Pentaerythrityl Tetranitrate and Nitroglycerin, but not Isosorbide Mononitrate, Prevent Endothelial Dysfunction Induced by Ischemia and Reperfusion

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Background—Short term exposure to nitroglycerin (GTN) has protective properties that are similar to ischemic preconditioning. Whether other organic nitrates such as pentaerythrityl tetranitrate (PETN) and isosorbide mononitrate (ISMN) have similar protective effects has not been explored.

Methods and Results—In a randomized, parallel, double blind, controlled trial, 37 healthy young volunteers received no therapy (n=10), transdermal GTN 1.2 mg for 2 hours (n=9), PETN 80 mg (n=9), or ISMN 40 mg (n=9). Twenty-four hours later, endothelium-dependent flow-mediated vasodilation (FMD) was measured before and after local exposure to ischemia and reperfusion (IR). In the no therapy group, IR blunted FMD (FMD after IR: 1.9±0.6%, P<0.05), an effect that was prevented by GTN (FMD after IR: 5.3±1.4%, P<0.05 compared with no therapy). PETN had the same protective effect (FMD after IR: 8.1±1.3%, P<0.05 compared with no therapy), whereas ISMN had no significant pharmacological preconditioning effect (FMD after-IR: 3.6±0.8%, P=ns compared with no therapy). While it blocked the effect of GTN, Vitamin C (n=8) did not modify PETN preconditioning (FMD after IR: 6.3±0.9%, P=ns compared with before IR), showing that this phenomenon is not mediated by oxygen free radical production. In an effort to identify the mechanism of PETN preconditioning, isolated human endothelial cells were incubated with PETN, GTN, or ISMN. Only PETN induced expression of the genes encoding for heme oxygenase and ferritin, which have been involved in ischemic and pharmacological preconditioning.

Conclusions—We show important differences among organic nitrates in their capacity to prevent IR-induced endothelial dysfunction. GTN and PETN, but not ISMN, have this preconditioning effect. The potential clinical implications of these data warrant further investigation. (Arterioscler Thromb Vasc Biol. 2007;27:1955-1959.)

Key Words: organic nitrates ■ nitric oxide ■ preconditioning ■ oxygen-derived free radicals ■ ischemia reperfusion injury

For more than a century, organic nitrates have been widely prescribed for the treatment of stable angina, acute coronary syndromes, and congestive heart failure. Currently, the most commonly prescribed nitrates include nitroglycerin (GTN) and isosorbides (particularly isosorbide-5-mononitrate, ISMN). Another organic nitrate, pentaerythrityl tetranitrate (PETN), although still in use in some European countries, is no longer marketed in North America.

Although organic nitrates continue to be widely prescribed for angina and congestive heart failure, their therapeutic utility is recognized to be problematic because the development of tolerance limits their clinical efficacy. Furthermore, multiple lines of evidence suggest that sustained administration of organic nitrates is associated with adverse effects on vascular function which appear to be mediated by an increase in nitrate-induced oxygen free radical bioavailability. Importantly, recent animal and human studies have documented previously unexpected, nonhemodynamic, protective effects of GTN, whereby a short (2 to 4 hours) exposure to GTN is associated with a reduced myocardial sensitivity to ischemia and reperfusion (IR) injury. The mechanism of this effect resides in the induction of a complex cascade of mediators that regulate intracellular adaptive protective factors and ultimately lead to the increased expression of protective molecules and enzymes such as the superoxide dismutase, heme-oxygenase, and the endogenous calcitonin gene-related peptide. Although exact nature of these pathways remain incompletely understood, recent human in vivo data suggest that the production of reactive oxygen species (ROS) from GTN may act as an important trigger of this effect.
Figure 1. Description of protocol 1. Subjects were allocated to 1 of 4 groups of treatment and underwent measurement of flow-mediated dilation before and after local ischemia and reperfusion 26 hours later.

To date, it remains unknown whether or not other organic nitrates, that have both different molecular structure and biotransformation pathways, have the same preconditioning mimetic antiischemic effects. Given the potential therapeutic importance of nitrate-induced preconditioning, and the large use of ISMN in patients with coronary syndromes and heart failure, this question has clinical as well as pharmacological implications.

Methods

The Ethics Committee of the University of Siena, Italy approved the protocol of the study, which was designed in conformity with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained in all cases.

Study Population

A total of 45 healthy male nonsmoking volunteers (25 to 35 years old) were enrolled in this study. All subjects were asked to abstain from caffeine-containing beverages and from any food for at least 4 hours before each study visit. Exclusion criteria were the presence of any active disease, the use of any drug (including supplemental vitamins) as well as risk factors for cardiovascular disease such as hypertension, smoking, hypercholesterolemia, and family history of early cardiovascular disease.

Protocol 1: The Preconditioning Effect of Organic Nitrates

The protocol of this parallel double blinded study is presented in Figure 1. After screening procedures and signing informed consent, subjects were randomized to receive transdermal GTN 0.6 mg/h for 2 hours (Minitrain, 3 mol/L, n = 9), PETN 80 mg per os (Pentalong, ISIS Pharma, n = 9) or ISMN 40 mg per os (Monoket, Chiesi, n = 9). This dosage of GTN has been previously shown to induce a significant preconditioning effect 24 hours after administration (ie, delayed preconditioning) in this experimental model. The doses of ISMN and PETN were chosen because they are the most commonly used clinically, and previous studies of our laboratory showed that they produce hemodynamic changes that are comparable to those of the GTN dosage used. To maintain a double-blind design, all subjects were given 1 capsule and 1 transdermal patch. Subjects in the GTN group received a transdermal system containing active GTN dosage used. To maintain a double-blind design, all

Protocol 2: The role of ROS in PETN Preconditioning

Eight volunteers were enrolled for this study. All subjects received 2 g of the antioxidant vitamin C intravenously, a dosage that has been shown to block GTN preconditioning. At the same time, all subjects were administered 80 mg PETN per os. Twenty-six hours later, they underwent the same protocol described above. An additional group (n = 8) received, in the same protocol, vitamin C and transdermal GTN as described above.

The Effect of PETN on the Induction of Heme-Oxygenase and Ferritin

RNA Isolation and Quantitative Reverse Transcription/Polymerase Chain Reaction

Data is reported in Figure 4. EA.hy 926 cells incubated for 8 hours with medium containing solvent (PETN, DMSO; NTG, EtOH; ISMN, H2O) or the organic nitrates (PETN, NTG, ISMN each 50 μmol/L end concentration) were washed twice with PBS, and total cellular RNA was isolated by guanidinium thiocyanate/phenol/chloroform extraction as described previously. One-step RT-PCR was performed in 25-μL reactions in a 96-well spectrofluorometric thermal cycler (iCycler; Bio-Rad, Munich, Germany). The oligonucleotides (listed below) were purchased from MWG-Biotech, Ebersberg, Germany.

Heme Oxygenase-I

sense AGGCCAAGACTGCGTTTCC, antisense GGCTCTGGTCCTGGTGTCAT, probe CTCAACATCCAGCTCTTTGAGGAGTTGCAG

Ferritin Heavy Chain

sense GTCACTACACAGTCAGGTTG, antisense TGGCCAGTTGTTCAGATC, probe CGGCGTCGAATGCAATGGATGTG

RNA Polymerase II Great Subunit

sense GCACACCTCAATGACAT, antisense GTGCCGGTCTGTCGATAT, probe TACCAGTCATCTCCTTTGATGGCTCTGTTCTAT

Each experimental reaction was performed in triplicate. All primer/probes sets had efficiencies of 100% (+10%). To calculate the relative expression of heme oxygenase-I (HO-1) and ferritin heavy chain (FeHc) mRNA in EA.hy 926 cells, the 2(-ΔΔC(T)) method was used. According to this method, the C(T) values for HO-1 and FeHc mRNA expression in each sample were normalized to the C(T) values of polymerase II great subunit (Pol 2A) mRNA (as housekeeping gene) in the same sample.

Statistical Analysis

All data are presented as mean±SE. A paired t test was used to compare pre-IR with post-IR FMD within each study group, and an analysis of variance followed by the Fisher probable least square difference test was used to assess for differences between nitrates. P<0.05 was set as the threshold for significance. Statview version 5 (SAS Institute Inc) was used for statistical analysis.

Results

Hemodynamic changes induced by the administration of nitrates are described in Table 1.
The Preconditioning Effect of Nitrates
Radial artery diameter and blood flow measurements are presented in Table 2. FMD data are reported in Figure 2. Resting radial artery diameters and blood flows were not different between groups and were not changed, in any group, by IR (Table 2). Similarly, reactive hyperemia was not different between groups before IR and was not affected by IR. In the no treatment group, IR blunted FMD (before-IR: 8.1 ± 0.9%, after-IR: 2.8 ± 0.8%, P = ns compared with before-IR, P < 0.05 compared with no treatment). Of the other nitrates tested, PETN was found to have the same protective effect as GTN (before-IR: 8.9 ± 1.6%, after-IR: 8.1 ± 1.3%, P = ns compared with before-IR, P < 0.05 compared with no treatment group and P = ns compared with GTN group, both after IR). Importantly, FMD was blunted by IR in the subjects that received ISMN (before-IR: 8.8 ± 1.5%; after-IR: 3.6 ± 0.8%, P < 0.05 compared with before-IR and to PETN after IR, P = ns compared no therapy group after IR).

The Role of ROS in PETN Preconditioning
Data are presented in Table 2 and Figure 3. Resting radial artery diameters and blood flows were not different between groups and were not changed, in any group, by IR. In the subjects who received vitamin C and PETN, IR did not modify FMD (before-IR: 7.3 ± 1.6%, after-IR: 6.3 ± 0.9%, P = ns compared with before-IR, P < 0.05 compared with no treatment, protocol 1). In contrast, FMD was significantly reduced after IR in the subjects that received vitamin C before transdermal GTN (before IR: 8.10 ± 0.9%, after IR: 2.8 ± 0.9%, P < 0.05 compared with vit C-PETN).

The Effect of PETN on the Induction of Heme-Oxygenase and Ferritin
Human endothelial cells incubated with PETN, but not those incubated with GTN or ISMN, showed an increase in the expression of the mRNA encoding for HO-1. Similarly, an increased expression of ferritin, although not statistically significant, was observed after PETN, whereas a decrease in the mRNA for ferritin was observed after both GTN and ISMN.

Discussion
Daiber et al. recently proposed the existence of at least 2 different pathways for the metabolism of organic nitrates. Molecules with 3 or more nitrate groups, such as PETN and

| TABLE 1. Changes in Standing Blood Pressure in Response to GTN, ISMN, and PETN |
|-----------------|-----------------|-----------------|
|                 | Basal            | Before IR       |
| Systolic blood pressure, mm Hg |                |                 |
| No therapy      | 120±4            | 115±2           |
| GTN             | 122±4            | 119±3           |
| PETN            | 122±5            | 125±4           |
| ISMN            | 118±4            | 115±3           |
| Diastolic Blood pressure, mm Hg |                |                 |
| No therapy      | 75±2             | 77±2            |
| GTN             | 78±4             | 80±2            |
| PETN            | 81±5             | 75±3            |
| ISMN            | 77±3             | 75±3            |

*P<0.05 compared to basal.

| TABLE 2. The Effect of IR in the Radial Artery Diameter and Blood Flow in the Four Treatment Groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Blood Flow (ml/min) | Radial Artery Diameter (mm) |
|                 | Basal | RH   | Before IR | Basal | ΔFMD   | Basal | Before IR | Basal | ΔFMD   |
| No therapy      | 31±4  | 211±99 | 22±2  | 182±40 | 2.36±0.07 | 0.19±0.03 | 2.38±0.11 | 0.05±0.01* |
| GTN             | 11±4  | 112±32 | 9±3   | 127±45 | 2.39±0.12 | 0.16±0.03 | 2.42±0.14 | 0.12±0.03   |
| PETN            | 28±10 | 208±83 | 22±11 | 185±70 | 2.29±0.16 | 0.20±0.02 | 2.10±0.15 | 0.17±0.03   |
| ISMN            | 25±14 | 131±30 | 23±11 | 133±33 | 2.12±0.19 | 0.18±0.02 | 2.21±0.20 | 0.07±0.02*  |
| GTN+vit. C      | 24±5  | 330±85 | 26±4  | 322±85 | 2.49±0.08 | 0.20±0.02 | 2.56±0.10 | 0.06±0.02*  |
| PETN+vit. C     | 14±5  | 96±16  | 14±6  | 101±18 | 2.56±0.14 | 0.19±0.04 | 2.55±0.16 | 0.15±0.02   |

RH indicates blood flow during reactive hyperemia; ΔFMD, diameter increase during reactive hyperemia. *P<0.001 compared to corresponding ΔFMD before IR, P<0.05 compared to after IR, PETN, and GTN groups.
GTN, undergo mitochondrial biotransformation in a process that is associated with uncoupling of the respiratory chain and production of ROS. In contrast, nitrates with 2 or less nitrate groups (eg, ISMN and ISDN) undergo metabolization through an extramitochondrial low affinity pathway that yields negligible quantities of ROS. This nitrate-induced mitochondrial ROS production appears to have complex biological effects: in the setting of short-term GTN administration, it is responsible for the induction of a protective effect that mimics ischemic preconditioning. During prolonged nitrate treatment, mitochondrial ROS, as well as ROS produced by enzymes such as the nitric oxide synthase, membrane oxidases, and the cytochrome P450, have been associated with the adverse effects of nitrates (which include endothelial and autonomic dysfunction) as well as with nitrate tolerance.

The data presented here are the first to report differences among organic nitrates in their antiischemic effects, as we demonstrate that there is a gradient in the capacity of these drugs to protect the endothelium against IR-induced endothelial dysfunction in a human in vivo model. Although both GTN and PETN prevented the decrease in FMD caused by IR, PETN appeared to be the most protective. These preconditioning effects of PETN are consistent with previous observations that its pharmacophysiologic effects are unique. While causing an increase in ROS production during its mitochondrial metabolism, this nitrate has also important intrinsic antioxidant properties attributable to the positive redox potential of its dinitrate metabolite and to its capacity to induce the expression of protective genes, which might contribute to limit the effect of IR. In the present article, we confirm that PETN, but not GTN and ISMN, causes induction of the genes for heme oxygenase and ferritin. Of interest, both these protective molecules have been attributed a key role in the cascade that leads to ischemic and pharmacological preconditioning. These proteins have been proposed to limit IR injury, respectively, by increasing the bioavailability of the antioxidant bilirubin and by scavenging free iron from the cytosol, therefore reducing Fenton reactions and ROS formation during IR. Additionally, increased bioavailability of the gaseous molecule CO, which is a byproduct of heme metabolism by heme oxygenase, has been proposed to act as an antiapoptotic messenger. Taken together, these protective effects of PETN are consistent with previous observations that prolonged PETN administration, as compared with GTN, does not cause either nitrate tolerance nor endothelial dysfunction, and with the present observation that PETN causes endothelial preconditioning via a ROS-independent mechanism.

Our data suggest that although the preconditioning properties of GTN are dependent on instantaneous mitochondrial ROS release, those of PETN are mediated by a ROS-independent upregulation of protective genes. In contrast, after administration of ISMN, which has an extramitochondrial activation pathway not leading to ROS release and does not cause genomic induction, this protective biological effect is markedly attenuated. In sum, nitrates are potent drugs with both hemodynamic and biological protective properties. Their use, however, is hampered by the development of tolerance and by potentially important side effects, such as endothelial and autonomic dysfunction. To date, although nitrates are treated as a class with a common mechanism and side effects, the organic nitrate most commonly used in clinical practice is ISMN, followed by GTN. The fact that the prolonged use of PETN is not associated with the development of tolerance and endothelial dysfunction, together with the present evidence that it possesses a

![Figure 3](image-url) **Figure 3.** The effect of vitamin C on PETN-induced endothelial protection. FMD was not modified by IR. *P* < 0.05 compared with no therapy, protocol 1, and to GTN + vit. C, after IR. †P < 0.05 compared with before IR, within group.

![Figure 4](image-url) **Figure 4.** Effect of GTN, ISMN, and PETN on the expression of mRNA for heme oxygenase-1 (A) and ferritin (B) in EA.hy 926 cells. mRNA expression is presented as a percentage of the corresponding vehicle.
potent preconditioning effect, suggest that the use of this drug should be reconsidered.

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**Disclosures**

None.

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