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Formation of Reactive Oxygen Species by Pentaerithrityltetranitrate and Glyceryl Trinitrate *In Vitro* and Development of Nitrate Tolerance[1]

S. DIKALOV, B. FINK, M. SKATCHKOV, D. STALLEICKEN and E. BASSENGE

Institute of Chemical Kinetics and Combustion, Novosibirsk 630090, Russia (S.D.), Institute of Applied Physiology, University Freiburg, Freiburg 79104, Germany (B.F., E.B.), and ISIS Pharma, Zwickau 08005, Germany (D.S.)

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ABSTRACT

Anti-ischemic therapy with organic nitrates is complicated by tolerance. Induction of tolerance is incompletely understood and likely multifactorial. Recently, increased production of reactive oxygen species (ROS) has been investigated, but it has not been clear if this is a direct consequence of the organic nitrate on the vessel or an *in vivo* adaptation to the drugs. To examine the possibility that nitrates could directly stimulate vascular ROS production, we compared the development of nitrate tolerance with the formation of ROS induced by pentaerithrityltetranitrate (PETN) or nitroglycerin (GTN) *in vitro* in porcine smooth muscle cells, endothelial cells, washed *ex vivo* platelets and whole blood. By examining cGMP formation, it was found that 24-hr treatment with GTN but not PETN induced significant nitrate tolerance, which was prevented by parallel treatment with Vit C. Incubation of vascular cells acutely with 0.5 mM GTN doubled the rate of ROS generation, whereas PETN had no such effect. The rate of ROS (peroxynitrite and $O_2^{\cdot-}$) formation detected by specific spin traps in tolerant smooth muscle cells, treated for 24 hr with 0.01 mM GTN, was substantially higher (30.5 nM/min) than in control cells acutely treated with 0.5 mM GTN (25 nM/min). In contrast to PETN, GTN induces nitrate tolerance and also increases the formation of ROS both in vascular cells and in whole blood. ROS formation is minimally stimulated by PETN comparable to data obtained in Vit C-suppressed GTN tolerance. ROS formation induced by organic nitrates seems to be a key factor in the development of nitrate tolerance.

Organic nitrates (*e.g.*, PETN and GTN) are used in the therapy of a large variety of cardiovascular diseases in which enhanced vasodilator responses of certain vascular sections are beneficial, such as in myocardial ischemia. The organic nitrates do not spontaneously release NO but must undergo a metabolic biotransformation to NO (Mülsch *et al*., 1995) before stimulating cGMP production and vascular relaxation (Mellion *et al*., 1983). Treatment with GTN and other organic nitrates is limited by the development of nitrate tolerance, especially during nonintermittent administration. Nitrate tolerance, a multifactorial phenomenon, is characterized by neurohormonal counterregulation, enhanced responses to vasoconstrictor agonists, as well as diminished responses to the endothelium-derived relaxing factor (Laursen *et al*., 1996). Despite many hypotheses and investigations, the mechanisms of nitrate tolerance have not yet been fully perceived.

Recently, it was found that a GTN treatment of animals increased the formation of superoxide ($O_2^{\cdot-}$) in blood vessels and that this seemed to inactivate NO (Münzel *et al*., 1994, 1995); other studies indicated that 3-day GTN treatment resulted in elevated vascular NADH-oxidase activity. The latter significantly enhances the basal generation of $O_2^{\cdot-}$ radicals (Griendling *et al*., 1994; Münzel *et al*., 1996) and promotes the inactivation of NO (Gryglewski *et al*., 1986) and the formation of peroxynitrite (Huie and Padmaja, 1993). Superoxide radicals ($O_2^{\cdot-}$) react effectively with NO ($k = 6.7\times10^9$ M^{-1} sec^{-1}) to form peroxynitrite (Huie and Padmaja, 1993), which is a strong oxidant (Pryor and Squadrito, 1995). The formation of peroxynitrite in GTN-treated *ex vivo* platelets (Skatchkov *et al*., 1996) and in human blood under GTN therapy was recently reported (Skatchkov *et al*., 1997). Thiols are principal targets of peroxynitrite in cells

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ABBREVIATIONS: CP, 3-carboxy-proxyl; CP-H, 1-hydroxy-3-carboxy-pyrrolidine; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMPO-OH, 2-hydroxy-5,5-dimethylpyrrolidine-N-oxyl; ESR, electron spin resonance; EC, endothelial cells; NO, nitric oxide; GTN, nitroglycerin; $O_2^{\cdot-}$, superoxide radical; PETN, pentaerithrityltetranitrate; ROS, reactive oxygen species; SMC, smooth muscle cells; SOD, erythrocyte superoxide dismutase; TMIO, 3,5,5-trimethyl-imidazole-1-oxide; TMIO-OH, 5-hydroxy-2,2,4,-trimethyl-3-imidazoline-1-oxyl; TEMPONE-H, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride; TEMPONE, 2,2,6,6-tetramethyl-4-oxo-piperidinoxyl; Vit C, vitamin C.

(Radi *et al*., 1991). Peroxynitrite is capable of modifying enzyme activities, for example by irreversibly oxidizing SH groups including those of sGC. The NO-mediated increase in the activity of sGC depends on the state of SH groups (Braughler, 1983). Peroxynitrite causes vascular dysfunction in isolated hearts (Villa *et al*., 1994). The action of GTN as an exogenous NO donor and a promoter of cGMP-dependent vasodilation depends on the specific balance between the concentrations of NO on one hand and the rate of the GTN-induced simultaneous ROS-formation (superoxide radicals and peroxynitrite) on the other hand.

It is still very difficult to quantify the formation of superoxide radicals and of peroxynitrite in biological systems. Recently, it was shown (Vásquez-Vivar *et al*., 1997) that the application of lucigenin-chemiluminescence for peroxynitrite quantification is unexpectedly associated with the presence of artifacts, for example with an additional lucigenin-induced formation of superoxide radicals. However, without an amplification of chemiluminescence (*e.g.*, with lucigenin) the GTN-induced formation of peroxynitrite and O_2^- in vascular cells remains below the detection limit. Therefore, in our experiments, we used ESR spectroscopy with spin trapping techniques.

To clarify the mechanisms involved in the development of nitrate tolerance and to elaborate and demonstrate a more effective and less detrimental vasodilator, it is important to compare different organic nitrates with regard to the development of nitrate tolerance and the concomitant formation of ROS (superoxide radical and of peroxynitrite). It has been reported that GTN treatment causes nitrate tolerance both *in vitro* and *in vivo* (Feelisch and Kelm, 1991; Tsutamoto *et al*., 1994). Fink and Bassenge (1997) reported that a nonintermittent PETN administration in dogs did not cause tolerance. As mentioned above, GTN induces ROS formation in vascular cells. However, no quantitative data are available hitherto quantifying the formation of superoxide radicals and peroxynitrite as a consequence of PETN and GTN metabolism. Consequently, we studied the effects of PETN and GTN on ROS formation in vascular cells and in blood.

It is known that treatment with organic nitrates induces vasodilation by increasing cGMP formation (Feelisch and Kelm, 1991). In nitrate-tolerant organs, the administration of organic nitrates does not cause vasodilation because the contents and release of cGMP cannot be enhanced (Tsutamoto *et al*., 1994). Therefore, we used the nitrate-induced effects on cGMP contents as a marker of the development of nitrate tolerance *in vitro*.

The aim of our study was to compare the development of nitrate tolerance *in vitro* with the concomitant formation of ROS (superoxide radicals and peroxynitrite) induced by PETN and GTN in suspensions of cultured SMC and EC in washed *ex vivo* platelets and in whole blood.

Four partly newly elaborated spin traps were used in our ESR experiments. To differentiate between the formation of superoxide radicals and peroxynitrite, the spin traps DMPO and TMIO were used. The rates of ROS formation were determined using TEMPONE-H and CP-H.

Materials and Methods

Processing of cells. EC and SMC were washed from porcine aortas as described by Hecker *et al*. (1994) and grown in culture (cat. no. 31095–029; GIBCO BRL Life Technologies, Eggenstein, Germany) as described previously (Campbell and Campbell, 1987). The purity of the EC and SMC cultures were checked as described by Hecker *et al*. (1994). Cell viability was checked by fluorescence microscopy and by measuring the increase in the cGMP induced by incubation with the NO donor sodium nitroprusside (2 μM, 3 min). Washed *ex vivo* platelets were obtained from trained, conscious dogs by venopuncture. Platelets were washed from plasma and resuspended in 50 mM phosphate buffer (PBS), pH 7.4, as in Bassenge and Fink (1996). EC (4000 cells/μl) and SMC (2500 cells/μl) obtained from cultures (fifth passage), as well as washed platelets (100,000/μl), were incubated with 0.5 mM GTN or PETN in PBS for 15 min at 20°C in the presence of cysteine (20 μM) as a cofactor of nonenzymatic systems involved in GTN metabolism (Weber *et al*., 1996). To induce tolerance to GTN or PETN both SMC and EC were incubated in culture with GTN, GTN with Vit-C (20 μg/ml) or with PETN for 24 hr at 37°C (initial concentration of nitrates was 10 μM) (Salvemini *et al*., 1993). The state of platelet function was analyzed by measuring the thrombin-induced increase in intracellular Ca^{++} using Ca^{++}-dependent fluorescence of FURA 2 dye as described by Bassenge and Fink (1996). The protein concentrations were determined using the Lowry method. Blood was drawn from the carotid artery of dogs into a citric acid solution (6:1 v/v) (Bassenge and Fink, 1996).

Spin trapping experiments. The cells were resuspended in 50 mM PBS, pH 7.4, containing 0.2 mM DTPA and 0.9% NaCl. Probes for ESR measurements were analyzed in quartz capillaries of an internal diameter of 1 mm. To inhibit the formation of hydroxyl radicals from H_2O_2 catalyzed by traces of transition metals potentially present in the buffer, DTPA was added to the cell suspensions (final concentration, 0.2 mM) (Rosen and Freeman, 1984). The absence of paramagnetic impurities was checked in stock solutions of spin traps by ESR spectroscopy. ESR measurements were performed at room temperature using an EMX-A ESR spectrometer (Bruker, Karlsruhe, Germany). The ESR settings were the following: field center, 3474 G; field sweep, 60 G; microwave frequency, 9.72 GHz; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2.0 G; conversion time, 655 msec; detector time constant, 1024 msec; magnetic field sweep time, 671 sec. The ESR spectra were recorded 5 min after equilibration of the samples in the ESR cavity.

Determination of cGMP contents. The cGMP contents were assayed in whole cells using radioimmunoassay (Biotrend, Cologne, Germany) as described by Bassenge and Fink (1996). The probes were prepared for the determination of cGMP formation induced by an acute addition of the nitrate (100 μM), according to the following protocol: cell suspensions were incubated for 1 min with different nitrate compounds and the process then stopped by the addition of trichloracetic acid (final concentration, 5%) after the probes were frozen in liquid nitrogen. To determine the basal level of cGMP in the control cells, after an incubation with nitrates in culture (24 hr), the probes were tested parallel to probes that had been acutely treated with nitrates (in control cells, saline was added instead of the solution-containing nitrates).

Preparation of CP-H and TEMPONE-H spin trap stock solutions. CP-H and TEMPONE-H were dissolved in oxygen-free (nitrogen bubbled) 0.1 M sodium phosphate buffer, pH 7.4, in the presence of 0.9% NaCl and 1 mM DTPA. DTPA was used to decrease the self-oxidation of hydroxylamines catalyzed by traces of transition metal ions. The concentration of CP-H and TEMPONE-H in the stock solutions amounted to 10 mM. Before the experiments, stock solutions were kept frozen or in a cool airtight place.

Superoxide radical determination. Superoxide radical generation in platelets, SMC and EC was determined using the spin trap DMPO (Rosen and Freeman, 1984) in a concentration of 0.1 M quantifying the DMSO-resistant DMPO-OH spin adduct (Dikalov *et al*., 1997a). DMSO (0.1% final concentration in probes) was used as a peroxynitrite scavenger. DMPO-OH formation in cell suspensions

was measured using the amplitude of the second low-field component of the ESR spectra of the DMPO-OH spin-adduct.

Peroxynitrite determination. Peroxynitrite formation was determined by the spin traps DMPO and TMIO. Using DMPO (0.1 M), the peroxynitrite formation in cell suspensions was quantified by the DMSO-inhibited DMPO-OH spin adduct (final concentration DMSO, 0.1%). Peroxynitrite also was determined by TMIO (0.2 M) by monitoring the ESR signal of the TMIO-OH spin-adduct (Dikalov *et al*., 1996) with a spectrum corresponding to the hyperfine interaction constants a_N = 14.3 G and a_H = 16.3 G. Solutions of known concentrations of peroxynitrite were used to obtain the corresponding calibration curve. For this purpose, small aliquots of peroxynitrite (pH 13) were mixed with 0.2 M TMIO dissolved in 50 mM phosphate buffer (pH 7.4) containing 0.2 mM DTPA.

No changes in platelet aggregation were observed when washed *ex vivo* platelets were incubated with any of the spin traps used (in the above mentioned concentrations) in the presence of 0.2 mM DTPA.

Hydroxyl radical formation in cell suspensions. The contribution of hydroxyl radicals formed from H_2O_2 to the formation of DMPO-OH and TMIO-OH spin adducts was tested using two methods: (1) by measuring the intensity of the ESR spectra of the spin adduct DMPO-CH°$_3$ in the presence of DMSO (a methyl radical CH°$_3$ is formed during the reaction of hydroxyl radicals with DMSO, which in turn forms the DMPO-CH_3° adduct from DMPO; Rosen and Rauckman, 1984); and (2) by the detection of a TMIO-OH spin adduct in the model system containing H_2O_2 and DTPA. It was found that the presence of 0.2 mM DTPA effectively inhibited the formation of the DMPO-CH°$_3$ spin adduct. Moreover, no ESR signal was observed in the samples containing 50 μM H_2O_2, 0.2 mM DTPA and 0.2 M TMIO in PBS (pH 7.4). Therefore, the presence of 0.2 mM DTPA effectively inhibited the iron catalyzed formation of hydroxyl radicals. Dismutation of superoxide radicals and the consequent formation of H_2O_2 did not contribute to the formation of TMIO-OH in the presence of DTPA. Thus, the contribution of hydroxyl radicals to the formation of TMIO-OH and DMPO-OH spin adducts was negligible under our experimental conditions.

Determination of rates of ROS formation. The determination of the rate of ROS formation by the DMPO spin trap is limited by the half-life period of the DMPO-OH spin adduct and usually the steady-state concentration of the DMPO-OH spin adduct is measured. Using CP-H or TEMPONE-H as spin traps (Dikalov *et al*., 1997a), the rate of ROS formation can be determined monitoring the time-dependent accumulation of the corresponding stable nitroxyl radicals TEMPONE or CP using ESR (Dikalov *et al*., 1997a). The rates of ROS formation (superoxide radical and peroxynitrite) *in vitro* were determined by the analysis of the oxidation of hydroxylamines CP-H (1 mM) and TEMPONE-H (1 mM) to nitroxyl radicals CP and TEMPONE, respectively. The rates of CP and TEMPONE formation were measured by the kinetics of nitroxyl radical generation, which were obtained by monitoring the amplitude of the low field component of the ESR spectrum. Experimental concentrations of CP and TEMPONE *in vitro* were determined using the dependence of the amplitude of the ESR spectrum on the concentration of CP and TEMPONE obtained from Sigma (Deisenhofen, Germany).

TEMPONE-H was used in suspensions of SMC and EC because TEMPONE-H is a more effective scavenger of superoxide radicals than CP-H. Platelets and blood contain a high concentration of ascorbate (Moser, 1987; Evans *et al*., 1982). Nitroxyl radicals, formed in the reaction of TEMPONE-H or CP-H with ROS, can be reduced by ascorbate to ESR-silent hydroxylamines. The CP radical is much more resistant to the reaction with ascorbate than TEMPONE nitroxyl radicals (Dikalov *et al*., 1997a). Therefore, we used CP-H to avoid an underestimation of the ROS formation both in platelets and in whole blood.

During GTN and PETN metabolism in vascular cells, it is possible that superoxide radicals and peroxynitrite could be formed (Münzel *et al*., 1996; Dikalov *et al*., 1997c; Skatchkov *et al*., 1997). TEMPONE-H reacts with both these ROS to form the nitroxyl radical TEMPONE. The formation of superoxide radicals can be tested using SOD as competitive reagent. SOD competes with TEMPONE-H for superoxide radicals, decreasing the TEMPONE formation. The formation of ROS was measured as TEMPONE generation in cultured SMC (2500 cells/μl) after the addition of 0.5 mM PETN, 0.5 mM GTN, and 0.5 mM GTN plus 1000 units/ml SOD, after 24-hr incubation with GTN.

The rate of ROS formation in suspensions of washed *ex vivo* platelets (100,000 cells/μl) was measured as the rate of CP formation with the addition of 0.5 mM PETN, 0.5 mM GTN or 1 mM hydralazine plus 0.5 mM GTN.

The calibrations were obtained using xanthine/xanthine-oxidase as a source of $O_2^{\cdot-}$ radicals. All measurements were performed in 50 mM PBS in the presence of 0.9% NaCl and 0.2 mM DTPA at pH 7.4 at 20°C.

Statistics. All values are expressed as mean ± S.D. Statistical significance was determined by Student's *t* test for paired data. Two groups of data were considered to be significantly different at $P < .05$.

Chemicals and drugs. PETN was obtained from ISIS Pharma (Zwickau, Germany). GTN was from Pohl Boskamp (Hohenlockstedt, Germany). Hydralazine (antihypertensive drug) was from Ciba-Geigy (Basel, Switzerland). The spin traps TMIO, TEMPONE-H and CP-H were from Alexis Corporation (San Diego, CA). The spin trap DMPO, nitroxyl radicals TEMPONE and CP, DMSO, cysteine, glutathione, DTPA, bovine erythrocyte SOD, catalase and xanthine were obtained from Sigma. Xanthine oxidase was supplied by Fluka (Neu-Ulm, Germany). Peroxynitrite was obtained from Alexis. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Results

Formation of ROS in suspensions of SMC, EC and platelets. The formation of the DMPO-OH spin adduct in suspensions of SMC is depicted in figure 1. An addition of GTN (0.5 mM) to SMC suspensions enhanced the DMPO-OH formation from 96 ± 10 to 180 ± 26 nM/mg protein (fig. 1), thus the 15-min incubation with GTN resulted in a significantly enhanced ROS formation in suspensions of SMC (fig. 1). Such an enhancement was not observed when PETN was used. The addition of 0.1% DMSO to the SMC suspension with 0.5 mM GTN reduced DMPO-OH formation from 180 ±

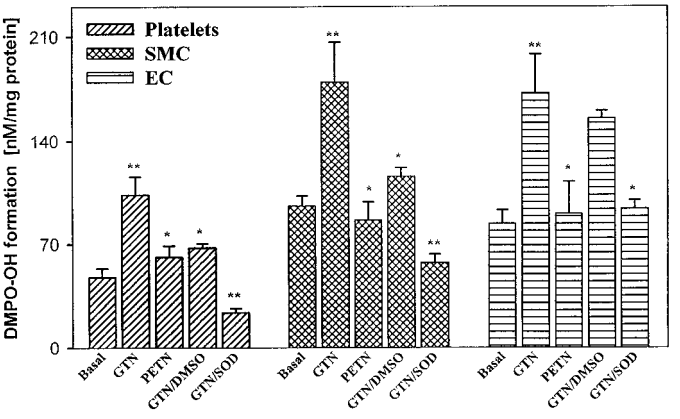

Fig. 1. Formation of ROS ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) measured as the formation of DMPO-OH spin-adducts in suspensions of SMC (basal level, 96 ± 10 nM/mg protein), platelets (basal level, 48 ± 6 nM/mg protein) and EC (basal level, 84 ± 9 nM/mg protein) incubated for 15 min at 20°C with 0.5 mM GTN or 0.5 mM PETN. All samples were in 50 mM sodium phosphate buffer (pH 7.4) with 0.9% NaCl and DTPA (0.2 mM). ESR settings were described in Materials and Methods. Data are mean ± S.D. (n = 8; *, $P < .05$ *vs*. GTN, **, $P < .01$ *vs*. control.

26 to 118 ± 12 nM/mg protein. SOD addition (1000 units/ml) to SMC suspensions inhibited the DMPO-OH formation to 58 ± 6 nM/mg protein.

A significant formation of the DMPO-OH spin adduct was observed in GTN-treated platelet suspensions (fig. 1). The addition of 0.5 mM GTN to platelet suspensions approximately doubled (from 48 ± 6 to 104 ± 13 nM/mg protein) the DMPO-OH formation (fig. 1). Addition of PETN to platelet suspensions elicited no rise in ROS formation (fig. 1). The addition of 0.1% DMSO or SOD (1000 units/ml) to the platelet suspensions with 0.5 mM GTN reduced the DMPO-OH formation from 104 ± 13 to 68 ± 6 or 24 ± 6 nM/mg protein, respectively.

The addition of GTN (0.5 mM) to EC suspensions substantially enhanced the DMPO-OH spin-adduct formation (from 84 ± 9 to 172 ± 13 nM/mg protein) (fig. 1). ROS formation in suspensions of EC with PETN (0.5 mM) was not statistically different from control (fig. 1). An administration of DMSO slightly decreased the formation of the DMPO-OH spin-adduct in suspensions of EC with 0.5 mM GTN (from 172 ± 13 to 138 ± 12 nM/mg protein). An addition of SOD (1000 units/ml) reduced the amount of the DMPO-OH adduct to 94 ± 10 nM/mg protein compared with the 181 ± 13 nM/mg protein of DMPO-OH detected in suspensions of EC treated with 0.5 mM GTN.

Spin trapping of peroxynitrite using TMIO in suspensions of SMC, EC and platelets. The spin trap TMIO does not trap superoxide radicals but reacts with peroxynitrite producing the TMIO-OH spin adduct (Dikalov *et al*., 1996). In our experiments, the spin trap TMIO was used for the determination of peroxynitrite formation during GTN and PETN metabolism (fig. 2). It was found that an acute addition of 0.5 mM GTN to the suspensions of vascular cells led to the formation of significant amounts of the TMIO-OH spin adduct. In contrast to GTN, the contents of the TMIO-OH spin adduct in suspensions of PETN-treated vascular cells did not differ statistically from the control.

No ESR signals corresponding to the TMIO-OH spin-adduct were detected when 0.5 mM GTN was added to cell-free PBS (pH 7.4) containing 0.2 M TMIO, 20 μM cysteine and 0.2 mM DTPA. The intensity of the ESR signals corresponding to TMIO-OH in cell suspensions was not affected by changes in the DTPA concentrations from 0.2 to 1.0 mM.

Determination of the rate of ROS formation in SMC, EC, platelets and whole blood. The addition of 0.5 mM GTN to suspensions of SMC increased the rate of ROS formation by 83 ± 8% (from 13.6 in control to 25 nM/min/mg protein). The presence of extracellular SOD (1000 units/ml) effectively inhibited the rate of TEMPONE formation (from 25 to 12 nM/min/mg protein). Acute treatment of SMC with PETN did not result in a significant increase in ROS formation (fig. 3).

The rate of ROS formation in a suspension of SMC incubated for 24 hr with 0.1 mM GTN and resuspended in a buffer without GTN was 30.5 ± 0.5 nM/min/mg protein, which is significantly higher than the rate of ROS formation in SMC acutely treated by 0.5 mM GTN (fig. 3). We assume that a long-term treatment with GTN results in a higher rate of ROS formation than an acute addition of GTN.

The rate of ROS formation in suspensions of EC (4000 cells/μl) was assayed as the rate of TEMPONE formation after the addition of 0.5 mM PETN, 0.5 mM GTN, 0.5 mM GTN plus 1% DMSO and 0.5 mM GTN plus 1000 units/ml SOD (fig. 4). It was found that the addition of 0.5 mM GTN increased the formation of ROS by 254% (from 11.8 to 30 nmol/mg protein/min). The addition of 1000 units/ml SOD effectively inhibited the formation of TEMPONE. The addition of DMSO (final concentration, 1%) inhibited TEMPONE formation (from 30 to 24 nmol/mg protein/min). The rate of ROS formation in suspensions of EC with PETN was not statistically different from the control data (fig. 4).

Blood plasma is rich in ascorbate (0.01 ± 0.1 mM) (Moser, 1987; Evans *et al*., 1982). Therefore, for the experiments with blood, we used the spin trap CP-H, which is more resistant to ascorbate than TEMPONE-H (Dikalov *et al*., 1997a). The formation of ROS in blood was measured as the rate of CP formation after the addition of 0.5 mM PETN, 0.5 mM GTN and hydralazine (1 mM) plus GTN (0.5 mM) as depicted in figure 5A. It was found that the addition of 0.5 mM GTN

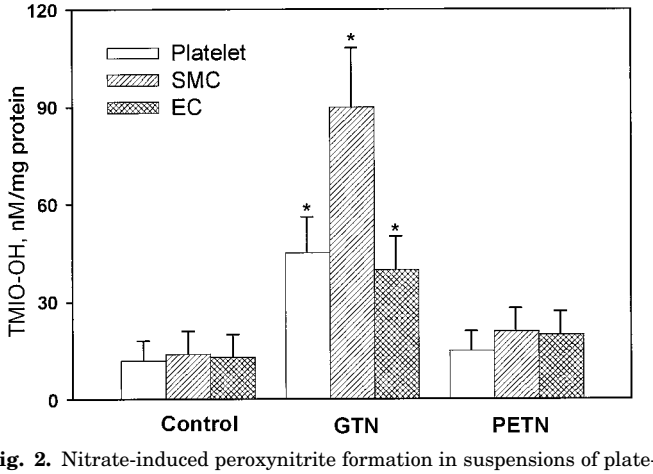

Fig. 2. Nitrate-induced peroxynitrite formation in suspensions of platelets, SMC or EC was determined by the detection of the TMIO-OH spin adduct using the spin trap TMIO (0.2 M). ESR signals were recorded 10 min after the addition of 0.5 mM GTN to cells. All samples were in 50 mM sodium phosphate buffer (pH 7.4) with 0.9% NaCl and DTPA (0.2 mM). ESR settings were described in Materials and Methods. Data are mean ± S.D. (n = 8; *, P < .05 *vs*. control).

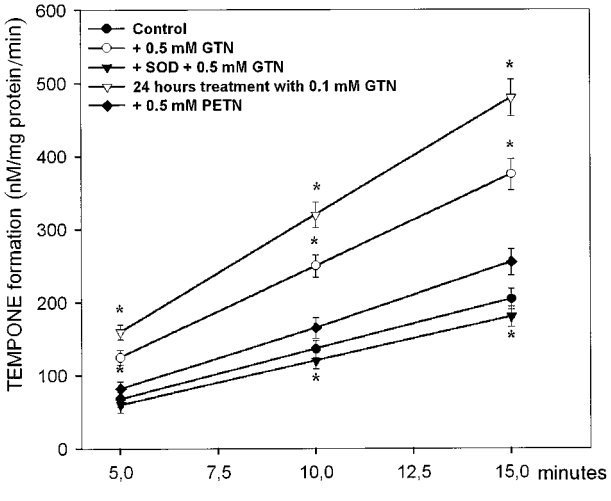

Fig. 3. Formation of ROS in SMC measured as the amount of TEMPONE radicals formed in cultured SMC (2500 cells/μl) in control (-●-); after the addition of 0.5 mM GTN (-○-), 0.5 mM GTN + 1000 units/ml SOD (-▼-) or 0.5 mM PETN (-◆-); and in cells after 24 hr of treatment with 0.1 mM GTN and resuspended in buffer-free from GTN (-▽-). Data are mean ± S.D.; n = 12; * P < 0.05 *vs*. PETN.

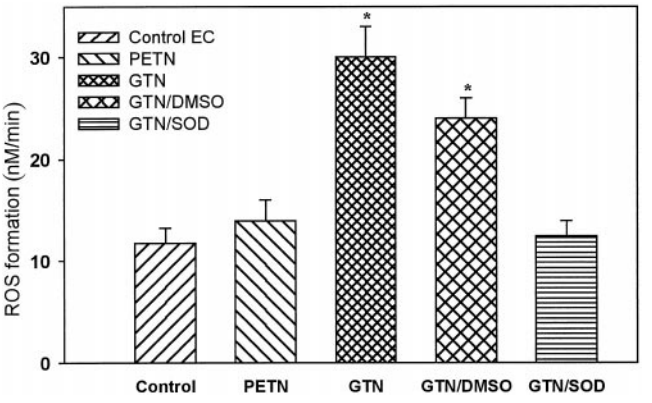

Fig. 4. Formation of ROS in cultured EC (4000 cells/μl) quantified as the rate of formation of nitroxyl radical TEMPONE after the addition of PETN (0.5 mM), GTN (0.5 mM), GTN (0.5 mM) + DMSO (0.1%) and GTN (0.5 mM) + 1000 units/ml SOD to suspensions of cells. Data are mean ± S.D. (n = 12; *, P < .05 *vs*. control).

augmented the formation of ROS up to 182 ± 7% (from 17 ± 1 to 31 ± 2 nmol/min). In contrast to GTN, the rate of ROS formation in blood with PETN was only slightly increased (115 ± 8%) (fig. 5A).

It was found in suspensions of platelets that an addition of 0.5 mM GTN increased the rate of ROS formation rate up to 365% (from 4 ± 0.4 to 14.6 ± 1.4 nmol/min) (fig. 5B). In contrast to GTN, the rate of ROS formation with PETN was not statistically different from that at control. The addition of 1000 units/ml SOD substantially decreased the CP formation (data not shown). It is interesting that an addition of hydralazine (inhibitor of NADH-oxidases; Münzel *et al*., 1996) to

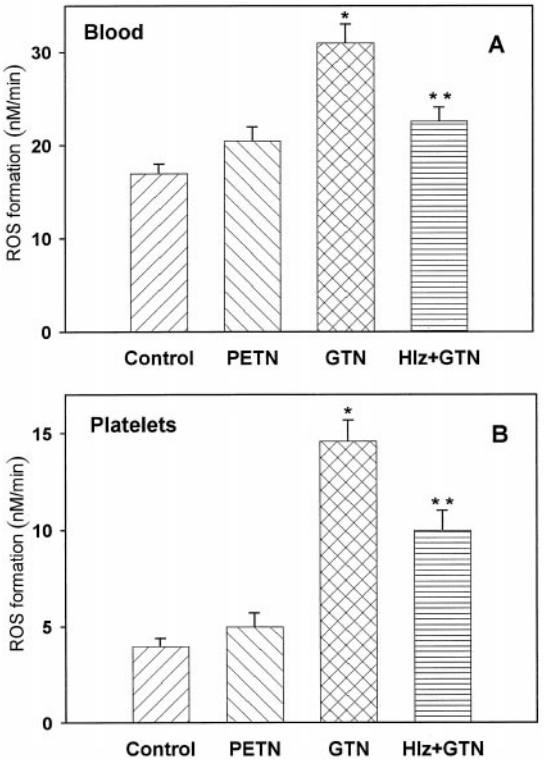

Fig. 5. Formation of ROS in whole blood (A) and in suspension of washed *ex vivo* platelets (100,000 cells/μl) (B) measured as the rate of formation of the nitroxyl radical CP after the addition of PETN (0.5 mM), GTN (0.5 mM) and 1 mM hydralazine (Hlz) + GTN (0.5 mM). Data are mean ± S.D. (n = 14; *P < .01 *vs*. control, **P < .05 *vs*. GTN).

platelets before GTN treatment reduced the rate of CP formation from 365% to 250%. However, a preincubation with hydralazine in a concentration up to 10 mM did not decrease the GTN-induced ROS formation to the basal level or to the level obtained by the addition of SOD to GTN-treated platelets (data not shown).

It is interesting that in our experiments the addition of hydralazine (1 mM) to the blood before GTN treatment reduced the formation of CP during GTN treatment from 182 ± 7% to 132 ± 6% (fig. 5A). This effect of hydralazine can be attributed to the inactivation of NADH-oxidases of blood cells in the same way as in washed *ex vivo* platelets and in isolated EC (Münzel *et al*., 1996).

Determination of cGMP contents. To compare the development of nitrate tolerance induced by PETN and GTN in vascular cells *in vitro*, we measured the formation of cGMP during acute administration and at the end of a 24-hr treatment with these nitrates. The formation of cGMP after acute treatment with nitrates was determined. It was found that the induction of cGMP formation in EC (similar to SMC) after a 1-min treatment with PETN or with GTN (concentrations varied from 10 to 500 μM) was almost the same (fig. 6, *left columns*). There was no significant difference between the PETN- or GTN-induced cGMP formation after a 24-hr treatment of EC cultures. The 24-hr treatment of EC with GTN showed clearly the development of tolerance, as neither GTN nor PETN stimulate the release of cGMP in EC (*center columns*). In contrast to GTN-treated cells, the formation of cGMP was stimulated both by GTN and PETN in EC treated for 24 hr with PETN (fig. 6, *right columns*). An incubation of EC with GTN plus an antioxidant (Vit C) significantly improved cGMP release from 0.8 pM/mg protein at control to 12 pM/mg protein after additional GTN/Vit-C treatment.

Discussion

Using a new, well validated technique for the detection of specific compounds in ROS formation, we could convincingly demonstrate a generation of both $O_2^{\cdot-}$ and peroxynitrite in various types of vascular cells during acute exposure to organic nitrates, especially after induction of tolerance after a 24-hr continuous exposure to organic nitrates. We found such formation in both SMC in EC in which tolerance was elicited before nitrate exposure and, surprisingly, even in platelets of animals. By using a combination of different nonspecific and several new specific spin traps, we could differentiate exactly between peroxynitrite and $O_2^{\cdot-}$ generation in various vascular cells. In addition, we found that different organic nitrates were associated with substantially different rates of ROS generation in all three vascular cell types tested, e.g., PETN produced substantially less ROS than GTN (only one fourth of the amount). Unexpectedly, our *in vitro* data closely resembled that of earlier *in vivo* data obtained not only by us (Fink and Bassenge, 1997) but also by others, especially with regard to the $O_2^{\cdot-}$-mediated generation of atheromatosis (Kojda *et al*., 1995).

As recent studies have shown, the development of tolerance to organic nitrates is a multifactorial phenomenon. Apart from the formation of ROS such as $O_2^{\cdot-}$ and $ONOO^-$, which have recently been shown to induce tolerance (Münzel *et al*., 1995; Dikalov *et al*., 1998), other changes have been recognized such as the reduction in the concentration of low

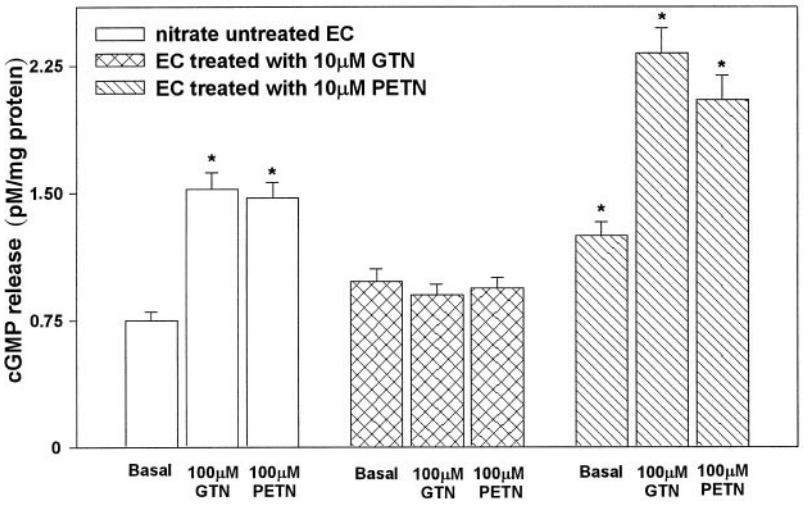

Fig. 6. Formation of cGMP in cultured EC in control cells, in cells cultured 24 hr with 10 μM GTN and in cells cultured 24 hr with 10 μM PETN. Effect of incubation during 1 minute with 100 μM GTN or 100 μM PETN. Content of cGMP was assayed as described in Materials and Methods. Data are mean ± S.D. (n = 9; $*P < .01$ *vs*. control).

molecular thiols (Needleman and Johnson, 1973) and the inhibition of sGC and the resulting decrease in cGMP formation and vasorelaxation (Schröder *et al*., 1988).

Previously, it was reported that treatment of vascular cells with GTN induces formation of ROS (Münzel *et al*., 1996). These data were obtained using lucigenin-chemiluminescence, which was recently criticized, because of substantial artifacts caused by lucigenin-mediated additional formation of superoxide radicals (Vásquez-Vivar *et al*., 1997). To avoid this problem, we therefore studied the nitrate-induced formation of ROS by ESR spectroscopy with spin trapping techniques, which are not limited by lucigenin-associated artifacts. The other advantage of using special spin traps such as TMIO (Dikalov *et al*., 1996), TEMPONE-H (Dikalov *et al*., 1997b) or CP-H (Dikalov *et al*., 1997a) lies in the fact that we were able to analyze not only the $O_2^{\cdot-}$ release during exposition to nitrates but also the formation of $ONOO^-$, which is known to inhibit various enzymes containing SH-groups such as sGC. In addition, by using the ESR technique, we were capable of measuring the rate of ROS release in cell suspensions and in blood in the presence of antioxidants and/or reductants. The data obtained confirmed the fact that GTN induces the formation of ROS both in isolated vascular cells and in whole blood. Moreover, it was shown in our study that GTN also induced ROS formation in whole blood treated with hydralazine [inhibitor of NAD(P)H-oxidoreductases] (Münzel *et al*., 1996), which only partially decreased the GTN-induced formation of ROS.

In addition to GTN we also studied PETN, an organic nitrate, the new interesting aspects of which were recently discussed by Hess *et al*. (1997). This study showed on one hand an $NO^{\cdot}$ release at a redox potential of +3 and on the other hand that a particular PETN metabolite (pentaerithrityldinitrate) could simultaneously act as a reductant. When we analyzed the effects of PETN on ROS formation in vascular cells and in whole blood, we could show for the first time that PETN only slightly increased ROS formation in vascular cells and in whole blood (not more than 20%). This could potentially be attributed to the superimposed action of this particular PETN-metabolite. Moreover, in contrast to PETN, the treatment of the vascular cells with GTN caused a 200% increase in ROS formation compared with nitrate-free control cell suspensions. Thus, it is reflected in our data that PETN treatment does not lead to the development of nitrate tolerance in vascular cells (and the effect of PETN on ROS formation is negligible), in contrast to GTN, which induced both ROS formation and substantial development of nitrate tolerance. This was shown as a decrease in stimulated cGMP release. Therefore, it seems rather likely that ROS formation is closely associated with the development of nitrate tolerance *in vitro*. This was also convincingly shown in newly developed assays using more specific spin traps, in addition to DMSO and SOD in different vascular cell preparations. Therefore, we assume that the enhanced *in vitro* formation of superoxide radicals and of peroxynitrite during long-term treatment with GTN could also play a key role in the development of nitrate tolerance under *in vivo* conditions.

Taking into account that the formation of hydrogen peroxide results from the dismutation of superoxide radicals, one cannot exclude a generation of hydroxyl radicals in our samples. However, the formation of hydroxyl radicals from hydrogen peroxide is a transition metal-catalyzed reaction, which can be suppressed by adding a chelating agent such as DTPA (Butler and Halliwell, 1982). In fact, in our probes, a formation of the TMIO-OH spin adduct was not detected in the presence of 0.2 mM DTPA and 50 μM H_2O_2, whereas a bolus addition of peroxynitrite (50 μM) to 0.2 M TMIO in the presence of 0.2 mM DTPA in PBS (pH 7.4) led to the formation of a strong ESR signal of the TMIO-OH spin-adduct. Thus, 0.2 mM DTPA effectively inhibited the iron-catalyzed reaction of hydroxyl radical formation from H_2O_2 but did not affect the formation of the TMIO-OH spin-adducts by peroxynitrite.

The detailed molecular mechanism of the effect of ROS formation on the development of nitrate tolerance is still not precisely known. However, the enhanced formation of superoxide radicals and of peroxynitrite in GTN-treated cells *in vitro* could lead to a pronounced oxidative damage of the cells. Such oxidative damage can contribute to the development of nitrate tolerance *via* an inactivation of certain enzymes involved in the NO-induced stimulation of cGMP formation and contents (particularly by damaging the SH-groups of soluble guanylyl cyclase; Braughler, 1983), which were substantially diminished in our cell suspensions incubated with GTN for 24 hr. This was in contrast to PETN-incubated cells as well as to non-nitrate-exposed cells and to

cells which were incubated with GTN along with the antioxidant Vit-C.

Such an additional supplementation with antioxidants could thus be important not only under our *in vitro* conditions (because of this excessive formation of ROS during therapy with organic nitrates) but also under *in vivo* conditions in which similar mechanisms were recently demonstrated (Bassenge and Fink, 1996; Fink *et al*., 1998).

Conclusions. GTN metabolism in vascular cells and in blood is associated with a drastically enhanced formation of ROS (superoxide radical and peroxynitrite) for which hydralazine-inhibited enzymatic systems may be partially responsible. A 24-hr treatment of the vascular cells with 10 μM GTN leads to nitrate tolerance, whereas PETN has no affect on the formation of cGMP. PETN causes a negligible rise in the formation of ROS and does not induce nitrate tolerance, a finding that we have also seen under *in vivo* conditions (Fink and Bassenge, 1997).

GTN, in contrast to PETN, induces both nitrate tolerance and an increase in the formation of ROS both in vascular cells and in whole blood. The formation of ROS, therefore, seems to be a key factor in the development of nitrate tolerance.

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Send reprint requests to: Prof. Dr. Eberhard Bassenge, Institute of Applied Physiology, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany. E-mail: angphys@ruf.uni-freiburg.de