Nitrate tolerance does not increase production of peroxynitrite in the heart

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Nitrate tolerance does not increase production of peroxynitrite in the heart. Am J Physiol Heart Circ Physiol 283: H69–H76, 2002. First published February 28, 2002; 10.1152/ajpheart.00817.2001. —Clinical studies have suggested that long-term nitrate treatment does not improve and may even worsen cardiovascular mortality, and the possible role of nitrate tolerance has been suspected. Nitrate tolerance has been recently shown to increase vascular superoxide and peroxynitrite production leading to vascular dysfunction. Nevertheless, nitrates exert direct cardiac effects independent from their vascular actions. Therefore, we investigated whether in vivo nitroglycerin treatment leading to vascular nitrate tolerance increases cardiac formation of nitric oxide (NO), reactive oxygen species, and peroxynitrite, thereby leading to cardiac dysfunction. Nitrate tolerance increased bioavailability of NO in the heart without increasing formation of reactive oxygen species. Despite elevated myocardial NO, neither cardiac markers of peroxynitrite formation nor cardiac mechanical function were affected by nitroglycerin treatment. However, serum free nitrotyrosine, a marker for systemic peroxynitrite formation, was significantly elevated in nitroglycerin-treated animals. This is the first demonstration that, although the systemic effects of nitroglycerin may be deleterious due to enhancement of extracardiac peroxynitrite formation, nitroglycerin does not result in oxidative damage in the heart.

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On the other hand, many studies (2, 6, 7, 15, 16, 26, 34, 37) show that NO donor compounds including nitroglycerin exert direct cardiac protective effects. We (7) have shown that nitroglycerin exerts a direct myo-
cardiac anti-ischemic effect in isolated rat hearts that is independent of the vascular effects of the drug and it is not diminished even in the presence of vascular nitrate tolerance. Furthermore, nitroglycerin and other NO donors have been shown to be cardioprotective by inducing delayed (or late) preconditioning via a possible free radical-mediated trigger mechanism (2, 26, 37).

The chemical nature of reactive oxygen species including peroxynitrite makes them very difficult to measure. This technical difficulty may result in the controversial findings in the literature as to whether nitrate treatment leads to enhanced oxidative stress and subsequent organ dysfunction or exerts protective effects in the cardiovascular system. Therefore, in the present study we systematically analyzed whether nitroglycerin treatment, leading to the development of vascular nitrate tolerance, influences formation of cardiac reactive oxygen species and thus myocardial contractile performance. We therefore measured cardiac levels of NO, superoxide, their reaction product peroxynitrite, and hydroxyl radical, a major toxic derivative of peroxynitrite and superoxide. We also determined activities of major enzymatic sources for NO and superoxide, i.e., NO synthases and xanthine oxidoreductase (11), activity of the major antioxidant enzyme SOD, as well as several parameters of myocardial contractile function.

**METHODS**

All investigations conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985), and was approved by the ethics committee at University of Szeged.

Nitrate treatment and induction and verification of vascular nitrate tolerance. Male Wistar rats (300–360 g) were given nitroglycerin (100 mg/kg sc) or its vehicle lactose 3 times a day for 3 days (9 repetitive injections) to induce vascular tolerance to nitroglycerin, as described in earlier studies (7, 16, 35). All of these animals were used for isolated heart preparations and biochemical measurements on the fourth day, ~12 h after the last injection. Development of vascular tolerance to nitroglycerin was confirmed by testing aortic rings for isometric tension as described (16, 36). Rats were deeply anesthetized with diethylether and the thoracic aorta was then carefully removed and cleaned. Rings (4 mm long) were exposed to increasing concentrations of norepinephrine (10⁻⁴–10⁻⁵ M) until a maximum contractile response was obtained. After being washed, rings were precontracted with an EC₅₀ concentration of norepinephrine in addition to a resting tension of 20 mN. Rings were then exposed to cumulative concentrations of nitroglycerin in half-log increments.

In separate experiments, rats were injected subcutaneously with a single dose of 100 mg/kg nitroglycerin to test whether acute treatment with a single dose of nitrate without the development of tolerance affects systemic peroxynitrite formation. Rats were anesthetized with diethylether 1, 5, and 12 h after nitroglycerin treatment (n = 6 in each group). The thorax was then quickly opened and immediately before the heart was excised; ~0.5–1 ml blood was drawn from the vena cava inferior for determination of serum tyrosine levels. Hearts were used for isolated heart preparations.

Measurement of cardiac function in isolated rat hearts. Rats were anesthetized with diethylether and given 500 U/kg heparin intravenously. Hearts were then isolated and perfused at 37°C in a working mode with recirculating Krebs-Henseleit bicarbonate buffer containing (in mmol/l) 118 NaCl, 4.3 KCl, 2.4 CaCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, and 11.1 glucose, gassed with 95% O₂-5% CO₂, and supplemented with 0.3 mmol/l L-tyrosine (7, 13). Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the experiments. After a 10-min normoxic, normothermic perfusion, cardiac mechanical functional and hemodynamic parameters including heart rate, coronary flow, aortic flow, left ventricular developed pressure and its first derivatives (±dP/dt₉₀), and left ventricular end-diastolic pressure were monitored as described (5, 7, 13). Coronary effluent and cardiac tissue were sampled and frozen in liquid nitrogen for further biochemical measurements.

Measurement of cardiac NO, superoxide, and hydroxyl radical formation. In separate experiments, NO content of ventricular tissue was measured using electron spin resonance (ESR) spectroscopy after loading the heart with the NO-specific spin trap Fe²⁺-N-methyl-D-glucosamine-dithiocarbamate (MGD) as described (5, 7). The spin trap for NO was prepared freshly before each experiment. MGD (175 mg) and 50 mg FeSO₄ dissolved in distilled water (pH 7.4, volume 6 ml) was infused into the aortic cannula under Langendorff perfusion (constant pressure at 9.8 kPa) for 5 min at a rate of 1 ml/min to measure basal cardiac NO content. Tissue samples from the apex of the heart (~150 mg) were collected at the end of the infusion of Fe²⁺ (MGD)₂ and placed into quartz ESR tubes and frozen in liquid nitrogen. ESR spectra of NO-Fe²⁺(MGD)₂ adducts were recorded with a spectrometer (model ECS106, Bruker; Rheinstetten, Germany) (ESR parameters: X band, 100 kHz modulation frequency, 160 K temperature, 10 mW microwave power, 2.85 G modulation amplitude, 3,356 G central field) and analyzed for NO signal intensity as described (5, 6).

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced luminescence as described (13). Approximately 150 mg of the heart apex were placed in 1 ml of air-equilibrated Krebs-Henseleit solution containing 10 mmol/l HEPES-NaOH (pH 7.4) and either 250 or 5 μmol/l lucigenin (13). Chemiluminescence was measured in a liquid scintillation counter with single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the superoxide scavenger nitro blue tetrazolium (200 μmol/l). Nitro blue tetrazolium-inhibitable chemiluminescence was considered an index of cardiac superoxide generation.

To estimate cardiac hydroxyl radical generation, 2,5-di-hydroxybenzoic acid formation was determined by high-performance liquid chromatography (HPLC) from coronary effluents following Langendorff perfusion (9.8 kPa) of the hearts with Krebs-Henseleit buffer containing 1 mmol/l salicylic acid in separate experiments (40). Twenty microliters of filtered coronary effluents were injected to a LiChrospher 100 RP-18 cartridge (model 50734, Merck; Darmstadt, Germany). The HPLC system consisted of a Gilson 307 pump, an ESA model 5011 analytical cell, and an ESA Coulochem II electrochemical detector. 2,5-Dihydroxybenzoic acid was eluted by a buffer containing 50 mM sodium acetate, 50 mM citric acid, and 25% methanol (pH 2.5) at a flow rate of 0.5 ml/min. The detection potential was maintained at 750 mV. Peaks were integrated and compared with values obtained with serial concentrations of standard 2,5-dihydroxybenzoic acid.
Measurement of cardiac NO synthase, xanthine oxidoreductase, and SOD activities. To estimate endogenous enzymatic NO production, Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent NO synthase activities in ventricular homogenates were measured by the conversion of L-[14C]arginine to L-[14C]citrulline as previously described (13). Powdered frozen ventricular tissue was placed in four volumes of ice-cold homogenization buffer (composition given in Ref. 33) and homogenized with an Ultra-Turrax disperser using three strokes of 20-s duration each. The homogenate was centrifuged (1,000 g for 10 min) at 4°C and the supernatant was kept on ice for immediate assay of enzyme activities. Samples were incubated for 25 min at 37°C in the presence or absence of EGTA (1 mM) or EGTA plus N<sup>ω</sup>-monomethyl-L-arginine (1 mM) to determine the level of Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent NO synthase activities, respectively. NO synthase activities were expressed in picomoles per minute per milligram of protein.

Activity of xanthine oxidoreductase (xanthine oxidase and xanthine dehydrogenase), the major source of superoxide in rat hearts (11), was determined from ventricular homogenates by a fluorometric kinetic assay based on the conversion of xanthine to isoxanthopterine in the presence (total xanthine oxidoreductase activity) and absence (xanthine oxidase activity) of the electron acceptor methylene blue, as described by Beckman et al. (4). Ventricular homogenates were prepared as for the measurement of NO synthase activity.

Total activity of SOD was measured by a spectrophotometric assay using a kit (Randox Laboratories). Approximately 100 mg ventricular tissue was homogenized in 10 volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). SOD activity in homogenates was determined by the inhibition of formazan dye formation due to superoxide generated by xanthine and xanthine oxidase.

Measurement of markers of peroxynitrite. We measured dityrosine by spectrophotofluorometry in the perfusate and free nitrotyrosine by enzyme-linked immunosorbent assay (ELISA) in the perfusate and in the serum as markers of peroxynitrite generation (41, 42). ELISA (Cayman Chemical; Ann Arbor, MI) was conducted as described (13). Briefly, serum or perfusate samples were deproteinized by the addition of 4 volumes of ice-cold ethanol. After centrifugation, the supernatants were evaporated in nitrogen flow, dissolved in phosphate buffer, and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman’s reagent.

Proteins containing nitrotyrosine residues were used as intracellular markers of peroxynitrite formation in ventricular sections obtained from control, nitrate tolerant, and positive control hearts. Positive controls were obtained by incubation of authentic peroxynitrite (1 mmol/l final concentration) for 10 min into the perfused hearts. The ventricles were frozen in isopentane cooled in liquid nitrogen and 6-μm-thick fresh frozen sections were made by a cryostate. The sections were fixed in acetone (5 min) and, after being washed, they were blocked for 15 min in Tris-buffered saline containing 1% bovine serum albumin, 0.1% Na-azide, and 0.5% Tween 20. Slices were incubated with a primary anti-nitrotyrosine antibody (rabbit polyclonal IgG, Upstate Biotechnology; Lake Placid, NY) for 2 h at room temperature. Bovine anti-rabbit IgG was used as a secondary antibody followed by fluorescein isothiocyanate-conjugated streptavidine labeling (DAKO; Glostrup, Denmark) (19). The sections were examined with the use of confocal laser scanning microscopy (Leica DMRE; Heidelberg, Germany).

Statistical analysis. Data were expressed as means ± SE and analyzed with unpaired t-test. P < 0.05 was accepted as indicating a statistically significant difference compared with the control group.

RESULTS

Vascular nitrate tolerance and cardiac function. To verify the development of nitrate tolerance after in vivo repetitive nitrate treatment, isolated aortic rings obtained from control and nitroglycerin-treated (9 × 100 mg/kg) animals were precontracted with an EC<sub>50</sub> concentration of norepinephrine. The nitroglycerin concentrations needed to produce half-maximal relaxation were 0.09 ± 0.01 μmol/l in rings obtained from control animals versus 1.79 ± 0.28 μmol/l in nitroglycerin-treated group (P < 0.05, n = 7 in both groups). This 20-fold decrease in sensitivity to nitroglycerin observed in the present study is consistent with previous observations (6, 17, 35).

Parameters of cardiac performance in isolated working rat hearts, such as heart rate, aortic flow, coronary flow, left ventricular developed pressure, left ventricular end-diastolic pressure, and ±dP/dt<sub>max</sub> were not affected by in vivo repetitive nitroglycerin treatment compared with the control group (Table 1).

In nontolerant rats (1, 5, or 12 h after treatment with a single dose of nitroglycerin), neither cardiac function (data not shown) nor nitroglycerin sensitivity of aortic rings (12-h group: EC<sub>50</sub> of nitroglycerin 0.1 ± 0.02 μmol/l, n = 5, nonsignificant vs. control) were significantly changed. Nitroglycerin sensitivity of aortic rings was not further tested in the 1-h and 5-h groups.

Cardiac NO content and NO synthase. Cardiac NO content was significantly elevated in the nitroglycerin-tolerant group when measured by ESR spectroscopy after ex vivo spin trapping of NO in isolated hearts (Fig. 1A).

To test whether elevated NO derives from exogenous nitroglycerin or endogenous enzymatic sources, we measured cardiac activities of NO synthases. Endogenous enzymatic sources of NO, Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent activities were not changed in the ventricular tissue due to development of nitrate tolerance (Fig. 1B).

Table 1. Basal myocardial functional parameters in isolated working rat hearts

<table>
<thead>
<tr>
<th>Control</th>
<th>Nitrate</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>278 ± 7</td>
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<tr>
<td>Coronary flow, ml/min</td>
<td>23.6 ± 0.8</td>
</tr>
<tr>
<td>Aortic flow, ml/min</td>
<td>47.8 ± 1.8</td>
</tr>
<tr>
<td>LVDP, kPa</td>
<td>18.9 ± 0.6</td>
</tr>
<tr>
<td>±dP/dt&lt;sub&gt;max&lt;/sub&gt;, kPa/s</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td>-dP/dt&lt;sub&gt;max&lt;/sub&gt;, kPa/s</td>
<td>882 ± 43</td>
</tr>
<tr>
<td>405 ± 30</td>
<td>453 ± 39</td>
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</tbody>
</table>

Values are means ± SE; n = 7 isolated hearts per group. LVDP, left ventricular (LV) developed pressure; LVEDP, LV end-diastolic pressure; ±dP/dt<sub>max</sub>, first derivatives of LV pressure. Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the perfusion.
Cardiac superoxide. To test whether nitrate tolerance increases cardiac superoxide generation, we performed lucigenin-enhanced chemiluminescence assay in fresh cardiac tissue. Cardiac superoxide generation remained unchanged due to repetitive nitroglycerin treatment compared with the control group (Fig. 2A). To further study markers of peroxynitrite, we performed nitrotyrosine immunostaining in ventricular slices. Nitrotyrosine immunostaining detected by confocal laser scanning microscopy was negligible in hearts of both control and nitrate-tolerant groups, whereas an intensive nitrotyrosine staining was observed in positive control hearts, which were treated with authentic peroxynitrite (images not shown).

In hearts of nontolerant rats (1, 5, and 12 h after treatment with a single dose of nitroglycerin), formation of dityrosine in the coronary perfusate was not significantly different compared with controls (data not shown).

Cardiac hydroxyl radical. We also tested whether the formation of hydroxyl radical, a toxic derivative of peroxynitrite and superoxide, is changed in the heart as a result of development of nitroglycerin tolerance. Isolated hearts from control and nitroglycerin-tolerant hearts were perfused with a buffer supplemented with 0.3 mmol/l L-tyrosine. Formation of dityrosine in the coronary effluent markers of peroxynitrite generation did not differ significantly between the two groups (Fig. 3A). To further study markers of peroxynitrite, we performed nitrotyrosine immunostaining in ventricular tissue of control and nitroglycerin-treated rats. *P < 0.05 vs. control (n = 4–7 in each group).

Cardiac peroxynitrite. To estimate the peroxynitrite-generating capacity of the heart, hearts were isolated from nitroglycerin-tolerant and control rats and were perfused with a buffer supplemented with 0.3 mmol/l L-tyrosine. Formation of dityrosine and nitrotyrosine in the coronary perfusate was not significantly different compared with controls (data not shown).
rats were perfused in the presence of 1 mmol/l salicylic acid. Formation of 2,5-dihydroxybenzoic acid from salicylate in the perfusate due to hydroxyl radical activity was not significantly different (Fig. 3B).

**DISCUSSION**

This is the first demonstration that repetitive nitrate treatment, which leads to the development of vascular nitrate tolerance, results in a sustained (>12 h) increase in serum nitrotyrosine. This, however, does not enhance production of reactive oxygen species including peroxynitrite in the heart. We have also shown here that nitroglycerin treatment increases the bioavailability of NO in the heart even when nitrate tolerance develops. We further observed that a single, acute treatment with nitroglycerin, which does not lead to nitrate tolerance, results in a transient (<5 h) increase in serum nitrotyrosine.

Many previous studies (9, 10, 22, 24) have suggested that nitrate treatment, especially when it results in the development of nitrate tolerance, is associated with increased free-radical generation in the vasculature. For instance, Münzel et al. (22) showed a twofold increase in total superoxide production and a significant decrease in vascular SOD activity in functionally damaged nitrate tolerant aortic rings. However, no studies have examined the influence of nitrate treatment and nitrate tolerance on cardiac generation of reactive oxygen species. In contrast to earlier studies on the vascular effects of nitrates and nitrate tolerance, here we have shown that the generation of cardiac superoxide, hydroxyl radical, and peroxynitrite, enzymatic superoxide synthesis by xanthine oxidoreductase and its detoxification by SOD, as well as the nonenzymatic breakdown of superoxide and peroxynitrite yielding hydroxyl radicals, were not changed in the heart after in vivo nitroglycerin treatment resulting in nitrate tolerance. Furthermore, myocardial mechanical function was not altered either as a result of vascular nitrate tolerance. This further supports the conclusion that superoxide and peroxynitrite genera-

![Fig. 3. Dityrosine and nitrotyrosine, markers of peroxynitrite (A), and 2,5-dihydroxybenzoic acid (DHBA), marker of hydroxyl radical (B), formation in perfusate from hearts perfused with 0.3 mmol/l L-tyrosine or 1 mmol/l salicylic acid in control and in vivo nitroglycerin-treated groups, respectively (n = 5–7 in each group).](image-url)

![Fig. 4. Free nitrotyrosine concentration in deproteinized sera of control, nitroglycerin (GTN)-tolerant, and nontolerant nitroglycerin-treated rats. Serum samples were taken 12 h after repetitive nitroglycerin treatment in the tolerant group and 1, 5, or 12 h after single nitroglycerin treatment. *P < 0.05 vs. control, n = 5–7 in each groups.](image-url)
tion in the myocardium and in the coronary vasculature were unaffected by nitroglycerin tolerance. Our data show that nitrate treatment does not enhance oxidative stress in the heart. It has been suggested that NO donors induce delayed preconditioning through the stimulation of free radical generation in the rabbit heart (26, 37). However, in those studies, a pharmacological approach (i.e., a single concentration of mercaptopropionyl glycine as a free radical scavenger) was used to investigate the involvement of free radicals in the cardioprotective effect of NO donors, and this may be less rigorous than biochemical measurements of several free radicals and their enzymatic production and breakdown as seen in the present study.

Although impairment of nitroglycerin bioconversion to NO and a decrease in NO bioavailability have been shown ex vivo in vessels from nitrate tolerant animals (23) and patients (31), these findings were refuted by others (20). Here we have demonstrated that cardiac NO accumulated after repetitive nitroglycerin treatment in vivo. This shows that nitrate treatment increases the bioavailability of NO in the heart even in the state of vascular nitrate tolerance. Because the cardiac activities of NO synthases did not change, the enhanced NO level must derive from nitroglycerin accumulated in the myocardium due to exogenous nitroglycerin treatment. This is in accordance with the observation by Torfgård et al. (39) showing the accumulation of nitroglycerin in cardiac tissue after long-term nitroglycerin treatment.

Increased cardiac NO due to nitrate treatment could result in enhanced formation of peroxynitrite in the present study. However, recent studies (18) suggest that peroxynitrite generation is mainly dependent on superoxide concentrations. Accordingly, the level of superoxide did not change after nitrate treatment in our experiments. Despite the increased level of cardiac NO, markers of peroxynitrite formation in the coronary perfusate (dityrosine and free nitrotyrosine) as well as in cardiac tissue (nitration of protein tyrosine residues) were not significantly affected by in vivo nitroglycerin treatment resulting in vascular nitrate tolerance. This shows that the generation of peroxynitrite either in the coronary vasculature or in the myocardium is not affected by nitrate treatment. Although enhanced vascular peroxynitrite formation has been reported in nitrate tolerance (9, 21), this was accompanied by increased superoxide generation in the vasculature. Nevertheless, in the heart (including coronary vasculature), we have not found any evidence of elevated superoxide or peroxynitrite formation. However, we did observe a significant increase in serum free nitrotyrosine concentration in nitrate-tolerant rats 12 h after the last nitroglycerin injection. This shows a sustained, increased production of peroxynitrite in extracardiac tissues, possibly in the extracardiac vasculature. This is in accordance with previous findings (9, 10, 21, 24) showing increased generation of reactive oxygen species in vascular tissue due to nitrate tolerance. To test whether increased generation of peroxynitrite is a general phenomenon of nitrate treatment or whether it is specific for nitrate tolerance, we also measured serum nitrotyrosine after a single dose of nitroglycerin, which does not lead to tolerance development. Increase in serum nitrotyrosine was found 1 h after administration of a single dose of nitroglycerin. However, this increase was not observed 5 and 12 h after treatment. This finding suggests that nitroglycerin treatment in the absence of nitrate tolerance leads to a transient increase in extracardiac peroxynitrite formation. This is in accordance with findings of Dikalov et al. (9), showing that the biotransformation of nitroglycerin is accompanied by superoxide formation in endothelial and smooth muscle cells in the absence of nitrate tolerance. The reason why nitrate tolerance extends the time frame of peroxynitrite formation is not clear; however, increased accumulation of tissue nitroglycerin is a plausible explanation (39).

Unfortunately, the endogenous formation of peroxynitrite cannot be directly detected in biological systems. Therefore, dityrosine and nitrotyrosine, products of the reaction between peroxynitrite and tyrosine, are the most often used markers of peroxynitrite, despite some criticism. Some in vitro biochemical data suggested that peroxynitrite does not cause tyrosine nitration at physiological pH (28), but this has been recently refuted (18, 30). Moreover, peroxynitrite-mediated nitration of tyrosine residues was proposed to be the most likely mechanism of nitrotyrosine formation in vivo (30). Myeloperoxidase activity in the presence of relatively high concentrations of nitrite may lead to nitrotyrosine formation (12). However, both myeloperoxidase activity and nitrite concentration were negligible because granulocyte-free, crystalloid-perfused hearts were used to test cardiac peroxynitrite formation in this study (13). In addition, concurrent measurement of nitrotyrosine and dityrosine decreases the possibility of false estimation of peroxynitrite generation (29). The possibility of enhanced myeloperoxidase activity in extracardiac tissues contributing to serum nitrotyrosine formation, however, cannot be excluded.

Clinical trials (17, 25, 38) have suggested that nitrate therapy may worsen the prognosis and survival in ischemic heart disease, although further studies are required to confirm these findings. Animal studies (9, 10, 22, 24) showed enhanced generation of oxygen free radicals and impaired vascular function associated with nitrate treatment. Moreover, vascular nitrate tolerance has been reported (36) to interfere with the cardioprotective action of classic preconditioning in rabbits in vivo. These observations may lead to a declining confidence in the use of nitrate therapy in patients. However, here we have provided several lines of evidence showing that in vivo nitroglycerin treatment leading to vascular nitrate tolerance does not result in oxidant stress and subsequent functional damage in cardiac tissue. Furthermore, we (7, 16) have shown previously that nitroglycerin exerts a direct anti-ischemic effect on the myocardium, which does not diminish even after the development of vascular nitrate tolerance. These findings may support the safety
of nitrate therapy even in the state of nitrate tolerance. However, nitroglycerin treatment, especially when leading to vascular nitrate tolerance, induces an increase in serum nitrotyrosine, a marker for systemic peroxynitrite formation. This shows that nitrate treatment may enhance oxidative stress in extracardiac tissues. The pathological significance of extracardiac oxidative stress due to nitrate treatment requires further investigation. Nevertheless, our results that nitroglycerin may induce oxidative stress in extracardiac tissue but not in the heart suggest that either cardio-selective nitrates, nitrate compounds with additional antioxidant activity, or combination therapy with nitrates and antioxidants may increase the safety of organic nitrates in the chronic treatment of ischemic heart disease. Combined treatment with nitroglycerin and the antioxidant vitamin C has been used successfully to attenuate the development of nitrate tolerance in animal studies and humans (3, 8). Our present study opens a new perspective for “nitrate-antioxidant” treatment in ischemic heart disease.

In conclusion, in vivo nitroglycerin treatment, which results in vascular nitrate tolerance, may enhance oxidative stress in extracardiac tissues, but does not increase formation of oxygen free radicals and peroxynitrite in the heart. Moreover, it increases the bioavailability of cardiac NO. These findings support the safety of nitrate therapy even in the state of vascular nitrate tolerance although the importance and possible risk of nitrate-induced increase in extracardiac peroxynitrite generation requires further investigation. Our results provide a rationale for the development of cardioselective nitrates and promote further clinical studies on combination therapy with nitrates and antioxidants.

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