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Title: Dissociation Between Superoxide Accumulation And Nitroglycerin- Induced Tolerance

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Running title: role of superoxide in nitroglycerin tolerance

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Abbreviations: cGMP, cyclic guanosine monophosphate; Ang II, angiotensin II; NTG, nitroglycerin; NOX, NADPH oxidase; superoxide, $O_2^{\bullet -}$; XOR, xanthine oxidoreductase

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Abstract

We hypothesize that superoxide ($O_2^{\bullet -}$) accumulation is not a crucial causative factor in inducing nitroglycerin (NTG) tolerance. In LLC-PK1 cells, pre-exposure to NTG resulted in increased $O_2^{\bullet -}$ accumulation and reduced cyclic guanosine monophosphate (cGMP) response to NTG vs. vehicle control. $O_2^{\bullet -}$ stimulated by NTG was reduced by oxypurinol (100 μM), a xanthine oxidase inhibitor. Exposure to angiotensin II (Ang II) increased $O_2^{\bullet -}$ but did not reduce cGMP response. The $O_2^{\bullet -}$ scavenger tiron reduced Ang II-induced $O_2^{\bullet -}$ production but did not increase NTG-stimulated cGMP production. Using $p47^{phox}$ (–/–) and $gp91^{phox}$(–/–) mice vs. their respective wild-type controls (WT), we showed that aorta from mice null of these critical NADPH oxidase subunits exhibited similar vascular tolerance after NTG dosing (20 mg kg^{-1} sc, tid for 3 days), as indicated by their *ex vivo* pEC_{50} and cGMP accumulation upon NTG challenge. *In vitro* aorta $O_2^{\bullet -}$ production was enhanced by NTG incubation both in $p47^{phox}$ null and WT mice. Pre-exposure of isolated mice aorta to 100 μM NTG for 1 hour resulted in vascular tolerance toward NTG and increased $O_2^{\bullet -}$ accumulation. Oxypurinol (1 mM) reduced $O_2^{\bullet -}$ but did not attenuate vascular tolerance. These results suggest that $O_2^{\bullet -}$ does not initiate either *in vitro* and *in vivo* NTG tolerance, and that the $p47^{phox}$ and $gp91^{phox}$ subunits of NADPH oxidase are not critically required. Increased $O_2^{\bullet -}$ accumulation may be an

effect, rather than an initiating cause, of NTG tolerance.

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Introduction

Nitroglycerin (NTG, glyceryl trinitrate) and other organic nitrates have been widely used to treat coronary artery disease in patients with stable and unstable angina, acute myocardial infarction and congestive heart failure. Given acutely, NTG relieves ischemic symptoms rapidly without serious side effects. However, its efficacy after repeated dosing is attenuated because of the development of pharmacologic tolerance. This phenomenon was first described by Stewart (Stewart, 1888) in the 19th century, but its underlying mechanism(s) still remained incompletely defined.

Nitrate tolerance is a complex phenomenon accompanied by a myriad of events, including decreased pharmacological response, reduced metabolism, increased oxidative stress, altered gene expression, etc. Consequently, several hypotheses have been proposed to account for these various phenomenon, including those of sulfhydryl depletion, impaired biotransformation of mitochondrial aldehyde dehydrogenase (ALDH2), and oxidative stress, as reviewed recently in (Fung, 2004; Munzel et al., 2005).

Munzel et al. (Munzel et al., 1995) found that prolonged period of NTG exposure in rabbits enhanced superoxide ($O_2^{\bullet -}$) production in the blood vessel wall, and proposed that nitrate

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tolerance was caused by increased $O_2^{\bullet-}$ formation. Further development of this mechanism, which became known as "the $O_2^{\bullet-}$ -oxidative stress hypothesis", suggested that chronic NTG treatment increases angiotensin II (Ang II) binding to endothelium angiotensin receptors, subsequently stimulating NADPH oxidase (NOX) to produce $O_2^{\bullet-}$. This oxygen free radical then reacts with nitric oxide (NO), the putative intermediate of NTG, to form peroxynitrite ($ONOO^-$), an oxidant and a weaker vasodilator than NO. Oxidative stress also brings about uncoupling of endothelial NO synthase (eNOS), resulting in decreased NO availability and further $O_2^{\bullet-}$ formation, and ultimately endothelial dysfunction (Gori and Parker, 2002). This hypothesis was supported by the attenuating effects of agents that inhibit $O_2^{\bullet-}$ production (e.g., antioxidants such as liposomal superoxide dismutase (Munzel et al., 1995), vitamin C (Bassenge et al., 1998), vitamin E (Watanabe et al., 1997) and hydralazine (Daiber et al., 2005), and drugs that inhibit the Ang II/NOX pathway (Wada et al., 2002). A later study suggested that reactive oxygen species (ROS) produced by NTG may inhibit ALDH2, one of several enzymes responsible for NTG bioactivation (Wenzel et al., 2007).

While the "$O_2^{\bullet-}$ -oxidative stress hypothesis" involving $O_2^{\bullet-}$ accumulation can explain several important findings associated with nitrate tolerance, the causative role of $O_2^{\bullet-}$ in this phenomenon was not always observed. Increased $O_2^{\bullet-}$ production was observed in tolerant

vessels obtained from patients with coronary artery disease, but NTG responsiveness was not altered, suggesting lack of critical involvement of $O_2^{\bullet -}$ in NTG tolerance (Sage et al., 2000). Using 8-iso-prostaglandin(PG)$F_{2\alpha}$ and circulating 3-nitrotyrosine and their metabolites as indicators for oxidative stress, Keimer et al. (Keimer et al., 2003) reported that sustained administrations of isosorbide dinitrate and pentaerythrityl tetranitrate did not enhance systemic oxidative stress in healthy volunteers. In addition, a major gap in the $O_2^{\bullet -}$ hypothesis continues to exist, as it is unknown how NTG would activate Ang II and NOX to produce $O_2^{\bullet -}$, and experimental evidence regarding this crucial initiation step is still not available.

In this study, therefore, we further examined the role of $O_2^{\bullet -}$ in NTG tolerance development, using two separate experimental systems: LLC-PK1 cells and NOX knockout mice. LLC-PK1 cells, derived from a porcine kidney epithelial cell line, have been used as a model for examining the cellular mechanisms of nitrate-induced activation and desensitization of cyclic guanosine monophosphate (cGMP) response in previous studies (Hinz and Schroder, 1998). We employed this *in vitro* system here to examine the roles of Ang II and $O_2^{\bullet -}$ in NTG tolerance. Since Ang II-mediated NOX stimulation was proposed to be critical for $O_2^{\bullet -}$ production to induce nitrate tolerance (Rajagopalan et al., 1996), two different strains of knockout mice, null of $p47^{phox}$ and $gp91^{phox}$ subunits of NOX, were used to explore their role in mediating $O_2^{\bullet -}$ production and

vascular tolerance.

Methods

Materials. LLC-PK1 cells were obtained from the American Type Culture Collection (Rockville, MD). Ham's F-12 medium, fetal bovine serum, 10^4 U ml^{-1} penicillin, 10 mg ml^{-1} streptomycin, dihydroethidium (DHE) and nitrocellulose membrane were purchased from Invitrogen (Carlsbad, CA). Adult $gp91^{phox}$ (–/–) mice and wild-type (WT) C57BL/6 controls, 7-9 weeks of age, were purchased from the Jackson's Laboratory (Bar Harbor, ME), while adult $p47^{phox}$ (–/–) and WT C57BL/6 mice were purchased from Taconic Farms Inc. (Germantown, NY). NTG (2 % powder, adsorbed on lactose) was kindly provided by Copperhead Chemical Company Inc. (Tamaqua, PA). The sources of other materials are indicated as follows: NTG solution (30% propylene glycol, 30% ethanol, Abbott Laboratories, North Chicago, IL); cyclic GMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI); ECL-enhanced chemiluminescence system (Pierce ,Rockford, IL). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell studies

Cell culture. LLC-PK1 cells were cultured in Ham's F-12 medium, containing 15% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. They were maintained in a

humidified incubator at 37^{o}C and 5% CO_2. Cells between passage 6 and 16 were used in all experiments.

Detection of cyclic guanosine monophosphate (cGMP). Cells subcultured in 35-mm culture dishes were grown to confluence, and preincubated in basal salt solution (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose, and 20 mM HEPES-NaOH, buffered to pH 7.3) for 4 hours containing vehicle control (5% dextrose), 1 μM NTG, or 1 μM Ang II, each with or without 10 mM tiron. At the end of the preincubation, cells were washed twice with phosphate-buffered saline (PBS), and further incubated in basal salt solution in the presence of 0.5 mM isobutylmethylxanthine (IBMX) for 10 min to inhibit cGMP degradation. The cells were then challenged with 3.16 μM NTG for 10 min to stimulate cGMP production, which was determined using an enzyme immunoassay kit, and normalized for protein content.

Superoxide measurement. Flow cytometry was used to quantify the fluorescence signals produced by DHE-superoxide complexes in cells (Munzel et al., 2002). Cells were grown in blank F-12 medium overnight and were suspended in blank medium (5×10^6 cells in 2 ml PBS) on the day of analysis. They were treated with 1 μM Ang II or 1 μM NTG for 2 hours. When tiron and oxypurinol were used, their final concentrations were set at 10 mM and 100 μM,

respectively. At the end of the incubation, cells were washed with PBS and bathed in fresh F-12 blank medium. A final concentration of 10 nM DHE was added to the medium and the cells are incubated for 20 min, then washed with ice-cold PBS and analyzed using a Becton Dickinson FACSCalibur flow cytometer, using excitation and emission wavelengths at 488 nm and 585 nm, respectively. BD CellQuest Pro software was used to quantify the fluorescence signal, which was expressed in arbitrary fluorescence units (FLU).

Animal studies

***In vivo* NTG tolerance induction.** Knockout and control mice were housed in sterile environment to prevent infection. Animals received a mixture of sulfamethoxazole (40 mg ml^{-1}) and trimethoprim (8 mg ml^{-1}) in their drinking water for 2 days, then switched to normal drinking water for 1 day before tolerance induction, as previously described (Wang et al., 2002). Briefly, animals received subcutaneous injections of either NTG (20 mg kg^{-1}) or vehicle (lactose solution) 3 times daily for 3 days. Animals were then sacrificed and thoracic aorta was removed for vascular relaxation studies and cGMP measurements (n = 6-7 each). All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

***Ex vivo* vascular relaxation.** The procedures for tissue bath relaxation studies were adopted from Wang et al. (Wang et al., 2002). Briefly, after tolerance induction, animals were anesthetized with a mixture of ketamine (90 mg kg^{-1}) and xylazine (9 mg kg^{-1}). The abdomen was cut open and the animal was exsanguinated via cardiac puncture. The thoracic aorta was removed and immersed in ice-cold Krebs buffer. The aorta was cleaned of connective tissue and cut into 3 mm wide ring segments. They were then suspended by stainless steel hooks in 10 ml tissue baths containing Krebs buffer and maintained at 37^{o}C with constant aeration of 5% CO_2 in O_2. The aorta rings were washed with fresh buffer and equilibrated for 1 hour. Following equilibration, the aortic rings were precontracted with 4 μM of phenylephrine. Once the contraction was stabilized, the cumulative NTG dose-response curves were obtained. Ring tensions were measured using isometric transducers and recorded using the PowerLab data acquisition system (ADInstruments, Colorado Springs, CO).

***Ex vivo* cGMP measurement.** After NTG tolerance induction, the thoracic aorta was collected as described, and equilibrated in Krebs buffer at 37^{o}C for 30 min in the presence of 0.5 mM IBMX, and then challenged with 1 μM NTG for 30 seconds. The preparation was immediately frozen in liquid nitrogen and homogenized in 1 ml 0.1 M hydrochloric acid. The supernatant was collected after centrifugation at 600 × g for 10 min at 4^{o}C, and analyzed for cGMP. The

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pellet was dissolved in 200 μL of 1 M sodium hydroxide, and centrifuged at 600 × g for 10 min at 4^{o}C to remove the insoluble content and the resultant supernatant was used for protein analysis. cGMP levels were determined by enzyme immunoassay, and normalized for protein content.

***In vitro* aortic superoxide measurement.** Thoracic aorta from untreated mice was collected as described. The dissected tissue was tied with thread and incubated in tissue bath in the presence of Krebs buffer for 20 min. Oxypurinol (1 mM) was added and incubated for 15 min, followed by addition of 10 μM NTG for an additional 1 hour incubation. The tissue was then cut open longitudinally and used for *ex vivo* vascular relaxation or $O_2^{\bullet-}$ measurements. Aortic $O_2^{\bullet-}$ accumulation was measured by lucigenin-enhanced chemiluminescence using a liquid scintillation counter (Hewlett Packard TRI-CARB 1900CA) set in out-of-coincidence mode. The assay medium contained 2 ml of Krebs buffer and 5 μM lucigenin; placed in glass scintillation vials and kept in the dark for 30 min. The chemiluminescence from several blank vials was first determined, and the ones with the lowest background counts were selected for use. The assay was initiated by adding the tissue in the dark-adapted scintillation vials, which were kept in the counter for 2 min prior to counting. After counting, 20 mM of the $O_2^{\bullet-}$ scavenger tiron was added, incubated in dark for 20 min, and the samples were counted again. Photon emission was measured every 1 min for 5 min. The tissue was dried at 90^{o}C overnight and the

result was expressed as counts per min per mg dry weight.

Statistical analysis. All data are presented as mean ± SD unless otherwise stated. Statistical differences between values were evaluated by either Student's t-test or one-way analysis of variance (one-way ANOVA), where appropriate. Statistical significance was determined by Tukey's post-hoc test (SPSS 11.x, Chicago, IL) after ANOVA. The EC_{50} from the NTG concentration-relaxation response curve was calculated by fitting the data with a sigmoid E_{max} model using SigmaPlot (v8.0, Systat Software, Inc., San Jose, CA). Differences with $p<0.05$ were denoted as statistically significant.

Results

Effects of Ang II or NTG on cGMP response in LLC-PK1 cells. Cells exposed to 1 μM NTG for 4 hours showed significantly reduced cGMP accumulation when stimulated by a challenge concentration of 3.16 μM NTG for 10 min (Table 1), suggesting development of nitrate tolerance ($p<0.01$). Co-incubation with 10 mM of vitamin C, but not with 100 μM of oxypurinol, partially restored this attenuated response (Table 1). Co-incubation with 10 mM tiron during the 4-hour exposure in both control and NTG-incubated samples ($p<0.01$). Cells exposed to 1 μM Ang II for 4 hours, with or without tiron, exhibited cGMP responses similar to those found in control samples (Table 1).

Effects of Ang II or NTG on superoxide production in LLC-PK1 cells. Fluorescence from DHE, a superoxide-trapping probe, was used to examine the effect of Ang II and NTG on $O_2^{\bullet-}$ accumulation in LLC-PK1 cells. DHE enters cells freely and, after reacting with $O_2^{\bullet-}$, is converted to ethidium which intercalates with DNA and produces nuclear fluorescence (Munzel et al., 2002). As shown in Table 1, cells treated with 1 μM Ang II showed higher $O_2^{\bullet-}$ accumulation compared to control ($p<0.01$). Addition of 10 mM tiron,

the $O_2^{\bullet-}$ scavenger, significantly reduced Ang II-stimulated $O_2^{\bullet-}$ accumulation ($p<0.01$). A similar profile was observed for the cells treated with 1 μM NTG (Table 1). Under tolerance conditions (exposure to 1 μM NTG for 4 hours), cells produced higher levels of $O_2^{\bullet-}$compared to control ($p<0.01$). Co-incubation with tiron reduced $O_2^{\bullet-}$ back to baseline. Co-incubation of NTG with 100 μM oxypurinol or 10mM vitamin C (Table 1) significantly abolished NTG-stimulated $O_2^{\bullet-}$ production ($p<0.01$).

Effect of *in vivo* NTG treatment on the *ex vivo* vascular relaxation of the mouse aorta.

The tolerance behavior of $gp91^{phox}$ (–/–) mice toward NTG was compared to that of WT mice. Fig. 1 shows that in both $gp91^{phox}$ (+/+) and $gp91^{phox}$ (–/–) animals (panels A and B, respectively), NTG pretreatment for 3 days produced a similar rightward shift in the concentration-relaxation response curves toward NTG over the concentration range of 0.1 nM to 1 mM. The pEC_{50} values obtained from both $gp91^{phox}$ (+/+) and $gp91^{phox}$ (–/–) mice are shown in Table 2.

Both $p47^{phox}$(+/+) and $p47^{phox}$(–/–) mice also exhibited vascular nitrate tolerance after NTG dosing (Fig. 2). The concentration-relaxation response curves were significantly right-shifted after pre-treatment with NTG for 3 days in both WT and knockout mice. The

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pEC_{50} values obtained from both $p47^{phox}$(+/+) and $p47^{phox}$(–/–) mice are shown in Table 2.

Effect of *in vivo* NTG treatment on cGMP accumulation in mouse aorta. The aortic cGMP levels after *in vivo* NTG tolerance induction was measured by stimulating the aorta with 1 μM NTG *ex vivo* for 30 seconds, as described previously (Wang et al., 2002). NTG-stimulated cGMP production trended higher in both untreated knockout animals than in their corresponding WTs, but without statistical difference. In both $gp91^{phox}$ (+/+) and $gp91^{phox}$ (–/–) mice, cGMP accumulation was significantly lower ($p<0.05$) in NTG-treated groups compared to vehicle-treated groups (Table 2). Similar to $gp91^{phox}$ gene knockout animals, cGMP accumulation in both $p47^{phox}$(+/+) and $p47^{phox}$(–/–) mice were significantly lower ($p<0.05$) in NTG-treated animals than vehicle-treated controls (Table 2).

Effect of *in vitro* NTG treatment on superoxide production in mouse aorta. To examine whether $O_2^{\bullet-}$ can be produced in $p47^{phox}$ WT and knockout animals, mice aorta was treated with NTG *in vitro* and $O_2^{\bullet-}$ accumulation was measured. As shown in Fig. 3, NTG significantly increased $O_2^{\bullet-}$ production in both $p47^{phox}$ (+/+) and $p47^{phox}$ (–/–) mice (**$p<0.01$ and *$p<0.05$, respectively). The $O_2^{\bullet-}$ produced by NTG can be quenched by 20 mM tiron ($p<0.01$, data not shown). NTG stimulated $O_2^{\bullet-}$ production in $p47^{phox}$ (+/+)

mice was significantly higher than those in $p47^{phox}$ (–/–) mice (**$p<0.01$).

Aorta from $p47^{phox}$ were used for further study regarding its ability to generate $O_2^{\bullet-}$ when exposed to Ang II and NTG *in vitro*. We found that $O_2^{\bullet-}$ production in aortas of WT animals treated with Ang II trended higher than vehicle controls, at $(1.99 \pm 0.59) \times 10^3$ vs. $(1.43 \pm 0.69) \times 10^3$ cpm mg^{-1} dry weight, respectively ($p>0.05$). In $p47^{phox}$ (–/–) mice treated with Ang II, $O_2^{\bullet-}$ concentration was $(1.09 \pm 0.29) \times 10^3$ cpm mg^{-1} dry weight, significantly lower than that found in WT mice ($p<0.05$), indicating that the $p47^{phox}$ knockout mice was indeed functionally unresponsive to Ang II stimulation.

To examine the possible source of $O_2^{\bullet-}$ production in mice, the effect of oxypurinol, a xanthine oxidase (XO) inhibitor, on NTG-stimulated $O_2^{\bullet-}$ was assessed. In $p47^{phox}$ (+/+) animals, NTG significantly increased $O_2^{\bullet-}$ production, while co-incubation of 1 mM oxypurinol significantly reduced NTG-stimulated $O_2^{\bullet-}$ production (Table 3, **$p<0.01$ control vs. NTG, *$p<0.05$ NTG vs. oxypurinol + NTG). The $O_2^{\bullet-}$ produced was quenched by 20 mM tiron in all groups. *In vitro* co-treatment with oxypurinol did not alter the extent of NTG vascular tolerance in isolated vessels, as the EC_{50} values were similar in the presence or absence of oxypurinol (Fig. 4 and Table 3).

Discussion

The present study only addressed one of the principal hypotheses of nitrate tolerance, viz., that of oxidative stress caused by $O_2^{\bullet -}$ accumulation, through the Ang II/NOX pathway (Munzel et al., 1996). *In vivo* Ang II treatment for 7 days in rats increased the activity and expression of NOX, leading to increased $O_2^{\bullet -}$ production, eNOS uncoupling, impaired NO/cGMP signaling, and endothelial dysfunction (Mollnau et al., 2002). In healthy human subjects, plasma Ang II increased after continuous transdermal NTG application (Watanabe et al., 1998), and in rabbits, 3 days continuous treatment with NTG patches resulted in an increase in Ang II type 1 (AT_1) receptor mRNA expression, and a marked increase in constrictions to Ang I and II (Kurz et al., 1999). These findings led the last group of investigators to conclude "a pathophysiological role for angiotensin II..... in the development of nitrate tolerance".

Despite these findings, the direct relevance of the Ang II/NOX/$O_2^{\bullet -}$ axis in causing vascular tolerance (so-called true tolerance) is not clear. Plasma Ang II levels remained elevated despite avoidance of tolerance via intermittent dosing (Watanabe et al., 1998), suggesting that the Ang II response might be a result of hemodynamic counter-regulation (so-called pseudo-tolerance). An obligatory involvement of the endothelium in nitrate

tolerance was indicated (Kurz et al., 1999), but several investigators have found that vascular NTG tolerance can be induced in the absence of the endothelium, and cross-tolerance between NTG and acetylcholine was not observed (Stewart et al., 1987; Mulsch et al., 1989). Nitrate tolerance in the rat thoracic aorta was not diminished by endothelium removal (Rapoport et al., 1987). Using knockout mice, we also showed that endothelial NOS was not critically required for the induction of nitrate tolerance (Wang et al., 2002).

Here we sought to examine the criticality of $O_2^{\bullet -}$ as an initiator of NTG tolerance, using both *in vitro* and *in vivo* approaches. We first examined the feasibility of using cultured human vascular smooth muscle cells (HuVSMC, ATCC CRL-1999) instead of LLC-PK1 cells Consistent with literature findings (Bennett et al., 1989), HuVSMC showed a flat and poor response toward NTG stimulation from 10^{-6} to 10^{-3} M. The expression of ALDH2 and the α-subunit of soluble guanylyl cyclase were significantly lower in HuVSMC vs. LLC-PK1 cells (Tsou and Fung, unpublished data). Thus, cultured HuVSMC cells were not suitable for the *in vitro* examination of nitrate metabolism and action.

We confirmed that *in vitro* NTG tolerance in LLC-PK1 cells was accompanied by increased $O_2^{\bullet-}$ accumulation (Table 1). Co-incubation with tiron, a $O_2^{\bullet-}$scavenger, expectedly quenched the $O_2^{\bullet-}$ response, but did not result in restoring cGMP responsiveness in the cells preincubated with NTG (Table 1). $O_2^{\bullet-}$ accumulation increased in LLC-PK1 cells exposed to Ang II, which was quenched by tiron (Table 1), but cGMP response toward NTG challenge was unchanged. In the presence of tiron, cells exposed to Ang II showed a diminished cGMP response toward NTG, similar to those observed in control cells and NTG-pretreated cells. Thus, tiron appeared to exert a general attenuating effect on the cGMP-producing effect of NTG. The use of tiron as a $O_2^{\bullet-}$ quencher to study nitrate tolerance is therefore complicated by this non-specific effect on cGMP production.

Our finding that Ang II can stimulate $O_2^{\bullet-}$ production in LLC-PK1 cells agrees with a previous report (Hannken et al., 1998), who suggested that the underlying mechanism involves the activation of angiotensin receptor type-1 (AT_1) and up-regulation of membrane NOX. The fluorescent probe DHE used here is reasonably specific to $O_2^{\bullet-}$ and does not react with O_2 and H_2O_2 (Benov et al., 1998).

While several studies showed that angiotensin-converting enzymes inhibitors (ACEIs) or

Ang II- receptor antagonists can reverse or prevent of nitrate tolerance (Cotter et al., 1998), this beneficial effect was not always observed (Dakak et al., 1990; Longobardi et al., 2004). In larger clinical trials, such as GISSI-3 and ISIS 4, there was no significant evidence that the effect of nitrate therapy was different in the presence or absence of ACEIs. Consistent with these latter findings, the present results indicate that Ang II-stimulated $O_2^{\bullet-}$ accumulation was not critically involved in producing cellular nitrate tolerance, at least in LLC-PK1 cells.

We employed NOX knockout mice to examine the critical participation of NOX in $O_2^{\bullet-}$ production and tolerance induced by NTG. The strains used here were $p47^{phox}$ and $gp91^{phox}$ knockout mice. Both $gp91^{phox}$ and $p47^{phox}$ are crucial for NOX to be functionally active. Our vascular relaxation results of the $gp91^{phox}$ and $p47^{phox}$ knockout mice showed similar right-ward shifts in NTG dose-response curves, with 3-6 fold changes in EC_{50} values after *in vivo* NTG treatment (Fig. 1, 2, and Table 2). NTG- stimulated cGMP levels in the aorta was measured as a second indicator for nitrate tolerance (Table 2). In both strains the cGMP levels were attenuated to a comparable extent after *in vivo* NTG treatment (about 55% decrease), consistent with the results obtained in the vascular relaxation experiments.

Ang II infusion caused a 2- to 3- fold increase in vascular $O_2^{\bullet -}$ production in $p47^{phox}$ (+/+), but not in $p47^{phox}$ (–/–) mice (Landmesser et al., 2002), indicating a "pivotal" role for $p47^{phox}$ in vascular oxidative and blood pressure response to Ang II *in vivo*. $p47^{phox}$ has only one homolog (NOXO1) which is mainly observed in the colon but not in vascular tissues (Quinn et al., 2006). The role of $gp91^{phox}$ in vascular relaxation is less definitive, since $gp91^{phox}$ knockout did not alter $O_2^{\bullet -}$ generation and vascular reactivity when compared to WT mice (Souza et al., 2001). In addition, in $gp91^{phox}$ knockout mice, other vascularly expressed homologs of $gp91^{phox}$, such as NOX1 or NOX4 (Bedard and Krause, 2007), may remain active. Our results also showed that $O_2^{\bullet -}$ production from aorta of $p47^{phox}$ knockout mice was significantly reduced when compared to WT. Significant amounts of $O_2^{\bullet -}$ were produced in both WT and knockout animals when treated with NTG compared to their corresponding vehicle control (Fig 3). While no difference in eNOS and XO protein expression in $p47^{phox}$ (–/–) mice vs. WT mice was observed in one study (Li et al., 2004), another study found that endothelial cells lacking the $p47^{phox}$ subunit had minimal XO protein and activity (McNally et al., 2003). Our finding that NTG induced less $O_2^{\bullet -}$ formation in $p47^{phox}$ knockout vs. WT mice (Fig. 3) is therefore more consistent with the latter observation.

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The ability of oxypurinol to reduce NTG-mediated $O_2^{\bullet-}$ production was seen in both LLC-PK1 cells (Table 1) and WT mice (Table 3), but it did not alter the extent of *in vitro* aorta tolerance (Fig. 4). Oxypurinol was chosen over its prodrug allopurinol which produces $O_2^{\bullet-}$ in the biotransformation process (Galbusera et al., 2006). Our result differed from that of Munzel et al. (Munzel et al., 1995) who found no effect of 1 mM oxypurinol on vascular $O_2^{\bullet-}$ production in NTG tolerant vessels isolated from rabbits which have been treated with NTG-patch (0.4 mg $hour^{-1}$) for 3 days. These dissimilar observations may have arisen from species and methodological differences.

The lack of crucial involvement of Ang II and NOX-mediated $O_2^{\bullet-}$ in this study supports our view that oxidative stress may be a by-product of nitrate tolerance rather than its cause. We hypothesize that protein S-oxidation, rather than the Ang II/NOX/$O_2^{\bullet-}$ pathway, may be the initiating step for NTG metabolism and tolerance (Fung, 2004). We suggest further that the increased $O_2^{\bullet-}$ observed in nitrate tolerance can result from protein S-oxidation, as indicated by our results in experiments involving XO. This enzyme, together with xanthine dehydrogenase (XDH), belongs to the xanthine oxidoreductase system (XOR). XDH is the predominant form of XOR and upon activation, it is converted to XO via reversible S-oxidation or irreversible partial proteolysis (Borges et al., 2002). Both XDH

and XO are capable of producing $O_2^{\bullet-}$. Being flavoproteins, XOR have been shown to reduce nitrates into inorganic nitrites under anaerobic conditions (Doel et al., 2001). The specific biochemical interactions between NTG and XDH/XO are currently under examination in our laboratory.

In conclusion, we showed that in two separate experimental systems, $O_2^{\bullet-}$ is not critically responsible for developing nitrate tolerance. Fig. 5A illustrates the involvement of Ang II and NOX in the classical $O_2^{\bullet-}$ -oxidative stress hypothesis of nitrate tolerance, in which chronic NTG exposure led to increased Ang II/NOX activity,and $O_2^{\bullet-}$ production, which initiates nitrate tolerance. Our present findings argue against this hypothesis, and suggest an alternative mechanism (Fig. 5B) in which the initiating step involves NTG-induced S-oxidation in multiple cysteine-sensitive proteins (P-SH). A component of these affected proteins is the XOR system, which induces the conversion of XDH to XO, leading to increased $O_2^{\bullet-}$ production. While it is likely that the accumulated $O_2^{\bullet-}$ would participate in some of the downstream events of nitrate tolerance, our results indicate that neither $O_2^{\bullet-}$ generation nor the involvement of the AngII/NOX pathway are critical for the initiation of this phenomenon, at least in the two experimental systems that we studied.

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Footnotes

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Legends for Figures

Fig. 1. *Ex vivo* concentration vs. relaxation curves of isolated mouse aorta toward NTG in gp91^{phox} (+/+) (A) and gp91^{phox} (–/–) mice (B). Closed symbols represent aorta response of vehicle treated controls, while open symbols represent those of NTG treated mice (3 days at 20 mg kg^{-1} tid s.c.). The dashed lines showed a rightward shift of the NTG-relaxation curves vs control, consistent with vascular tolerance development. Data are expressed as mean ± SD, n = 6-7 animals.

Fig. 2. *Ex vivo* concentration vs. relaxation curves of isolated mouse aorta toward NTG in p47^{phox} (+/+) (A) and p47^{phox} (–/–) mice (B). Closed symbols represent aorta response of vehicle treated controls, while open symbols represent those of NTG treated mice (3 days at 20 mg kg^{-1} tid s.c.). The dashed lines showed a rightward shift of the NTG-relaxation curves vs control, consistent with vascular tolerance development. Data are expressed as mean ± SD, n = 6-7 animals.

Fig. 3. Effects of *ex vivo* NTG treatment on superoxide production in mouse aorta upon 10 μM NTG challenge in p47^{phox} (+/+) and p47^{phox} (–/–) mice. Closed bars represent

response of vehicle-treated controls, while open bars represent those of NTG-treated aorta. NTG significantly increased superoxide production in both $p47^{phox}$ (+/+) and $p47^{phox}$ (–/–) mice (**p<0.01 in $p47^{phox}$ (+/+), *p<0.05 in $p47^{phox}$ (–/–) mice, Student's t-test). NTG stimulated superoxide production in $p47^{phox}$ (+/+) mice was significantly more than those in $p47^{phox}$ (–/–) mice (**p<0.01, Student's t-test). Data are expressed as mean ± SD, n = 4-7 animals.

Fig. 4. *Ex vivo* concentration vs. relaxation curves of isolated mouse aorta toward NTG in $p47^{phox}$ (+/+) mice. (●) represents aorta treated with vehicle; (○) represents aorta treated with NTG; (▼) represents aorta pretreated with oxypurinol followed by vehicle; (△) represents aorta pretreated with oxypurinol followed by NTG. The dashed lines showed a rightward shift of the NTG-relaxation curves vs their corresponding control, consistent with vascular tolerance development. Data are expressed as mean ± SD, n = 6-7 animals.

Fig. 5. Mechanisms for nitrate tolerance and oxidative stress. (A) Representation of the $O_2^{\bullet -}$ -oxidative stress hypothesis: NTG increases the action of Ang II through AT_1 receptor and NOX activation. The $O_2^{\bullet -}$ produced through this process initiates nitrate tolerance. (B) Representation of mechanism suggested by this study: the Ang II/NOX pathway is not

critically involved in nitrate tolerance. Instead through S-oxidation of various cysteine-containing proteins (PSH), NTG releases its metabolites (GDN: dinitrates, NOx: NO-related species). Tolerance is induced through this process and production of $O_2^{\bullet -}$ is mediated by S-oxidization of XOR.

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Table 1. Effects of NTG and Ang II incubation on NTG-stimulated cGMP accumulation and superoxide production in LLC-PK1 Cells.

Incubation with	Other agents added in incubation	NTG-stimulated cGMP accumulation (pmol mg^{-1} protein)	Superoxide (fluorescence units)
Vehicle	none	151 ± 56	45.6 ± 6.5
	10 mM tiron	109 ± 13	45.4 ± 13.3
	10 mM vitamin C	221 ± 54^{**c}	30.3 ± 2.3
	100 µM oxypurinol	116 ± 13	42.1 ± 5.0
NTG	none	27.5 ± 9.2^{**cd}	63.2 ± 14.0^{**a}
	10 mM tiron	9.3 ± 3.1^{**c}	42.3 ± 1.9^{*b}
	10 mM vitamin C	67.9 ± 14.0^{**ad}	23.8 ± 0.1^{**b}
	100 µM oxypurinol	24.0 ± 6.5^{*ad}	42.8 ± 2.6^{*b}
Ang II	none	159 ± 55^{*e}	138 ± 18^{**a}
	10 mM tiron	74.2 ± 34^{*a}	62.1 ± 2.6^{**b}

*p<0.05; **p<0.01; N.D = not determined;

^{a}vs. control; ^{b}vs. corresponding treatment (NTG, Ang II); ^{c}vs. control + tiron; ^{d}vs. control + vitamin C; ^{e}vs. Ang II + tiron;

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Table 2. Aorta vascular relaxation (pEC_{50}) and cGMP responses after *in vivo* vehicle or NTG treatment (20 mg kg^{-1} sc, tid for 3 days). Data expressed as mean ± SD, n = 6-7.

	pEC_{50} (-Log M)		cGMP (pmol mg^{-1} protein)	
	vehicle	NTG	vehicle	NTG
$p47^{phox}$(+/+)	7.34 ± 0.14	6.80 ± 0.39*	136 ± 58	75.7 ± 36.7*
$p47^{phox}$(–/–)	7.57 ± 0.28	6.80 ± 0.63*	207 ± 60	137 ± 48*
$gp91^{phox}$(+/+)	7.42 ± 0.20	7.00 ± 0.31*	117 ± 51	66.5 ± 24.3*
$gp91^{phox}$ (–/–)	7.73 ± 0.29	6.92 ± 0.39*	196 ± 89	103 ± 39*

*$p<0.05$ NTG treatment vs. control

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Table 3. *In vitro* vascular relaxation (pEC_{50}) and superoxide production in $p47^{phox}$(+/+) mice aorta after *in vitro* NTG (10 μM for 1 hr) and oxypurinol (1 mM for 15 min) treatments. Data expressed as mean ± SD, n = 6-7.

	Vehicle	NTG	Oxypurinol	Oxypurinol + NTG
pEC_{50} (-Log M)	7.32 ± 0.14	6.59 ± 0.24^{**a}	7.31 ± 0.03	6.40 ± 0.16^{**b}
Superoxide × 10^{-3} (cpm mg^{-1} dry weight)	0.26 ± 0.07	3.12 ± 0.07	0.87 ± 0.43^{**a}	0.39 ± 0.13^{*c}
Superoxide× 10^{-3} (cpm mg^{-1} dry weight) in the presence of tiron	0.16 ± 0.05	0.38 ± 0.19^{**d}	0.15 ± 0.05	0.08 ± 0.02

*p<0.05; **p<0.01

^{a}vs. vehicle; ^{b}vs. vehicle and oxypurinol only; ^{c}vs. oxypurinol + NTG;

^{d}vs. NTG-superoxide production without tiron

Fig.1A

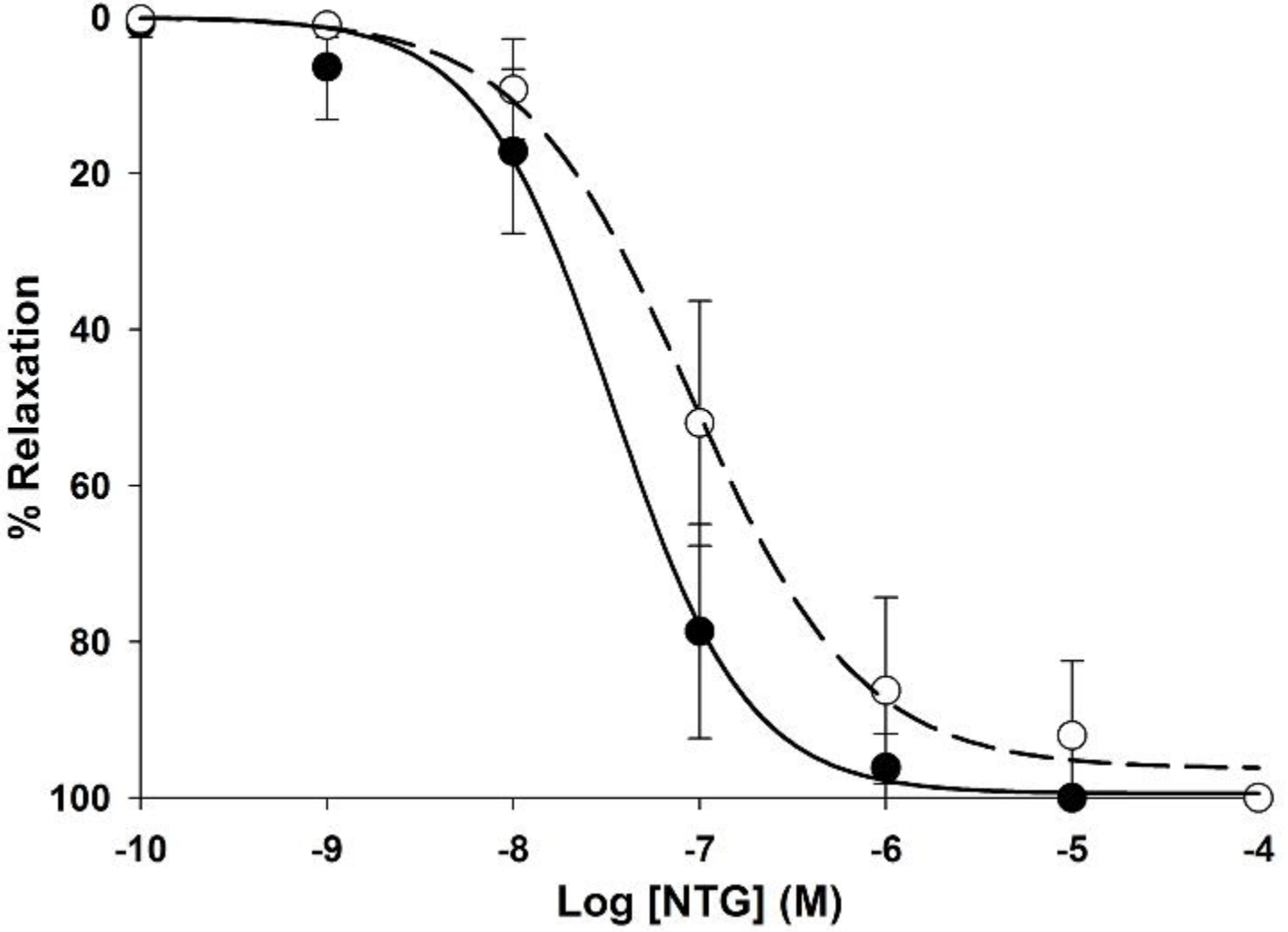


Fig.1B

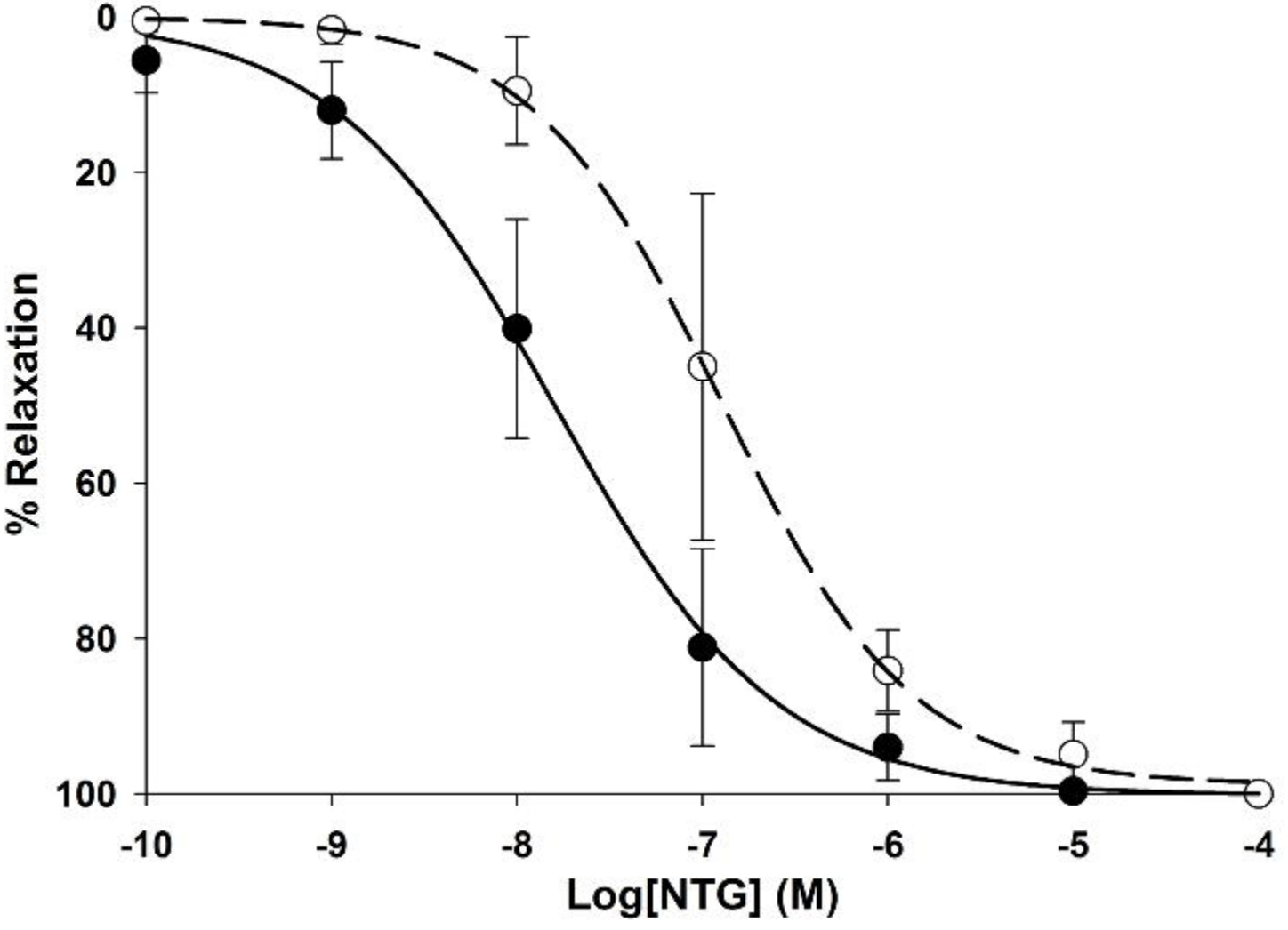


Fig.2A

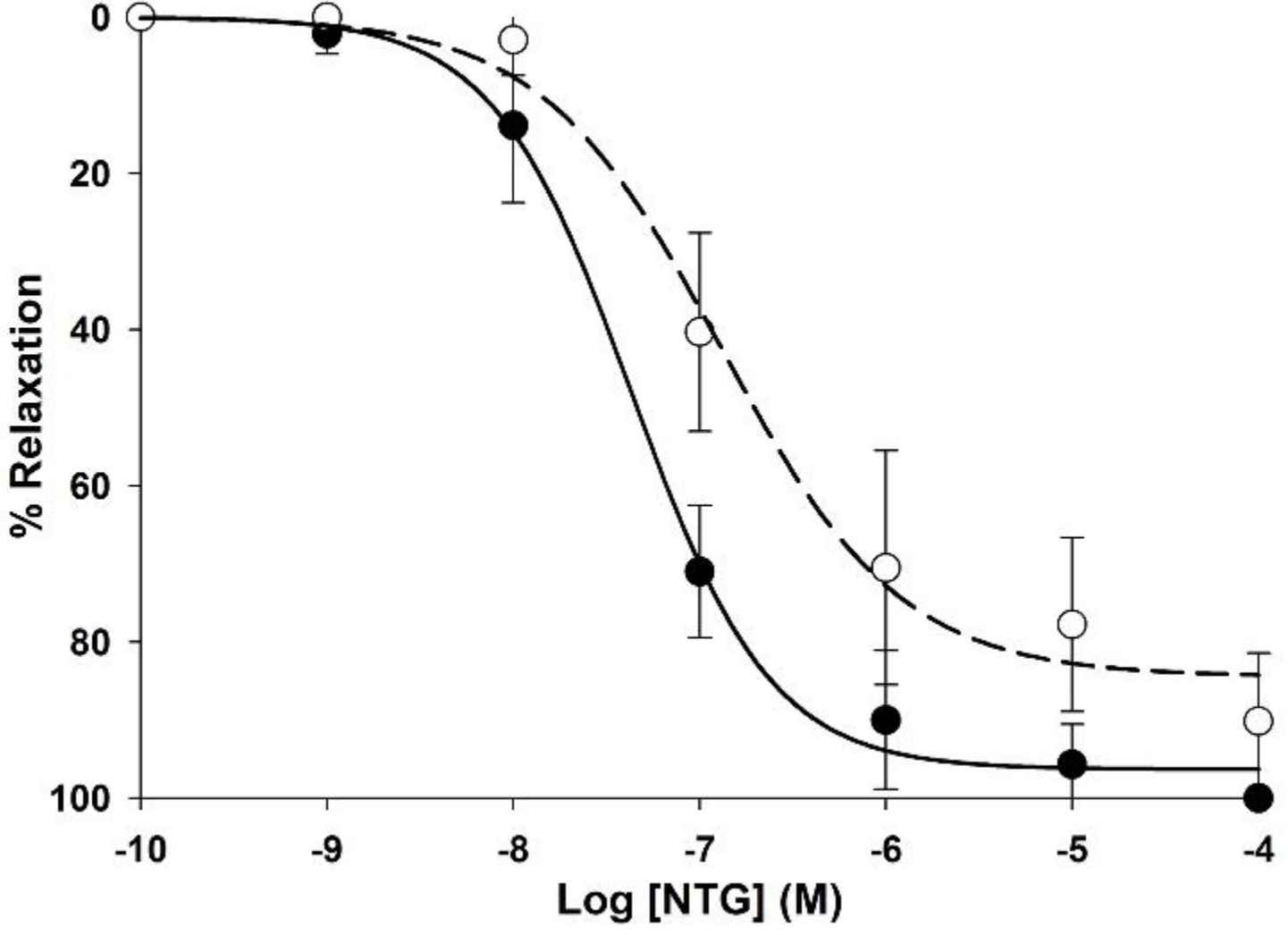


Fig.2B

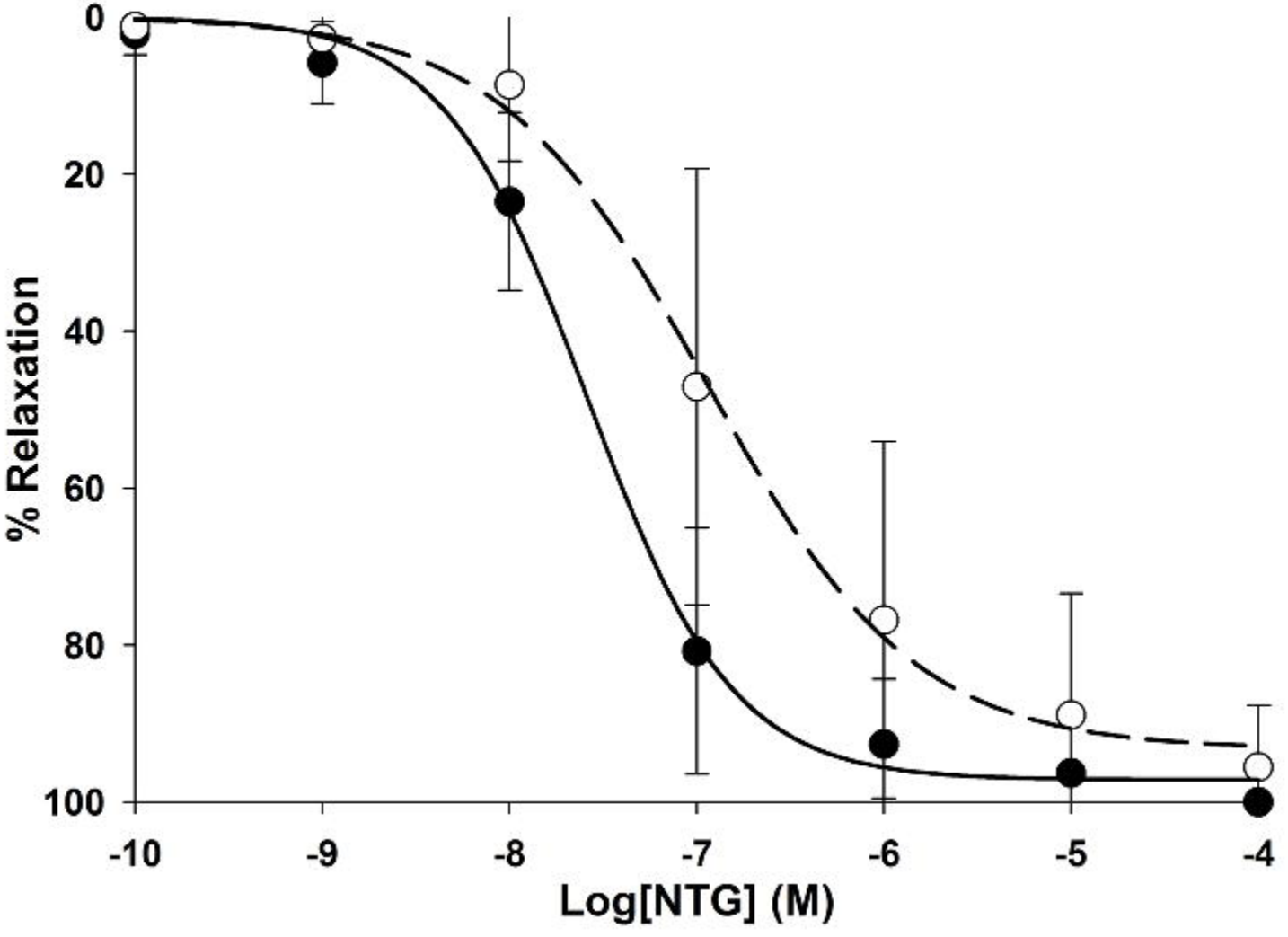


Fig.3

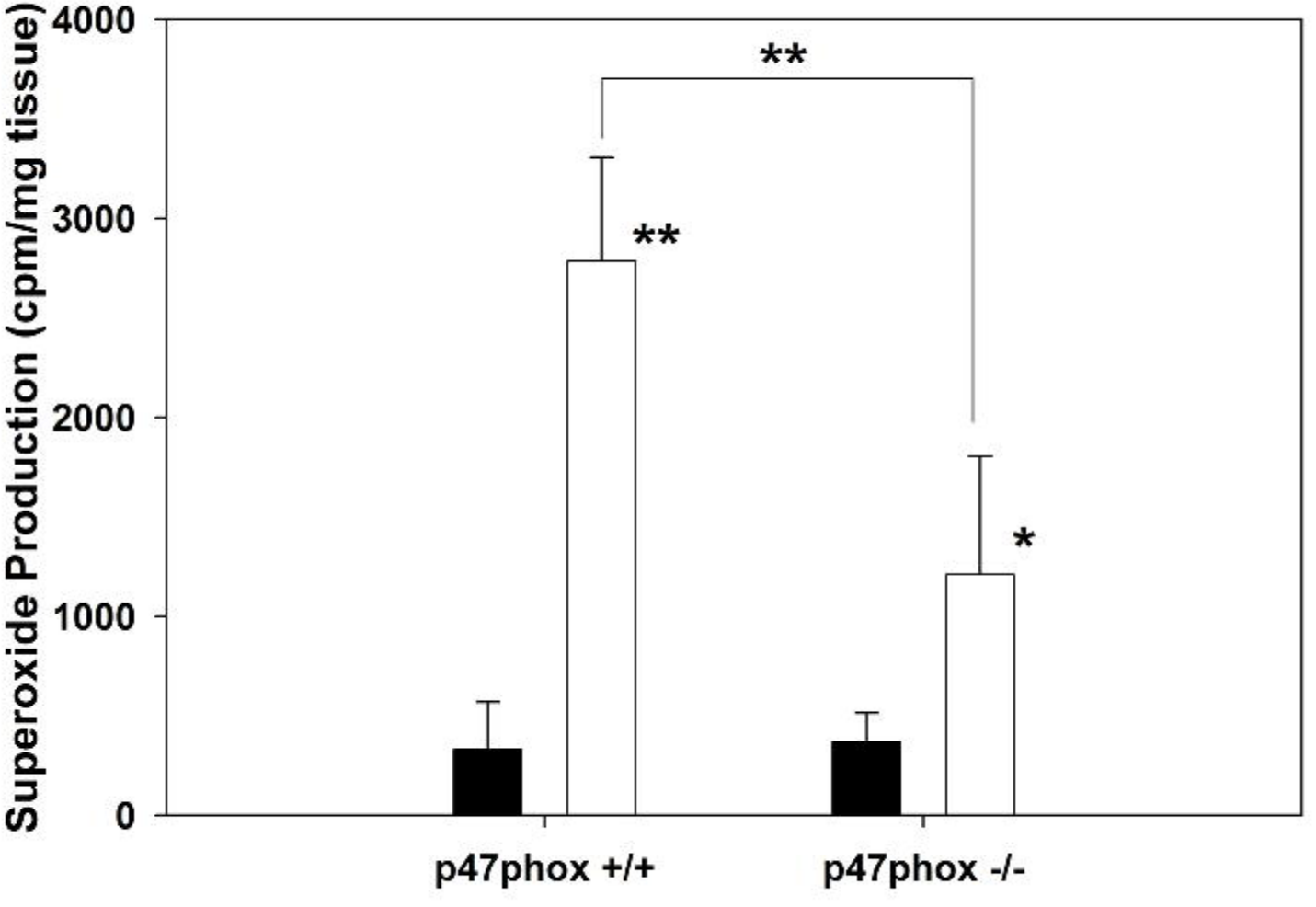
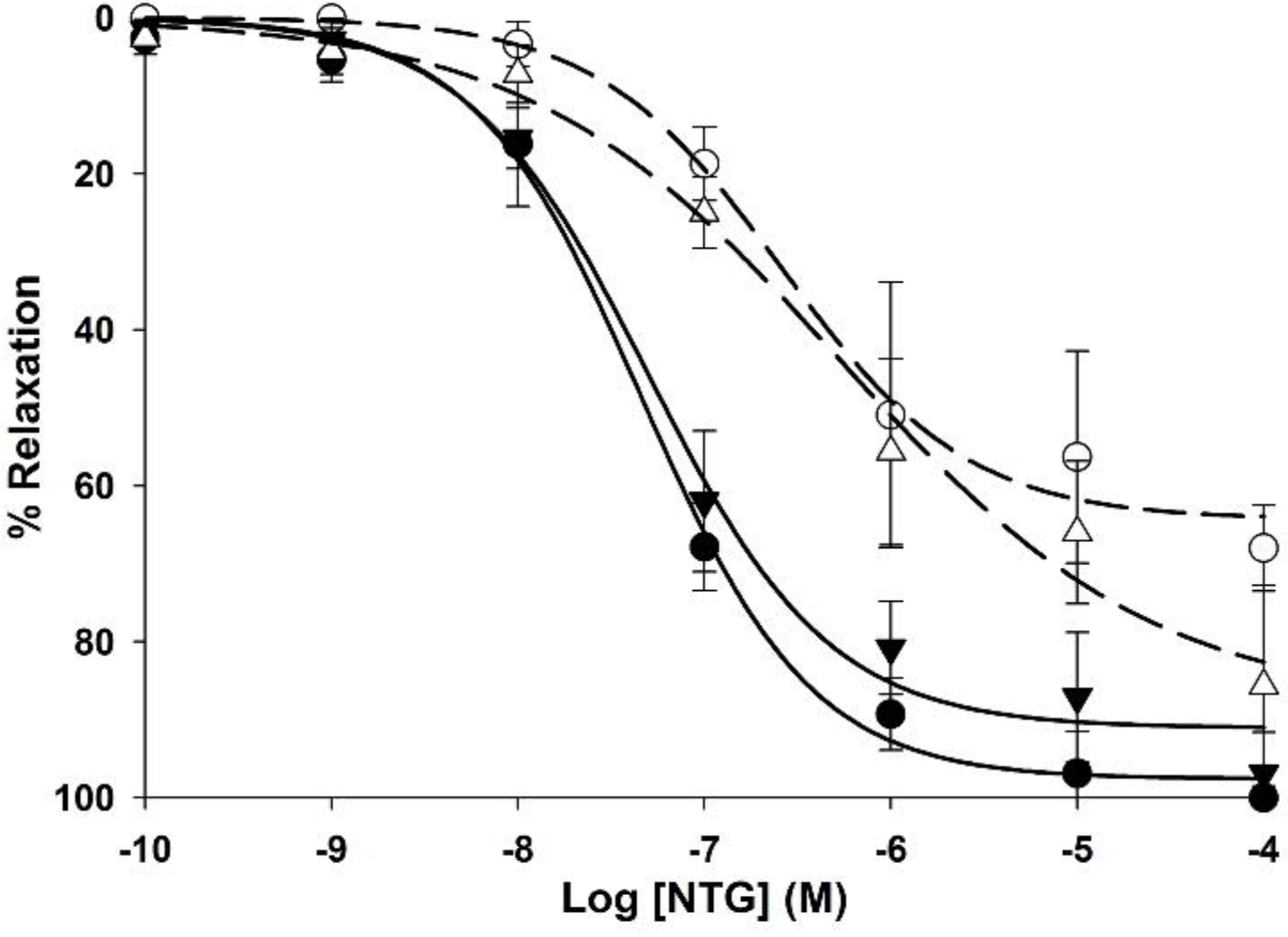
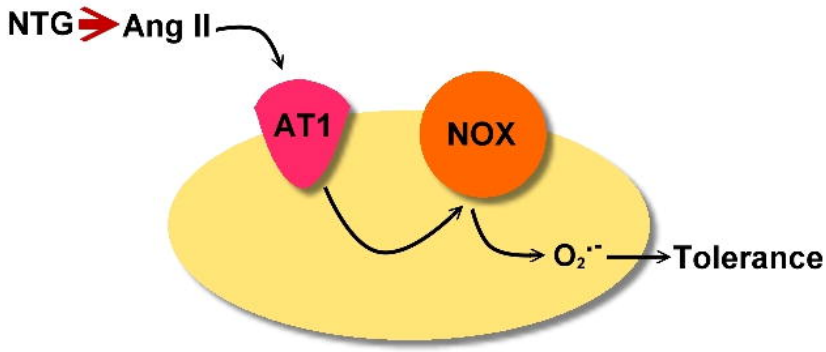

Superoxide Production (cpm/mg tissue)
4000
3000
2000
1000
0
**
**
*
p47phox +/+
p47phox -/-

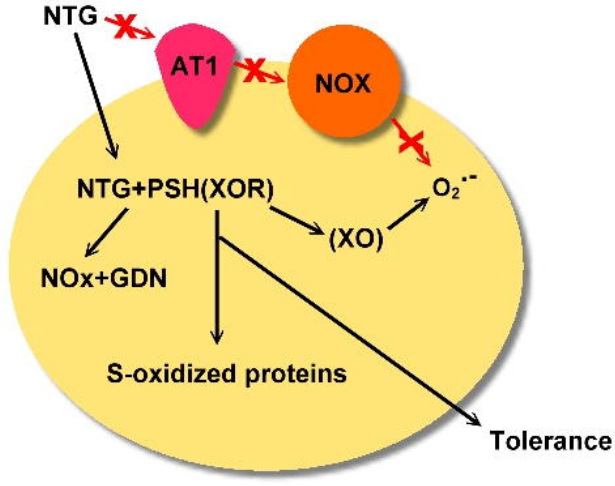
Fig.4



A


NTG
Ang II
AT1
NOX
$O_2^{\cdot-}$
Tolerance

B


NTG
AT1
NOX
NTG+PSH(XOR)
$O_2^{\cdot-}$
(XO)
NOx+GDN
S-oxidized proteins
Tolerance