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Evidence for a relationship between mitochondrial Complex I activity and mitochondrial aldehyde dehydrogenase during nitroglycerin tolerance: Effects of mitochondrial antioxidants

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ABSTRACT

The medical use of nitroglycerin (GTN) is limited by patient tolerance. The present study evaluated the role of mitochondrial Complex I in GTN biotransformation and the therapeutic effect of mitochondrial antioxidants. The development of GTN tolerance (in rat and human vessels) produced a decrease in mitochondrial O_2 consumption. Co-incubation with the mitochondria-targeted antioxidant mitoquinone (MQ, 10^{-6} mol/L) or with glutathione ester (GEE, 10^{-4} mol/L) blocked GTN tolerance and the effects of GTN on mitochondrial respiration and aldehyde dehydrogenase 2 (ALDH-2) activity. Biotransformation of GTN depended on the mitochondria being functionally active, particularly mitochondrial Complex I. Tolerance induced mitochondrial ROS production and oxidative stress, though these effects were not detected in HUVECρ^0 cells or Complex I mutant cells. Experiments performed to evaluate Complex I-dependent respiration demonstrated that its inhibition by GTN was prevented by the antioxidants in control samples. These results point to a key role for mitochondrial Complex I in the adequate functioning of ALDH-2. In addition, we have identified mitochondrial Complex I as one of the targets at which the initial oxidative stress responsible for GTN tolerance takes place. Our data also suggest a role for mitochondrial-antioxidants as therapeutic tools in the control of the tolerance that accompanies chronic nitrate use.

1. Introduction

Nitroglycerin (glyceryl trinitrate, GTN) has long been the principal therapeutic agent for the treatment of acute angina and congestive heart disease [1]. Its action is generally attributed to its bioconversion into the relaxant agent nitric oxide (NO), which acts on the enzyme soluble guanylate cyclase (sGC) [1–3]. However, most studies supporting the existence of such a pathway have demonstrated increases of NO only when GTN concentrations considerably exceeded the plasma levels reached during clinical dosing [4]. Several enzymes have been proposed as catalysts of the bioactivation of GTN [5], but some evidence suggests that aldehyde dehydrogenase-2 (ALDH-2) is essential for GTN-triggered vasodilation in rodents and humans [6,7], and that this reaction is accelerated by an allosteric action of NAD^+.

The medical use of GTN is limited by tolerance, which develops following prolonged administration or high dosage. This phenomenon has been related to various mechanisms, in particular desensitization of sGC [8] and impairment of GTN biotransformation by inhibition of ALDH-2 [6,7,9]. These actions, like others associated with GTN, have been linked to an increase in the production of reactive oxygen species (ROS) [10], which seems to depend on the decreased function of the mitochondrial electron transport chain (ETC) [11].

Although antioxidants have been shown to be effective in diminishing oxidative stress during the inflammatory process, there is a lack of conclusive evidence regarding the clinical relevance of these beneficial effects in many pathological settings. This may be due to the limited capacity of these molecules to reach and/or accumulate within mitochondria. The most abundant cellular antioxidant is glutathione (GSH), which has been attributed multiple redox-related functions in the cell. As the cellular up-take of this molecule is rather inefficient, several strategies have been developed to increase its intracellular levels, one of which is glutathione ester (GEE), a molecule that is converted intracellularly into GSH with a 1:1 molar

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stoichiometry. GEE is effective in that it can reach mitochondria without difficulty. Selective mitochondria-targeted compounds constitute another group of intracellular low molecular weight molecules with antioxidant properties. One example is mitoquinone Q (MQ), which consists of the lipophilic TPP (triphenylphosphonium) cation covalently linked to ubiquinone [12]. Due to this conjugation with TPP, MQ is readily transported into the cell and can concentrate several hundred-fold within mitochondria attracted by the large mitochondrial inner membrane potential [13]. The active antioxidant form of MQ is the reduced form of ubiquinol, which is regenerated by the ETC and selectively blocks mitochondrial oxidative damage by detoxifying ROS [14]. This and other mitochondria-targeted antioxidants have been shown to protect mitochondria from oxidative stress in different models [15–17].

The present study confirms and expands the theory that tolerance to GTN is related to the oxidative stress that causes inhibition of ALDH-2. Furthermore, using antioxidants such as GEE and the mitochondria-targeted antioxidant MQ, inhibitors of Complex I of the ETC such as rotenone, and a cellular model of Complex I mutant fibroblasts, we show that mitochondrial Complex I plays a key role in the aforementioned effect of ALDH-2. In fact, the NADH generated during the ALDH-2-catalyzed reaction is reoxidized back to NAD^+ by Complex I (NADH-ubiquinone oxidoreductase) of the respiratory chain. Thus, the ability to metabolize GTN and accelerate this reaction may depend on the capacity of mitochondrial Complex I to reoxidize the NADH that is converted into NAD^+. Moreover, we have identified mitochondrial Complex I as one of the targets at which the initial oxidative stress responsible for GTN tolerance takes place.

2. Materials and methods

2.1. Experimental models

Human umbilical cords were obtained from the Department of Gynaecology (University Hospital Doctor Peset, Valencia, Spain) and cut into rings. Upon anesthesia with sodium pentobarbital (65 $mg \cdot kg^{-1}$, i.p.), male Sprague–Dawley rats (200–250 g; Harlan, Barcelona) were sacrificed and their thoracic aorta cut into 5 mm rings. Rings from thoracic aorta and human umbilical arteries or veins were suspended in an organ bath containing Krebs solution (37 °C), ([in $\times 10^{-3}$ mol/L] NaCl 118, KCl 4.75, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10.1) as described previously [11].

Human umbilical vein endothelial cells (HUVEC) and human umbilical artery endothelial cells (HUAEC) were obtained from fresh umbilical cords and cultured in medium 199 (Cambrex, Walkersville, MD) as previously described [18]. In accordance with the Declaration of Helsinki, all donors of umbilical cords were informed of the purpose, risks, procedures and possible benefits of the study and gave their express consent. The study was approved by the local ethics committee.

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA) and was employed in this study as an additional cell type due to its capacity for induction of GTN tolerance, as already described [6]. RAW 264.7 cell cultures were maintained at sub-confluence at 37 °C in a 5% CO_2 humidified atmosphere using RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 10 μg/mL streptomycin.

For the generation of HUVECρ^0, 50 ng/mL ethidium bromide was added to the medium for 7 days to inhibit mitochondrial gene transcription and, consequently, mitochondrial activity [9]. Pyruvate (110 mg/mL) and uridine (50 μg/mL) were used as alternative sources of energy and nucleotide. Lack of mitochondrial gene expression was confirmed by Western blot analysis for cytochrome *c* oxidase subunit II and the absence of respiratory function was evaluated by measuring mitochondrial oxygen (O_2) consumption.

Complex I mutant cells (Y204C/C206G) were kindly donated by Dr. A Rotig (INSERM, Paris, France). This human fibroblast cell line carries a mutation in the subunit Ndufv1 of the mitochondrial Complex I that causes a considerable reduction in mitochondrial Complex I activity (a residual activity of 18%).

2.2. Vascular contractility studies

Rings were suspended in an organ bath containing Krebs solution (37 °C), as described previously [11]. GTN-tolerance was induced *in vitro* by incubating rings for 3 h with GTN (5×10^{-6} mol/L) following the protocol reported in a previous study [11], and the Complex I inhibitor rotenone (10^{-9} mol/L) was added for 3 h to assess the role of Complex I in GTN tolerance. Relaxation–response curves were obtained by adding cumulative concentrations of GTN (10^{-10} to 10^{-4} mol/L) or the commonly used NO donor, DETA-NO ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; 10^{-8} to 10^{-4} mol/L). Some experiments were performed in aortic denudated rings and, when necessary, MQ (10^{-6} mol/L) was added 1 h prior to incubation with GTN or rotenone and maintained thereafter. In preliminary experiments the lipophilic cation linkers TPP (5×10^{-6} mol/L) or TPMP (5×10^{-6} mol/L), the former of which is responsible for targeting MQ to mitochondria, produced no effect on vascular responses. The adequate concentration ($-\log$ [M]) for producing 50% relaxation (pEC_{50}) was established using a non-linear regression analysis with Graph Pad Software.

2.3. ALDH-2 activity

ALDH-2 activity was determined in homogenates of aortic rings and mitochondrial extracts from HUVEC, Raw 264.7 or Complex I mutant cells (treated as described above) by monitoring NADH formation from NAD^+ using a Uvikon 941 dual-beam spectrophotometer at room temperature at 340 nm. Isolation of mitochondria was performed using "Mitochondria isolation kit" (BioChain Institute, Inc.) according to the manufacturer's instructions. Deep-frozen aortic rings were homogenized for 30 s in a liquid nitrogen precooled dismembranator (B. Braun Biotech International GmbH, Melsungen, Germany). The powdered tissue was dispersed in 5 vol ice-cold aqueous 30 mM potassium phosphate buffer (deoxygenated with nitrogen gas), pH 7.5, vortexed, sonicated, and centrifuged at 10,000 *g* for 10 min.

The assay mixture (0.5 mL) contained 100 mM Tris–HCl (pH 8.5), 1 mM NAD^+, 1 mM 4-methylpyrazole, and 100 μg protein. The reaction was started by addition of 1 mM propionaldehyde to the cuvette, and absorbance changes were recorded for 10 min. The mean rate of absorbance change was taken as a measure of ALDH-2 activity (0.0125 A_{340} was equivalent to 1 nmol/mg/min). The ALDH-2 inhibitor benomyl (10^{-5} mol/L) was used as a negative control. The presence of ALDH-2 in the homogenates was also evaluated by Western blot.

2.4. Protein extracts and immunoblotting

Whole-cell protein extracts were obtained from t-25 flask cell cultures by lysing cell pellets in 50–100 μL complete lysis buffer (2×10^{-2} mol/L HEPES pH = 7.4, 4×10^{-1} mol/L NaCl, 20% (v/v) glycerol, 1×10^{-4} mol/L EDTA, 1×10^{-5} mol/L Na_2MoO_4, 1×10^{-3} mol/L DTT) supplemented with protease inhibitors ("Pefabloc" and "Complete Mini" protease inhibitor cocktail, both from Roche Diagnostics) and phosphatase inhibitor (10^{-2} mol/L $NaVO_3$, 10^{-5} mol/L NaF, 10^{-2} mol/L p-nitrophenylphosphate and 10^{-2} mol/L β-glycerolphosphate). Samples were vortexed, incubated in ice for 15 min, vortexed once again and centrifuged at 16,100 *g* for 15 min at 4 °C. Protein content was quantified with the "BCA Protein Assay Kit" (Pierce, Thermo Scientific, Rockford, IL). SDS-PAGE and WB

were performed following a standard procedure (BioRad, Hercules, CA), using 50 μg of the protein extract. The following primary antibodies were employed: polyclonal anti-ALDH-2 (1:4000), kindly donated by Dr. Stamler (Duke University, North Carolina, USA); anti-actin at 1:500 (Sigma-Aldrich, Steinheim, Germany); the monoclonal anti-Complex IV subunit II, at 1:1000 (Molecular Probes, Invitrogen, Eugene, OR); and anti-tubulin at 1:5000 (Sigma-Aldrich, Steinheim, Germany). Secondary peroxidase-labeled antibodies included anti-mouse antibody (Dako, Glostrup, Denmark) at 1:2000 and anti-rabbit IgG (Vector laboratories, Burlingame, CA) at 1:5000. Immunolabeling was detected using the enhanced chemiluminescent reagent ECL (Amersham, GE Healthcare, Little Chalfont, UK) or Super-Signal WestFemto (Pierce, Thermo Scientific, Rockford, IL). The signal was visualized with a digital luminescent image analyzer (FUJIFILM LAS 3000, Fujifilm), and densitometric analysis was performed using ImageQuant software v. 4.0.

2.5. Measurement of O_2 consumption, NO production, ATP and cGMP levels

Rat aortas (98 ± 1.05 mg wet weight) and human umbilical arteries or veins (195 ± 2.6 or 198 ± 3.2 mg wet weight, respectively) were cut into rings and suspended in Krebs solution. HUVEC, HUVECρ^0, HUAEC, Raw 264.7 cells and Complex I mutant cells were resuspended (5×10^6cells/mL) in Krebs supplemented with L-arginine (3×10^{-4} mol/L) and HEPES (25×10^{-3} mol/L). Induction of GTN tolerance, treatment with rotenone and use of antioxidants were the same as in the vascular reactivity studies. When administered acutely, the concentration of GTN employed (5×10^{-6} mol/L) had no effect on mitochondrial O_2 consumption, despite producing maximal relaxation of vascular rings (data not shown). The aforementioned tissues were then placed in a gas-tight chamber and O_2 consumption was measured with a Clark-type O_2 electrode (Rank Brothers, Bottisham, UK) [19]. Sodium cyanide (10^{-3} mol/L) was employed to confirm that O_2 consumption was mainly mitochondrial (95% to 99%). Measurements were collected using the data-acquisition device Duo.18 (WPI, Stevenage, UK). A hyperbolic function was employed to describe the relationship between O_2 concentration and the rate of O_2 consumption (V_{O2}). The maximal rate of O_2 consumption (V_{O2max}) was calculated according to its analogy with the Michaelis–Menten constant. A Trypan blue exclusion test revealed no alterations of cell viability.

NO concentration in the cell medium was monitored throughout the 3 h incubation period with GTN with the aid of an NO electrode (ISO-NOP; WPI, Stevenage, UK), as described previously [20]. The NO donor DETA-NO (10^{-5} mol/L) was added at the end of each experiment as an internal control. Changes in intracellular NO were also evaluated by incubating cells with the fluorescent probe diaminodifluorofluorescein diacetate (DAF-FM DA, 10^{-6} mol/L). For these experiments, the medium was changed to HBSS supplemented with glucose (20×10^{-3} mol/L), L-arginine (3×10^{-4} mol/L) and DAF-FM, incubated for 30 min, and measured using a Fluoroskan plate reader (TL, Franklin, MA). ATP concentration (nmol/mg protein) was determined by fluorimetry and was compared with a standard curve for ATP using an ATP Bioluminescence Assay Kit HSII (Roche, Mannheim, Germany) and a Fluoroskan microplate reader (Thermo Labsystems, OH). cGMP levels were measured using the Biomol assay kit (Plymoth Meeting, PA) [11].

2.6. Measurement of ROS production, GSH content and Complex I activity

Different methods were employed to evaluate ROS. Total ROS production was assessed by fluorimetry following incubation (30 min) with the fluorescent probe (5×10^{-6} mol/L) 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), as described elsewhere [21]. Quantitative assessment of hydrogen peroxide (H_2O_2) was performed with the Amplex Red^R H_2O_2/peroxidase assay kit (Molecular Probes, Eugene, OR) [22].

GSH content was measured by confocal microscopy (Leica, Heidelberg, Germany) following incubation (30 min) with the fluorescent probe 5-chloromethylfluorescein diacetate (CMFDA, 10^{-6} mol/L).

A more accurate determination of GSH and GSSG was performed using an HPLC method. For GSSG determination, samples (0.5 mL) were treated at 4 °C with 0.5 mL ice-cold perchloric acid (6%) containing 40×10^{-3} mol/L NEM (N-ethylmaleimide; Sigma Chem. Co., St. Louis, MO) in order to prevent GSH oxidation, and 2 mM BPDS (bathophenanthroline disulfonic acid, Sigma Chem. Co., St. Louis, MO), as described by Asensi et al. [23]. To measure total glutathione, 0.5 mL was incubated at 4 °C with 0.5 mL trichloroacetic acid (30%). Samples were then centrifuged at 15,000 *g* for 5 min at 4 °C and the acidic supernatants were employed for measurement of GSH, GSSG and total glutathione.

GSSG was measured by HPLC as previously described [23]; 0.5 mL of the acidic supernatants (see above) were derivatized by adding 50 μL of 1 mM γ-glutamyl-glutamate (Sigma Chem. Co., St. Louis, MO) prepared in 0.3% perchloric acid. Subsequently, pH was adjusted to 8.0 with KOH (2 mol/L)/morpholinopropane sulfonic acid (0.3 mol/L), samples were centrifuged, and an aliquot of 25 μL of the supernatant was mixed with 50 μL of 1% 1-fluoro-2,4-dinitrobenzene (Sigma Chem. Co., St. Louis, MO). Derivatization was completed in 45 min and desiccated samples were maintained stable at −20 °C for several weeks until injection.

The activity of mitochondrial Complex I was assessed by calculating the NADH oxidation rate [24], measured with the Multiscan Plate Reader Spectrophotometer as the decrease in absorbance at 340 nm. In short, a cellular homogenate (20 μL, 0.3 mg) was added to 1 mL of potassium phosphate buffer (10^{-2} mol/L) containing NADH (10^{-4} mol/L) at 37 °C. Basal absorbance was recorded for 1 min, after which 5 μL of decylubiquinone (10^{-2} mol/L) were added. The rate of NADH oxidation (interpreted as Complex I activity) was measured over 2 min and confirmed by inhibition with rotenone (6×10^{-6} mol/L). The NADH oxidation rate was calculated from the time-dependent decrease of the slope of absorbance using a combined NADH-ubiquinone extinction coefficient of 6.81×10^{-3} mol/L cm^{-1} at 340 nm. Complex I-dependent respiration was evaluated in digitonin-permeabilized cells using a Clark-type O_2 electrode (Rank Brothers, Bottisham, UK) [21]. To do this, O_2 consumption was monitored in the presence of the Complex I substrates malate (0.4×10^{-3} mol/L) and glutamate (3×10^{-2} mol/L), the Complex II substrate succinate (10^{-2} mol/L), or the Complex I inhibitor rotenone (6×10^{-6} mol/L) [25].

2.7. Drugs and solutions

Phenylephrine (Phe), sodium cyanide, KCl, NADH, ubiquinone, succinate, rotenone, glutamate, malate, digitonine, arginine, HEPES, uridine, NEM, TPP, TPMP, glucose, DTNB, trypan blue, NADH, glutathione reductase, γ-glutamyl-glutamate, BPDS, methypyrazole, H_2O_2 and hemoglobin were obtained from Sigma-Aldrich (St. Louis, MO). Propionaldehyde was supplied by Fluka (Milano, Italy). The GTN employed in this study is a clinically-used preparation (Solinitrina®, Allmirall, Barcelona, Spain). GTN patches were obtained from Schering-Plough (Madrid, Spain). Na-pyruvate was supplied by Gibco (BRL, Gaithesburg, MD). Ethidium bromide was purchased from SERVA (Heidelberg, Germany), and HBSS and M199 were provided by Cambrex (Verviers, Belgium). DETA-NO was obtained from Alexis (San Diego, CA), and DAF-FM, DHR and DCFH-DA were purchased from Calbiochem (San Diego, CA). CMFDA was obtained from Molecular Probes (Eugene, OR). MQ was synthesized according to the published method [26]. The concentration of 10^{-6} mol/L of MQ used in the present study was selected from concentration–effect experiments (0.001–2 μM) to assess the capacity for scavenging ROS

in a model of GTN tolerance in HUVEC cells (Supplementary Fig. 1). In addition, we have previously found this concentration to have beneficial effects in different models of oxidative stress [11,27].

MQ was used alone in all of the parameters studied in the present work and did not induce significant changes, which rules out the possibility of secondary effects. The characterization of this molecule is described in Supplementary Fig. 2.

2.8. Data analysis

Unless stated otherwise, all values are expressed as the mean ± S.E.M. of at least 5 experiments. Statistical analysis was performed with one-way ANOVA with post-hoc corrections, followed by the Student's *t* test for unpaired samples (Graph Pad Software). Significance was defined as $P<0.05$.

3. Results

3.1. Tolerance to GTN vasorelaxation

GTN treatment induced vascular tolerance in several endothelium models. Fig. 1 shows dose-dependent relaxation curves by GTN (10^{-10} to 10^{-4} mol/L) in precontracted (Phe 10^{-6} mol/L) rings of rat aorta and human umbilical artery and vein. (Fig. 1A, B and C respectively). The vessels were pre-treated with GTN (5×10^{-6} mol/L) or rotenone (10^{-9} mol/L), alone or in the presence of MitoQ (10^{-6} mol/L). The pEC_{50} values are summarized in Table 1. Continuous treatment with GTN caused a significant rightward shift in the GTN concentration–response curves, indicating the development of GTN-induced vascular tolerance. In GTN-tolerant vessels, the maximal GTN relaxation values were significantly diminished. Administration of rotenone, an irreversible inhibitor of Complex I, caused a significant rightward shift in the GTN-induced relaxation of the studied vessels similar to that observed in the presence of GTN. When rotenone was added in the presence of GTN, the result was similar to that obtained with rotenone alone.

Treatment with MQ during the induction of tolerance restored the acute vasorelaxant effect of GTN, but not that of rotenone, suggesting that inhibition of Complex I is as relevant as that of mitochondrial ROS in the development of GTN-tolerance. Similar results were obtained in all the vessels studied and in the presence of another mitochondrial antioxidant, GEE (Table 1).

pEC50 values with DETA-NO (10^{-8} to 10^{-4} mol/L) were similar in controls (6.03 ± 0.09), in GTN-tolerant (5.97 ± 0.04) and rotenone-treated (5.96 ± 0.02) aortas. Incubation with DETA-NO (5×10^{-6} mol/L, 3 h) did not induce tolerance to the effects of GTN (7.87 ± 0.07) when administered later (data not shown). These results argue against desensitization of sGC as a possible mechanism of GTN tolerance.

3.2. GTN biotransformation

The activity and expression of ALDH-2, the enzyme responsible for GTN biotransformation, is represented in Fig. 2. ALDH-2 activity (Fig. 2A) was significantly ($P<0.001$) diminished in GTN-tolerant aortas and in cellular models of HUVEC and Raw 264.7 cells treated with GTN with respect to that in controls. In a similar manner, ALDH-2 activity was inhibited in the presence of rotenone or in Complex I mutant cells ($P<0.001$), which confirms that functional Complex I is essential for GTN biotransformation. This inhibition was blocked by the presence of MQ during the induction of GTN tolerance in all three control experimental models; however, MQ did not restore the inhibition induced by rotenone. The presence of the antioxidant GEE produced similar effects to those observed with MQ (data not shown).

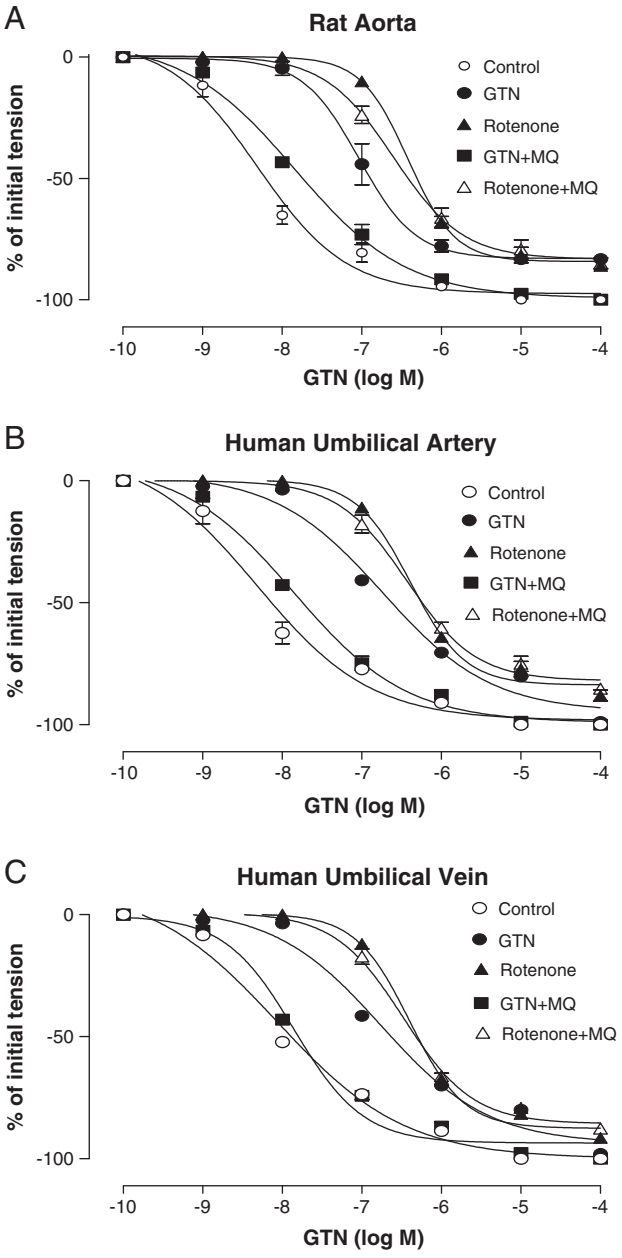

Fig. 1. Dose-dependent relaxation curves for the effects of GTN (10^{-10} to 10^{-4} mol/L) on precontracted (Phe 10^{-6} mol/L) vascular rings of rat aorta (A) and human umbilical artery (B) and vein (C), in which GTN tolerance was induced *in vitro*. The effect of cotreatment with the mitochondria-targeted antioxidant MitoQ (MQ 10^{-6} mol/L) or the Complex I inhibitor rotenone (10^{-9} mol/L) is shown. Each point represents the mean ± SEM of 5–7 separate experiments.

The presence of ALDH-2 was confirmed by Western blot (Fig. 2B) using whole-cell protein extracts of all the cellular models assayed (Raw 264.7, HUVEC and Complex I mutant cells). Densitometry revealed no significant differences between any of the treatments (Fig. 2B).

3.3. Mitochondrial O_2 consumption

As shown in Fig. 3 (A, representative traces and B, data quantification) and Table 2, the rate of O_2 consumption decreased in vessels and cells in which GTN-tolerance was induced (rat aorta, human umbilical artery and vein, HUVEC and Raw 264.7 cells). A rotenone dose–response curve demonstrated that 10^{-9} mol/L rotenone

Table 1
pEC_{50} of the dose-dependent relaxation curves by GTN in rat aorta and human umbilical cord artery and vein rings.

	Rat aorta	HUA	HUV
Control	8.31 ± 0.10	8.25 ± 0.08	8.03 ± 0.06
MQ	8.04 ± 0.07	8.12 ± 0.09	8.05 ± 0.08
Rot	6.41 ± 0.05*	6.38 ± 0.05*	6.43 ± 0.06*
Rot + MQ	6.56 ± 0.06*	6.41 ± 0.04*	6.47 ± 0.04*
Rot + GTN	6.38 ± 0.06*	6.36 ± 0.04*	6.41 ± 0.05*
GTN	7.03 ± 0.07*	6.85 ± 0.06*	6.87 ± 0.06*
GTN + MQ	7.83 ± 0.06†	7.84 ± 0.06†	7.85 ± 0.05†
GTN + GEE	7.80 ± 0.04†	7.88 ± 0.08†	7.89 ± 0.07†

Rat aortic rings, human umbilical artery (HUA) and human umbilical cord vein (HVA) rings were precontracted with Phe (10^{-6} mol/L). GTN tolerance was induced *in vitro* and relaxation was determined upon the addition of GTN (concentration range of 10^{-10} to 10^{-4} mol/L) in all samples. Vascular rings were preincubated with MQ (10^{-6} mol/L), GEE (10^{-4} mol/L), rotenone (10^{-9} mol/L), GTN (5×10^{-6} mol/L) or a combination of Rot + MQ, Rot + GTN, GTM + MQ, GTN + GEE or underwent no pretreatment (control). Data are represented as the mean ± SEM of 5–7 independent experiments.
* P<0.001 vs control.
† P<0.001 vs GTN.

produced a similar inhibition of mitochondrial respiration to that obtained with GTN (10^{-6} mol/L) (Supplementary Fig. 3). Treatment with MQ during the induction of tolerance prevented these inhibitory effects of GTN in all the control samples, but not in Complex I mutant cells or HUVECρ^0, or after treatment with rotenone. GEE reproduced the result obtained with MQ (data not shown). As expected, the inhibition of mitochondrial O_2 consumption during GTN tolerance and after administration of rotenone produced a decrease in ATP levels in HUVEC cells (around 50% in both cases, data not shown). Treatment with MQ during the induction of tolerance prevented the inhibitory effect of GTN on ATP generation but did not produce any changes after treatment with rotenone (data not shown).

In accordance with previous results [11], the addition of GTN (5×10^{-6} mol/L) in HUVEC did not induce any NO signal, whereas a robust increase was noted when DETA-NO (10^{-5} mol/L) was measured with an NO electrode and by fluorescence microscopy (data not shown).

3.4. Role of mitochondria in GTN-induced cGMP production

HUVECρ^0 cells were employed to confirm the role of functional mitochondria and, particularly, the role of Complex I in GTN bioactivity and ALDH-2 activity. The rho^o phenotype was verified when the absence of the Cytochrome *c* oxidase subunit II was confirmed by WB. (Fig. 4B). Additionally, we employed cells treated with rotenone or human Complex I mutant cells (Y204C/C206G), which display only 18% Complex I activity. The acute addition of GTN increased cGMP levels in HUVEC but not in HUVECρ^0 or in Complex I mutant cells, or when cells were pre-incubated with GTN, or after treatment with rotenone (Fig. 4A). Co-incubation with MQ blocked the effect induced by incubation with GTN in controls but not in Complex I mutant cells or in HUVECρ^0. GEE had a similar effect (data not shown). HUVEC, HUVECρ^0 and Complex I mutant cells exhibited an increased cGMP production following addition of DETA-NO (10^{-4} mol/L), which rules out the possibility that sGC was affected. Preincubation of HUVEC with DETA-NO (5×10^{-6} mol/L, 3 h) did not modify the increase in cGMP that followed the acute addition of GTN (data not shown). These data highlight the key role of mitochondrial Complex I in the biotransformation of GTN.

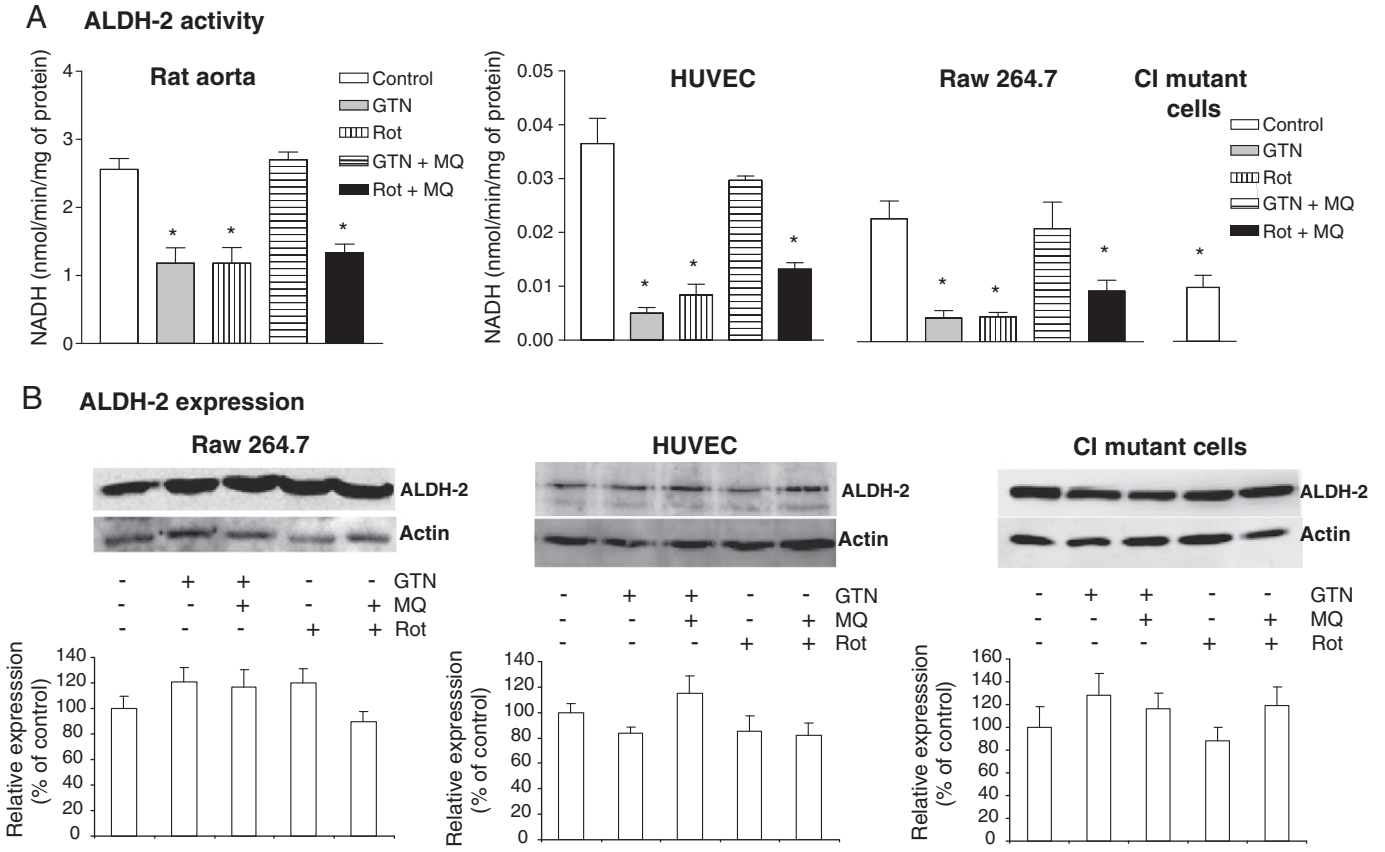

Fig. 2. Effects of pre-incubation with GTN (5×10^{-6} mol/L, 3 h), GTN + MQ (10^{-6} mol/L), rotenone (10^{-9} mol/L) or rotenone + MQ on ALDH-2 activity in rat aorta, HUVEC, Complex I mutant and Raw264.7 cells (A) and ALDH-2 protein expression studied by Western blot (B) in Raw264.7 cells, HUVEC and Complex I mutant cells. Data are represented as mean ± SEM of 5–7 experiments.*P<0.001 vs. control.

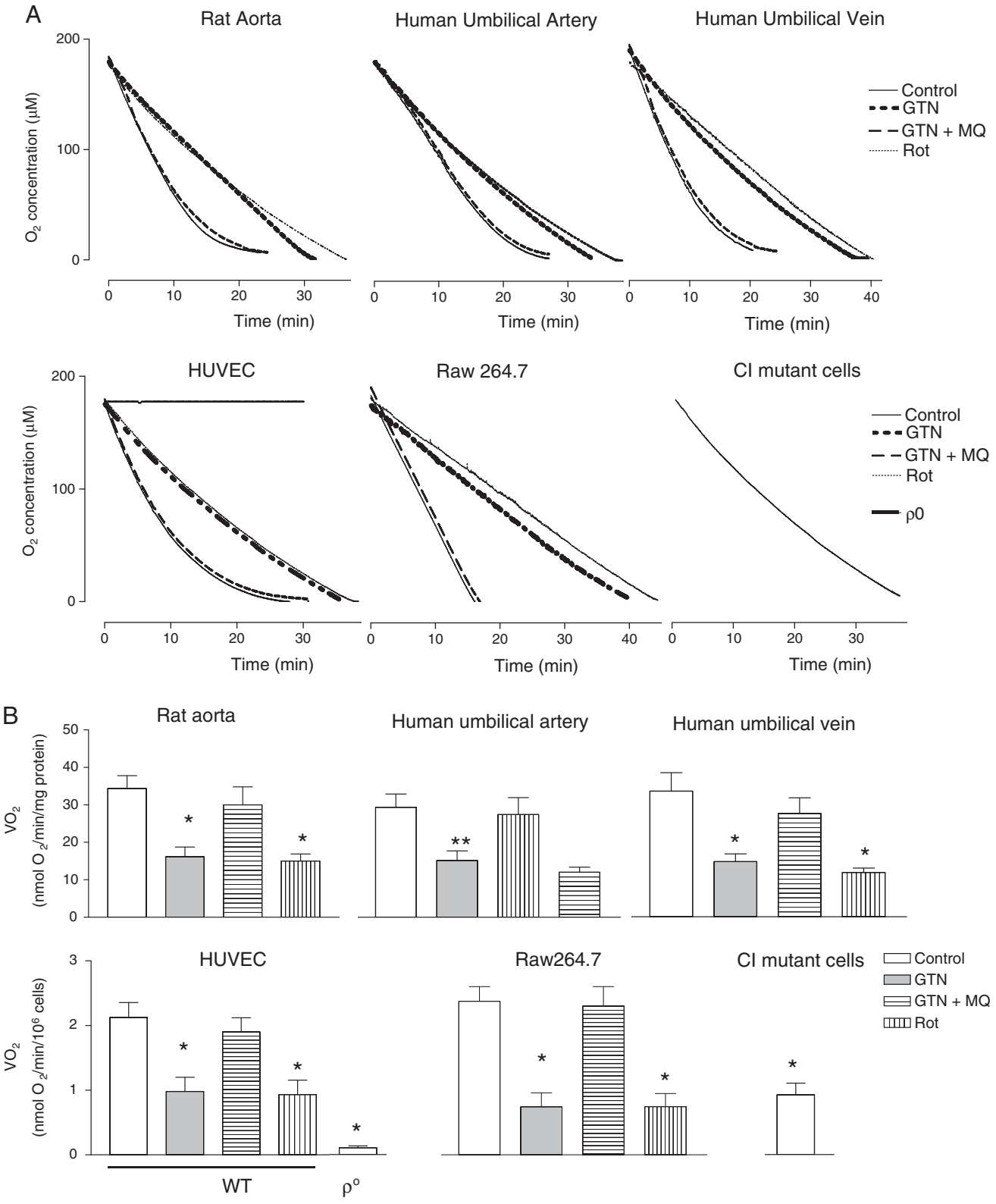

Fig. 3. Representative traces (A) and summarizing bar charts (B) showing O_2 consumption rate in rat aorta, human umbilical artery, human umbilical vein, HUVEC, Complex I mutant cells and Raw264.7 cells. Samples were pre-incubated with GTN (5×10^{-6} mol/L, 3 h), in the absence or presence of MQ (10^{-6} mol/L), or with rotenone (1×10^{-9} mol/L). Data are represented as mean ± SEM of experiments.*P<0.001 vs. control.

3.5. ROS production and GSH levels

Pre-incubation of HUVEC with GTN significantly increased ROS generation. This was detected with the ROS-sensitive fluorescent probe DCFH-DA and the aid of confocal microscopy (Fig. 5A) and fluorimetry (Fig. 5B). A similar ROS increase resulting from GTN pre-incubation was also observed when H_2O_2 concentration was evaluated (Fig. 5C). Co-incubation with MQ reversed the effect of GTN. Similar

Table 2
Modulation of the V_{O2max} in rat aortic rings, human umbilical cord artery rings, human umbilical cord vein rings, HUVEC and Raw264.7.

	Rat aorta	HUA	HUV	HUVECs	Raw264.7
Control	32.1 ± 1	33.3 ± 2	32.8 ± 3	31.4 ± 2	36.5 ± 2
Control + MQ	31.9 ± 1	32.6 ± 2	31.6 ± 2	30.5 ± 2	35.6 ± 2
Rot	17.6 ± 2*	20.8 ± 2*	19.3 ± 2*	20.4 ± 2*	18.5 ± 2*
Rot + MQ	18.3 ± 2	22.1 ± 2	20.5 ± 2	20.9 ± 2	20.4 ± 2
GTN	18.4 ± 1*	21.1 ± 2*	20.2 ± 4*	21.1 ± 2*	19.3 ± 1*
GTN + MQ	33.2 ± 2†	32.8 ± 3†	31.5 ± 3†	30.3 ± 2†	35.6 ± 1†

Vascular rings or cell cultures underwent no preincubation (control), were pre-incubated with GTN (5×10^{-6} mol/L, 3 h), or with rotenone (1×10^{-9} mol/L) in all three cases in the absence or in the presence of MQ (10^{-6} mol/L). Data are shown as mean ± SEM of 5–7 independent experiments.
* P<0.001 vs controls.
† P<0.001 vs GTN.

results were obtained in HUAEC (data not shown). Neither incubation of HUVECρ^0 cells with GTN nor acute administration of GTN to HUVEC, HUVECρ^0 or Complex I mutant cells produced any increase in ROS-related fluorescence.

Oxidative stress is related to both an increase in ROS production and a decrease in antioxidant content. Using different detection methods, we found that the level of the major cellular antioxidant GSH in HUVEC was significantly lower (P<0.05) after pre-incubation with GTN, as shown in Fig. 5D and E. No changes in GSH concentration were observed in HUVECρ^0 or Complex I mutant cells following treatment with GTN or in rotenone-treated HUVEC. Treatment with MQ reversed the effect of GTN. In addition, we also evaluated the GSH:GSSG ratio, which is often employed as a reliable indicator of redox stress. Fig. 5F reveals that incubation of HUVEC with GTN led to a decrease in the GSH:GSSG ratio, a pattern that was not reproduced in rotenone-treated cells in HUVECρ^0 or Complex I mutant cells treated with GTN. Again, co-incubation with MQ reversed the effects of GTN. As suggested by these data, rotenone at the concentration of 10^{-9} M does not affect the redox status of HUVEC cells.

3.6. Complex I activity

The specific effect of GTN on Complex I activity was also evaluated. As represented in Fig. 6A, pre-incubation with GTN exerts an inhibitory effect on mitochondrial Complex I activity in HUVEC, calculated from the rate of NADH oxidation. As expected, a major drop in Complex I activity was observed in the presence of rotenone and in Complex I mutant cells which were used as controls. Of note, co-incubation with either MQ or GEE reversed the inhibitory effect of GTN. The Complex I-specificity of the action of GTN was further characterized through an alternative method involving cells permeabilized with digitonin and measurement of Complex I-dependent respiration. As was to be expected, respiration of permeabilized HUVEC after administration of the Complex I substrates malate (0.4×10^{-3} mol/L) and glutamate (30×10^{-3} mol/L) was inhibited by almost 91% with the Complex I inhibitor rotenone (6×10^{-6} mol/L), as shown in Fig. 6B. HUVEC incubated with GTN or Complex I mutant cells respired very poorly with malate and glutamate, while rotenone-sensitive respiration did not differ to that observed in the absence of the inhibitor. When succinate (10×10^{-3} mol/L), a Complex II electron donor, was added in order to bypass Complex I-dependent respiration, GTN-treated cells exhibited O_2 consumption rates similar to those of controls. Collectively, these data suggest that Complex I is the main target of GTN preincubation. Co-treatment with MQ or GEE (not shown) prevented the action exerted by GTN preincubation on Complex I-dependent respiration. MQ had no effect on Complex I mutant cells (data not shown).

4. Discussion

The present study demonstrates that pre-incubation with GTN results in a reduced vascular relaxation resembling the clinical induction of GTN tolerance [9,28]. Specifically, the findings presented endorse the fundamental role that mitochondrial dysfunction plays in the appearance of GTN tolerance. In addition, through the use of mitochondrial antioxidants such as MQ, and GEE, and by employing Complex I mutant cells and inhibitors of the electron transport chain (rotenone), we have shown that mitochondrial Complex I is a key factor in the regulation of ROS production and ALDH-2 activity during the biotransformation of GTN.

We postulate that GTN is metabolized depending, in part, on the ability of mitochondrial Complex I to reoxidize NADH as our data show that dysfunction of Complex I (presumably through the generation of NAD^+ deficit and/or altered NADH/NAD^+ ratio) exerts an inhibitory effect on aldehyde dehydrogenase independently of any ROS participation. This is supported by the fact that ALDH-2 activity is inhibited by rotenone at a concentration at which this inhibitor compromises Complex I without substantial generation of ROS. In addition, MQ cannot reverse this effect of rotenone ruling out the

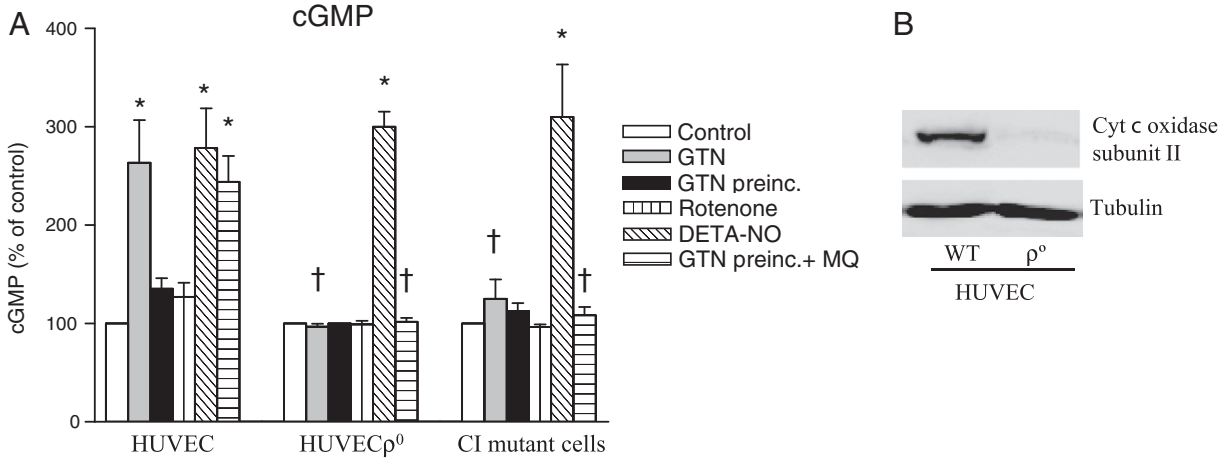

Fig. 4. (A) cGMP levels in HUVEC, HUVECρ^0 and in Complex I mutant cells. In some cases cells were pre-incubated with GTN (5×10^{-6} mol/L). Co-incubation with MQ (10^{-6} mol/L) blocked the effect of GTN tolerance on cGMP levels in HUVEC but not in HUVECρ^0 or in Complex I mutant cells. Addition of DETA-NO (10^{-4} mol/L) increased cGMP levels in all the cell types. *P<0.001 vs. control. †P<0.001 vs HUVEC cells. (B) Western blot analysis of the presence of cytochrome *c* oxidase subunit II in HUVECρ^0 cells using tubuline as a control charge.

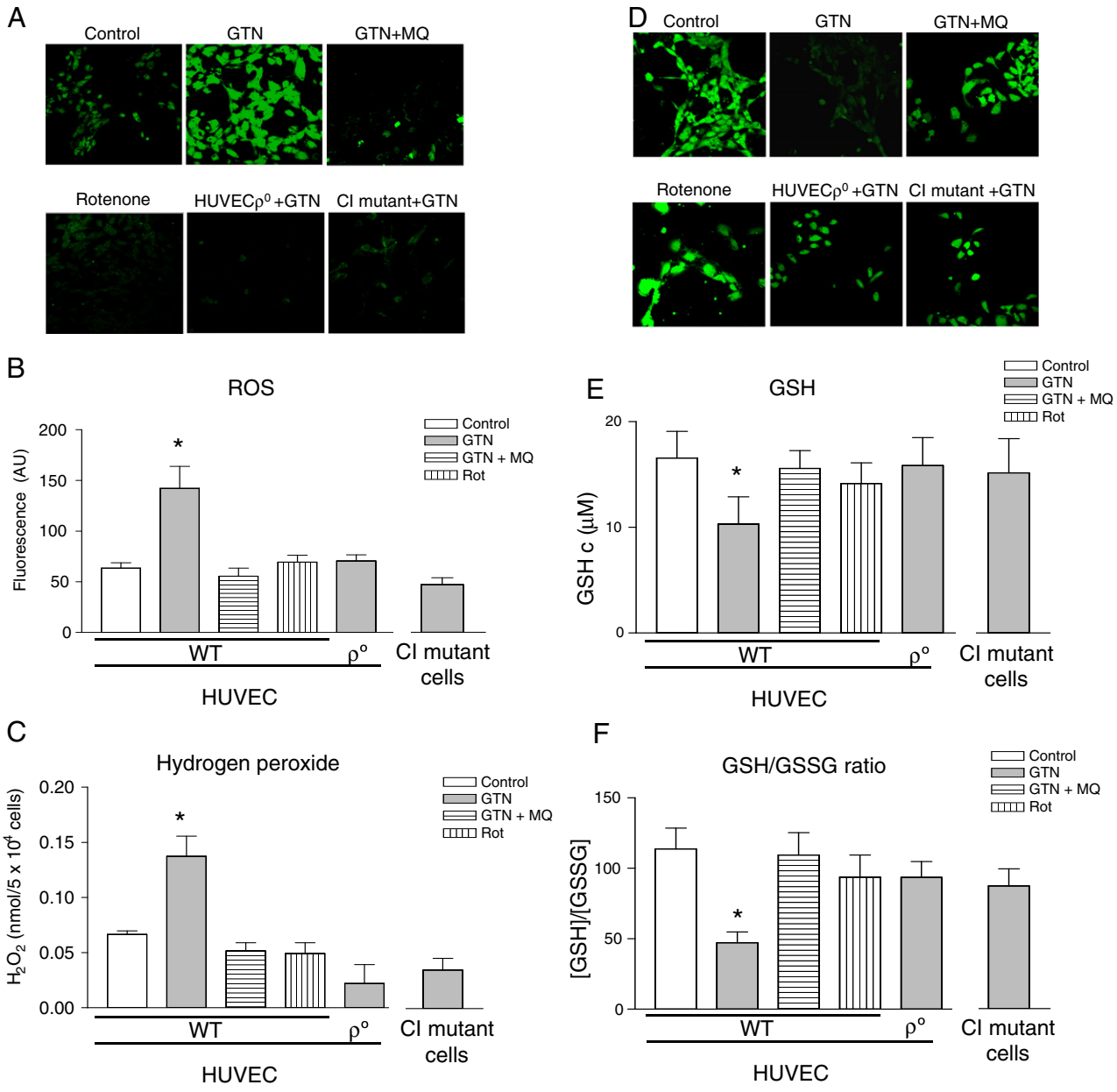

Fig. 5. Effects of pre-incubation of HUVEC, HUVECρ^0 and Complex I mutant cells with GTN (5×10^{-6} mol/L, 3 h), GTN + MQ (10^{-6} mol/L) or rotenone (1×10^{-9} mol/L) on cellular redox status. Total ROS production was studied by fluorescence microscopy employing the fluorescent probe DCFH-DA (A, representative images), by fluorimetry (B, changes in fluorescence) and by evaluating hydrogen peroxide (H_2O_2) levels (C). Total GSH levels were detected by confocal microscopy (D representative images). GSH content (E) and GSH:GSSG ratio (F) were assessed by HPLC. Data are shown as mean ± SEM of 5–7 experiments. *P<0.001 vs. control.

participation of ROS. However, the inhibitory action on ALDH-2 is most likely dual, involving both the described phenomenon and ROS-induced damage of this enzyme. In this sense, we identify mitochondrial Complex I as one of the targets at which the initial oxidative stress responsible for GTN tolerance takes place. It is thus likely that prolonged exposure to GTN tolerance manifested as increased ROS generation accompanied by alterations in NAD^+ availability and/or altered NADH/NAD^+ ratio, might provoke conformational (and other) changes in ALDH-2 undermining its activity.

GTN (5×10^6 mol/L) was capable of reducing mitochondrial O_2 consumption in vascular tissue and endothelial cells in which GTN-tolerance had been induced *in vitro* and this inhibition occurred in a non-competitive manner. Administration *in vitro* of either MQ or GEE blocked these effects, except when rotenone was present.

We further explored these processes by employing HUVECρ^0 cells, a mitochondrial antioxidant and mitochondrial Complex I mutant cells, and found mitochondrial Complex I activity to be essential for proper ALDH-2 function. Collectively, these findings endorse the fundamental role that mitochondrial dysfunction plays in the appearance of nitrate tolerance, thus underlining the vital role of mitochondrial Complex I in the biotransformation of GTN.

The dose of GTN (5×10^{-6} mol/L) used for inducing tolerance did not cause NO release, thus confirming our previous hypothesis that biotransformation of these clinically relevant concentrations of GTN does not cause free NO to be released [19]. The acute addition of GTN increased cGMP levels and ALDH-2 activity in HUVEC, while this response was absent following pre-incubation with GTN, thus suggesting an inhibition of this enzyme's activity. These results are in accordance with those of a previous report [9] which suggested that tolerance-inducing concentrations of GTN *in vitro* lead to inhibition of both ALDH-2 activity and GTN biotransformation (1,2-GDN formation). Co-incubation with either MQ or GEE prevented the

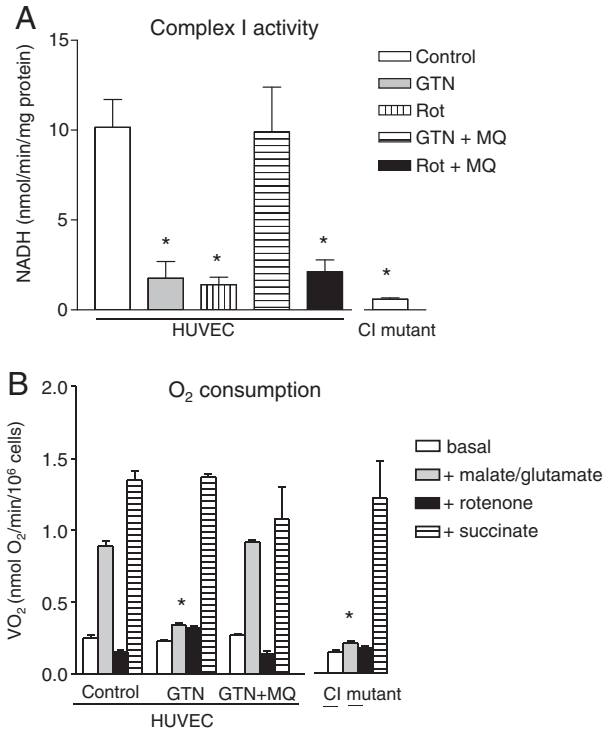

Fig. 6. Effects of pre-incubation with GTN (5×10^{-6} mol/L, 3 h), GTN + MQ (10^{-6} mol/L) or rotenone (10^{-9} mol/L) (A) on the activity of Complex I in HUVEC and Complex I mutant cells, measured by calculating the NADH oxidation rate; or by the measurement of (B) isolated Complex I-dependent respiration in digitonin permeabilized HUVEC in the presence of the Complex I substrates malate (0.4×10^{-3} mol/L) and glutamate (3×10^{-2} mol/L), the Complex II substrate succinate (10^{-2} mol/L), or the Complex I inhibitor rotenone (6×10^{-6} mol/L). Data are shown as mean ± SEM of 5–7 experiments. *P<0.001 vs. control.

effects induced by incubation with GTN. In HUVECρ^0 cells, Complex I mutant cells and HUVEC treated with rotenone, GTN-stimulated increases in cGMP were absent, which confirmed the metabolization of GTN by functional mitochondria and, particularly, Complex I. Considered as a whole, this evidence gives weight to the idea that ALDH-2 contributes to GTN bioconversion and the subsequent increase in cGMP levels, whereas inhibition of this enzyme is implicated in GTN-tolerance.

It is widely postulated that prolonged exposure to GTN leads to an increase in the production of ROS [29–32], which is related to the development of tolerance and cross-tolerance [9]. The results that we have obtained with different fluorescence methods confirm this hypothesis, since incubation with GTN increased the release of ROS in vascular cells and tissues. Mitochondria seem to be the origin of this ROS production, as it was absent in HUVECρ^0 cells. Furthermore, treatment with MQ and GEE reversed the increase in ROS and the oxidative stress that followed incubation with GTN. The oxidative stress and Complex I impairment that followed long-term treatment with GTN could inactivate ALDH-2 or inhibit the ALDH-2 repair system, both of which effects cause an impairment of GTN bioactivation. In addition, a potential interaction with superoxide could decrease the bioavailability of the vasodilator released following GTN bioactivation. This central role of mitochondrial ROS in nitrate tolerance can be explained by the structure of ALDH-2. This enzyme has three adjacent cysteine thiol-groups in its active site [33]. One of these thiol groups participates directly in the enzymatic catalysis (aldehyde breakdown) of ALDH-2. Therefore, oxidation of these thiol groups leads to inactivation of the enzyme and formation of a disulfide or, at the very least, a sulfenic acid group. The inhibition of ALDH-2 by oxidative stress provides the missing link between nitrate tolerance and a diminished organic nitrate bioactivation [34]. During the catalytic cycle of GTN bioactivation, the drug is de-nitrated, which leads to the release of 1,2glyceryldinitrate and the formation of thionitrate ($-SNO_2$) intermediate [35]. Upon nucleophilic attack of a second adjacent thiol-group, nitrite is released and a disulfide is formed. Since a detectable amount of the enzyme cannot be reactivated by DTT or dihydrolipoic acid upon challenge with GTN *in vivo* or *in vitro*, it has been suggested that GTN-triggered ROS production causes irreversible inhibition of the enzyme via formation of the sulfonic acid ($-SO_3H$) group [36].

ROS are highly toxic to various sites of the mitochondrial respiratory chain, and inhibition of Complex I seems to be the most likely consequence of this toxicity. We have demonstrated that GTN-tolerance is accompanied by a marked reduction in NADH oxidation, which is indicative of a reduction in Complex I activity and is prevented by mitochondrial antioxidants. Further experiments that we have carried out to analyze isolated Complex I-dependent respiration in permeabilized HUVEC have demonstrated that, in the presence of succinate, a Complex II electron donor added in order to bypass Complex I-dependent respiration, GTN-treated cells exhibit O_2 consumption rates similar to those of controls. This confirms that continuous exposure to GTN mainly affects Complex I, although we cannot discard that a decrease in the respiration of GTN-treated cells could indicate mitochondrial permeability transition pore (PTP) opening. In this sense, it has been described that matrix pyridine nucleotide release following a permeability transition leads to respiratory inhibition with Complex I substrates, and PTP opening induces a specific conformational change of Complex I that (*i*) dramatically increases H_2O_2 production so long as electrons are provided to Complex I, and (*ii*) inhibits the physiological pathway of electrons inside Complex I [37]. Importantly, we did not detect induction of apoptosis nor changes in the viability of the cells after treatment with GTN which rules out the presence of a major opening of PTP at least within the time frame studied.

Once again, MQ and GEE blocked the effects of GTN, highlighting ROS-mediated damage of Complex I as the likely cause of respiration deficiency. In addition, it has been described that sustained treatment with GTN increases ischemic damage, thereby inducing ALDH-2 inactivation. This is highly relevant given that ALDH-2 activity is known to be crucial for cardioprotection from ischemia [38].

5. Conclusion

The present study demonstrates that prolonged exposure to GTN induces oxidative stress, and highlights the mitochondria as both its source and its target. Our results lead us to propose that the activity of mitochondrial Complex I is undermined following continuous incubation with GTN, and that this Complex plays a key role in the adequate regulation of ROS production during GTN biotransformation; in other words, GTN metabolization depends on the ability of mitochondrial Complex I (NADH-ubiquinone oxidoreductase) to reoxidize the NADH generated from NAD^+ by ALDH-2 (see supplementary Fig. 4). Our data provide fresh insight into the mechanisms responsible for nitrate tolerance and suggest that enhancing the activity of ALDH-2 and Complex I is a potentially beneficial strategy to adopt in the case of patients with cardiac ischemia and those receiving chronic treatment with GTN.

6. Abbreviations

ALDH-2 Aldehyde dehydrogenase 2
BPDS Bathophenanthroline disulfonic acid
CMFDA 5-Chloromethylfluorescein diacetate
DAF-FM Diaminodifluorofluorescein diacetate
DCFH-DA 2′,7′-Dichlorodihydrofluorescein diacetate

DETA-NO	1-[N-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DTNB	5-5′-Dithiobis[2-nitrobenzoic acid
ETC	Electron transport chain
FBS	Fetal bovine serum
GEE	Glutathione ester
GSH	Glutathione
GSSG	Oxidized glutathione
GTN	Glyceryl trinitrate
H_2O_2	Hydrogen peroxide
HUAEC	Human umbilical aortic endothelial cells
HUVEC	Human umbilical vein endothelial cells
MQ	Mitoquinone
NEM	N-ethylmaleimide
NO	Nitric oxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
Phe	Phenylephrine
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
TPP	Triphenylphosphonium
TPMP	Methyltriphenylphosphonium

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