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Pharmacology

Differential Effects of Pentaerythritol Tetranitrate and Nitroglycerin on the Development of Tolerance and Evidence of Lipid Peroxidation: A Human In Vivo Study

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OBJECTIVES	We investigated the development of nitrate tolerance after continuous exposure to nitroglycerin (GTN) as compared with pentaerythritol tetranitrate (PETN) in humans.
BACKGROUND	Sustained therapy with GTN causes tolerance and has been associated with increased production of free oxygen radicals by the endothelium. Pentaerythritol tetranitrate is an organic nitrate that has been used in the therapy of angina. There have been no investigations concerning the development of tolerance to PETN in humans. Animal investigations suggested that continuous therapy with PETN does not cause increased free radical production or hemodynamic tolerance.
METHODS	We randomized 30 healthy volunteers to continuous GTN (0.6 mg/h/24 h), long-acting PETN (60 mg orally three times a day) or no treatment (control group) for seven days. We studied systemic blood pressure responses and venous volume responses to GTN with strain-gauge plethysmography. The levels of cytotoxic aldehydes and isoprostanes were measured as markers of free radical-mediated lipid peroxidation.
RESULTS	Tolerance, as demonstrated by blood pressure and forearm plethysmography, developed in the GTN group and was absent in the PETN group ($p < 0.05$). Therapy with GTN was associated with a significant increase in plasma markers of lipid peroxidation. This response was not observed in those treated with PETN (isoprostanes: control: 38 ± 5; GTN: 59 ± 6; PETN: 38 ± 3 μg/ml; $p < 0.005$).
CONCLUSIONS	Treatment with PETN does not cause tolerance and is not associated with evidence of increased free radical production. (J Am Coll Cardiol 2001;38:854–9) © 2001 by the American College of Cardiology

Organic nitrates have been used for more than 100 years in the treatment of myocardial ischemia and congestive heart failure, but the development of tolerance still represents a limitation to their use. Tolerance can be defined as loss of nitrate effects on symptomatic benefit or hemodynamic parameters after continuous exposure. There is clear evidence that tolerance develops to the effects of nitroglycerin (GTN) as well as with isosorbide mononitrate and dinitrate when they are administered in a fashion that leads to sustained therapeutic plasma concentrations.

Pentaerythritol tetranitrate (PETN) is an organic nitrate that has been used extensively in Europe for many years. Although this compound has been available in North America, its clinical application in that market has been

limited. Investigations concerning the clinical efficacy of this nitrate drug have been limited, and there have been no human studies addressing the issue of tolerance during therapy with this compound. Interestingly, recent animal data suggest that PETN might have antiatherosclerotic effects (1–3) and that continuous exposure to this compound does not cause tolerance (4). Furthermore, animal experiments have suggested that PETN therapy is not associated with increased free radical production by the endothelium (3,5,6), a phenomenon that has now been repeatedly demonstrated with GTN (7–9). This study was designed to investigate the hemodynamic and biochemical effects of continuous therapy with PETN as compared with transdermal GTN or no treatment.

METHODS

Study population. Thirty healthy male nonsmoking volunteers (age range 19 to 39 years) were included in the study. Subjects were asked to abstain from medications or supplemental vitamins during the study period and from alcohol or caffeine-containing beverages for at least 12 h before each study visit. A 3-h fasting period preceded visits.

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Abbreviations and Acronyms

ANOVA = analysis of variance
ES/MS = on-line electrospray mass spectrometry
FBV   = forearm venous blood volume
GTN   = nitroglycerin
HPLC   = high-pressure liquid chromatography
NO   = nitric oxide
PETN   = pentaerythritol tetranitrate
ROS   = reactive oxygen species

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The study protocol was approved by the University of Toronto Human Subjects Review Committee, and written informed consent was obtained in all cases.

Study protocol. VISIT 1. On visit 1, after subjects were screened for admission into the study, standing arterial blood pressure and heart rate were measured using an automatic calibrated sphygmomanometer (Critikon Company LLC, Tampa, Florida). All results represented the average of three different measurements taken at 1-min intervals. Forearm venous blood volume (FBV) and responses to sublingual GTN (0.6 mg) were then recorded using forearm venous occlusion mercury-in-silastic strain-gauge plethysmography. The details of this technique are described in the following text.

VISIT 2. One week later, subjects arrived at our laboratory at the same time as for visit 1. Standing blood pressure and heat rate measurements were repeated. The subjects were then randomized in an investigator-blind, parallel design to receive transdermal GTN 0.6 mg/h (GTN group), PETN 60 mg orally every 8 h (PETN group) or no treatment (control group). Nitroglycerin patches were obtained from Novartis Pharmaceuticals (Dorval, Québec, Canada); PETN pills were obtained from Dexo S.A. (Nanterre, France). The first dose was administered in the laboratory, and repeat blood pressure and heart rate measurements were taken 3 h after the initial dose in order to measure the response to acute administration. Subjects randomized to nitrates were instructed by the randomizing nurse to wear the patch continuously, changing it every morning at the same time. Subjects were instructed not to reveal their treatment allocation to the investigator. The randomizing nurse did not take part in any other aspect of study procedures or data analysis. Subjects randomized to PETN were asked to take 60 mg PETN orally every 8 h for the following six days. This dosing regimen of PETN was chosen in order to prevent a nitrate-free interval. The half-life of PETN and its metabolites in humans is reported to be 8 h (10), and the formulation used was a long-acting preparation designed to be given twice daily. Subjects were then given a seven-day supply of the assigned study drug and discharged home.

VISIT 3. After seven days of continuous treatment, subjects returned to the laboratory, and blood samples were ob-

tained. Blood pressure and heart rate were taken again with the same procedure, and FBV was measured as in visit 1.

Visits were conducted at mid-day in order to ensure that the subjects had received their morning dose of the study drug.

Measurement of FBV. Forearm venous blood volume at baseline and in response to sublingual GTN was measured using strain-gauge venous occlusion plethysmography as previously reported (11). Briefly, hand circulation was excluded by inflating a wrist cuff to 200 mm Hg during measurement periods. The upper arm cuff was inflated to 30 mm Hg and was kept inflated until a plateau was reached, representing the baseline FBV. Once a baseline measure had been taken, 0.6 mg sublingual GTN were administered, and FBV was recorded for at least 10 min until a new plateau had been reached. Forearm venous blood volume was measured in ml/100 ml of forearm tissue, and the change in FBV in response to acute administration of 0.6 mg sublingual GTN was expressed as percent from baseline value. Forearm plethysmographic measurements were made using a Hokanson EC4 Plethysmograph (D. E. Hokanson Inc., Bellevue, Washington) and recorded on a multichannel recorder (Gould 5900 Card Cage, Gould Instrument Systems, Inc., Valley View, Ohio). All measurements and visits were performed in a quiet, temperature and humidity-controlled laboratory.

Markers of oxidative stress. The levels of cytotoxic aldehydes and isoprostanes were determined as previously described (12). Total lipid extract of plasma was prepared by the method of Folch et al. (13). The total lipid extract was chromatographed on a normal phase 5-μm Spherisorb high-pressure liquid chromatography (HPLC) column (250 × 4.6 mm inner diameter, Alltech Associates, Deerfield, Illinois). The column was installed into a Hewlett-Packard (Andover, Massachusetts) model 1090 liquid chromatograph and eluted with a linear gradient of 100% solvent A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by volume) to 100% solvent B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by volume) in 14 min and then at 100% solvent B for 10 min (14). The flow was set at 1 ml/min. The peaks were monitored by on-line electrospray mass spectrometry (ES/MS). Normal phase HPLC with ES/MS was performed by splitting the HPLC flow by 1/50, resulting in 20 μl/ml being admitted to a Hewlett-Packard model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface (HP 59987A) (15). Arachidoyl phosphatidylcholine was added as internal standard. This method allowed us to measure the levels of four cytotoxic aldehydes (16:0-C_5 aldehyde phosphatidylcholine, 18:0-C_5 aldehyde phosphatidylcholine, 16:0-C_9 aldehyde phosphatidylcholine and 18:0-C_9 aldehyde phosphatidylcholine) and three isoprostanes (16:0-E_2/D_2 isoprostane phosphatidylcholine, 16:0-F_2 isoprostane phosphatidylcholine and 18:0-E_2/D_2 isoprostane phosphatidylcholine). All of these chemical compounds represent the result of nonenzymatic lipid

Table 1. Blood Pressure and Heart Rate Responses to Study Drug in the Three Groups

	Control	GTN	PETN
Blood pressure			
Before randomization	116 ± 2	115 ± 3	116 ± 2
After acute administration	114 ± 2	104 ± 3*	107 ± 3*
After chronic administration	116 ± 3	113 ± 3	109 ± 3*
Heart rate			
Before randomization	82 ± 2	76 ± 4	74 ± 4
After acute administration	79 ± 3	88 ± 6*	82 ± 3*
After chronic administration	82 ± 3	80 ± 5	80 ± 3*

*$p < 0.05$ compared with before randomization, one-way analysis of variance.
GTN = nitroglycerin; PETN = pentaerythritol tetranitrate.

Table 2. Forearm Blood Volume Results: Baseline Values and Absolute Increases in Response to Sublingual GTN

	Control	GTN	PETN
Before randomization			
Baseline	2.57 ± 0.34	2.70 ± 0.29	2.39 ± 0.24
Delta after sublingual GTN	0.60 ± 0.09	0.79 ± 0.17	0.71 ± 0.13
After randomization			
Baseline	1.65 ± 0.21	2.44 ± 0.29	2.26 ± 0.30
Delta after sublingual GTN	0.54 ± 0.08	0.43 ± 0.06*	0.68 ± 0.10†

*$p < 0.05$ compared with before randomization (paired t test); †$p < 0.05$ compared with GTN group (analysis of covariance).
GTN = nitroglycerin; PETN = pentaerythritol tetranitrate.

peroxidation and, as such, are felt to be specific markers of free radical-mediated lipid injury (16–18).

Statistical analysis. All variables were normally distributed (Shapiro-Wilk). Changes in blood pressure and heart rate within groups were evaluated using a one-way analysis of variance (ANOVA) for repeated measures. The Bonferroni test was used for multiple comparisons. Differences in venous volume responses between study days within each group were compared using a paired t test. Between group differences in the response of venous blood volume were compared using analysis of covariance. Oxidative stress data were analyzed with a one-way ANOVA for nonrepeated measures. All analyses were performed in SAS version 8.1 (SAS Institute Inc., Cary, North Carolina). All differences were considered to be significant when $p < 0.05$. All results are expressed as mean ± SE.

RESULTS

Blood pressure and heart rate responses. All results are presented in Table 1. Standing heart rate and systolic blood pressure did not differ among groups at baseline. Blood pressure did not change significantly in the control group throughout the study.

Transdermal GTN caused a significant fall in standing systolic blood pressure 3 h after patch application. On visit 3, after sustained therapy with transdermal GTN, standing systolic blood pressure had returned to baseline values, indicating the development of tolerance ($p < 0.05$). In the PETN group, a significant decrease in systolic blood pressure was observed 3 h after acute administration ($p < 0.05$), and this decrease was still present after continuous therapy ($p < 0.05$ vs. before randomization, $p =$ NS vs. after acute administration).

Similar results were obtained with heart rate. Heart rate increased 3 h after the first administration of GTN and returned to lower levels after one week of treatment ($p < 0.05$). In the PETN group, heart rate increased after acute administration and remained higher after continuous exposure ($p < 0.05$). There was no change in the control group.

FBV responses. Venous blood volume responses and the results of the statistical analysis are reported in Table 2 and Figure 1. Venous blood volume (ml/100 ml of forearm volume) increased similarly among groups before random-

ization in response to sublingual GTN. In the control group, the increase in FBV in response to sublingual GTN was similar at each visit. Continuous treatment with transdermal GTN caused a significant decrease in both absolute and percent increase in volume in response to sublingual GTN compared with before randomization ($p < 0.05$). In the PETN group, after continuous therapy, the response to sublingual GTN was similar to that obtained on visit 1 ($p =$ NS). The analysis of covariance revealed that the differences in absolute, delta and percent changes in blood volume between groups on visit 3 were significant ($p < 0.05$).

Markers of oxidative stress. Plasma levels of cytotoxic aldehydes are expressed individually and as the sum of the four compounds measured. After the treatment period, in the GTN group the plasma concentrations of the aldehydes were significantly higher than those in the control and PETN groups. Furthermore, there was no difference observed in the plasma concentration of these compounds when values in the control and PETN groups were compared. ($p < 0.02$; Table 3, Fig. 2). Similar results were obtained with isoprostanes ($p < 0.005$, Table 3, Fig. 3). Individual values for each compound are also reported in Table 3.
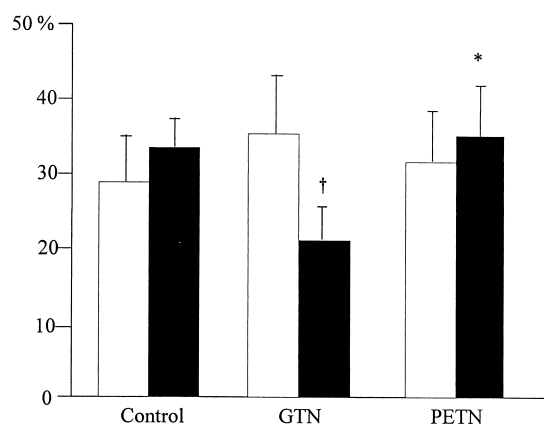

Figure 1. Percent change in forearm blood volume in response to 0.6 mg sublingual nitroglycerin (GTN) in the three groups. *$p < 0.05$ compared with GTN group, analysis of covariance. †$p < 0.05$ compared with before randomization, paired t test; $p =$ NS before randomization versus after chronic administration for control and pentaerythritol tetranitrate (PETN) groups, paired t test. **Open bar** = before randomization; **closed bar** = after chronic administration.

Table 3. Aldehydes and Isoprostanes: Individual Levels and Totals of the Compounds Measured

	$16:0\text{-}C_5$ Ald PC	$18:0\text{-}C_5$ Ald PC	$16:0\text{-}C_9$ Ald PC	$18:0\text{-}C_9$ Ald PC	Total
Aldehydes					
Control	2.6 ± 0.2	1.9 ± 0.5	1.0 ± 0.2	1.2 ± 0.2	6.7 ± 0.9
GTN	3.3 ± 0.4	$2.7 \pm 0.5^*$	$2.0 \pm 0.4^*$	$2.7 \pm 0.5^*$	$10.7 \pm 0.8†$
PETN	3.7 ± 1.2	0.8 ± 1.2	0.9 ± 0.2	1.0 ± 0.4	6.4 ± 1.5

	$16:0\text{-}F_2$ IsoP PC	$16:0\text{-}E_2/D_2$ IsoP PC	$18:0\text{-}F_2$ IsoP PC	Total
Isoprostanes				
Control	10.2 ± 1.2	18.1 ± 3.3	9.7 ± 0.7	38.0 ± 4.5
GTN	$19.6 \pm 2.8^*$	$25.8 \pm 3.3^*$	$13.7 \pm 2.4^*$	$59.1 \pm 5.9‡$
PETN	11.4 ± 1.6	19.1 ± 1.7	7.8 ± 0.6	38.3 ± 2.9

$^*p < 0.05$; $†p < 0.02$; $‡p < 0.005$ compared with control and PETN, one-way analysis of variance.
Ald PC = aldehyde phosphatidylcholine; GTN = nitroglycerin; IsoP PC = isoprostane phosphatidylcholine; PETN = pentaerythritol tetranitrate. All values in $\mu g/ml$.

DISCUSSION

Evidence for a role of oxidative stress in nitrate tolerance.
Nitrate tolerance remains a major limiting factor in the treatment of congestive heart failure and coronary artery disease. The mechanism of nitrate tolerance remains elusive and is probably multifactorial. Traditionally, reduced bioconversion of nitrates was implicated (19), although later animal experiments did not support that view (20). Most recently, Sage et al. (21), using human vascular tissue, proposed that reduced bioconversion could be involved, especially in the venous circulation. Although a unifying hypothesis of nitrate tolerance has remained elusive over the past several years, a great deal of research has focused on the role of the endothelium in the development of nitrate tolerance. A number of reports have suggested that exposure to GTN is associated with increased production of reactive oxygen species (ROS) by the endothelium and that this response may play a role in the development of tolerance. Several reports have confirmed that GTN therapy leads to endothelial dysfunction and increased production of superoxide anion (7–9). An increased production of superoxide anion has also been demonstrated in the presence of all risk factors for coronary artery disease, and its negative effects on vasomotion have been extensively investigated (22).

Interestingly, Münzel et al. (8) demonstrated that the endothelium plays an obligatory role in the development of tolerance, potentially through the synthesis of oxygen free radicals. It has been demonstrated that superoxide anion can bind to nitric oxide (NO), which mediates the activity of nitrates, thus reducing its bioavailability. The end product of the reaction is peroxynitrite (23), which, at doses in the micromolar range, induces vasoconstriction (24) and inhibits the uptake of levo-arginine and the activity of endothelial NO synthase (25,26). Thus, it appears that the bioavailability of NO, both endogenous and delivered by GTN, might depend on the GTN-induced local formation of superoxide anion.

Results from ex vivo animal studies demonstrate that PETN does not induce vascular tolerance (4) and does not cause an increase in vascular production of ROS as was seen with GTN therapy. One in vitro study provides a potential mechanism, where PETN induced the production of ferritin, a potent antioxidant protein, in endothelial cells (27).
Summary of results. The results of this study confirm that PETN, as compared with GTN, does not induce hemodynamic tolerance or evidence of oxidative stress in humans in vivo. To the best of our knowledge, this is the first human study to examine the impact of sustained PETN therapy on the development of tolerance.
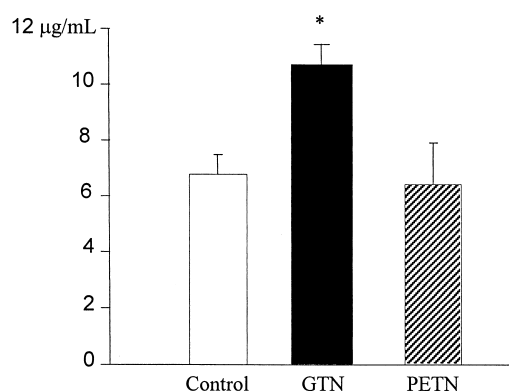

Figure 2. Cytotoxic aldehydes (total of the four compounds measured) after continuous treatment in the three groups. $^*p < 0.02$ compared with control and pentaerythritol tetranitrate (PETN), analysis of variance. GTN = nitroglycerin.
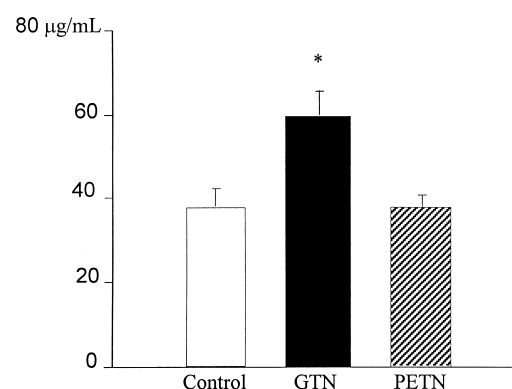

Figure 3. Isoprostanes (total of the three compounds measured) after continuous treatment. $^*p < 0.005$ compared with control and pentaerythritol tetranitrate (PETN), analysis of variance. GTN = nitroglycerin.

Blood pressure measurements demonstrate that, after continuous treatment, PETN continues to have a significant hemodynamic effect, while GTN does not. This indicates that tolerance does not develop to the systemic hemodynamic effects of PETN. The venous volume responses demonstrate that cross-tolerance to GTN, given acutely, does not develop during PETN therapy.

Our biochemical data show that GTN induces a significantly higher production of ROS, while, after PETN treatment, the levels of oxidized lipids do not differ from normal.

Importance of cytotoxic aldehydes and isoprostanes. Isoprostanes and cytotoxic aldehydes are products of oxidative modification of membrane-bound or low-density lipoprotein-bound phospholipids through a free radical catalyzed reaction (16–18). Although the importance of these compounds is still not completely clear, it has been suggested that they may have adverse effects on endothelial and smooth muscle cell function (28). Increased levels of isoprostanes have been detected in the presence of many risk factors for cardiovascular disease, including smoking, diabetes mellitus and hypercholesterolemia (29–31). Furthermore, some isoprostane species have been shown to have potent vasoconstrictor effects (32) as well as significant antinatriuretic effects (33), which might contribute to the phenomenon of plasma expansion that follows continuous GTN exposure and to tolerance itself. The importance of free radical production in the development of tolerance is supported by the results of studies in which antioxidant supplementation prevented tolerance or reduced free radical production during nitrate therapy (6,34,35). The vasoconstrictor activities of isoprostanes might also be implicated in the development of tolerance and supersensitivity to vasoconstrictor stimuli (36) and in the pathophysiology of rebound phenomena (37). Furthermore, it has been hypothesized that isoprostanes, due to the stiffness of their molecular structure, may significantly affect the fluidity and the integrity of cell membrane bilayers (18).

Previous findings from our laboratory argued against a role for oxidative stress in the pathogenesis of nitrate tolerance (11). In this report, the levels of a short chain isoprostane, 8-iso-PGF$_{2\alpha}$, were not modified by GTN treatment. We believe that our present observations may be more reliable. Long-chain isoprostanes are less water soluble and more stable than short-chain species and, thus, may be more sensitive as markers of lipid peroxidation. In our previous report (11), tolerance to GTN was not restored with the acute administration of vitamin C. We do not believe that this is entirely inconsistent with the present findings. If the mechanism underlying nitrate tolerance is mediated by oxidative stress, antioxidant supplementation might be able to prevent this process while being unable to reverse it.

Study limitations. In this study, the effect of an oral formulation of PETN was compared with that of transdermal GTN. Indeed, our study represents a follow-up of other animal studies comparing GTN to PETN (1–4). Nitroglycerin given orally is poorly bioavailable, and there is essentially no evidence concerning the development of tolerance or even the acute clinical effectiveness of GTN in this formulation. On the other side, PETN is not available as a transdermal preparation. A formulation and dosage regimen that was designed to maintain therapeutic plasma PETN concentrations throughout the 24-h period was chosen. Future studies are now needed to compare the effects of PETN with those of other orally available nitrates, such as isosorbide dinitrate and mononitrate.

Importance of the present findings. We believe our findings to have potential, direct clinical relevance. First, this study demonstrates the absence of tolerance to PETN in a human model. This finding warrants a reconsideration of the utility of PETN in clinical practice, and further clinical studies investigating the effects of PETN versus GTN in the setting of heart failure or angina are now required. Second, we demonstrated that the appearance of tolerance to GTN is associated with the production of ROS that are able to oxidize membrane lipids. These findings have potential implications concerning the long-term effects of therapy with GTN.

Understanding the nature of the biochemical responses to nitrates at the level of the endothelium appears to be providing clear insights into the mechanism of tolerance. A series of investigations now suggests that GTN causes abnormalities in NOS function that lead to increased ROS production. Further studies are required to investigate the mechanisms of these changes and to develop strategies to counteract them. Our observations with PETN suggest that there are important nitrate-specific differences in these responses.

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