic arch via the left carotid artery. After 1-week recovery, mice were placed above the telemetric receivers, and MAP was recorded every 5 minutes. GTN treatment (50 g/L, UNIKEM) was achieved with a FLUOstar microplate reader (BMG Labtech GmbH). Solvent propylene glycol served as a vehicle control. In some experiments, auranofin was administered (20 mg/kg IP daily) for 2 days before implantation of GTN minipumps.

**Small-Vessel Myography**

Thoracic aorta vascular rings and third-order mesenteric vessels were mounted for isometric tension recordings in a tension myograph (Danish Myo Technology), stretched to the optimal pretension condition with a DMT normalization module, and bathed in Krebs solution at 37°C with a 95% O2–5% CO2 environment. Vasodilator responses to auranofin (1 nmol/L to 10 μmol/L), AT1 (1 nmol/L to 300 μmol/L), atrial natriuretic peptide (300 μmol/L to 1 μmol/L), 8-Br-cGMP (1–300 μmol/L), or N-nitroso-N-acetyl-D,L-penicillamine (SNAP; 1 nmol/L to 300 μmol/L) were assessed with endothelium-intact isolated mouse vessels, and the plateau responses of precontracted (by α-adrenoceptor agonist phenyl-e phrine [EC80] or thromboxane mimetic compound U46619 [EC80]) vessels to the potentially vasodilatory drugs were deter-
Nitroglycerin (GTN) induces protein kinase G (PKG)-1α disulfide dimerization in both in vitro and ex vivo preparations. A, Time-dependent increase in PKG1α disulfide dimer after A10 smooth muscle cells were treated with GTN (3–300 μmol/L) every hour for 4 hours. B, Aortic rings were treated with GTN (3–300 μmol/L) for 6, 18, or 24 hours. PKG1α disulfide increased in a dose-dependent manner at each of the time points. C, Mesenteric vessels were treated with GTN (3–300 μmol/L) for 30 minutes, the highest concentration being effective in inducing PKG1α disulfide.

Results

Nitroglycerin Induces PKG1α Disulfide Dimerization In Vitro

To evaluate the ability of GTN to induce PKG1α disulfide dimerization in vitro, we used cultured smooth muscle cells, isolated mouse aortic rings, and mesenteric vessels. A10 cells treated with GTN 100 μmol/L every hour for 4 hours showed a time-dependent increase in PKG1α disulfide dimerization (Figure 1A). Mouse aortic rings then were exposed to increasing concentrations of GTN (3–300 μmol/L) for 6, 18, or 24 hours, which resulted in a dose- and time-dependent increase in PKG1α disulfide dimer formation (Figure 1B). Treatment of isolated mesenteric vessels with GTN (3–300 μmol/L) resulted in PKG1α disulfide dimerization, albeit only at the highest concentration examined (Figure 1C).

The GTN concentration required to induce a stable increase in disulfide PKG1α (ie, >100 μmol/L) is not clinically relevant; however, redox cycling of oxidized PKG1α back to the reduced state is anticipated to occur, and this would limit the accumulation of the disulfide product. To determine whether disulfide PKG1α is indeed redox cycled, we treated aortic rings with the thioredoxin reductase inhibitor auranofin (1–3 μmol/L), which alone significantly increased oxidation of the kinase independent of the addition of GTN (Figure 2A). Furthermore, when aortic rings were preincubated with auranofin (3 μmol/L), it significantly potentiated GTN-induced disulfide formation (Figure 2A). Consistent with disulfide PKG being redox recycled by the thioredoxin-thioredoxin reductase system, we observed loss of NADPH from aortas of both genotypes after GTN treatment (Figure 2A).

This effect of auranofin was of particular note because it caused stable accumulation of disulfide PKG1α at much lower concentrations of GTN (ie, 3 and 30 μmol/L) than was achieved by GTN alone, which required 300 μmol/L. These observations are again consistent with PKG1α oxidation occurring at low GTN concentrations, but the reductive recycling limited our ability to observe this on immunoblots. The implication is that GTN-induced vasodilation may occur through oxidative activation of PKG1α, but the accumulation of substantive disulfide is prevented by reductive recycling. Because PKG1α disulfide dimerization couples to vasodilation,7–10,12 we reasoned that comparing the responses of WT and KI vessels would be illuminating with regard to the role of PKG1α oxidation. Indeed, there was a dose-dependent relaxation in WT aortic rings in response to auranofin (0.001 μmol/L to 10 μmol/L) that was fully absent in PKG1α KI mice (Figure 2B), consistent with the anticipated complete absence of disulfide dimerization basally or after auranofin (3 μmol/L) treatment in aortic rings from KI mice (Figure 2B). In addition, auranofin sensitized WT aortas to GTN-induced relaxation (EC50 =0.9±0.2 μmol/L versus 2.9±1.1 μmol/L for vehicle-treated aortas; Figure 2C). In contrast, auranofin failed to sensitize KI aortas to GTN-induced relaxation (EC50 =4.2±1.3 μmol/L versus 6.1±0.9 μmol/L for vehicle-treated; Figure 2C).

Statistical Analysis

Results are presented as mean±SEM. Differences between groups were assessed by repeated-measures ANOVA followed by a Bonferroni i test. Differences were considered significant at the 95% confidence level. Analyses were performed with GraphPad Prism5.

In some experiments, auranofin (3 μmol/L) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (20 μmol/L) was added to the bath for 30 minutes before preconstriction and determination of drug dose-response.
Next, we tried to assess the role of PKG1α disulfide activation on blood pressure lowering by GTN in vivo; again, the Cys42Ser redox-dead PKG1α KI mouse, which cannot form a disulfide in response to oxidants, was used. When establishing a model of chronic GTN treatment, we initially tested administering it by subcutaneous injections, as used by others.15 We envisaged that 3 injections per day (every 8 hours) would replicate sustained treatment and generate the anticipated classic blood pressure profile of an initial hypotension that eventually is lost as the tolerance phenomenon ensues. However, the first injection of GTN (either 50 or 100 μg) caused an immediate increase in MAP and not the hypotension expected (online-only Data Supplement Figure IA). This hypertensive response was likely caused by a stress-induced catecholamine surge and occurred in both WT and KI mice. Essentially the same profile was observed again in both genotypes after the second GTN injection; however, the 50-μg dose caused a statistically significant (P<0.05)
greater hypotension in the WT than the KI mice. This differential effect between genotypes was observed again after the third injection and was now also clearly prominent in the group treated with 100 μg GTN. It may be that with repeated handling, the stress response of the mice declines because of a training effect, which allows the vasodilatory actions of GTN to occur and the anticipated differential response between the 2 genotypes to become apparent. When high doses of GTN (200 μg) were trialed, it caused a pronounced hypotension, which was again less marked in the KI than the WT mice, which is consistent with our hypothesis that kinase oxidation contributes to vasodilation. However, this high dose also caused a marked bradycardia, which at least in part would overdrive the hypertensive stress response to result in a net hypotension (online-only Data Supplement Figures IA and IB). Nevertheless, despite the complexities caused by the stress response during the subcutaneous injection procedure, there is clear evidence that the GTN-induced hypotension is attenuated in KI compared with WT littermate controls in conscious free-moving mice (online-only Data Supplement Figures IC and ID). In complementary studies, we also compared the effect of inhibiting the soluble guanylate cyclase–cGMP-NO-pathway with ODQ on GTN-induced hypotension in both genotypes in vivo. ODQ prevented the peak MAP decrease after treatment with GTN 100 μg in KI mice but not in WT mice (online-only Data Supplement Figure IE). This differential genotype response further supported a role for PKG oxidation in GTN-dependent vasodilation, but the observations and conclusions are again limited by the repeated stress response associated with the injections. To overcome the limitations of administering GTN or vehicle by serial injections, we established a model that used an osmotic minipump to deliver the drug. Figure 3A shows the MAP profile averaged every 3 hours for 24 hours before (baseline) and then 72 hours after GTN infusion was initiated. Because mice are nocturnal, they have higher blood pressure at night; it is therefore important to segregate the averaged day MAP from that at night. Another important consideration is that mild surgical pain causes a stress response on the day of minipump implantation, which alone increases MAP. Thus, implantation of minipumps that delivered vehicle alone into C57BL/6 mice immediately increased their MAP, and this was sustained for ∼8 hours (Figure 3A). MAP was also increased in the GTN group, but this was not as marked, which is consistent with a counterbalancing vasodilatory action of the drug. To avoid confusion as a result of this stress response, which can be variable depending on the presence of GTN or vehicle, this initial period was omitted in subsequent graphs of the MAP profile. Figure 3B is a further simplified trace in which MAP has been 12-hour averaged, and it clearly demonstrates the anticipated hypotensive response in the GTN-treated group but not the vehicle-treated controls. Furthermore, Figure 3B also shows that tolerance began from the second day of continued GTN treatment. We assessedPKG1α disulfide formation in aorta at 12, 24, and 72 hours after vehicle control or GTN treatment. This analysis showed PKG1α disulfide dimerization increased 2-fold after 12 hours of GTN treatment, when tolerance was clearly present, PKG1α disulfide dimerization returned to baseline. GTN treatment in vivo induced disulfide formation in aortas, and this was potentiated by cotreatment with auranofin. Veh indicates vehicle; Aur, auranofin.
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The GTN minipump delivery model was used again to compare the responses of the WT and KI mice. There was a clear and significant (P<0.05) hypotension in WT mice (Figure 4A), and this was comparable to that observed with standard C57BL/6 mice described above (Figures 3A and 3B). In contrast, the KI mice had a much attenuated response to GTN compared with WT littermates, with only a marginal decline in blood pressure after treatment (Figure 4B). Moreover, WT mice showed the tolerance profile expected and observed in standard C57BL/6 mice, but this was fully abrogated in the KI mice. However, it is perhaps illogical to conclude tolerance was absent in KI when they did not initially vasodilate to the GTN intervention.

In a separate cohort of WT and KI mice, minipumps were used to compare the responses to GTN with a vehicle control over a 48-hour period. Once again, we found vehicle infusion did not modulate MAP and that WT mice were the only genotype to show a hypotensive response to GTN before becoming tolerant. To establish whether the differential responses of WT and KI mice to GTN were a result of intrinsic properties of their blood vessels, both mesenteries and aortas were isolated and analyzed by myography. These measurements were made in vessels from both genotypes after 48 hours of vehicle or drug treatment, during which only WT mice administered GTN became tolerant. A GTN dose-response curve was constructed in U-46619 preconstricted vessels and compared between genotypes treated with vehicle or GTN for 48 hours. Isolated mesenteric resistance vessels from KI were markedly less sensitive to GTN-induced vasodilation than WT. Mesenteric vessels from “tolerant” WT mice were less sensitive to GTN (essentially matched the KI responsiveness). The responsiveness of KI mesenteries was not altered from baseline by the tolerance protocol. The aortas of KI mice were also basally less responsive to GTN than were WT aortas. The tolerance protocol decreased the sensitivity of WT aortas to GTN, but this tolerance also occurred in KI aortas. GTN treatment induced vasodilator-stimulated phosphoprotein phosphorylation (pVASP) in WT but not KI aortas. Veh indicates vehicle; toler, tolerant; and au, arbitrary units.
ing no differences between WT and KI mice on NO synthase activity. In addition, there was no difference in SNAP-induced vasorelaxation in mesenteric vessels of either genotype. Although several enzymes may be capable of bioactivating GTN, ALDH is widely considered the predominant enzyme responsible. Bioactivation generates several end products, including nitrosothiols, which are capable of inducing protein disulfide formation. Previously, we found that nitrocysteine can induce PKG1α oxidation, which logically would occur via transient formation of an $\text{S-N}$-nitrosylation intermediate of one of the Cys42 thiols before reduction by the other Cys42 on the opposite chain in the kinase homodimer to yield a disulfide. The likelihood of such a mechanism is supported by studies that show that GTN induces protein $\text{S-N}$-nitrosylation in vivo. However, because $\text{S-N}$-nitrosylated PKG1α would be extremely short-lived because of rapid reduction, to demonstrate that this intermediate occurs in vivo may be practically impossible with available technology, such as gas phase chemiluminescence or the ascorbate-dependent biotin-switch method. Overall, it is clear that there is a rational molecular basis for hypothesizing that GTN would induce oxidative activation of PKG1α. Furthermore, we indeed observed the anticipated increased in kinase oxidation in cells, tissues, and animals exposed to GTN.

Although our observations strongly support a role for oxidative activation of PKG in GTN-dependent vasodilation, this conclusion is at odds with reports that relaxation by this nitrate is efficiently blocked by ODQ. We have 2 potential explanations that may reconcile these seemingly opposing observations. First, it is conceivable that as the concentration of GTN is elevated, it increasingly signals via soluble guanylate cyclase, becoming gradually more sensitive to ODQ. Although Kleschsov et al. found that GTN relaxed aortas without elevating cellular NO levels, because the nitrate concentration was increased, NO became measurable. Second, although resistance vessels are the primary determinant of blood pressure, most studies investigate aortas. Here, we assessed mesenteries and found that WT and KI vessels relax identically to SNAP and that this is fully abrogated by blockade of soluble guanylate cyclase with ODQ in both genotypes. In notable contrast, ODQ did not fully abrogate the GTN-induced relaxation of WT mesenteries, consistent with it being able to relax by the kinase oxidation mechanism. Furthermore, ODQ blockade of GTN-induced relaxation was more marked in KI than WT mice, which was anticipated because the KI cannot recruit vasodilation via PKG disulfide.

In addition to nitrosothiols, there may be additional mechanisms that contribute to the oxidative activation of PKG1α, consistent with GTN treatment being historically associated with oxidative stress. Indeed, thiol-based antioxidants such as N-acetylcysteine (NAC) have been suggested as a therapy to limit oxidative damage and perhaps also prevent GTN tolerance. However, because NAC might be expected to react with (and so scavenge) nitrosothiols or other oxidants generated during GTN bioactivation, we might anticipate such an intervention may actually be ineffective and limit GTN-induced blood pressure lowering by attenuating PKG1α oxidation. Indeed, clinical trials have shown that NAC is ineffective at limiting GTN tolerance, as might be predicted from our new mechanistic understanding. Although it is evident that GTN can directly activate oxidase enzymes to induce oxidative stress, for example, by uncoupling endothelial NO synthase to yield superoxide, this can also occur indirectly as a consequence of the bioactivation process. During bioactivation, ALDH forms an oxidized disulfide product that is catalytically dead and cannot metabolize another GTN molecule. However, a reduced lipoic acid molecule thiol-disulfide exchanges with ALDH to regenerate the dehydrogenase so it can bioactivate another GTN molecule. This redox cycling reaction yields oxidized (disulfide) lipoic acid, which may potentially react with other thiols to induce $\text{S-thiolation}$ oxidation products. Oxidized lipoic acid is also reduced back to its thiol state by a NADPH-dependent reductase enzyme. Depletion of NADPH during chronic GTN treatment would therefore be expected to broadly compromise cellular reducing systems. This indirectly induces an oxidative stress that may contribute to the accumu-
lation of disulfide PKG1α and to the phenotypic difference observed between WT and KI mice.

One enzyme that uses substantial amounts of NADPH-reducing equivalents is thioredoxin reductase, which reduces oxidized thioredoxin back to the reduced state. Reduced thioredoxin generically reduces disulfide-oxidized proteins back to their reduced state but can also cause the denitrosylation of proteins. Indeed, thioredoxin was considered the prime candidate responsible for converting reducing disulfide PKG1α to baseline, serving as an “off switch” to provide regulation of oxidant-induced kinase activity. To establish whether thioredoxin reduces oxidized PKG1α, we undertook studies with the thioredoxin reductase inhibitor auranofin, which alone was sufficient to induce disulfide kinase in aortic rings. However, auranofin induced oxidation of a relatively small proportion of total PKG, and it was important to consider whether a low stoichiometry can be functionally significant. Because PKG1α disulfide dimerization couples to vasodilation, we reasoned that comparing the responses of WT and KI vessels would be illuminating with regard to the functional impact of the limited PKG1α oxidation induced by auranofin. These studies confirmed that although auranofin only maximally induced 5% to 10% of total PKG1α to form disulfide, there was a prominent differential effect between genotypes, such that the WT relaxed in a dose-dependent manner and the KI did not relax at all. That only a small proportion of PKG1α had to be oxidatively activated for vasodilation to occur is consistent with our previous observation that an endothelium-derived hyperpolarizing factor protocol that efficiently induced vasodilation only increased disulfide PKG from ~4% to ~9% to 10%. This essentially matches the increase observed with auranofin 3 μmol/L, which clearly demonstrated that only a small amount of disulfide-activated kinase is required to induce vasodilation. It is difficult to know how disulfide activation compares with the classic cGMP activation in terms of the stoichiometry of activation required to achieve a comparable vasodilation. However, because oxidative PKG activation involves the lowering of the enzyme’s affinity for substrate, whereas cGMP increases the maximum reaction rate (Vmax), it is conceivable that disulfide formation requires a smaller proportion of the total kinase pool to be activated compared with the classic pathway to achieve vasodilation. It is noteworthy that gold-containing compounds, such as auranofin or sodium aurothiomalate, which are used to treat rheumatoid arthritis, include vasomotor-like reactions characterized by facial flushing and occasionally hypotension in so-called nitritoid reactions.

Clearly, endothelium-derived hyperpolarizing factor and auranofin couple to vasodilation despite only inducing ~9% to 10% PKG1α oxidation. Thus, although GTN at the time of maximal hypotension only induced ~6% PKG1α oxidation in vivo, this would be enough to account for the hypotensive response, because auranofin doses that cause comparable oxidation are adequate to relax WT but not KI blood vessels ex vivo. It is also notable that basally, WT mice have ~2% to 3% disulfide PKG1α, whereas KI mice have 0%, and this alone results in an MAP difference of ~10 to 15 mm Hg. In addition, the ~6% PKG1α oxidation measured in vivo after GTN treatment may be an underestimate caused by redox recycling back to the reduced state. When increasing amounts of GTN are applied or longer treatment durations are used, it may be intuitive to expect proportionately more PKG1α to accumulate. However, because the rate of reduction can match or indeed be faster than the oxidation step, minimal accumulation can occur despite significant flux through the disulfide-activated state. Increasing the blood vessel PKG1α disulfide content to ~6% was associated with a 7-mm Hg drop in MAP. We should be mindful that GTN was administered to healthy mice with “normal” blood pressure. Clinically, GTN would be given to hypertensive patients; should we have treated hypertensive mice, then a much greater drop in MAP would likely be observed, but this would have added significant complexity.

To the best of our knowledge, this is the first report showing that PKG1α oxidation significantly mediates GTN-induced vasodilation and blood pressure lowering. Our conclusions are robustly supported by studies with a redox-dead Cys42Ser KI mouse that essentially fails to become hypotensive in response to GTN treatment. The response to GTN in vivo was studied with radiotelemetry, which, despite now being the “gold standard” for blood pressure measurements in mice, has scarcely been used in the study of tolerance. This technology overcomes many technical problems associated with other approaches, providing a robust and continuous readout of blood pressure in a model that replicates the clinical use of the drug, and also clearly shows the tolerance phenomenon. Complementary studies comparing the vasorelaxation of vessels from the KI mice further corroborated our conclusions, because they were significantly less responsive to GTN than WT preparations. Our observations are consistent with GTN lowering blood pressure by stimulating the same pathway as the endogenous vasodilating endothelium-derived hyperpolarizing factor stimuli, which we previously showed operate significantly via PKG1α oxidation. Indeed, preparations taken from tolerant animals are deficient in the response to GTN treatment, as well as to an endothelium-derived hyperpolarizing factor protocol. Our studies also provide some mechanistic insight to the tolerance phenomenon, but our conclusions are limited by the fact that KI mice do not respond to GTN. GTN initially caused PKG1α oxidation (at 12 hours) and lowered MAP in WT mice. Because the loss of GTN-induced MAP lowering was associated with a loss of PKG1α oxidation, we would suggest that tolerance occurs because of a failure to bioactivate GTN. Indeed, tolerance is associated with a loss of the bioactivation pathway. Loss of bioactivation would prevent the formation of oxidizing species such as nitrosothiols and prevent disulfide activation of PKG1α, resulting in a failure of GTN to lower MAP (ie, tolerance), which is what we observed.

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Disclosures
None.
GTN Oxidizes PKG1α to Induce Hypotension

Rudyk et al

References


CLINICAL PERSPECTIVE

Nitroglycerin (GTN) remains a treatment for unstable angina and hypertension after >130 years of clinical use. Despite this, the mechanism of action still is not fully understood. Here, we present evidence that GTN induces the oxidation of protein kinase G (PKG)-1α such that it forms an interprotein disulfide bond, directly activating the kinase, which couples to blood vessel dilation. This occurs independent of the classic mode of stimulating PKG, in which nitric oxide stimulates the production of cGMP, which then binds to PKG to activate it. Thus, although GTN is an organic nitrate, its mode of kinase activation at therapeutic concentrations is principally through oxidative activation and not cGMP binding. Prolonged (>24 hours) treatment with GTN commonly results in the well-recognized “tolerance” phenomenon, in which the vasodilatory and antianginal effects of GTN are lost, thus hampering its clinical use. Our studies show tolerance to nitroglycerin is associated with loss of PKG oxidation. If pharmacological interventions can be identified that maintain GTN-induced PKG oxidation during chronic use, this may potentially counteract the development of tolerance.
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Figure S1. KI mice are deficient in their GTN-induced hypotensive response compared to WT as measured in vivo by radiotelemetry. (A) The mean arterial pressure (MAP) response
of each genotype to three consecutive subcutaneous injections of 50, 100 or 200 µg of GTN is shown. It is clear that administering GTN by subcutaneous injection induces a stress response that elevates MAP, meaning the vasodilatory actions of GTN are superimposed on a pressor response. This adds significant complexity to the interpretation of this dataset. (B) The heart rate response to three consecutive subcutaneous injections 50, 100 or 200 µg of GTN is shown. (C) Comparison of the delta decrease in MAP of the WT and KI following GTN treatment at each of the three doses studied. (D) The delta decrease in MAP following GTN treatment was more marked in WT than KI when the responses after the third consecutive subcutaneous injection of drug were compared. (E) The delta decrease in MAP following a single 100 µg of subcutaneous GTN injection was compared in WT and KI. Pretreatment with the soluble guanylate cyclase inhibitor ODQ before injection of 100 µg GTN revealed a prominent differential effect between the genotypes, with the WT showing a decrease in MAP, whereas the KI showed an increase.
Figure S2. WT and KI vessels have identical responses to oxidant-independent PKG activators. (A-B) 8Br-cGMP and ANP relaxed aortae and mesenteries from both genotypes identically. (C) SNAP relaxed mesenteries from both genotypes identically GTN. In contrast
GTN-induced relaxation was attenuated in WT compared to KI. Although ODQ attenuated GTN-induced relaxation of WT mesenteries, relaxation still could still be achieved at higher concentrations. This residual ODQ-insensitive relaxation was less prominent in the KI compared to WT.