Vascular Peroxynitrite Formation during Organic Nitrate Tolerance

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ABSTRACT

Nitroglycerin (NTG) is an important cardiovascular agent, but tolerance during continuous administration limits its clinical utility. Increased vascular superoxide production may mediate nitrate tolerance via a reduction in nitric oxide availability. Because superoxide anion and nitric oxide react avidly to form peroxynitrite, an aggressive cellular toxicant that nitrates protein tyrosine residues, we tested the hypotheses that protein nitration, indicative of peroxynitrite formation, occurs during vascular tolerance, and that protein nitration participates in tolerance development. Preincubation of rat thoracic aorta segments with NTG (22 \( \mu \)M, EC_{50} for 30 min) caused a significant shift in NTG relaxation response (EC_{50}, 6.7 \pm 1.7 versus 0.50 \pm 0.13 \( \mu \)M, NTG versus vehicle, \( p < .05 \)). After functional evaluations, tissues were fixed in formalin for immunohistochemistry and digital image analysis. NTG-induced vascular tolerance was associated with increased immunoprevalence of 3-nitrotyrosine (3NT, stable biomarker of protein nitration; 11.41 \pm 2.48 versus 0.04 \pm 0.02% positive pixels, NTG versus vehicle, \( p < .05 \)). Staining was observed throughout vascular smooth muscle layers. Addition of 500 \( \mu \)M free tyrosine to the preincubation medium did not alter tolerance development (NTG EC_{50}, 6.5 \pm 3.0 \( \mu \)M) but abolished 3NT immunoprevalence (0.16 \pm 0.10%). No significant relationship between NTG potency and 3NT immunoprevalence was observed. These data support the hypothesis that protein nitration occurs during nitrate vascular tolerance, however, it apparently does not mediate this phenomenon.

Although first introduced as an angina therapy over 100 years ago, the organic nitrate vasodilators, such as nitroglycerin (NTG), remain an important class of cardiovascular agents (Bauer et al., 1995). Organic nitrates are prodrugs of the potent vasorelaxant nitric oxide (NO), requiring vascular metabolism for their pharmacological activities (Chung and Fung, 1990; Feelisch and Kelm, 1991). Their unique hemodynamic profile (i.e., predominant relaxation of capacitance vessels at low doses; Bassenge and Zanzinger, 1992; Bauer and Fung, 1996) makes these drugs effective agents for treating both angina pectoris and congestive heart failure (Murrell, 1879; Abrams, 1992). However, rapid tolerance development to both the hemodynamic and therapeutic effects of organic nitrates limits the utility of this otherwise useful drug class (Ahlner et al., 1991; Bauer and Fung, 1994).

Although the clinical problem of nitrate tolerance has been well described, a unifying mechanism of this phenomenon has not been established (Bauer and Fung, 1994). Previous studies by us and others have suggested that physiological responses to selective preload reduction and/or metabolic alterations in vascular tissues may participate in the pharmacodynamic tolerance observed (Needleman and Johnson, 1973; Parker et al., 1991; Feelisch and Kelm, 1991). A recently proposed mechanism of nitrate tolerance implicates increased vascular superoxide anion formation (O_{2}^{-}), reducing NO bioavailability and its relaxation effects (Munzel et al., 1995). O_{2}^{-} interacts with NO in a diffusion rate-limited reaction to form peroxynitrite (ONOO^{-}), a potent cellular toxicant (Pryor and Squadrito, 1991; Feelisch and Kelm, 1991). Thus, rapid tolerance development may be a consequence of increased vascular superoxide anion formation.

O_{2}^{-} is apparently formed in disease settings, including vascular dysfunction during renal transplant rejection (MacMillan-Crow et al., 1996), and that this agent is a potent inhibitor of enzyme processes in vitro (Zou et al., 1997; Mihm and Bauer, 1998). ONOO^{-} induced protein nitration has been demonstrated as a mechanism of this enzymatic inhibition (Yamakura et al., 1998). Finally, recent evidence suggests that ONOO^{-} is formed in vascular smooth muscle homogenates during NTG tolerance development, as measured by electron spin resonance spectroscopy (Dikalov et al., 1998).

Given the recent evidence that O_{2}^{-} may mediate vascular...
tolerance to organic nitrates together with its established chemical interactions with NO, here we hypothesized that nitrate tolerance is associated with increased protein nitration, a stable biomarker of ONOO⁻ formation. Additionally, we evaluated the potential contribution of vascular protein nitration as a mediator of nitrate vascular tolerance.

Materials and Methods

Isolated Vascular Function. Healthy male Sprague-Dawley rats (300–400 g) were euthanized by pentobarbital overdose, and the thoracic aorta was rapidly isolated and prepared as described previously (Bauer and Fung, 1991; Bauer et al., 1997). Vascular segments (2–3 mm) were suspended by stainless steel hooks in 10-ml tissue baths containing Krebs’ buffer at 37°C, oxygenated by constant bubbling of a 95/5% O₂/CO₂ mixture. In vitro vascular tolerance was induced by a 30-min preincubation period with 22 μM NTG (EC₉₅ concentration, Nitrocine; Schwarz Pharma, Mannheim, Germany). Vehicle control consisted of Krebs’ buffer + 100 μM 5% dextrose vehicle to account for time course and solvent effects. After a series of five rapid washes, vessels were precontracted with 2.5 μM phenylephrine to a magnitude between 1.3 and 1.9 g tension. After precontraction, NTG concentration-effect data were obtained by cumulative addition of NTG in 100-μl aliquots over a concentration range of 4 × 10⁻¹¹ to 2 × 10⁻⁴ M. Vessel tension data were collected by DigiMed Tissue Force Analyzer and System Integrator model 210 (Micro-Med, Louisville, KY).

In separate experiments, free 500 μM L-tyrosine (L-TYR, Sigma, St. Louis, MO) was added to the incubation medium to probe the functional relevance of ONOO⁻ formation in tolerance development. Tissues receiving TYR treatment were exposed to this solution throughout all phases of the experiment, and NTG tolerance was evaluated as described above. TYR addition did not affect Krebs’ buffer pH or composition.

Vascular Immunohistochemistry. Immediately after functional assessment, vessel segments were immersed in 10% formaldehyde (Formalin) for 48 h, then transferred to tissue cassettes for standard dehydration and paraffin infiltration in an automated tissue processor (Fisher Histomatic model 166; Fisher Scientific, Pittsburgh, PA). After paraffin embedding, 5-μm sections were mounted onto Fisher Scientific ProbeOn Plus slides for immunohistochemical treatment. Tissue sections were heated to 60°C for 30 min followed by immersion in clearing fluid (Hemo-D; Fisher Scientific) to remove paraffin wax. The tissue was partially rehydrated by alcohol gradient (100 to 70%) then immersed in 3% hydrogen peroxide/methanol solution for 10 min to block endogenous peroxidase activity. Slides were rinsed and reheated in citrate buffer (pH 6.0) to recover antigenicity. Tissues were blocked in 10% goat serum (Vector Laboratories, Burlingame, CA/PBS blocking solution for 30 min, then incubated with rabbit anti-mouse polyclonal antibodies directed against 3NT (1:400 dilution; Upstate Biotechnology, Lake Placid, NY). Staining (isotypic) control tissues were exposed for the same duration to nonimmune rabbit IgG (1:200; Vector Labs) in place of primary antibody. After primary antibody incubation, tissues were washed, then exposed to biotinylated goat anti-rabbit secondary antibody (Vector Labs) for 20 min. Peroxidase enzymes were linked to the antibody complex by incubation in a 1:200 dilution of ABC Elite reagent (Vector Labs) in PBS. Diaminobenzidine (0.06% w/v) was used to provide visualization of immunoreactivity, followed by methyl green counterstaining. Preliminary experiments were conducted to verify the specificity of immunostaining in our laboratory (Mihm and Bauer, 1998). Preincubation of primary antibody with 1 mM free 3NT completely quenched positive tissue staining, whereas 1 mM TYR had no effect. Nonimmune serum (isotypic) controls also showed no detectable immunoreactivity in any treatment group.

Digital Photomicroscopy and Image Analysis. Immunostained tissues were visualized using light microscopy at 200× magnification (Olympus BX40), digitally captured (Pixera digital camera, 1440 × 760 pixel resolution), and transferred into Image Pro Plus software (Media Cybernetics, Silver Spring, MD) for analysis. Image analysis was conducted using methods similar to Russ (1998). Cross-sectional images of a particular vascular segment encompassed at least 75% of the total circumference. Relative immunoreactivity of the vascular smooth muscle layer was then evaluated by applying intensity thresholding analysis using identical total tissue areas (75,000 μm²). Colored images were converted to gray-scale via extraction of the green channel to remove interference from methyl green counterstaining. Intensity threshold of 150 was predetermined as background staining in control tissues; therefore, the percentage of total pixels in an image falling into the 0 to 150 range was used as a relative measure of immunoreactivity.

In preliminary studies we evaluated our image analysis approach through the use of tetranitromethane (TNM, a well established chemical nitrating agent at pH 8.0; Sokolovsky et al., 1966)-exposed aortic segments as a positive control. Aortic segments similar to those used in functional experiments were treated with 8 M TNM (in 100 mM Tris buffer, pH 8.0) for 30 min to obtain saturated, uniformly nitrated control segments. Positive immunoreactivity was linearly related to total analyzed area (see Fig. 1) and preincubation of primary antibody to 2 mM free 3NT completely quenched immunoreactivity. These data suggested that extent of 3NT immunoreactivity was independent of the selected vascular area and that the primary antibody used was specific. Using the digital image analysis methods described above, we determined intra- and interobserver variability to be less than 4 and 8%, respectively (n = 3 observers evaluating 20 vessel segments).

Data Analysis. Cumulative relaxation data were expressed as a percentage of initial precontraction; relaxation data from each vessel segment was fitted to a sigmoidal E₅₀ model using GraphPad Prizm Software (San Diego, CA). EC₅₀, E₉₀, and Hill slope were determined for each treatment group. Statistical analyses were performed using one-way ANOVA. Statistical significance was assigned at p < .05.

Results

Isolated Vascular Function. NTG tolerance was rapidly induced by 30-min preincubation in EC₉₅ concentration of NTG. Figure 2A illustrates the average concentration-effect data for NTG in control versus tolerant vessels. NTG preincubation resulted in a significant loss of NTG potency, but no
loss in the maximal ability of NTG to relax the vessels, as illustrated in Fig. 2A. The fitted vascular relaxation parameters are presented in Table 1. Tolerant vessels demonstrated a 13-fold loss of NTG potency compared with control, as determined by comparison of fitted EC_{50} values. No difference in fitted E_{max} was observed among treatment groups.

To probe the functional relevance of ONOO^- formation in tolerance development, free L-TYR was used as a scavenger of ONOO^- . Phenylephrine precontraction was unchanged in TYR-containing buffer relative to control. Shown in Fig. 2B are average NTG response data (control versus NTG preincubated) in the presence of TYR. TYR treatment did not affect NTG potency or vascular tolerance development under either treatment condition (Table 1).

3NT Immunohistochemistry. After functional assessment, vessel segments were fixed and prepared for immunohistochemical analysis to determine the extent of smooth muscle protein nitration. Control segments demonstrated negligible 3NT immunoprevalence, and TYR incubation alone did not influence this parameter; therefore, the two control groups were merged for further statistical comparisons. Representative images are displayed in Fig. 3. Using digital image analysis, control tissues exhibited only 0.08 ± 0.04% positive immunoreactive area (n = 60 images from 12 total vascular segments).

In the absence of TYR in the incubation medium, NTG-tolerant tissues demonstrated 3NT immunoreactivity that was extensive and uniform throughout vascular smooth muscle (see Fig. 3B). Percent immunoreactive area from these NTG-tolerant vascular segments was 11.41 ± 2.48% (n = 14 vascular segments, p < .05 when compared with pooled control; Fig. 4). In contrast, the presence of 500 µM TYR during vascular studies abrogated vascular smooth muscle protein nitration to control levels, evinced by an absence of positive staining in the TYR-exposed, NTG-tolerant group (Fig. 3C). Positive immunoreactivity was similar to pooled control levels, yielding 0.16 ± 0.10% immunoreactive area (n = 8; N.S. from pooled control, statistically significant from NTG-tolerant, p < .05).

Discussion

Organic nitrate tolerance limits the long-term efficacy of an otherwise valuable cardiovascular drug class (Zimrin et al., 1988; Fung et al., 1998). Despite decades of investigation, the mechanisms by which nitrate tolerance develops are poorly understood (Munzel and Harrison, 1997). Recent evidence suggests that increased vascular free radical formation may participate in this phenomenon (Munzel et al., 1995). For example, in vivo experiments and isolated vascular studies have shown that antioxidants such as ascorbic acid (Bassenge et al., 1998) and α-tocopherol (Watanabe et al., 1997) can prevent or reduce nitrate tolerance development. In addition, recent studies by Munzel et al. (1995) have shown that vascular tissues from NTG-tolerant rabbits have elevated basal formation of O2^- and that this change functionally reduces the vasoactivity of NTG and other NO donors in vitro. These investigators also demonstrated that NTG vascular tolerance was reversible via addition of superoxide dismutase to the incubation medium. Finally, Dikalov et al. (1998) have provided indirect evidence, using electron spin resonance spectroscopy, of ONOO^- formation in vascular smooth muscle homogenates after acute, high concentration NTG (500 µM) incubation. Although these previous studies suggest a role for O2^- in the contribution of ONOO^- to the toxic end-product of NO destruction by O2^- during nitrate tolerance has not been investigated. Here we hypothesized that vascular protein nitration, indicative of ONOO^- formation, occurs during nitrate tolerance.

Increased O2^- formation could contribute to nitrate tolerance by a number of mechanisms. O2^- might simply serve to shunt NO produced from NTG away from guanylate cyclase and its vascular smooth muscle activities. Moreover, ONOO^- is an established inhibitor of several enzymatic and biochemical processes (Freeman, 1994). Because conversion of NTG to NO requires enzymatic participation (Ignarro et al., 1981; Chung and Fung, 1990), we hypothesized that the ONOO^- formed during nitrate tolerance (due to increased vascular O2^- formation) may play a functional role in the phenomenon by inhibiting the enzyme(s) required for NO formation.

Using conditions similar to those previously used, we observed significant vascular tolerance to NTG after only 30-min incubation with 22 µM NTG. Associated with this tolerance development was extensive evidence of vascular protein nitration when compared with control tissues. These findings provide the first experimental evidence of vascular protein nitration during organic nitrate tolerance.

Although recent studies have suggested the existence of other potential biological pathways of TYR nitration, these studies have demonstrated that the chemistries responsible are dependent on neutrophil infiltration and activation (Sampson et al., 1998; Eiserich et al., 1998). Because tolerance induction occurred so rapidly and our histochemical analysis of the tissues demonstrated no immune cell infiltration into the vascular smooth muscle, the protein nitration in these studies was most likely derived from ONOO^- formation in the vasculature. Additionally, previous studies demonstrate that cardiovascular tissue homogenates require exogenous addition of neutrophil myeloperoxidase (5 µM), 1
mM concentrations of nitrite and hydrogen peroxide, and long incubation times (>1 h) to produce detectable protein nitration (Sampson et al., 1998). In contrast, NO and O$_2^-$ are known to form ONOO$^-$ at a diffusion-limited rate, even at very low concentrations. Therefore, under our experimental conditions the observed protein nitration may be most simply explained by increased ONOO$^-$ formation, rather than more complicated or less efficient chemical processes.

Studies by Munzel et al. (1995) have suggested that the vascular endothelial cell layer is a major production site for O$_2^-$ during nitrate tolerance. In our studies, protein nitration (suggestive of ONOO$^-$ formation) was observed consistently throughout the entire smooth muscle layer after NTG incubation. Given the high instability and rapid reactivity of ONOO$^-$ with intracellular proteins, the observed staining pattern suggests that ONOO$^-$ was likely formed throughout the vascular wall during tolerance development. Identification of the primary cellular site(s) of O$_2^-$ formation during nitrate tolerance (endothelial layer versus vascular smooth muscle) may provide further insight into the biochemical mechanisms involved, as well as provide basic understanding of the intra-and intercellular reactivity of ONOO$^-$.

We evaluated the potential role of protein nitration in nitrate tolerance development by the addition of 500 µM TYR to the vascular incubation media. Free TYR was added as a competitive nitration site for ONOO$^-$ at a concentration 5- to 10-fold in excess of physiologic intracellular levels (Thalhammer et al., 1982). TYR (500 µM) had no effect on NTG potency in control or tolerant vessel segments. However, TYR addition completely protected vascular smooth muscle from protein nitration, most likely by scavenging ONOO$^-$ away from vascular smooth muscle sites. These results indicate that protein nitration apparently does not directly participate in NTG tolerance. Investigating protein nitration as the functional endpoint of ONOO$^-$ formation does not account for alternative biochemistries by which ONOO$^-$ may exert effects (e.g., sulfhydryl oxidation). However, we were able to completely reverse the extensive protein nitration associated with nitrate tolerance (via TYR addition), while leaving NTG potency in the vessel unchanged. By eradicating all protein nitration in the vasculature, it is likely that a majority of the ONOO$^-$ formed during tolerance was scavenged by TYR addition, with no alteration in functional response to NTG. Therefore, it is unlikely that alternative ONOO$^-$-mediated biochemistries participate in tolerance development.

Skatchkov et al. (1997) recently demonstrated evidence of ONOO$^-$ formation during nitrate tolerance in vivo. In a dog model of nitrate tolerance, these investigators demonstrated that urinary levels of 3NT were 4-fold higher in nitrate-tolerant dogs compared with control (Skatchkov et al., 1997). Our observations are consistent with these in vivo studies and consistent with the hypothesis that O$_2^-$ contributes to nitrate tolerance. Additionally, Dikalov et al. (1998) have demonstrated ONOO$^-$ formation during NTG incubation, and have suggested that formation of reactive oxidative species such as ONOO$^-$ is a key mediator of NTG tolerance development. Although NO-related biochemistry may be altered during tolerance development, our observed dissociation between extent of protein nitration and NTG potency...
suggests that vascular protein nitration does not mediate this phenomenon.

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References


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