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First Evidence for a Crosstalk Between Mitochondrial and NADPH Oxidase-Derived Reactive Oxygen Species in Nitroglycerin-Triggered Vascular Dysfunction

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ABSTRACT

Chronic nitroglycerin treatment results in development of nitrate tolerance associated with endothelial dysfunction (ED). We sought to clarify how mitochondria- and NADPH oxidase (Nox)-derived reactive oxygen species (ROS) contribute to nitrate tolerance and nitroglycerin-induced ED. Nitrate tolerance was induced by nitroglycerin infusion in male Wistar rats (100 μg/h/4 day) and in C57/Bl6, p47$^{phox-/-}$ and gp91$^{phox-/-}$ mice (50 μg/h/4 day). Protein and mRNA expression of Nox subunits were unaltered by chronic nitroglycerin treatment. Oxidative stress was determined in vascular rings and mitochondrial fractions of nitroglycerin-treated animals by L-012 enhanced chemiluminescence, revealing a dominant role of mitochondria for nitrate tolerance development. Isometric tension studies revealed that genetic deletion or inhibition (apocynin, 0.35 mg/h/4 day) of Nox improved ED, whereas nitrate tolerance was unaltered. Vice versa, nitrate tolerance was attenuated by co-treatment with the respiratory chain complex I inhibitor rotenone (100 μg/h/4 day) or the mitochondrial permeability transition pore blocker cyclosporine A (50 μg/h/4 day). Both compounds improved ED, suggesting a link between mitochondrial and Nox-derived ROS. Mitochondrial respiratory chain-derived ROS are critical for the development of nitrate tolerance, whereas Nox-derived ROS mediate nitrate tolerance-associated ED. This suggests a crosstalk between mitochondrial and Nox-derived ROS with distinct mechanistic effects and sites for pharmacological intervention. *Antioxid. Redox Signal.* 10, 1435–1447.

INTRODUCTION

The anti-ischemic effects of organic nitrates are largely due to venous and coronary artery dilation, as well as improvement of collateral blood flow, which decrease myocardial oxygen consumption and are mediated by nitric oxide or a related species (2). However, the usefulness of organic nitrates is limited by tolerance and endothelial dysfunction (cross tolerance), which develops shortly upon continuous treatment. The mechanisms underlying tolerance and cross tolerance remain incompletely understood and are most likely multifactorial (33). An early adaptation to long-term nitrate therapy involves neurohumoral adjustments, including activation of the renin-angiotensin-aldosterone system (40, 41), retention of sodium and water, and volume redistribution (15). These mechanisms impair the vasodilator and preload-reducing effects of the organic nitrates. Previously, we found that 3 day treatment of experimental animals with nitroglycerin (GTN) doubles vascular su-

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peroxide ($O_2^{\cdot -}$) production (38), which was also confirmed in human bypass material of GTN-treated patients (43). Subsequent studies of particulate fractions of vascular homogenates stimulated with NADH suggested that the source of $O_2^{\cdot -}$ in these vessels is a membrane-bound NADH/NADPH oxidase (Nox) (24, 36, 44).

Upon identification of the mitochondrial aldehyde dehydrogenase (ALDH-2) as a nitrate bioactivating enzyme (7), a follow-up study in ALDH-2-deficient mice (ALDH-$2^{-/-}$) stressed the importance of this enzyme for bioactivation and accordingly for the vasodilator potency of GTN and other organic nitrates (6, 49). Our laboratory further substantiated this concept in an animal model of *in vivo* tolerance and extended previous observations by demonstrating that mitochondria are a major source of ROS formation in response to both acute and chronic GTN challenges (10, 45). The importance of the ALDH-2 concept for clinical nitrate tolerance was further substantiated by two independent clinical studies in Asian subjects with a point mutated, dysfunctional ALDH-2 (27, 30). Since ALDH-2 is oxidatively inhibited by GTN administration (45, 48), these data provide a link between the oxidative stress and the impaired GTN biotransformation concept (33).

Further evidence for an important role of mitochondrial superoxide production in causing tolerance was provided with heterozygous deficiency in mitochondrial superoxide dismutase (Mn-$SOD^{+/-}$, which show increased basal levels of mitochondrial oxidative stress), where tolerance as well as cross tolerance were clearly enhanced when the activity and the expression of this free radical scavenging enzyme was decreased by heterozygous knockout (12, 32). Further, a recent study of Esplugues *et al*. demonstrated a role of mitochondria in the development of nitrate tolerance, since this phenomenon was associated with dysfunction of mitochondrial complex I and tolerance was normalized by administration of mitochondria-targeted antioxidants such as glutathione-ester and mito-quinone (16). A role of mitochondria for the development of nitrate tolerance was also proposed in a study by Gori et al., based on interference of cyclosporine A (CsA) with nitroglycerin-mediated preconditioning (18).

With this background, we hypothesized that ROS derived from the mitochondrial respiratory chain and from the NADPH oxidase might have differential effects on the development of nitroglycerin-triggered vascular dysfunction depending on their source of generation, and that they might interfere with each other in a crosstalk fashion.

MATERIALS AND METHODS

Reagents

For isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). For induction of *in vivo* tolerance, GTN was used from a solution in ethanol (102 g/l) which was obtained from UNIKEM (Copenhagen, Denmark). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H, 3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany).

Animals and in vivo *treatment*

Male Wistar rats (250 g), (Charles River, Sulzfeld, Germany) were equipped with micro-osmotic pumps 1003D from Alzet (Cupertino, CA) containing 450 mM GTN (100 μg/h, 6.6 μg/kg/min in ethanol or the solvent as a control). GTN-infusion was performed as previously described (45) with or without the NADPH oxidase inhibitor apocynin (33.6 mg/kg/day, s.c.). Another co-treatment was GTN along with the inhibitor of K_{ATP}-channels glibenclamide (1 mg/kg/day), the inhibitor of mitochondrial complex I rotenone (10 mg/kg/day) or the blocker of mPTP opening CsA (5 mg/kg/day) as well as ethanol along with the opener of K_{ATP}-channels diazoxide (5 mg/kg/day) for 4 days. GTN and inhibitors were administrated in separate osmotic pumps. All animal treatment was in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health and was granted by the Ethics Committee of the University Hospital Eppendorf and the University Hospital Mainz. We also used male mice aged 7–10 months on a C57/Bl6 background. Experiments were performed with 12–18 wt, $p47^{phox-/-}$ or $gp91^{phox-/-}$ mice. The $gp91^{phox}$ knockout mice were obtained from Jackson laboratories (Bar Harbor, Maine), whereas the $p47^{phox}$ knockout mice which are now available at Jackson laboratories, too, were provided by Steven M. Holland (NIH, Bethesda, MD). The animals were bred at the animal facility of the faculty of medicine at Frankfurt University. *In vivo* tolerance was induced by chronic infusion of mice with GTN in ethanol (50 μg/h, 120 nmol/min/kg for 4 days) by implanted micro-osmotic pumps (Alzet, model 1007D, 0.5 μl/h for 7 days) from Durect Corp. (Cupertino, CA). Infusion of the solvent ethanol served as a control. After this period, the animals were sacrificed, and heart and aorta were subjected to further analysis as described (12).

Isometric tension studies

Vasodilator responses to ACh and GTN were assessed with endothelium-intact isolated murine and rat aortic rings mounted for isometric tension recordings in organ chambers, as described previously (10, 38). The mouse aorta was preconstricted with prostaglandin $F_{2\alpha}$, whereas the rat aorta was preconstricted with phenylephrine.

Detection of oxidative stress in mitochondria and isolated aorta

Isolated mitochondria were prepared from rat hearts according to a previously published protocol, and ROS/RNS formation was detected by L-012 (100 μM)-enhanced chemiluminescence as described (10, 12, 42). L-012 has a higher specificity for peroxynitrite than for superoxide (9), which may provide the basis for the high sensitivity of L-012 for GTN-triggered oxidative stress (11). Briefly, hearts from Wistar rats were glass/glass homogenized in HEPES buffer and subjected to centrifugation steps at 4°C of 1500 *g* for 10 min and 2,000 *g* for 5 min (the pellets were discarded). The resulting supernatant was centrifuged at 20,000 *g* for 20 min, and the pellet was resuspended in 1 ml of Tris buffer. The last centrifugation step was repeated and the pellet was finally resuspended in 1 ml of Tris buffer. The protein content was determined by the Lowry

method. Mitochondrial suspensions were diluted to a final protein concentration of 0.1 mg/ml in 0.5 ml of PBS buffer containing L-012 (100 μM). ROS/RNS production was determined after stimulation with succinate (5 m*M* final concentration), malate/glutamate (2.5 m*M* each), malate/oxaloacetate which decays to pyruvate (2.5 m*M* each), or α-ketoglutarate (2.5 m*M*). In some experiments, we used modulators of mitochondrial function diazoxide (100 μM), glibenclamide (10 μM), rotenone (5 μM), and CsA (200 n*M*). The chemiluminescence (CL) was registered at intervals of 30 s over 5 min with a chemiluminometer (Berthold Techn., Bad Wildbad, Germany) and the signal was expressed as counts/min at 5 min. Vascular superoxide production was determined using the highly selective chemiluminescence indicator reagent Diogenes™, a luminol-peroxidase based assay (National Diagnostics, Atlanta, GA; 50% of total reaction volume) (8, 25). Briefly, aortas were isolated in chilled buffer, cut into 3 m*M* segments and incubated for 30 min at 37°C in 96-well plates (segments from one aorta were measured in triplicates) in Hanks' buffered salt solution (PAA Laboratories, Pasching, Austria). Afterwards a mixture of Diogenes™ containing dimethylsulfoxide (0.1%, vehicle for PMA) and PMA (final concentration 100 nM; Calbiochem, San Diego, CA) was added to the plate. Chemiluminescence was measured using a Mithras Microplate Luminometer (Berthold). 10-s readings were obtained for each ring over 40 min. The photon counts were normalized for the dry weight of aortic tissue and expressed as percentage change of control.

Calibration of oxidative stress (chemiluminescence) in mitochondria by HPLC-based measurement of 2-hydroxyethidium

Superoxide was also measured by a modified HPLC-based method to quantify ethidium and 2-hydroxyethidium levels, as previously described (51). Briefly, heart mitochondria were incubated with 50 μM DHE for 20 min at 37°C in PBS buffer and stored at −80°C. Upon thawing, DHE oxidation products were extracted by addition of 50% acetonitrile, centrifuged, and 50 μl of the supernatant were subjected to HPLC analysis. The system consisted of a control unit, two pumps, mixer, detectors, column oven, degasser and an autosampler (AS-2057 plus) from Jasco (Groβ-Umstadt, Germany) and a C_{18}-Nucleosil 100-3 (125 × 4) column from Macherey & Nagel (Düren, Germany). A high pressure gradient was employed with acetonitrile and 25 m*M* citrate buffer pH 2.2 as mobile phases with the following percentages of the organic solvent: 0 min, 36%; 7 min, 40%; 8–12 min, 95%; 13 min, 36%. The flow was 1 ml/min and DHE was detected by its absorption at 355 nm, whereas 2-hydroxyethidium and ethidium were detected by fluorescence (Ex. 480 nm/Em. 580 nm). The signal was normalized on protein content of the mitochondrial preparations. The 2-hydroxyethidium data was calibrated by different xanthine oxidase concentrations for which the superoxide formation rate was determined by the cytochrome c assay (for details, see Fig. 1).

GTN-induced ROS in Nox1 or Nox4 transfected cells

HEK293 cells were seeded into 12-well dishes and transfected with 0.3 μg mouse Nox1, Noxa1, and Noxo1 (the Nox1 analogs of $p47^{phox}$ and $p67^{phox}$), Nox4, or GFP (as a control)

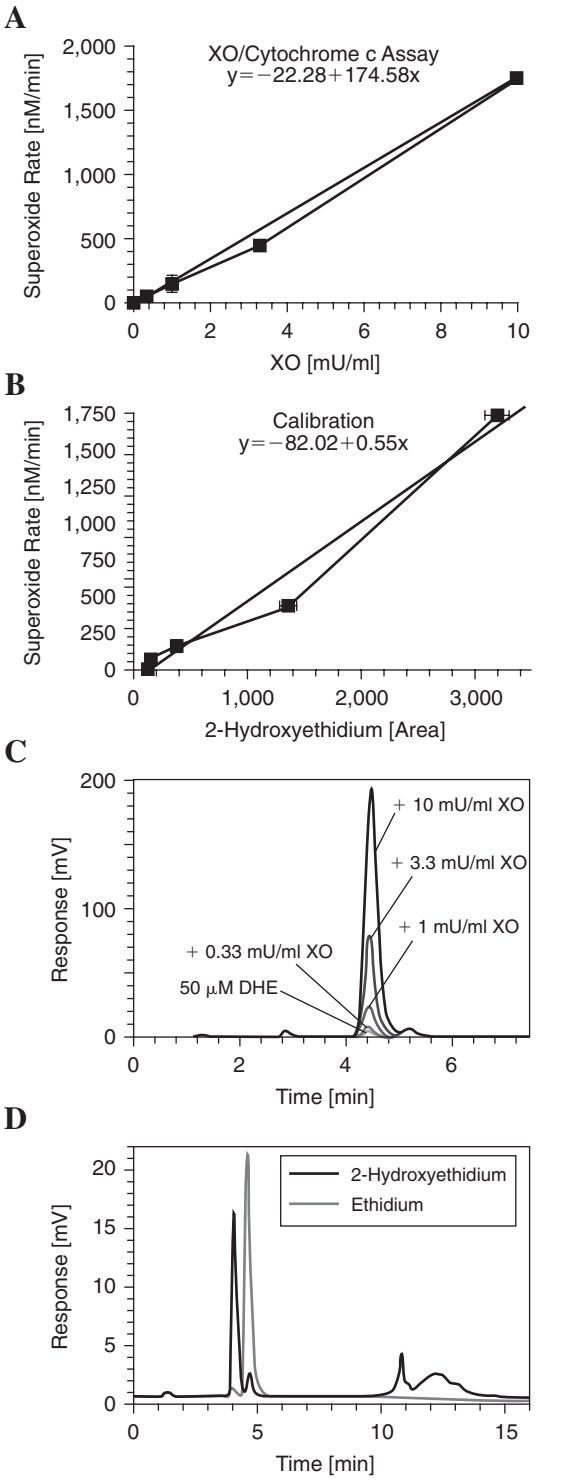

FIG. 1. Calibration of superoxide detection via 2-hydroxyethidium measurement by the cytochrome c assay. (**A**) Quantification of superoxide formation rates from xanthine oxidase (0, 0.33, 1, 3.3, and 10 mU/ml) and hypoxanthine (1 m*M*) in PBS with cytochrome c (100 μM). The concentration range 0.01–10 mU/ml XO was used for linear regression. Rate [dA/min] is transformed to rate [nM $O_2^{\cdot -}$/min] using E = ε_{550}*c*d using ε_{550} = 19.5 $mM^{-1}cm^{-1}$ (23). (**B**) Superoxide formation rates were correlated with 2-hydroxyethidium (2-HE) formation using identical XO concentrations. (**C, D**) Representative chromatograms are shown for each XO concentration and for 2-HE and ethidium standards (each 500 n*M*).

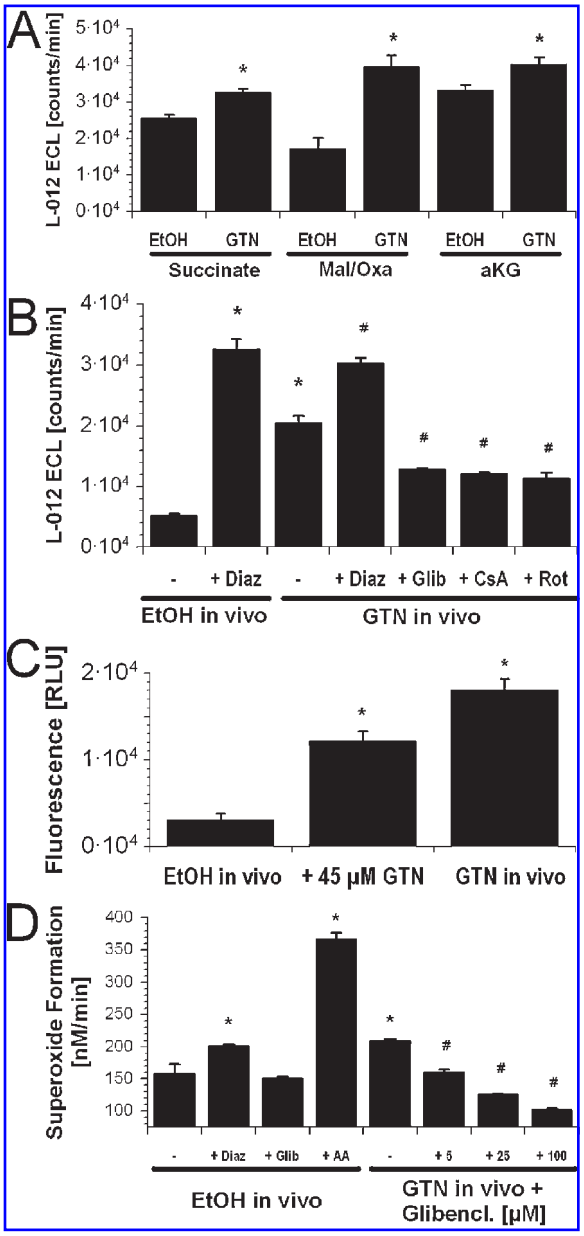

FIG. 2. Effects of GTN *in vivo* treatment on ROS and RNS formation in isolated mitochondria. (**A**) Oxidative stress in cardiac mitochondria was measured by L-012 (100 μM) enhanced chemiluminescence in the presence of malate/oxalacetate which decays to pyruvate (Mal/Oxa, each 2.5 mM), α-ketoglutarate (aKG, 2.5 mM), or succinate (5 mM). Data are mean ± SEM of 15–24 independent experiments. $^{*}p < 0.05$ vs. EtOH *in vivo* treatment. (**B**) Malate/glutamate (each 2.5 mM) dependent ROS and RNS formation in isolated mitochondria was measured by L-012 (100 μM) enhanced chemiluminescence in the presence of diazoxide (Diaz), glibenclamide (Glib), cyclosporin A (CsA), or rotenone (Rot). Data are mean ± SEM of 4 independent experiments; $^{*}p < 0.05$ vs. solvent control, $^{\#}p < 0.05$ vs. GTN in vivo. (**C**) Oxidative stress in cardiac mitochondria (0.1 mg/ml final protein) measured by amplex red (50 μM) and horseradish peroxidase (0.1 μM) fluorescence in the presence of malate/glutamate (2.5 mM). The signal was background corrected with respect to signal without mitochondria. Data are mean ± SEM of 4 independent experiments; $^{*}p < 0.05$ vs. EtOH in vivo. (**D**) Superoxide formation rate was measured by the DHE oxidation product 2-hydroxyethidium using an HPLC method. Superoxide yield was determined in response to succinate (5 mM), diazoxide (Diaz, 25 μM), glibenclamide (Glib, 10 μM), or antimycin A (AA, 20 μg/ml) in mitochondria from control (EtOH) rats as well as to glibenclamide (0–100 μM) in mitochondria from tolerant (GTN) rats. Data are mean ± SEM of 4–5 independent experiments; $^{*}p < 0.05$ vs. solvent control, $^{\#}p < 0.05$ *vs.* GTN *in vivo*.

using lipofectamine. ROS were determined by luminol (100 μM) and horseradish peroxidase (1 U/ml)-driven chemiluminescence to detect extracellular hydrogen peroxide or L-012 (100 μM)-enhanced chemiluminescence to detect intracellular peroxynitrite and superoxide anions in trypsinized cells in 500 μl HEPES-modified Tyrode's solution using a chemiluminescence reader (LB9505 Berthold) as reported previously (3). ROS formation was determined in the presence or absence of GTN (500 μM) and normalized to the signal obtained in the presence of solvent.

Western blot analysis

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking, immunoblotting was performed with antibodies against a-aktinin (the loading control), Nox1 (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA) and Nox2 (gp91^{phox}, 1:1000, Transduction Laboratories, Heidelberg, Germany). Detection was performed by ECL with peroxidase conjugated anti-rabbit/mouse secondary antibodies (1:10,000, Vector Lab., Burlingame, CA). The antibody-specific bands were quantified by densitometry.

RT-PCR

mRNA expression was analyzed by quantitative real-time RT-PCR using an iCyclerTM iQ system (Bio-Rad Laboratories, Munich, Germany). Briefly, total RNA from rat aorta and heart was isolated according to the manufacturer's protocol of the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). 0.5 μg of total RNA was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for GAPDH were purchased as probe and primer sets. Primers for Nox2 (gp91^{phox}), Nox4, and p22^{phox} were obtained from MWG-Biotech (Ebersberg, Germany). Sequences of the primers and TaqMan® probes were (forward, reverse, and probe) CTTCTTGGGTCAGCACTGGC, GCAGCAAGATCAGCATGCAG, and CACCTGCAGCCTGCCTGAATTTCA for Nox2; CTGTCCTGAACCTCAACT-GCAG, TGTGATCCGCGAAGGTAAGC, and CTTTTACCCATGTGCCGCACAGTCCT for Nox4; TACCTGACCGCTGTGGTGAAG, GCAGTAAGTGGAGGACAGCCC, and TGTTCGGGCCCCTCACCAGAAATTAC for p22^{phox}. The comparative Ct

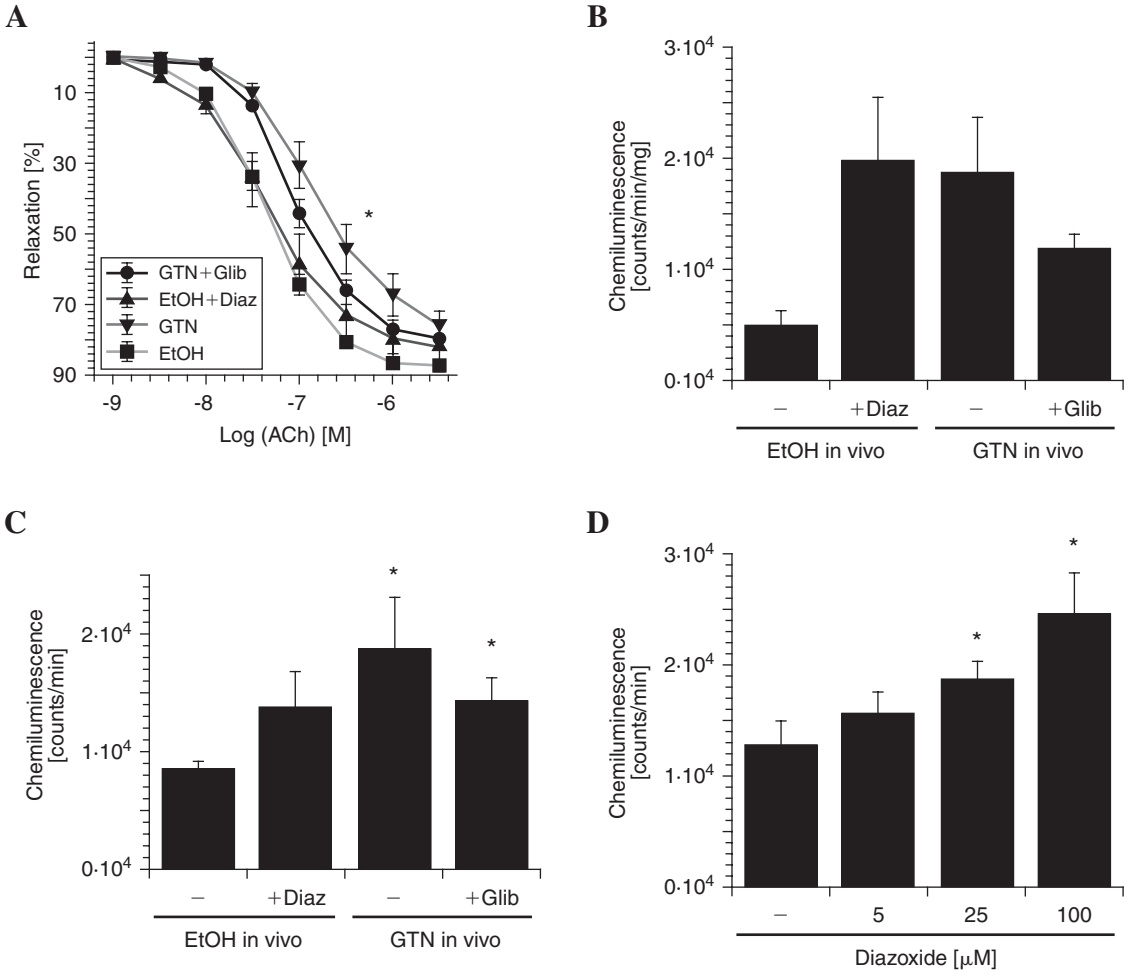

FIG. 3. Formation of reactive oxygen species (ROS) and vasodilator responses of isolated aortic vessel segments upon chronic treatment of Wistar rats with solvent (EtOH), EtOH/diazoxide, GTN, or GTN/glibenclamide. (**A**) Effects of *in vivo* co-treatment with diazoxide or glibenclamide on endothelial function of intact aorta. Concentration-relaxation curves for acetylcholine (ACh) were obtained by isometric tension recordings of aortic segments. Data are mean ± SEM of 8 independent experiments with aortae from three rats/group. (**B** and **C**) ROS formation in isolated aortic rings (**B**) or M/G stimulated heart mitochondria (**C**) was measured by L-012 (100 μM) enhanced chemiluminescence. Data are mean ± SEM of 4 (**B**) or 8 (**C**) independent experiments with tissue from three rats/group. (**D**) Effect of diazoxide (0–100 μM) on mitochondrial ROS formation was determined in succinate stimulated heart mitochondria by L-012 ECL. Data are mean ± SEM of 8 independent experiments, $*p < 0.05$ vs. solvent control.

method was used for relative mRNA quantification (28). Gene expression was normalized to the endogenous control, GAPDH mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

Statistical analysis

Results are expressed as mean ± SEM. One-way ANOVA (with Bonferroni's or Dunn's correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy, L-012-, lucigenin- and Diogenes-derived chemiluminescence, as well as protein or mRNA expression, cellular ROS/RNS detection, and mitochondrial respiration. The EC_{50} value for each experiment was obtained by log-transformation. P values < 0.05 were considered significant. * indicates significance *vs*. solvent control.

RESULTS

ROS/RNS formation in isolated mitochondria and pharmacologic inhibition of complex I of the mitochondrial respiratory chain and the mitochondrial permeability transition pore (mPTP)

We tested different substrates of mitochondrial respiration and observed that in addition to succinate (complex II), malate/oxaloacetate (complex I), and α-ketoglutarate (a substrate of the lipoyl-dehydrogenase subunit of α-ketoglutarate dehydrogenase generating NADH which feeds into complex I) were all able to significantly increase the L-012-derived chemiluminescence signal in isolated mitochondria from GTN-treated

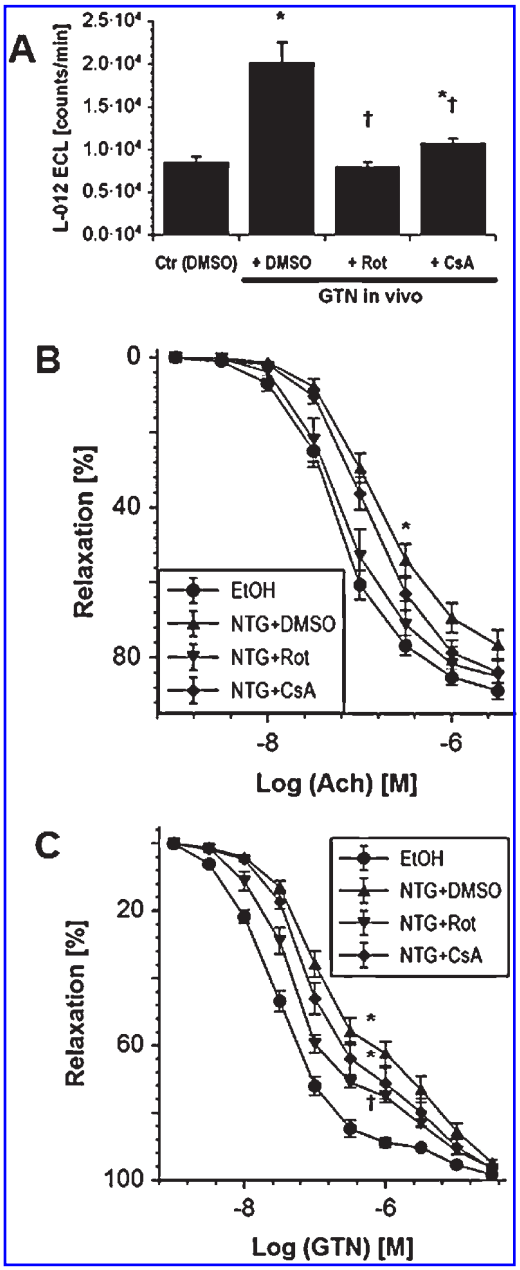

FIG. 4. Formation of reactive oxygen and nitrogen species (ROS/RNS) and vasodilator responses of isolated aortic vessel segments upon chronic treatment of Wistar rats with GTN, GTN/rotenone, or GTN/cyclosporine A. (**A**) ROS/RNS formation in isolated mitochondria was measured by L-012-enhanced chemiluminescence. Data are mean ± SEM of 16 independent experiments; $^{*}p < 0.05$ vs. solvent control, $^{\dagger}p < 0.05$ vs. GTN-DMSO. (**B** and **C**) Effects of *in vivo* co-treatment with rotenone or CsA on tolerance and cross-tolerance of intact aorta. Concentration-relaxation curves for acetylcholine (ACh) (**B**) and nitroglycerin (GTN) (**C**) were obtained by isometric tension recordings of aortic segments. Data are mean ± SEM of 12–16 (**B**) and 15–16 (**C**) independent experiments. For statistical analysis see Table I (**B** and **C**); $^{*}p < 0.05$ vs. solvent control and $^{\dagger}p < 0.05$ *vs.* GTN-DMSO (**A**).

rats (Fig. 2A). However, one should note that oxaloacetate decays to pyruvate and that freshly prepared stocks yielded significantly lower signals than older stocks (not shown). Malate/glutamate (complex I)-dependent ROS/RNS formation was significantly increased in the GTN *in vivo* treated group (Fig. 2B). This increase could be mimicked by *in vitro* incubation of control mitochondria with the K_{ATP}-channel opener diazoxide. The K_{ATP}-channel inhibitor glibenclamide attenuated the GTN-induced increase (Fig. 2B). These observations were further supported by investigation of the concentration-dependency of the diazoxide effect starting at 5 μM (Fig. 3D) and *in vivo* administration of diazoxide in control animals and glibenclamide in GTN-treated rats (Fig. 3A–C). *In vivo* diazoxide treatment slightly impaired endothelial function (Ach reponse) and increased vascular and mitochondrial ROS/RNS formation in control (EtOH) rats, whereas *in vivo* glibenclamide therapy improved endothelial dysfunction and oxidative stress in tolerant (GTN) animals. Also rotenone and CsA blocked the GTN-induced increase (Fig. 2B). We also detected increased GTN-induced mitochondrial ROS/RNS formation using amplex red/horseradish peroxidase detection (Fig. 2C). In addition, superoxide was specifically measured by 2-hydroxyethidium detection (Fig. 2D) and formation rates were determined by calibration with xanthine oxidase and cytochrome c (Fig. 1). Diazoxide caused a small but significant increase in superoxide formation in mitochondria from control (EtOH) rats, glibenclamide had no effect, antimycin A yielded almost 2-fold more superoxide and GTN *in vivo* treatment significantly increased superoxide formation rate, which was dose-dependently inhibited by glibenclamide. *In vivo* co-treatment with the complex I inhibitor rotenone or the mPTP blocker CsA significantly improved GTN-induced mitochondrial oxidative stress (Fig. 4A). Moreover, these compounds improved GTN-triggered ED and nitrate tolerance (Fig. 4B and C). Rotenone completely normalized the impaired acetylcholine response upon GTN-treatment, whereas CsA only slightly improved endothelial function. For EC_{50} values and statistics, see Table 1. In conclusion, we demonstrate here that GTN treatment triggers mitochondrial ROS/RNS formation, which was blocked by CsA, rotenone, and glibenclamide, and mimicked by diazoxide. Most importantly, CsA and rotenone not only affected the tolerance but also endothelial dysfunction of aorta, suggesting a causal role of mtROS/RNS for both phenomena.

ROS/RNS formation in isolated aortic segments and pharmacologic inhibition of Nox proteins

It has been previously shown that GTN in vivo treatment increases vascular ROS/RNS formation in intact aortic rings. We here demonstrate that the vascular ROS/RNS formation in response to activation of protein kinase C with phorbol myristate (PMA) is substantially increased in aortas from GTN-treated rats as compared to the solvent controls (Fig. 5A). This observation may indicate that Nox, which is activated upon PKC stimulation, may contribute to increased oxidative stress in the setting of tolerance. To gain further insight in the importance of a potential Nox -dependent mechanism, GTN-treated rats were co-infused with the Nox inhibitor apocynin.

TABLE 1. VASODILATOR POTENCY OF ACH AND GTN IN VESSELS FROM WILD TYPE, p47$^{phox-/-}$ OR gp91$^{phox-/-}$ MICE, AS WELL AS WISTAR RATS ± APOCYNIN, ROTENONE, OR CYCLOSPORINE A UPON CHRONIC TREATMENT WITH GTN

	Potency, EC_{50} (-log M)		*Efficacy, max. relaxation (%)*	
In vivo *treatment*	*ACh*	*GTN*	*ACh*	*GTN*
WT + EtOH	6.9 ± 0.1 ($n = 7$)	6.6 ± 0.1 ($n = 7$)	72 ± 5 ($n = 7$)	84 ± 3 ($n = 7$)
WT + GTN	6.9 ± 0.1 ($n = 7$)	5.9 ± 0.1 ($n = 7$)*	52 ± 4 ($n = 7$)*	78 ± 2 ($n = 7$)
p47$^{phox-/-}$ + EtOH	7.0 ± 0.1 ($n = 11$)	6.9 ± 0.1 ($n = 12$)	72 ± 5 ($n = 11$)	80 ± 4 ($n = 12$)
p47$^{phox-/-}$ + GTN	7.0 ± 0.1 ($n = 11$)	5.9 ± 0.1 ($n = 9$)*	67 ± 4 ($n = 11$)	79 ± 3 ($n = 9$)
gp91$^{phox-/-}$ + EtOH	7.0 ± 0.1 ($n = 9$)	6.2 ± 0.1 ($n = 9$)	63 ± 4 ($n = 9$)	70 ± 2 ($n = 9$)
gp91$^{phox-/-}$ + GTN	7.1 ± 0.1 ($n = 10$)	5.7 ± 0.1 ($n = 10$)*	60 ± 2 ($n = 10$)	63 ± 3 ($n = 10$)*
Control (EtOH)	7.2 ± 0.1 ($n = 14$)	7.4 ± 0.1 ($n = 16$)	88 ± 2 ($n = 14$)	98 ± 1 ($n = 16$)
GTN + DMSO	6.8 ± 0.2 ($n = 16$)	6.6 ± 0.2 ($n = 16$)*	77 ± 4 ($n = 16$)*	95 ± 1 ($n = 16$)
GTN + rotenone	7.2 ± 0.2 ($n = 15$)	7.1 ± 0.1 ($n = 15$)†	85 ± 4 ($n = 15$)	96 ± 1 ($n = 15$)
GTN + CsA	6.9 ± 0.1 ($n = 12$)	6.9 ± 0.2 ($n = 16$)*	84 ± 3 ($n = 12$)	96 ± 1 ($n = 16$)
Control + apocynin	7.5 ± 0.1 ($n = 8$)	7.7 ± 0.1 ($n = 7$)	89 ± 3 ($n = 8$)	100 ± 0 ($n = 7$)
GTN	7.0 ± 0.1 ($n = 11$)‡	7.2 ± 0.1 ($n = 14$)‡	74 ± 3 ($n = 11$)‡	98 ± 1 ($n = 14$)
GTN + apocynin	7.2 ± 0.1 ($n = 10$)	7.0 ± 0.1 ($n = 14$)‡	83 ± 3 ($n = 10$)§	97 ± 2 ($n = 14$)

*$p < 0.05$ *vs.* solvent (EtOH) group; $^{\dagger}p < 0.05$ *vs.* GTN-DMSO, and $^{\ddagger}p < 0.05$ *vs.* Ctr/apocynin, and $^{\S}p < 0.05$ *vs.* GTN.

GTN *in vivo* treatment resulted in a significant increase in vascular reactive oxygen and nitrogen species [most probably the RNS peroxynitrite (1, 21, 47), see Fig. 7 as well as results on Nox-transfected cells] formation, which was completely normalized by apocynin co-infusion (Fig. 5B). Apocynin co-infusion partially corrected endothelial dysfunction (cross-tolerance), whereas importantly tolerance was completely unaffected by the Nox inhibitor (Fig. 5C and D, Table 1). In conclusion, we here demonstrate that GTN treatment increases vascular oxidative stress which was normalized by apocynin. Most importantly, apocynin only affected the endothelial dysfunction of aorta but failed to improve the tolerance, suggesting that NADPH oxidases only play a causal role for endothelial function.

Effect of genetic deficiency in Nox subunits p47^{phox} or gp91^{phox} on vasodilator responses in response to organic nitrate treatment

To define the role of Nox for the development of nitrate tolerance, we used mice with genetic deficiency in the cytosolic subunit p47^{phox} or the membrane-located subunit gp91^{phox}. In aortas from C57/Bl6 control mice, GTN *in vivo* treatment induced a marked degree of cross-tolerance (impaired responsiveness to the endothelium-dependent vasodilator ACh) as well as tolerance (impaired responsiveness to GTN) (Fig. 6A). Interestingly, cross-tolerance was absent in p47^{phox-} and gp91^{phox-} deficient mice but the vasodilator potency (EC_{50}) of GTN (both acutely and after tolerance induction) was unaffected, pointing to a specific role of Nox in the development of GTN-induced endothelial dysfunction (Fig. 6B and C). In conclusion, we here demonstrate that genetic deletion of essential NADPH oxidase subunits eliminates GTN-triggered endothelial dysfunction, whereas tolerance is not affected.

ROS formation in Nox1/Nox2-transfected cells

The effect of acute GTN administration on cultured cells was determined with L-012 as a measure of intracellular ROS/RNS (most probably peroxynitrite) formation or with luminol/horseradish peroxidase as a measure of extracellular hydrogen peroxide. It was previously shown that GTN *in vivo* treatment of animals and cells results in significantly increased 3-nitrotyrosine levels (1, 21, 47) which is a footprint of peroxynitrite formation (a strong nitrating agent) (4). Moreover, the inhibitors used in these studies are commonly employed to scavenge peroxynitrite and free radicals (NO_2-radical and HO-radical) derived from it: uric acid, hydralazine, and ebselen (1, 11, 21). Finally, L-012 has a higher specificity for peroxynitrite than for superoxide (9). GTN enhanced L-012 chemiluminescence in control cells (transfected with GFP) as well as in cells transfected with subunits of Nox1 or Nox4 consisting NADPH oxidase leading to a 5- to 15-fold increase as compared to unstimulated control (Fig. 7A). The overall L-012 signals in Nox1-transfected cells were increased almost 250-fold over GFP-transfected control (compare left and middle panel), whereas transfection with the constitutive Nox4 isoform only resulted in a 2- to 6-fold increase in signal (compare left and right panel). In contrast, GTN treatment caused no increase in hydrogen peroxide levels (Fig. 7B). Also preincubation of the cells for 4 h with GTN (50 μM) caused no increased Nox activity in these cells (not shown).

Effect of in vivo *GTN treatment on protein and mRNA expression of Nox subunits*

The effect of GTN *in vivo* on Nox1 and Nox2 was determined by protein expression. No significant changes could be established (Fig. 8A). Likewise, vascular mRNA expression of

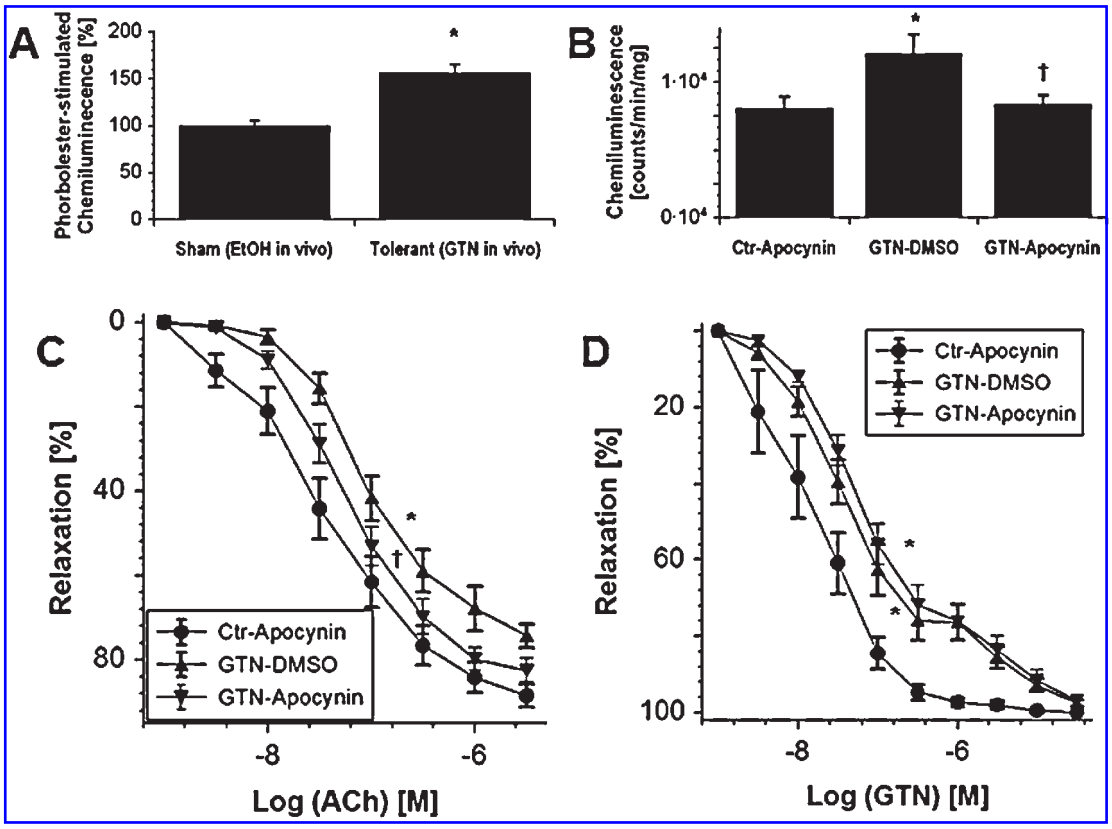

FIG. 5. Effects of GTN *in vivo* treatment on phorbol ester-stimulated vascular oxidative stress and vasodilator responses of isolated aortic vessel segments upon chronic treatment of Wistar rats with GTN or GTN/apocynin. (**A**) ROS formation in isolated aortic segments was measured by Diogenes-enhanced chemiluminescence upon stimulation with PMA (100 n*M*). Data are mean ± SEM of 6 independent experiments and the signal was normalized to the dry weight of the tissue; $*p < 0.05$ vs. solvent control. (**B**) Effects of *in vivo* co-treatment with the NADPH oxidase inhibitor apocynin on ROS/RNS production of intact vascular rings. ROS/RNS production was quantified using L-012 (100 μM) enhanced chemiluminescence. (**C** and **D**) Effects of *in vivo* co-treatment with apocynin on tolerance and cross-tolerance of intact aorta. Concentration-relaxation curves for acetylcholine (ACh) (**C**) and nitroglycerin (GTN) (**D**) were obtained by isometric tension recordings of aortic segments. Data are mean ± SEM of 6–10 (**B**) and 7–14 (**C** and **D**) independent experiments. For statistical analysis see Table I (**C** and **D**) or $*p < 0.05$ vs. Ctr-Apocynin and $^{\dagger}p < 0.05$ *vs*. GTN-DMSO (**B**).

Nox2, Nox4, and the cytosolic subunit $p22^{phox}$ was not affected by the GTN treatment (Fig. 8B). Similar observations were made with cardiac tissue (Fig. 8C).

DISCUSSION

With the present study, we show for the first time that a crosstalk of ROS/RNS exists between mitochondrial respiratory chain and vascular NADPH oxidase in nitroglycerin-triggered vascular dysfunction. We provide evidence that development of tolerance in response to GTN *in vivo* treatment depends primarily on mitochondrial ROS/RNS formation, whereas GTN-induced endothelial dysfunction involves the vascular Nox activity. Importantly, GTN-induced endothelial dysfunction was clearly attenuated by *in vivo* treatment with the NADPH oxidase inhibitor apocynin and was prevented in Nox subunit knockout animals. These results clearly demonstrate that nitrate tolerance and cross tolerance are different entities and are mediated by two different enzymatic superoxide sources.

Mitochondrial ROS/RNS sources rather than the vascular NADPH oxidases mediate nitrate tolerance

Previously, we have demonstrated that stimulation of tissue homogenates from tolerant animals with NADH results in increased ROS formation in the setting of tolerance (NADH was used to demonstrate involvement of the previously so-called NADH/NADPH oxidase) (36). Since the increased superoxide signal of intact vessels of GTN-treated animals was inhibited by diphenyleneiodonium (36), we speculated that vascular Nox may account primarily for increased oxidative stress in the setting of tolerance. It is also important to note that the previously observed increase in NADH oxidase activity was obtained in homogenates using lucigenin in a concentration of 250 μM and

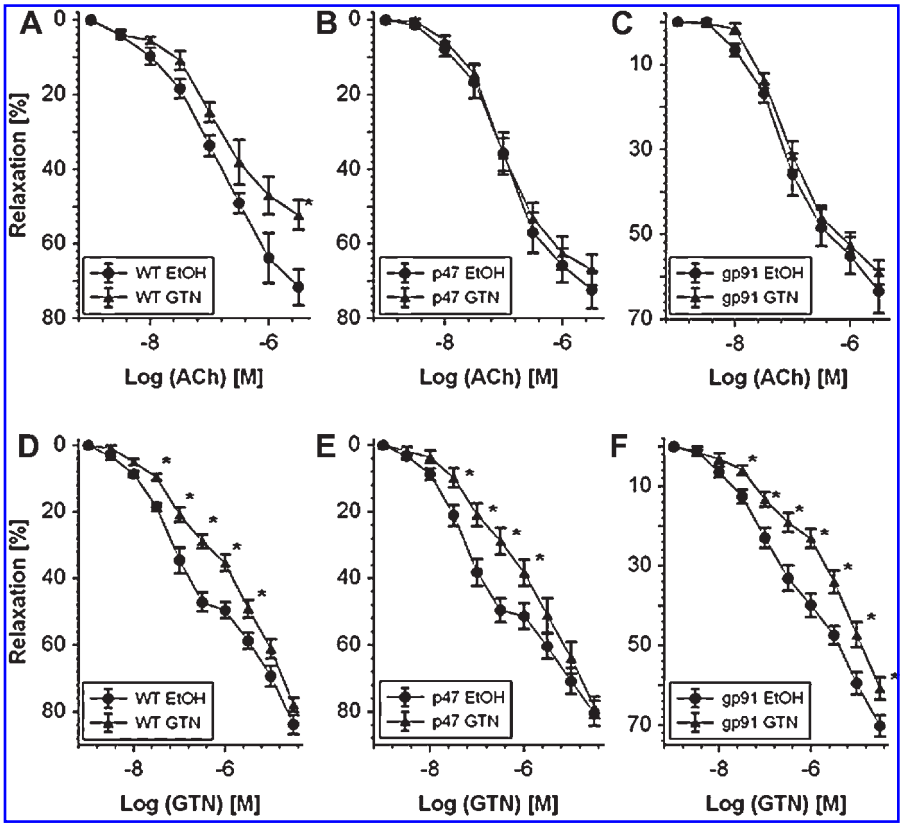

FIG. 6. Effects of NADPH oxidase subunit knockout ($p47^{phox-/-}$ and $gp91^{phox-/-}$ mice) on the development of endothelial dysfunction (A–C) and nitrate tolerance (D–F) in response to GTN *in vivo* treatment. Concentration-relaxation curves for acetylcholine (ACh, **A–C**) and nitroglycerin (GTN, **D–F**) were obtained by isometric tension recordings of aortic segments from wild type (**A, D**), $p47^{phox-/-}$ (**B,E**), and $gp91phox^{-/-}$ (**C, F**) mice. Data are mean ± SEM of 7 (wild type), 9–12 ($p47phox^{-/-}$), and 9–10 ($gp91^{phox-/-}$) independent experiments; $*p < 0.05$ vs. Ctr-EtOH.

therefore in a concentration where redox cycling between lucigenin and NADH has been reported (22, 26).

With the present studies we can demonstrate that mitochondrial ROS/RNS formation in response to acute and chronic GTN administration was increased in the presence of substrates for mitochondrial complexes I and II (Fig. 2A–C), pointing to an important role of this subcellular compartment for development of tolerance. GTN treatment clearly increased mitochondrial ROS/RNS production which was inhibited by rotenone and CsA *in vitro* (Fig. 2B). Moreover, the opener of K_{ATP}-channels diazoxide increased the mitochondrial ROS/RNS signal to a similar extent as compared to the GTN-treated group, and the inhibitor of K_{ATP}-channels glibenclamide decreased the ROS/RNS signal in the GTN-treated group. Since diazoxide at higher concentrations has been demonstrated to interfere with mitochondrial respiration (14) (*e.g.*, via blockade at complex II), we have performed experiments on diazoxide concentration-dependent ROS formation which point to a specific role of K_{ATP}-channel opening for nitrate tolerance and endothelial dysfunction (Fig. 3). Diazoxide *in vivo* treatment mimicked GTN-triggered tolerance, whereas glibenclamide co-therapy improved GTN-induced dysfunction (Fig. 3). Therefore, one may speculate that K_{ATP}-channels in mitochondria are important for development of tolerance. An important role of mitochondria was further supported by beneficial *in vivo* effects of the complex I inhibitor rotenone and the mPTP blocker CsA on GTN-induced oxidative stress and tolerance (Fig. 4).

Further support for an important role of mitochondria in the development of nitrate tolerance was provided by our previous studies in Mn-$SOD^{+/-}$ mice where we could demonstrate that these mice were clearly more susceptible to nitrate tolerance development (12, 32). In addition, recent studies revealed that mitochondria-targeted antioxidants efficiently improve nitrate tolerance (16) and that GTN treatment mimics ischemic preconditioning (a process which obviously involves mitochondria) (18). The possible sources of mitochondrial ROS are summarized in the hypothetical scheme (Fig. 9). Previous studies have reported an increase in mitochondrial respiration in response to organic nitrates and swelling of mitochondria (39).

Vascular NADPH oxidases mediate endothelial dysfunction in response to nitroglycerin treatment

Previously, we have shown that tolerance, cross tolerance and increase in sensitivity to vasoconstrictors was improved by *in vitro* and *in vivo* treatment with inhibitors of protein kinase C (34, 37). With the present studies, we can show that upon incubation of intact aortic rings from control and tolerant aortas with a direct activator of protein kinase C, PMA, the luminol-derived chemiluminescence was clearly enhanced in tolerant aorta as compared to control, which might be compatible with the view that *in vivo* GTN treatment leads to an increase in Nox activity and/or expression (Fig. 5A).

To test whether GTN can activate the vascular Nox in vitro, HEK 293 cells were transfected with the NADPH oxidase subunits Nox1- or Nox4 (Fig, 7). The results clearly show that transfection with Nox subunits does not further enhance GTN-

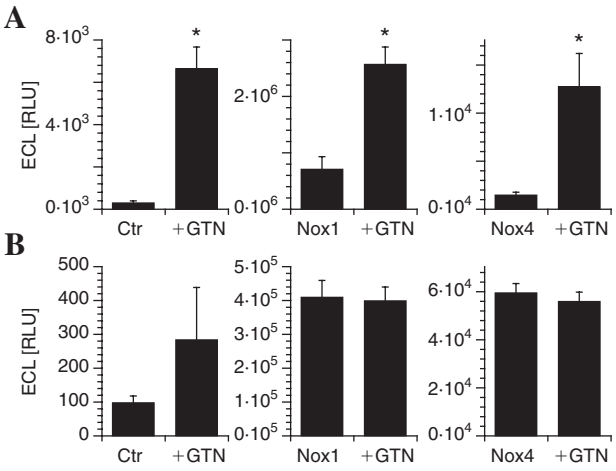

FIG. 7. Effects of GTN *in vitro* treatment on hydrogen peroxide or peroxynitrite formation in cultured cells. Formation of peroxynitrite (L-012) (**A**) or hydrogen peroxide (luminol/HRP) (**B**) in GFP- (control), Nox1- or Nox4-transfected cells in response to acute (500 μM) GTN challenges. Data are mean ± SEM of 6 independent experiments; $*p < 0.05$ *vs*. GTN-treated group.

induced ROS/RNS production. Also preincubation of HEK 293 cells with GTN for 4 h did not result in an activation of Nox (not shown), which suggests that GTN is not able to activate the oxidase *in vitro*. One possible explanation might be that the activation of vascular Nox is linked to a stimulation of the circulating renin–angiotensin–aldosterone-system (RAAS), where increases in circulating angiotensin II and/or aldosterone may increase mRNA or protein expression of vascular NADPH oxidase subunits in aortas from *in vivo* GTN-treated animals or may cause an activation of these oxidases. To address this issue, we quantified mRNA expression of NADPH oxidase subunit in aortas from *in vivo* GTN-treated animals. In accordance with previous results at the mRNA level (46), the mRNA and protein expression of all tested subunits/homologues Nox2 ($gp91^{phox}$), Nox4, and $p22^{phox}$ was not altered or even decreased in response to GTN treatment (Fig. 8), arguing against an involvement of the oxidase in nitrate tolerance development. Further evidence for a lack of involvement of the NADPH oxidase in contributing to tolerance development was provided by the fact that the Nox inhibitor apocynin failed to modify tolerance and that knockout of NADPH oxidase subunits such as $p47^{phox}$- and $gp91^{phox}$ did not result in any modification of the degree of nitrate tolerance.

On the contrary, the NADPH oxidase inhibitor apocynin completely suppressed the L-012 chemiluminescence signal (Fig. 5) in vessels from *in vivo* GTN-treated animals. Likewise, *in vivo* treatment with apocynin had a significant effect on endothelial dysfunction induced by the organic nitrate. To further analyze the potential role of the NADPH oxidase in being involved in GTN-induced endothelial dysfunction, we treated $p47^{phox}$- and $gp91^{phox}$-deficient mice with GTN. Importantly, these mice did not exhibit any cross tolerance to endothelium-dependent vasodilators at all, pointing towards a crucial role of the vascular NADPH oxidase in mediating GTN-induced endothelial dysfunction (Fig. 6). Thus, it became apparent that the two major side effects of GTN such as tolerance and GTN-induced endothelial dysfunction are mediated by two different superoxide producing enzymatic systems. Of these, the one located in mitochondria appears to be important for tolerance development; the other is the vascular NADPH oxidase, which is important for mediating the "side effect" endothelial dysfunction.

Despite the more recent demonstration that the high affinity pathway of GTN metabolism is mediated by the ALDH-2, there is no doubt that GTN-induced ROS/RNS production is one of the key events causing the phenomenon of nitrate tolerance (45). The ROS concept of tolerance was further substantiated by our recent findings that increasing the activity of the free radical scavenging enzyme heme oxygenase-1 prevents tolerance as well as cross tolerance in response to GTN (50).

Although we understand to some extent the mechanisms underlying increased mitochondrial superoxide production in response to GTN treatment, the precise pathway of activation of

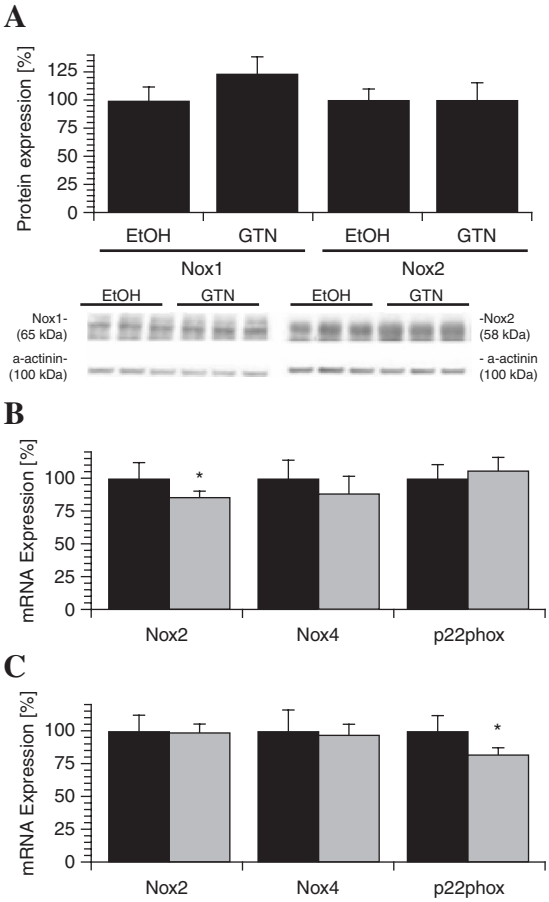

FIG. 8. Effects of *in vivo* treatment with GTN on the expression of Nox subunits. (**A**) Nox1 and Nox2 protein expression was measured in response to ethanol or nitroglycerin (GTN) *in vivo* treatment. Data are mean ± SEM of 8–11 independent experiments and original blots are representative for three of them. Nox2, Nox4, and $p22^{phox}$ mRNA expression in aortic (**B**) or heart tissue (**C**) was measured in response to ethanol (*black*) or GTN (*gray*) *in vivo* treatment. Data are mean ± SEM of 10–12 independent experiments; $*p < 0.05$ vs. ethanol *in vivo*.

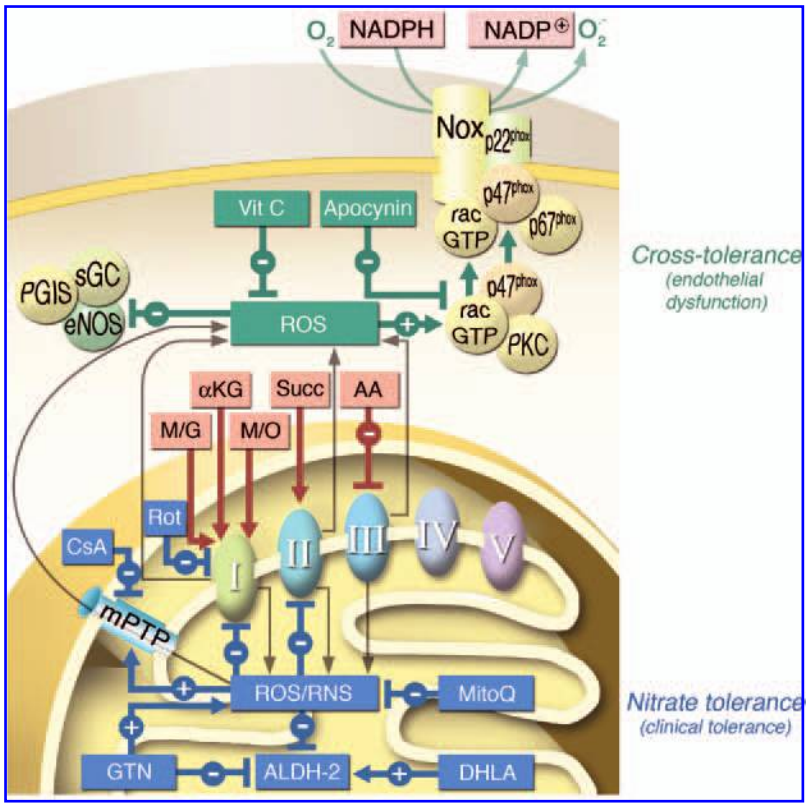

FIG. 9. Hypothetical mechanisms of a crosstalk between mitochondrial and Nox-derived ROS in vascular dysfunction. Nitroglycerin (GTN) is bioactivated by the mitochondrial aldehyde dehydrogenase (ALDH-2) that is directly inhibited by GTN or indirectly inactivated by GTN-triggered mitochondrial ROS formation. Since the stimulatory effect of GTN on ROS formation is independent of ALDH-2 function (GTN induces ROS in the presence of sensitive ALDH-2 inhibitors), it may be assumed that ROS originate from direct interaction of GTN with mitochondrial proteins, probably components of the respiratory chain. Most likely, GTN causes uncoupling of complex I (16) or interacts with complex II and III. Increased ROS formation in response to GTN *in vivo* treatment was observed in the presence of the mitochondrial substrates malate/glutamate (M/G), α-ketoglutarate (αKG), malate/oxaloacetate which decays to pyruvate (M/O) and succinate (Succ). These signals could be further increased by antimycin A (AA). Dihydrolipoic acid (DHLA) is able to reactivate ALDH-2 upon GTN or ROS challenges and the mitochondria-targeted antioxidant MitoQ was able to prevent GTN-induced ROS formation and tolerance. Most interestingly, the present and previous results provide strong evidence that GTN-induced mitochondrial ROS/RNS are transported outside the mitochondrium, where it may activate vascular Nox through a PKC-dependent process, inhibit important vascular signalling enzymes such as soluble guanylyl cyclase (sGC), endothelial NO synthase (eNOS) and prostacyclin synthase (PGI_2-S), all of which contributes to cross-tolerance (GTN-induced endothelial dysfunction). The latter processes are inhibited by vitamin C and apocynin, whereas mitochondrial ROS formation and release are prevented by rotenone (Rot) and cyclosporine A (CsA).

the vascular NADPH oxidase, as evidenced by the strong inhibitory effect of apocynin on vascular oxidative stress, remains obscure. Previous experimental studies have shown that increased oxidative stress in cellular tissue per se is able to activate the oxidase in a positive feedback fashion (17). Thus, GTN-induced mitochondrial superoxide production may cause a secondary activation of Nox. One may also speculate that via its hypotensive action, GTN may cause an activation of the renin–angiotensin–aldosterone system (35), leading to increased circulating levels of angiotensin-II and aldosterone, and therefore to an activation of the NADPH oxidase. This concept is further corroborated by the demonstration that *in vivo* treatment with an AT_1 receptor blocker was able to prevent the development of GTN-induced endothelial dysfunction in an animal model of nitrate tolerance (24). In addition, our present findings could explain why treatment with an AT_1 receptor blocker was not able to prevent the development of GTN-induced nitrate tolerance in human subjects (31, 29).

The present study demonstrated that mitochondria are an important source of oxidative stress in the setting of nitrate tolerance and are critically involved in the pharmacology of nitrate tolerance. Importantly, nitrate tolerance was not affected by the Nox inhibitor apocynin or knockout of NADPH oxidase subunits. These observations clearly point to a dominant role of mitochondrial ROS/RNS for the development and maintenance of nitrate tolerance. In contrast, GTN-induced cross tolerance to the endothelial dependent vasodilator acetylcholine (*e.g.*, endothelial dysfunction) was markedly attenuated in vessels from mice deficient in the critically important NADPH oxidase subunits $gp91^{phox}$ and $p47^{phox}$ and in animals co-treated with the NADPH oxidase inhibitor apocynin. This finding provides strong evidence for the crucial role of the NADPH oxidase in the development of endothelial dysfunction in chronic nitroglycerin treatment. Most interestingly, endothelial dysfunction could be attenuated either by blocking the formation of ROS at complex I of the mitochondrial respiratory chain or by hindering oxygen radicals from leaving the mitochondrion by blocking the mitochondrial permeability transition pore. There seems to be crosstalk of oxygen radicals between two distinct sources of oxidative stress with distinct mechanistic effects (*i.e.*, mitochondria being responsible for nitrate tolerance and NADPH oxidase being responsible for endothelial dysfunction). In this crosstalk, the activation of the vascular NADPH oxidase seems to be driven by ROS/RNS derived from the mitochondrial respiratory chain. Recently, Dikalov and co-workers reported on a similar crosstalk between mitochondria and NADPH oxidases (in both directions) in angiotensin-II induced hypertension (13).

With the knowledge of our present findings, investigators will be encouraged to identify other examples of crosstalk between mitochondrial and Nox-dependent sources of ROS in var-

ious disease states of vascular oxidative stress. It is well established that vascular NADPH oxidase is a primary source of superoxide formation in cardiovascular disease (19, 20) and Nox-derived ROS may stimulate mitochondrial ROS formation (5). However, the concept of mitochondrial-triggered activation of NADPH oxidases is novel and is made more attractive for other researchers by the results of the current study. Additionally, the differential effects of distinct sources of oxygen radicals implicate the importance of targeted antioxidant strategies. In the setting of nitroglycerin-triggered vascular dysfunction, future strategies to prevent GTN-induced side effects should therefore target mitochondrial as well as Nox-dependent oxidative stress or interfere with the crosstalk of ROS between mitochondria and Nox.

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ABBREVIATIONS

ACh, acetylcholine; ALDH-2, mitochondrial aldehyde dehydrogenase; CsA, cyclosporine A; GTN, glyceryl trinitrate (nitroglycerin); L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d] pyridazine-1,4-(2H,3H)dione sodium salt; Mn-SOD, manganese superoxide dismutase (mitochondrial isoform); Mn-$SOD^{+/-}$, heterozygous Mn-SOD deficiency; mPTP, mitochondrial permeability transition pore; Nox, NADPH oxidase; PMA, phorbol myristate; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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