

Roles of Superoxide, Peroxynitrite, and Protein Kinase C in the Development of Tolerance to Nitroglycerin

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ABSTRACT

A current hypothesis states that tolerance to nitroglycerin (GTN) involves increased formation of superoxide (O_2^-). Studies showing that inhibitors of protein kinase C (PKC) prevent tolerance to GTN suggest the involvement of PKC activation, which can also increase O_2^- . We examined the roles of O_2^- , peroxynitrite ($ONOO^-$), and PKC activation in GTN tolerance. Pre-exposure of rat aortic rings to GTN (5×10^{-4} M) for 2 h caused tolerance to the vasodilating effect of GTN, as evidenced by a substantial rightward shift of GTN concentration-relaxation curves. This shift was reduced by treatment of the rings with the antioxidants uric acid, vitamin C, or tempol or the PKC inhibitor chelerythrine. We also found that O_2^- generation via xanthine/xanthine oxidase in the bath induced tolerance to GTN. How-

ever, responses to nitroprusside were not affected. In vivo tolerance produced in rats by 3-day i.v. infusion of GTN was also almost completely prevented by coinfusion of tempol. In bovine aortic endothelial cells (EC), addition of GTN produced a marked increase in tyrosine nitrosylation, indicating increased $ONOO^-$ formation. This action was blocked by prior treatment with uric acid, superoxide dismutase, N^G -nitro-L-arginine methyl ester, or chelerythrine. We also demonstrated that GTN translocates the α - and ϵ PKC isoforms in EC. However, PKC ζ was not affected by GTN treatment. In conclusion, tolerance to GTN involves enhanced production of O_2^- and $ONOO^-$ and activation of NO synthase. Furthermore, sustained activation of α - and ϵ PKC isozymes in EC by GTN may play a role in development of tolerance.

Nitroglycerin (glyceryl trinitrate; GTN) is a vasodilator used in the therapy of cardiovascular diseases, such as angina pectoris, congestive heart failure, and hypertension. However, use of GTN and other organic nitrates is limited by the development of nitrate tolerance, especially during continuous administration. Mechanisms of this tolerance have been extensively investigated and are thought to involve both vascular and humoral effects (Munzel et al., 1996; Parker and Parker, 1998). Well established vascular mechanisms include desensitization of smooth muscle guanylate cyclase (Molina et al., 1987), enhanced cGMP breakdown due to increased phosphodiesterase activity (Pagani et al., 1993), and impaired GTN biotransformation (Slack et al., 1989). Humoral mechanisms include increases in plasma levels of

catecholamines, vasopressin, angiotensin II, and aldosterone, which elevate vascular resistance and blood volume (Stewart et al., 1986). Recently, it has been shown that GTN treatment of animals and cells increased the formation of superoxide (O_2^-) (Munzel et al., 1995; Dikalov et al., 1998; Kaesemeyer et al., 2000). Superoxide can be generated via multiple systems, including xanthine oxidase, NADH/NADPH oxidase(s), and nitric-oxide synthase (NOS). It rapidly reacts with and inactivates nitric oxide (NO) (Gryglewski et al., 1986) and produces peroxynitrite ($ONOO^-$). L-Arginine may also play a role in tolerance to organic nitrates in that reduced availability of L-arginine to NOS may occur from the direct inhibitory effects of NO, O_2^- , and/or $ONOO^-$, formed by GTN, on the transport of L-arginine into endothelial cells (Patel et al., 1996; Abou-Mohamed et al., 2000; Ogonowski et al., 2000). In support of this, our previous experiments have demonstrated that supplementing L-arginine to rat aorta and humans partially prevents the development of tolerance to GTN (Abou-Mohamed et al., 2000; Parker et al., 2002).

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ABBREVIATIONS: GTN, glyceryl trinitrate/nitroglycerin; PKC, protein kinase C; O_2^- , superoxide; $ONOO^-$, peroxynitrite; CRC, concentration-response curve; BAEC, bovine aortic endothelial cells; ROS, reactive oxygen species; NO, nitric oxide; NOS, nitric-oxide synthase; PE, phenylephrine; pD_2 , negative log of the dose producing 50% of maximal response; BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin; L-NAME, N^G -nitro-L-arginine methyl ester; M199, medium 199; SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; eNOS, endothelial nitric-oxide synthase.

A previous study has reported that a protein kinase C (PKC) inhibitor can prevent tolerance to GTN (Zierhut and Ball, 1996). The PKC family includes at least 11 isozymes (Nishizuka, 1986). Upon activation, PKC isozymes translocate from the cytoplasm to particulate structures, where they are thought to mediate their catalytic functions (Kraft and Anderson, 1983). Recent cardiovascular studies have revealed substantial "cross-talk" between the NO and PKC signaling pathways (Li et al., 1998; Liang and Knox, 1999; Ping et al., 1999; Balafanova et al., 2002; Tazi et al., 2002). For example, treatment with NO donors triggers the translocation of the α PKC isozyme to the particulate fraction in kidney cells (Liang and Knox, 1999). Nitric oxide has also been shown to directly stimulate PKC activities in heart tissue (Yoshida et al., 1999). NO donors have been shown to activate the PKC signaling pathway via a mechanism involving production of ONOO⁻ and tyrosine nitration of the ϵ PKC isozyme (Balafanova et al., 2002). This nitration enhances the interaction of ϵ PKC with its intracellular receptor that targets ϵ PKC to its active cell locus. In addition, O₂⁻ has been reported to be capable of activating PKC (Gopalakrishna and Anderson, 1989; Knapp and Klann, 2000). The activation and subsequent translocation of PKC trigger additional formation of O₂⁻ (Christiansen, 1988). Thus, a "positive feedback" loop may result.

In the present study, we have, therefore, investigated the interactive roles of GTN, reactive oxygen species (ROS), and PKC isozymes in the development of tolerance to GTN. Our work has therapeutic implications in the prevention of GTN tolerance and may be beneficial in the treatment of cardiovascular diseases.

Materials and Methods

Vascular Tone Studies

In Vitro Induction of Tolerance. Male Sprague-Dawley rats (200–250 g) were lightly anesthetized with ketamine and sacrificed. The descending thoracic aorta was removed, cleaned from the adjacent tissues, and cut into ring segments of approximately 4 mm in length. Two metal hooks were passed carefully through the lumen of each ring, and the tissues were then mounted under 2×10^{-3} newtons of tension in 25-ml organ baths containing Krebs' solution. Tissues were left to equilibrate for 90 min with intermittent changing of the solution every 15 min. Krebs' solution had the following composition: 118 mM NaCl, 4.75 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1.19 mM KH₂PO₄·H₂O, 2.54 mM CaCl₂·2H₂O, and 11 mM glucose. The bathing solution was kept at 37°C and was continuously aerated with 5% CO₂ and 95% O₂ gas mixture. After equilibrium, a submaximal dose of phenylephrine (PE; 3×10^{-7} M final, producing ~86% of maximum contraction) was added to the tissue bath, and the full response was allowed to develop. A concentration-response curve (CRC) for GTN was then constructed. The bathing solution was drained and replaced with fresh solution. The tissues were allowed to return to the baseline. Tolerance to GTN was developed by incubating the tissues with 5×10^{-4} M of GTN for 1 h followed by a replenishment of the same dose for a second hour.

In one series of experiments, 1) L-ascorbic acid (10^{-3} M), a general antioxidant; 2) uric acid (10^{-3} M), a scavenger of ONOO⁻, peroxyl, and OH⁻; 3) 4-hydroxy tempol (10^{-3} M), an intracellular scavenger of O₂⁻ and OH⁻; or 4) chelerythrine (10^{-6} M), an inhibitor of PKC, or the combination of 4-hydroxy tempol (10^{-3} M) and chelerythrine (10^{-6} M) was present in half of the tissue baths throughout the incubation time with GTN, whereas the other baths had only regular Krebs' solution. In other experiments, only the actions of the antioxidants

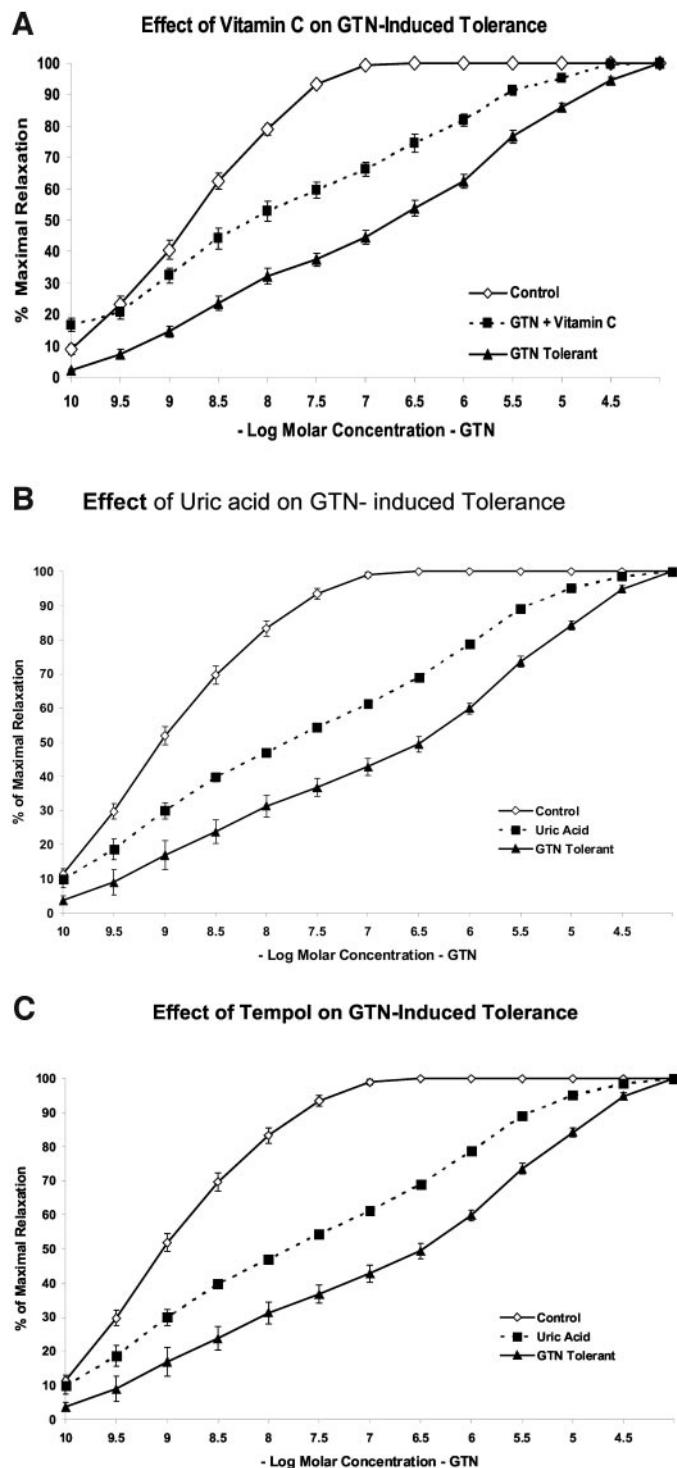


Fig. 1. Induction of tolerance to GTN in isolated rat aortic ring segments and the effects of prior treatment with vitamin C (10^{-3} M; A), uric acid (10^{-3} M; B), and 4-hydroxy tempol (10^{-3} M; C). Vessels made tolerant to GTN (\blacktriangle) were incubated for 2 h with GTN (5×10^{-4} M, replenished after 1st h). Control responses to GTN (\diamond) were obtained in vessels that were not exposed to GTN (5×10^{-4} M) for 2 h. Effects of concurrent treatment of vessels exposed to GTN (5×10^{-4} M, 2 h) with each of the three antioxidants are displayed as dashed lines (\blacksquare).

on the CRC to GTN were examined. Tissues were washed extensively every 15 min for 1 h after incubation. Rings were recontracted with PE, and a second series of CRC for GTN were constructed.

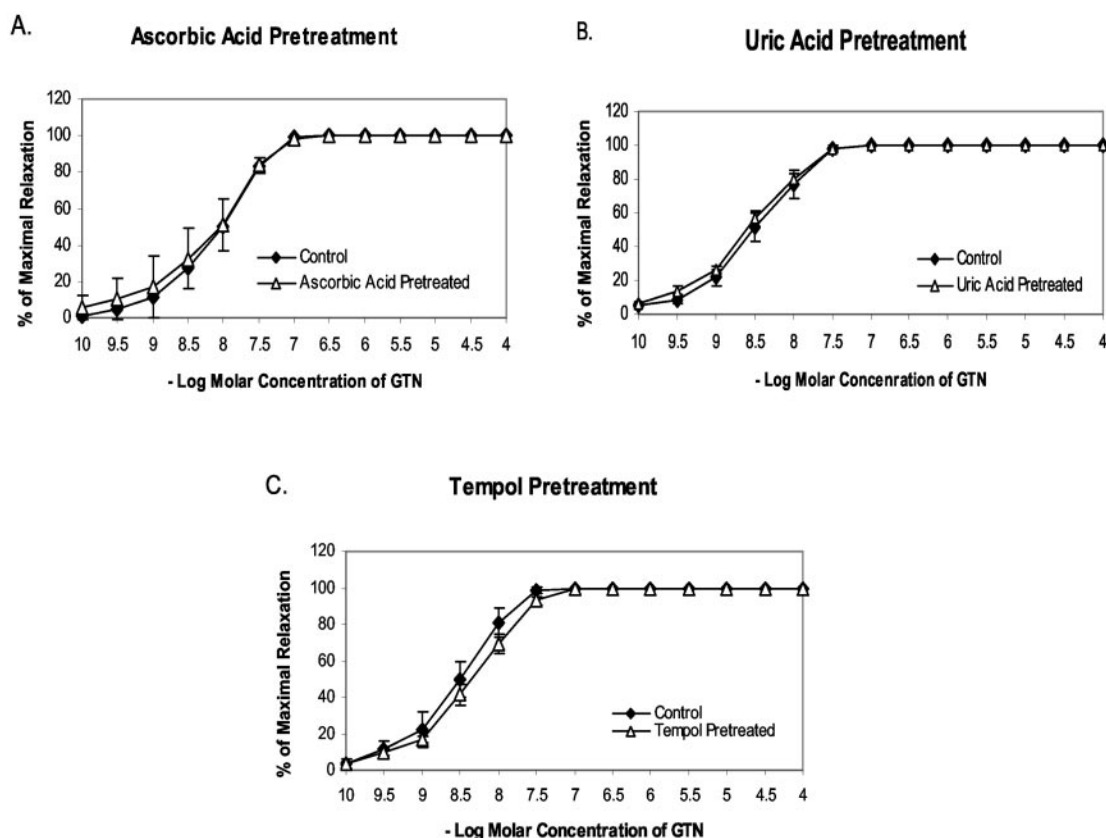


Fig. 2. Effect of antioxidants alone on the vasorelaxant responses to GTN. Control responses to GTN (\blacklozenge) were obtained in vessels not exposed to GTN for 2 h. Effects of vitamin C (10^{-3} M; A), uric acid (10^{-3} M; B), or 4-hydroxyl tempol (10^{-3} M; C) in the bath upon responses to GTN are shown in the lines with the symbol (\triangle).

To examine the effect of superoxide generation on GTN-induced relaxation, a second series of experiments were conducted. The tissues were contracted with PE, and concentration-response curves were constructed. The rings were washed and exposed to a free radical-generating system of xanthine/xanthine oxidase (10^{-5} M/0.5 mU/ml). The treatment was replenished every 10 min for 1 h. The aortic rings were washed three times, and cumulative dose-response curves for GTN and sodium nitroprusside (SNP) were constructed. The pD_2 values for GTN (negative log of the dose producing 50% of the maximal response) were determined for each treatment. For each experiment, four ring segments were obtained from the rat and randomly assigned to one of our experimental treatments. All responses were expressed as a percentage of the response produced by a time control to PE alone.

In Vivo Induction of Tolerance. Male Sprague-Dawley rats 200 to 250 g were utilized. Rats were anesthetized by an injection of mixture of xylazine and ketamine (40 mg/kg and 5 mg/kg, i.p., respectively). The jugular vein was isolated and cannulated with a heparinized saline-filled PE-10 catheter. The catheters were advanced under the skin to the back of the neck and supported by a swivel and coiled wire conduit to the outside of the cage. The skin was sutured under aseptic conditions. After complete recovery, animals were replaced in their cages. Three days later, rats were infused continuously with either saline or GTN at a dose of 15 mg/kg/day for 3 days with or without 4-hydroxy tempol (13.3 mg/kg/day) by means of syringe infusion pumps (Harvard Apparatus Inc., Holliston, MA). The development of tolerance was tested in vitro after 3 days of treatment as follows. Rats were sacrificed, and their descending thoracic aortas were removed and set up in tissue baths where concentration-response curves for GTN were constructed as described above.

Cell Culture Experiments

Cell Culture. Bovine aorta endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, Washington) and maintained in medium 199 (M199) with 10% fetal bovine serum and penicillin and streptomycin through passages 4 through 7.

cGMP Formation. BAEC were grown to $\sim 90\%$ confluence in 24-well plates and incubated in serum-free and arginine-deficient medium (M199) for 2 h. To determine the short-term effects of GTN on cGMP levels, cells were further incubated with or without GTN (10^{-6} M) for 10 min in this M199 medium containing 3-isobutyl-1-methylxanthine (0.5 mM; Sigma-Aldrich, St. Louis, MO). Following removal of medium, cell contents were harvested using 0.5 ml of 0.1 N HCl and 0.1% Triton X-100. To study the effects of tolerance-inducing concentrations of GTN on cGMP level, cells were treated with GTN (10^{-6} M) for 10 min before and after exposure to a tolerance-inducing concentration of GTN (10^{-5} M) for 1 h. At the end of the treatments, cells were harvested as described above. The cGMP levels were measured using enzyme-linked immunoassay commercial kit (Assay Designs, Inc., Ann Arbor, MI). The values are expressed as picomole(s) per milliliter.

Nitrotyrosine Formation. The relative amounts of proteins nitrated on tyrosine were measured by immunocytochemistry. BAECs were grown until $\sim 90\%$ confluent. Cells were incubated in serum-free and arginine-deficient M199 for 2 h. Cells were treated with GTN (10^{-5} M) for 30 min in the absence or presence of superoxide dismutase (SOD; 80 U/ml), uric acid (10^{-3} M), *N*^G-nitro-L-arginine methyl ester (L-NAME; 5×10^{-4} M), or chelerythrine (10^{-6} M). Cultures were fixed with 4% paraformaldehyde and then reacted with a polyclonal rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Oregon green-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) was used to visual-

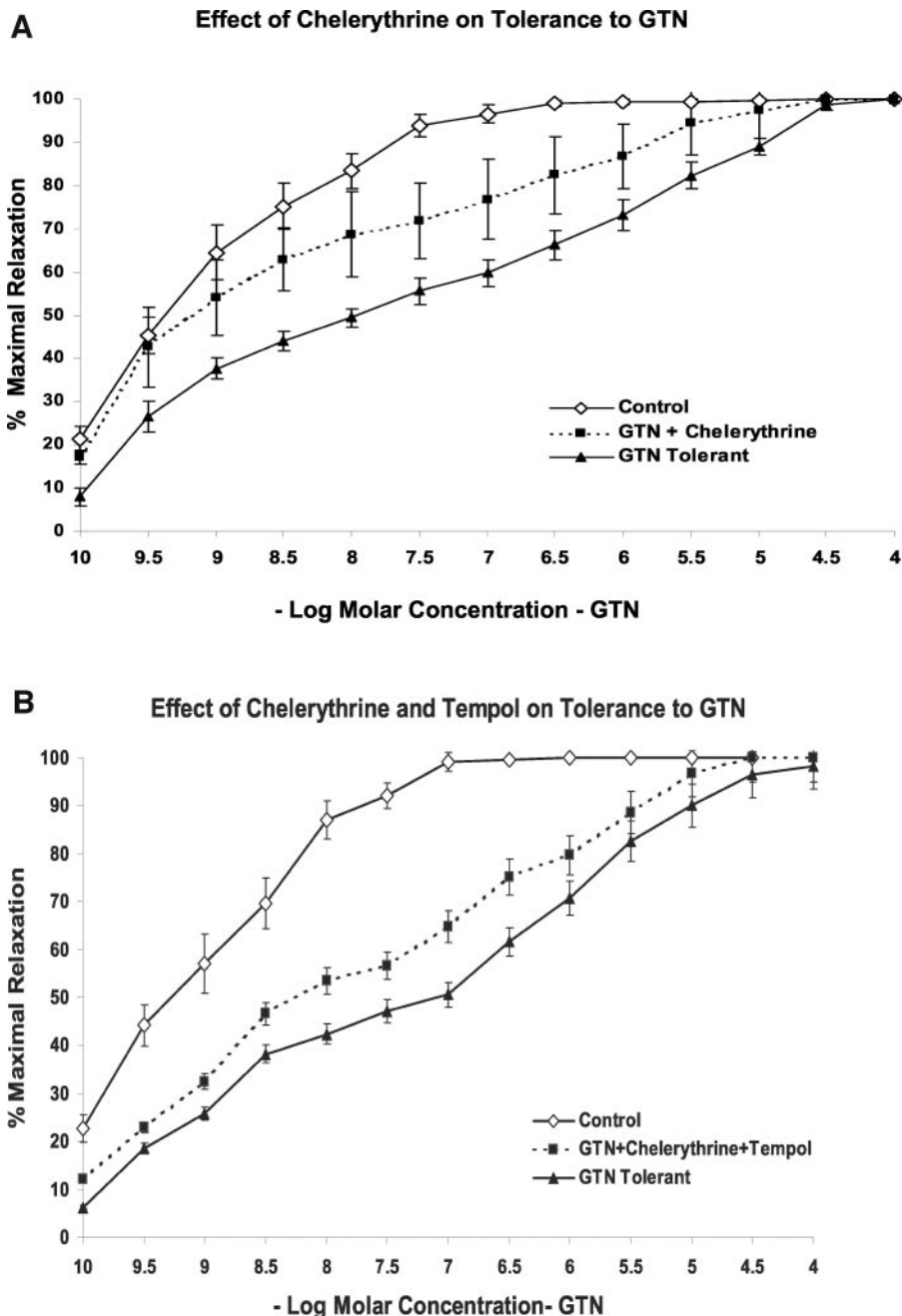


Fig. 3. Effect of chelerythrine (A) or chelerythrine and 4-hydroxy tempol (B) on tolerance to GTN. Vessels made tolerant to GTN (\blacktriangle) were incubated for 2 h with GTN (5×10^{-4} M, replenished after 1st h). Control responses to GTN (\diamond) were obtained in vessels not exposed to GTN for 2 h. Effects of concurrent treatment of vessels exposed to GTN (5×10^{-4} M, 2 h) with chelerythrine (10^{-6} M) or chelerythrine (10^{-6} M) and 4-hydroxy tempol (10^{-3} M) are displayed as dashed lines (\blacksquare).

ize the primary antibody. Data were analyzed using Ultra View morphometric program and fluorescence microscopy to quantify intensity of immunostaining.

Protein Kinase C Activation. BAEC were incubated in serum-free and arginine-deficient M199 for 2 h. Cells were then treated with 10^{-5} M GTN for 0, 15, 30, 60, 120, and 180 min. One group of cells was treated with 10 nM PMA for 15 min as a positive control. At the end of treatment period, the media from one 100-mm dish of BAEC was removed, and 0.5 ml of chilled homogenization buffer [10 mM Tris (hydroxymethyl-aminomethane), 1 mM EDTA, 1 mM EGTA, and 20 μ g/ml of phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, leupeptin, and aprotinin, pH 7.4] was added to each dish. The cells were scraped from the plates and triturated three times with a syringe and 22-gauge needle. The resulting lysates were centrifuged at 4°C for 20 min at 100,000g. The supernatants were concentrated, using a Centricon 30 concentration device (Millipore Corp., Bedford, MA), to a volume of 250 μ l. The pellets were resus-

ended in 250 μ l of homogenization buffer. Protein concentrations were measured by the Bio-Rad micro protein assay (Bio-Rad, Hercules, CA), and samples equated for total protein were subjected to SDS-polyacrylamide gel electrophoresis. Western blot analysis with PKC isozyme-selective antisera, 125 I-labeled protein A detection methods, and autoradiography were conducted as previously described (Johnson and Mochly-Rosen, 1995; Johnson et al., 1996). All antisera were from BD Transduction Laboratories (Lexington, KY) and were used at 1:500 dilutions. Data were analyzed by densitometry ($n = 2-3$ samples).

Chemicals. All chemicals were made fresh on the day of use. Acetylcholine chloride, phenylephrine HCl, sodium nitroprusside, L-ascorbic acid, uric acid, 4-hydroxy tempol, chelerythrine, L-NAME, xanthine, and xanthine oxidase were purchased from Sigma-Aldrich. Xylazine and ketamine were purchased through the Animal Resources Division of the Medical College of Georgia. Nitroglycerin

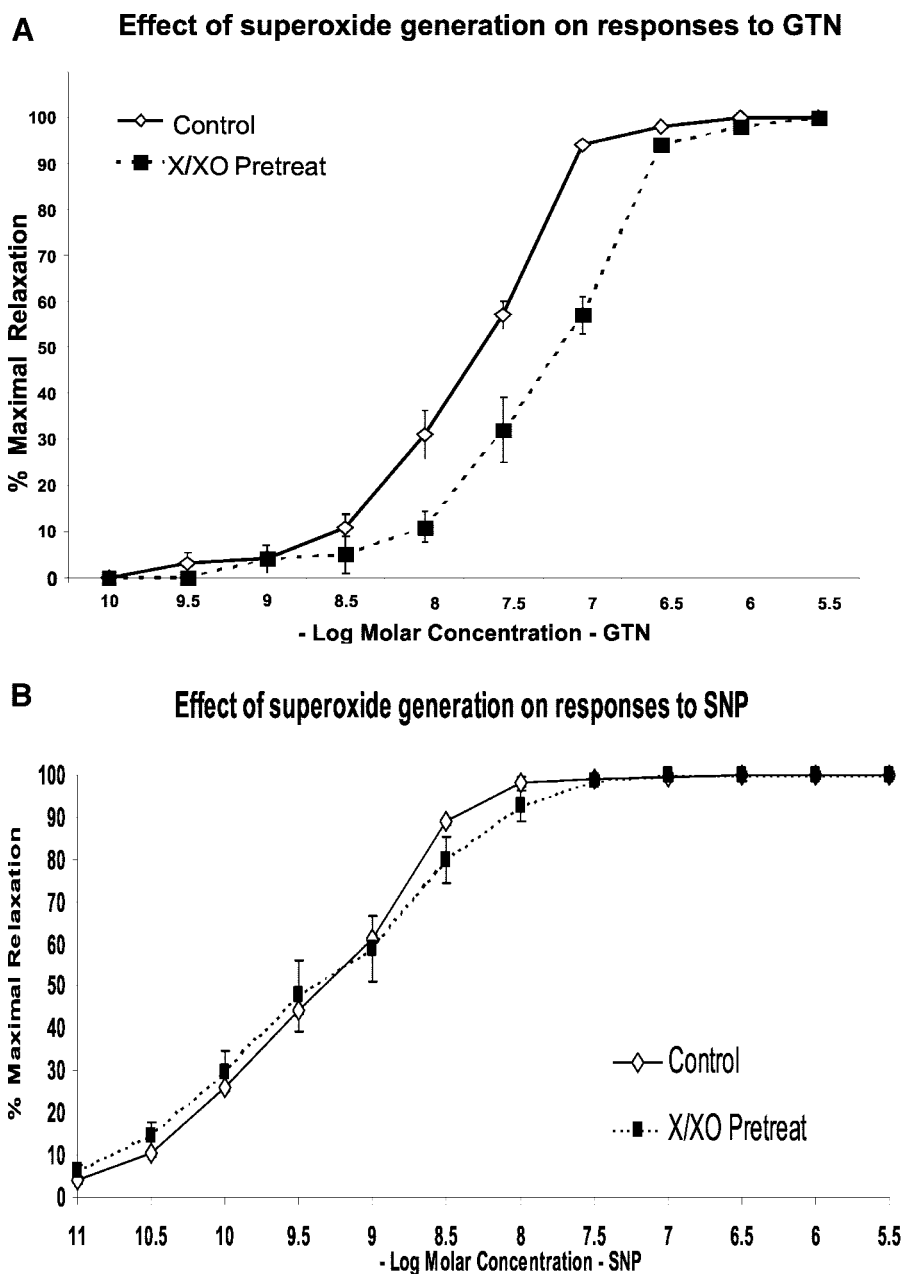


Fig. 4. Effect of xanthine/xanthine oxidase (10^{-5} M/0.5 mU/ml), replenished every 10 min for 1 h, on GTN-induced (A) and SNP-induced (B) relaxation of rat aortic ring segments. Control responses to GTN or SNP are displayed in solid line (\diamond); responses after xanthine/xanthine oxidase treatment and tissue washes are shown as dashed line (\blacktriangle).

(SDM27) was a kind gift from Astra-Zeneca Pharmaceuticals LP (Wilmington, DE).

Statistical Analysis. Data are presented as mean \pm S.E. of the indicated number of observations (n), and the difference between groups was assessed using the paired t test or analysis of variance, when appropriate. A probability value (P) less than 0.05 was considered to be statistically significant.

Results

Development of tolerance to GTN is associated with superoxide production in rat aorta. Nitroglycerin produced a concentration-dependent relaxation of constricted rat aortas with a pD_2 value of 8.83 ± 0.07 . As shown in Fig. 1, incubation with a high concentration of GTN (5×10^{-4} M) for 2 h rendered the vessels less responsive to GTN. In these tolerant vessels, GTN produced a relaxation with a pD_2 value of 6.57 ± 0.2 . Coincubating vessels with the tolerance-inducing

concentration of GTN along with the general antioxidant vitamin C (10^{-3} M) significantly reduced tolerance development (pD_2 value of 8.18 ± 0.15) (Fig. 1A). In a similar way, a peroxynitrite, peroxy, and OH⁻ scavenging agent, uric acid (10^{-3} M), significantly reduced tolerance development. The pD_2 value for GTN after uric acid was 7.8 ± 0.16 (Fig. 1B). Moreover, the water-soluble SOD mimetic, 4-hydroxy tempol (10^{-3} M), diminished the effect of a tolerance-inducing concentration of GTN. As a result, the pD_2 value for GTN was increased in the presence of 4-hydroxy tempol to 7.65 ± 0.16 as compared with 6.57 ± 0.2 in its absence (Fig. 1C). Exposure of vessels to any of the three antioxidants without the tolerance-producing concentration of GTN did not alter or shift the CRCs to GTN (Fig. 2). These results implicate ROS species in the development of GTN tolerance in rat aortic rings.

The presence of chelerythrine (10^{-6} M) during the tolerance-producing exposure to GTN also reduced development

Effect of Tempol on GTN-Induced Tolerance In Vivo

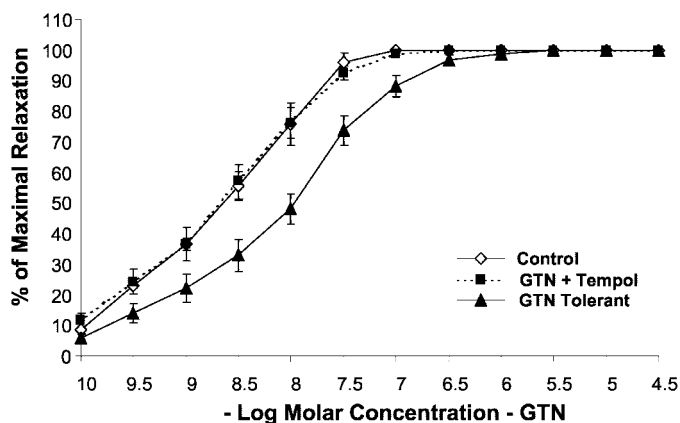


Fig. 5. Effects of induction of tolerance to GTN in vivo by continuous i.v. infusion of GTN into rats for 3 days (15 mg/kg/day) on the vasorelaxant responses of GTN in aortic ring segments isolated from these rats (\blacktriangle). These GTN concentration-response curves were compared with those obtained using vessel segments from rats treated with a 3-day i.v. infusion of 4-hydroxy tempol (13.3 mg/kg/day) in addition to GTN infusion (dashed line, \blacksquare) and vessels from control rats not receiving infusion of either agent (\diamond).

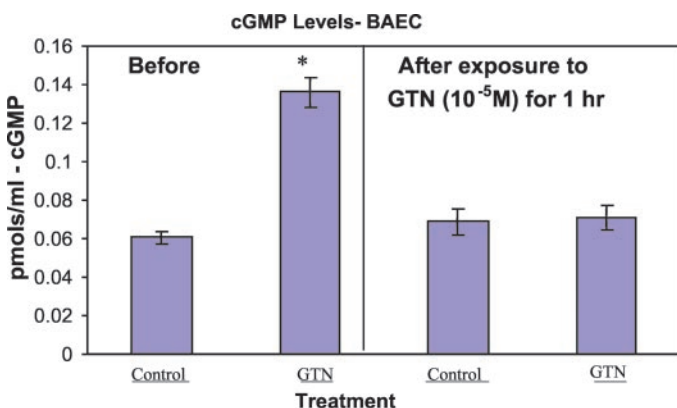


Fig. 6. Effect of challenge dose of GTN (10^{-6} M) or vehicle control on formation of cGMP in BAEC before and after 1-h exposure to a 10^{-5} M concentration of GTN. Values are pmol/ml/5 min (*, $P < 0.05$ versus control levels).

of tolerance (Fig. 3A). Coincubating vessels with the tolerance-inducing concentration of GTN along with the general PKC inhibitor chelerythrine (10^{-6} M) significantly reduced tolerance development. The pD_2 values for the CRC to GTN were 9.38 ± 0.14 , 7.87 ± 0.09 , and 9.24 ± 0.26 , respectively, for control, GTN tolerant, and GTN plus chelerythrine. Concurrent incubation of vessels with chelerythrine (10^{-6} M) and 4-hydroxy tempol (10^{-3} M) along with GTN also reduced tolerance to a similar degree (Fig. 3B). The pD_2 values for the CRC to GTN were 9.33 ± 0.21 , 7.04 ± 0.12 , and 8.25 ± 0.19 , respectively, for control, GTN tolerant, and GTN plus chelerythrine and tempol.

To examine the role of superoxide in the production of tolerance to GTN, aortic rings were incubated with a xanthine/xanthine oxidase mixture and washed before a second CRC was constructed. As shown in Fig. 4A, vessels exposed to superoxide were less sensitive to GTN than nontreated vessels as shown by 3.6-fold parallel shift in the CRC (pD_2 values are 7.20 and 7.76, respectively). In contrast, vasorelaxant responses to SNP were not altered (Fig. 4B).

To further confirm the role of reactive oxygen species in in vivo development of tolerance to GTN, in vivo tolerance was achieved by continuous i.v. infusion of GTN into rats for 3 days. Aortae were then tested in vitro. This treatment resulted in a 6.6-fold decrease in responsiveness to GTN in isolated aortic rings. When 4-hydroxy tempol was coinfused with GTN, it protected the rats against GTN tolerance. The pD_2 values for GTN on isolated rings were 8.63 ± 0.13 , 7.81 ± 0.13 , and 8.65 ± 0.16 , from control rats treated with saline (control), GTN alone, or GTN and 4-hydroxy tempol, respectively (Fig. 5). These results demonstrate that this antioxidant prevents tolerance to GTN administered in vivo.

GTN Induces Tolerance to Its Ability to Increase cGMP in Endothelial Cells. Effects of GTN were also examined in endothelial cells. In BAEC not previously exposed to GTN, there was a marked increase in cGMP levels with a challenge dose of GTN (GTN, 10^{-6} M) for 5 min compared with vehicle (Control) (Fig. 6). After a 1-h exposure of cells to a higher concentration of GTN (10^{-5} M) and application of fresh media, cells exhibited similar basal (Control) levels of cGMP but did not produce a rise in cGMP in response to the second challenge dose of GTN (GTN, 10^{-6} M, 5 min). Thus, BAEC exhibit tolerance to GTN-induced cGMP responses.

Inhibiting NOS or Scavenging Superoxide Blocks Peroxynitrite Formation. Since concurrent elevation of NO and superoxide is associated with increased peroxynitrite levels, we investigated the effect of GTN on formations of peroxynitrite in BAEC (Fig. 7). $ONOO^-$ is a short-lived molecule at physiologic pH, but it produces stable nitration of protein tyrosine residues. Thus, nitrotyrosine immunoreactivity can be used as an indicator for $ONOO^-$ formation. Our time course studies showed that GTN increases the $ONOO^-$ formation in BAEC in a time-dependent manner, reaching the maximum between 15 to 30 min (Fig. 7A, inset). Afterward, the levels of nitrotyrosine decreased, gradually returning to basal levels after 180 min. A sample photomicrograph illustrates cellular staining at + 30 min of GTN exposure (Fig. 7B). This rise in $ONOO^-$ formation was investigated further by determining the effects of inhibiting NOS, scavenging superoxide, or $ONOO^-$. Treatment of BAEC with GTN (10^{-5} M) for 30 min increased $ONOO^-$ formation, as indicated by a 3-fold increase in nitrotyrosine levels (Fig. 7A). Pretreatment with the NOS inhibitor L-NAME (5×10^{-4} M), SOD (polyethylene glycol-linked, 100 U/ml), or the peroxynitrite scavenger uric acid (10^{-3} M) also prevented the rise in nitrotyrosine in response to GTN. Uric acid totally blocked the GTN-induced nitrotyrosine formation. Moreover, treatment with either L-NAME or superoxide dismutase was equally as effective as uric acid in preventing nitration of tyrosine. The PKC inhibitor chelerythrine (10^{-6} M) also prevented the rise in nitrotyrosine in BAEC in response to GTN (Fig. 8). Chelerythrine alone did not alter nitrotyrosine formation. These results suggest that NOS and PKC are involved in peroxynitrite formation in endothelial cells with exposure to GTN.

GTN Induces Sustained Translocation of the α - and ϵ PKC Isozymes to the Particulate Cell Fraction in Endothelial Cells. The translocation of PKC isozymes from the cell supernatant to the cell particulate fraction has been used as an indirect measure of their activation in many cell types. We examined the effect of GTN on examples of three forms of isozymes of PKC: classic (α), novel (ϵ), and atypical (ζ). As a

A Effect of GTN on Nitrotyrosine Formation

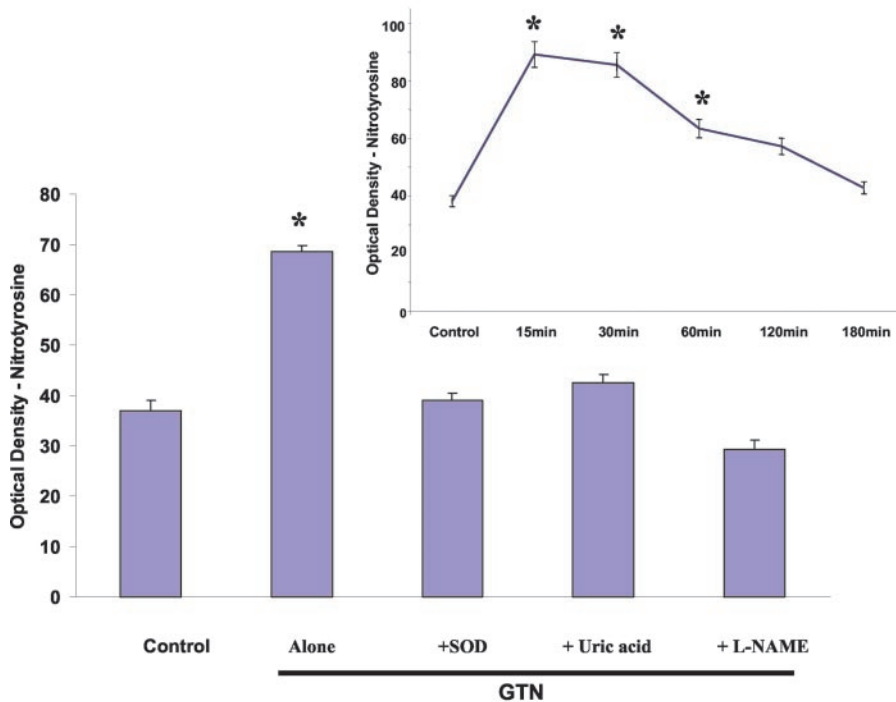
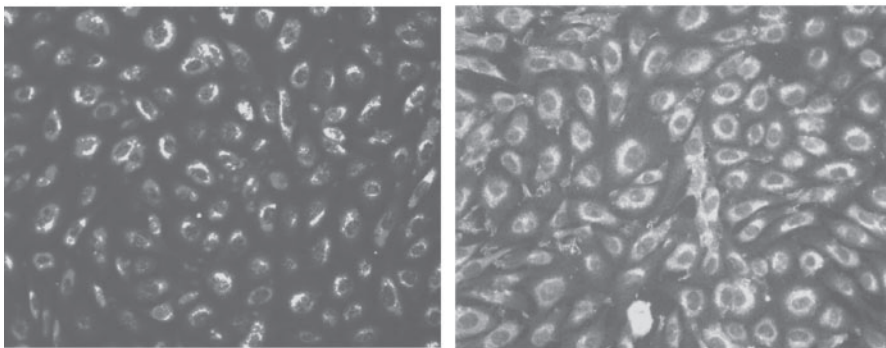


Fig. 7. Effects of GTN (10^{-5} M) exposure of BAEC on formation of nitrotyrosine. Inset displays the time course of the increase in nitrotyrosine levels (optical density) after GTN treatment (*, $P < 0.05$ versus control levels) (A). Main graph displays effects of exposure to GTN for 30 min alone or in the presence of SOD (80 U/ml), uric acid (10^{-3} M), or L-NAME (5×10^{-4} M) on nitrotyrosine levels (*, $P < 0.05$ versus control). Sample micrograph illustrates cellular staining after 30 min of GTN exposure.

B Nitrotyrosine - BAEC



Control

GTN

positive control for induction of PKC isozyme translocation, one group of BAEC was treated for 15 min with PMA (10^{-8} M). PMA triggered a robust translocation of the α - and ϵ PKC isozymes, but not the ζ PKC isozyme, to the particulate cell fraction (Fig. 9, lane 7). Other groups of BAECs were treated with GTN (10^{-5} M) for times ranging from 15 min to 1 h, and PKC isozyme translocation was monitored (Fig. 9, lanes 2–6). A second GTN (10^{-5} M) treatment was then administered at 60 min, and PKC isozyme distribution was monitored for an additional 2 h. Samples were taken at the times shown in Fig. 9. Cells were then lysed, fractionated, and subjected to Western blot analyses. As shown in Fig. 9 (lane 1), the α PKC isozyme was found predominantly in the cell supernatant (cytosolic) fraction prior to GTN treatment. Af-

ter a 15-min exposure to GTN, there was a complete redistribution of α PKC from the supernatant to the cell particulate fraction (Fig. 9, lane 1 versus 2). However, by 30 to 60 min of GTN treatment, α PKC immunoreactivity declined in the particulate fraction, but was still greater than in the control, and reappeared in the cell supernatant fraction. Since GTN is rapidly metabolized, we hypothesized that the decline in α PKC translocation could be due to diminished GTN levels in our experiments. For that reason, a second GTN bolus was delivered to the cells at 60 min, which caused α PKC to once again repartition to the particulate cell fraction (Fig. 9, lanes 5 and 6). The percentages of the α PKC isozyme found in the particulate cell fraction after 15 min, 1-, and 3-h GTN treatments were 100, 48, and 95%, respectively.

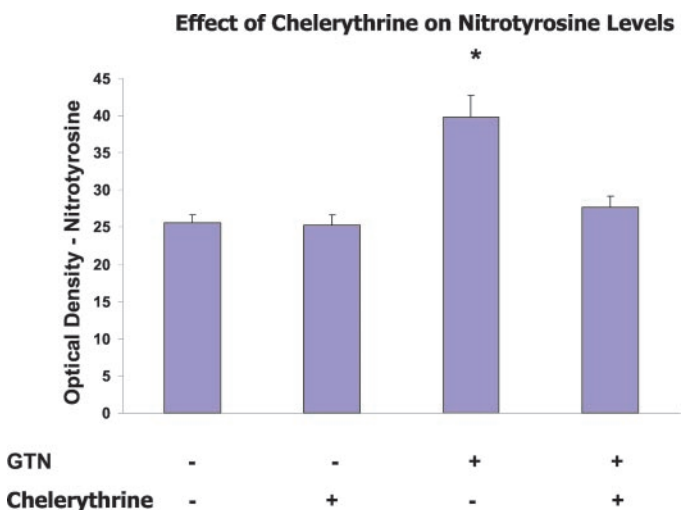


Fig. 8. Effects of GTN (10^{-5} M) exposure of BAEC in the presence of chelerythrine (10^{-6} M) on formation of nitrotyrosine (*, $P < 0.05$ versus control levels).

In contrast, the ϵ PKC isozyme was found to be evenly distributed between the cell supernatant and particulate fractions prior to GTN treatment. Figure 9 demonstrates that GTN (10^{-5} M) treatment stimulated the translocation of the supernatant-localized ϵ PKC to the particulate fraction. Similar to our findings with the α PKC isozyme, ϵ PKC translocation attained a maximal level following 15 min of GTN exposure. However, ϵ PKC remained in the particulate fraction longer than α PKC. There was a return of ϵ PKC immunoreactivity to the supernatant fraction at 30 to 60 min of GTN exposure (Fig. 9, lanes 3 and 4), but this effect was smaller in magnitude than was observed for the α PKC isozyme. A second treatment of BAECs with GTN (10^{-5} M) triggered the retranslocation of the ϵ PKC isozyme to the particulate fraction (Fig. 9, lanes 5 and 6). Unlike the α - and ϵ PKC isozymes, the ζ PKC isozyme was found almost entirely in the cell supernatant fraction before and after GTN treatments (Fig. 9, bottom). There was no significant redistribution of ζ PKC in response to PMA or GTN in these cells.

Discussion

We have examined the roles of O_2^- , $ONOO^-$, and PKC activation in vascular GTN tolerance. Tolerance to the vasorelaxation effects of GTN was studied by determining GTN concentration-response curves in rat aortic rings before and after 2-h treatment with GTN (5×10^{-4} M). Pretreatment of rings with GTN caused substantial tolerance to the vasodilating effect of GTN, as evident by a large rightward shift of the CRC. This effect was substantially inhibited by concurrent treatment of the rings with the antioxidants uric acid, vitamin C, or tempol or by the PKC inhibitor chelerythrine. Our experiments, which showed that the combination of tempol and chelerythrine similarly reduced GTN tolerance, suggest that both agents are working through reduction of oxidant levels; tempol is scavenging superoxide and other ROS, and chelerythrine is reducing oxidant formation by preventing PKC activation. Our findings confirm previous reports of involvement of oxidative stress and PKC activity in GTN tolerance (Zierhut and Ball, 1996; Munzel et al., 2000). Our finding that O_2^- generation via treatment with xanthine/xan-

thine oxidase also induces a tolerance to GTN supports the role of O_2^- as a mediator of GTN tolerance. The fact that the xanthine/xanthine oxidase treatment did not affect responses to the nitrovasodilator SNP demonstrates the specificity of the effect for GTN. This result is consistent with our earlier finding that GTN-tolerant vessels remain sensitive to the vasodilating effects of another potent nitrovasodilator, *S*-nitroso-*N*-acetyl penicillamine (Abou-Mohamed et al., 2000). The role of O_2^- in GTN tolerance was further confirmed by our in vivo study showing that GTN tolerance produced in rats by a 3-day i.v. infusion of GTN was also almost completely prevented by the concurrent infusion of tempol.

We also examined endothelial mechanisms that might contribute to tolerance to GTN. We first demonstrated that GTN induces tolerance to its ability to increase cGMP in cultured bovine aortic endothelial cells. Our data also showed that this effect is closely correlated with increases in tyrosine nitration of endothelial cell proteins and with translocation of the α - and ϵ PKC isozymes to the particulate fraction. Previous studies showing that tyrosine nitration of ϵ PKC enhances its interaction with its intracellular receptor and movement to its active cell locus (Balafanova et al., 2002) suggest that these events are causally related. We next determined that the free radical scavengers SOD and uric acid completely abolished GTN-induced tyrosine nitration. These results indicate that GTN has marked oxidant effects on endothelial cells. These results are in concert with our previous studies (Abou-Mohamed et al., 2000; Kaesemeyer et al., 2000). This endothelial dysfunction and tolerance to GTN may be mediated, at least in part, by ROS such as superoxide and peroxynitrite, tyrosine nitration, and the α - and ϵ PKC isozymes. In addition, we found that the selective NOS inhibitor L-NAME also abolished tyrosine nitration of proteins in these cells, implying that NOS activation is required for this effect. This result is consistent with our previous study showing a role for NOS activation in the action of GTN on endothelial cells (Abou-Mohamed et al., 2000). The importance of NOS activation in GTN tolerance is further demonstrated by the fact that other NO donors that do not induce vascular tolerance, such as sodium nitroprusside and *S*-nitroso-*N*-acetyl penicillamine, do not activate NOS or increase reactive oxygen species. Finally, we observed that the PKC inhibitor chelerythrine prevented tyrosine nitration, indicating that PKC activation is also involved in the enhanced $ONOO^-$ formation caused by GTN.

It is important to note that $ONOO^-$ has the prominent ability to oxidize tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) (Milstein and Katusic, 1999). Loss of BH_4 leads to further uncoupling of eNOS and more O_2^- formation (Stroes et al., 1998). Therefore, antioxidants and L-arginine, by reducing $ONOO^-$ levels or formation, can be expected to promote normal coupling of eNOS by the further action of maintaining BH_4 levels. This may partially explain a recent observation that folic acid prevents tolerance to GTN (Gori et al., 2001). In addition to being a precursor for BH_4 synthesis and a necessary cofactor for proper NOS function, folic acid and BH_4 are also antioxidants (Nakamura et al., 2001; Verhaar et al., 2002).

Previous studies have shown that treating endothelial cells with GTN leads to increased O_2^- formation (Dikalov et al., 1998; Kaesemeyer et al., 2000). Both vitamin C and the normal substrate for NOS, L-arginine, reduce O_2^- levels or

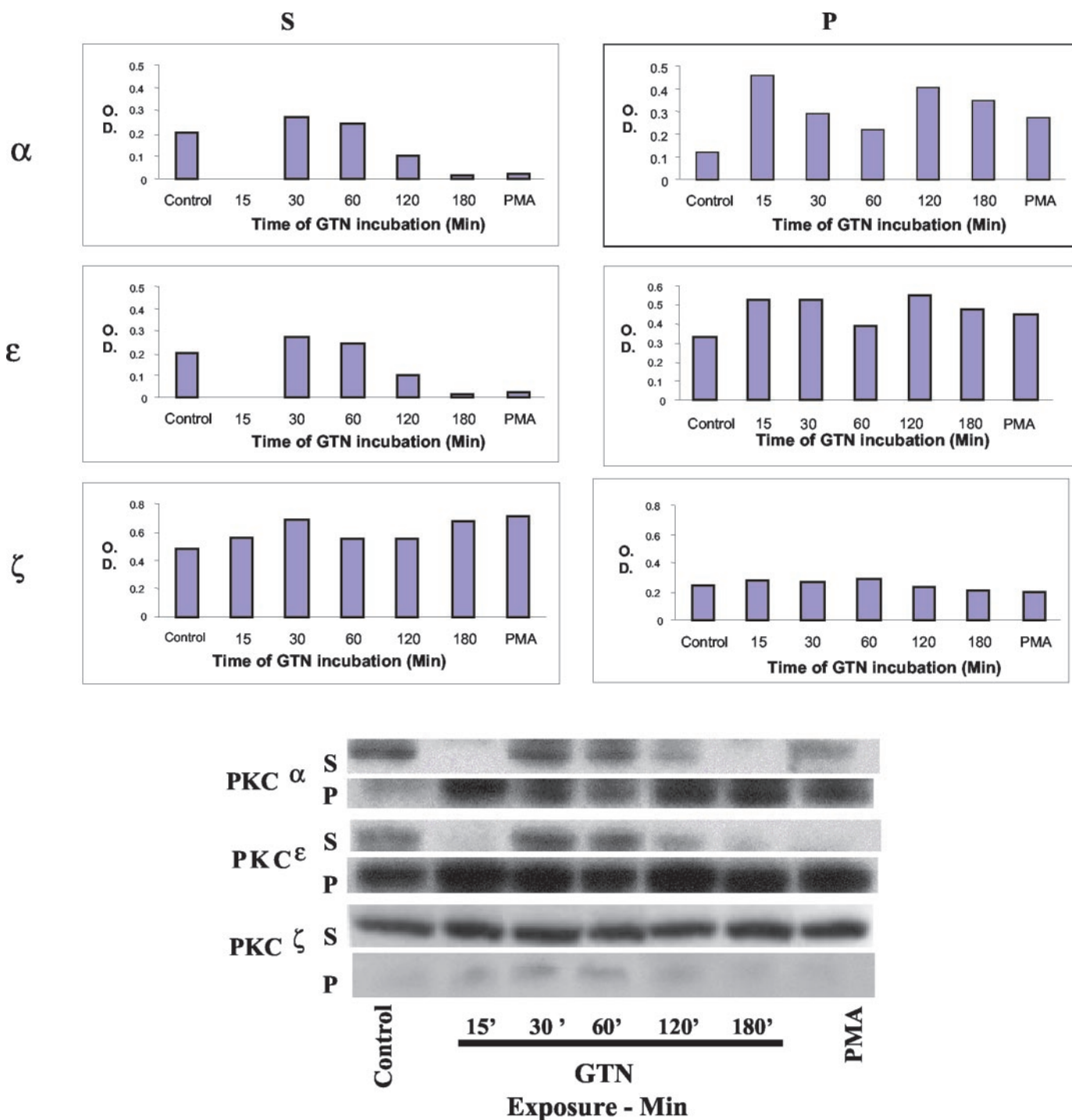


Fig. 9. Effects of GTN (10^{-5} M) treatment at time 0 and +60 min on the translocation of the α -, ϵ -, and ζ PKC isozymes in BAECs. Cells were isolated and cultured as described under *Materials and Methods* and treated with GTN for the times shown. Cells were then lysed and fractionated into cell supernatant (S) and particulate (P) fractions by centrifugation. Western blots were then conducted on each fraction with antisera selective for the α -, ϵ - or ζ PKC isozymes as indicated on the left side of the figure. Data are the mean of two to three experiments. Western blot detection was conducted using 125 I-protein A and autoradiography as described under *Materials and Methods*. In the top panels of the figure, optical density (O.D.) values for PKC isozyme immunoreactivity in the S and P fractions are plotted. These values were obtained by densitometric analysis of autoradiographs as described under *Materials and Methods*. The bottom portion of the figure is a representative autoradiograph demonstrating the changes in the redistribution PKC isozymes induced by GTN treatment. A typical autoradiograph from one of three experiments is shown.

production. It has also been shown that GTN both activates eNOS and inactivates the transporter system y^+ for uptake of arginine (Abou-Mohamed et al., 2000). Therefore, arginine can become depleted at the site of eNOS activity at caveolae of endothelial cells where eNOS and the L-arginine trans-

porter (system y^+) are colocalized. As this occurs, eNOS increasingly utilizes O₂ as its substrate, and O₂⁻ production increases. O₂⁻, once formed, freely reacts with NO to form ONOO⁻. Both of these reactive species are capable of producing further damage and suppression of the system y^+

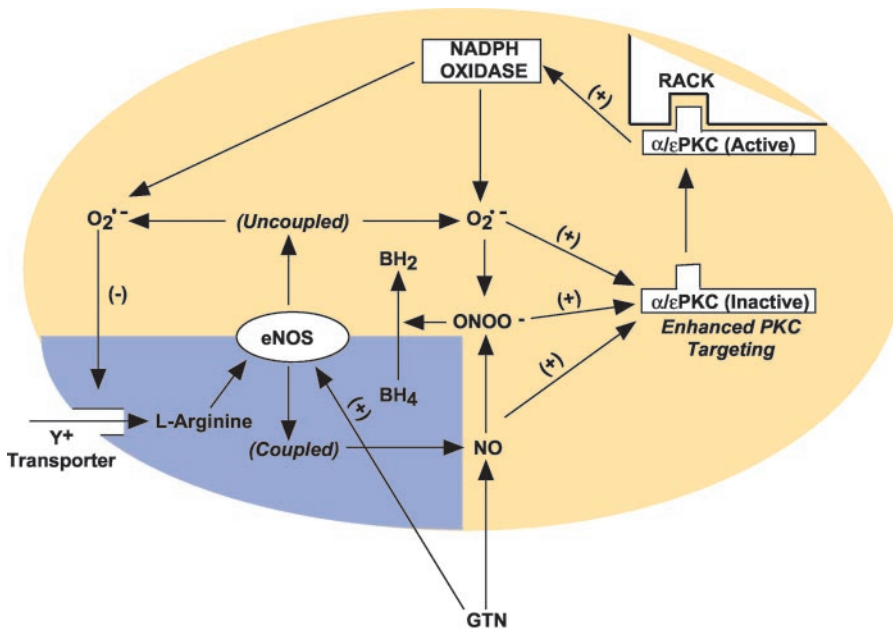


Fig. 10. Proposed signaling events involved in GTN-induced tolerance in endothelial cells. Physiologic endothelial cell NO production is shown in the bottom left of the figure (in the blue/shaded area). Under these conditions, eNOS has adequate L-arginine substrate and BH₄ cofactor from which NO can be synthesized. After prolonged elevation of NO and activation of NOS, the cells develop tolerance to subsequent GTN treatment. The hypothetical signaling events involved in tolerance are shown in the yellow/nonshaded area. GTN may increase production or levels of either O₂⁻ or NO. The O₂⁻ can come either from uncoupled NOS or another source. The NO may come from denitration of GTN or its activation of coupled NOS. NO rapidly reacts with O₂⁻ to form the highly reactive peroxynitrite (ONOO⁻), which can promote the uncoupling of eNOS by oxidizing BH₄ to BH₂ and oxidizing the y⁺ (L-arginine) transporter and reducing its function. When eNOS is “uncoupled”, it produces O₂⁻. Peroxynitrite and O₂⁻ have been shown to prompt translocation and activation of PKC isozymes. Our results have demonstrated enhanced translocation of the α- and εPKC isozymes to the cell particulate fraction (RACK, Receptor for Activated C-Kinase) following challenge with GTN. Active PKC isozymes have also been shown to enhance O₂⁻ production by NADPH oxidase in these cells, exerting a positive feedback system for increasing O₂⁻.

transporter. Our observation that L-NAME blocked nitrotyrosine formation is consistent with our prior finding that eNOS is a site of superoxide formation in endothelial cells treated with GTN (Kaesemeyer et al., 2000).

Antioxidants protect y⁺ transport function (Patel et al., 1996) and thereby provide arginine to the site of eNOS activity and interrupt this cycle. The y⁺ system transporter also has recently been reported to be regulated by PKCα in porcine pulmonary artery endothelial cells (Krotova et al., 2003). With activation of PKCα, an initial suppression (≤1 h) and a secondary increase (4–18 h) in L-arginine transport were observed. These investigators suggest that translocated PKCα may inhibit L-arginine uptake by phosphorylation of the transporter. However, our data suggest that enhancement of O₂⁻ and ONOO⁻ production by PKC and GTN may oxidize the transporter and inhibit its function.

Involvement of endothelial cells in GTN tolerance has been supported by earlier studies. Munzel et al. (1995) demonstrated that removal of endothelial cells from rabbit aorta made tolerant to GTN markedly improved the vasorelaxation to GTN. Furthermore, an increase in vascular levels of O₂⁻ in response to GTN was reduced with endothelial cell denudation. More recently, Munzel et al. (2000) have shown that 3-day treatment of rats with GTN enhanced eNOS (type III) expression and O₂⁻ production from eNOS. Furthermore, treatment of aortic rings with PKC inhibitors reduced O₂⁻ production and tolerance to GTN. Our previous study also demonstrated the depressive action of GTN on L-arginine uptake in endothelial cells (Abou-Mohamed et al., 2000). In our present study, we also have noted that an increase endothelial cell production of cGMP in response to GTN is absent after sustained exposure to GTN (10⁻⁵ M for 1 h). Thus, endothelial cells do exhibit a tolerance to an action of GTN.

A recent report using eNOS knockout and wild-type mice has suggested that endothelial NOS may not be critically involved in GTN tolerance because the degrees of tolerance to GTN in these two mouse strains were similar (Wang et al.,

2002). We do not believe that this is the case, because others have shown that in eNOS knockout mice, expression of neural (type I) NOS is enhanced within the endothelial cells and vessels and is capable of maintaining vasodilator responses to increased flows (Huang et al., 2002). Thus, it is likely that this vascular-associated neural NOS contributed to the development of tolerance in the eNOS-deficient mice.

Previous studies have indicated a role for PKC activation in GTN-induced vascular tolerance as inferred from the beneficial effect of chemical inhibitors of PKC in preventing the development of tolerance. Our data, for the first time, present evidence for the activation of PKC isozymes by GTN in endothelial cells and suggest a role for PKC activation by GTN in the development of tolerance. Activation of PKC has been shown to raise O₂⁻ levels via activation of NADPH oxidase (Bankers-Fulbright et al., 2001). Thus, a positive feedback loop appears likely, with O₂⁻ from NADPH oxidase subsequently further inactivating system y⁺ uptake of arginine, leading to reduced arginine levels at the site of eNOS activity and more superoxide as the end result. Inhibitors of PKC, as well as antioxidants and L-arginine, would be expected to ameliorate tolerance to GTN by interrupting this vicious cycle. A proposed scheme of events is given in Fig. 10.

In summary, tolerance to GTN in humans limits its therapeutic capacity, as its effects wane with continuous use. The molecular mechanisms contributing to this tolerance have been studied extensively in vascular smooth muscle, but the contribution of vascular endothelial cells to this phenomenon has received less attention. We, therefore, have investigated this aspect of GTN-induced tolerance in intact rat aorta preparations and in isolated bovine aortic endothelial cells in culture. Our results indicate that GTN tolerance occurs at the level of endothelial cells and is associated with 1) activation of NOS and PKC and 2) production of superoxide and peroxynitrite free radicals, which occur temporally with the activation of the α- and εPKC isozymes. These findings suggest potential therapeutic roles for agents that reduce ROS

levels and selective inhibitors of PKC isozymes in attenuating nitrate tolerance.

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