Nitроглицерин затормаживает дифференцировку и выживаемость клеток эндотелиального предшественника

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ABSTRACT
Endothelial progenitor cells (EPCs) participate in angiogenesis and the response to chronic ischemia. Risk factors and cardiovascular disease attenuate EPC number, function, and survival. Continuous therapy with nitroglycerin (glyceryl trinitrate; GTN) is associated with increased vascular oxidative stress, leading to nitrate tolerance and endothelial dysfunction. Thus, GTN therapy may also affect EPCs. The purpose of this study was to determine whether continuous exposure to GTN in vivo or during ex vivo expansion affects the circulating number and functional characteristics of human EPCs. To determine the effects of continuous in vivo GTN exposure, EPCs isolated from 28 healthy males before and after receiving 0.6 mg/h GTN (n = 17) or no treatment (n = 11) for 1 week were expanded for 6 days and compared. To determine the effects of continuous ex vivo GTN exposure, EPCs isolated before randomization were expanded for 6 days in medium supplemented with 100 nM, 300 nM, or 1 μM GTN. EPCs expanded without GTN served as controls (n = 10). In vivo, GTN exposure significantly increased the percentage of circulating cells expressing the EPC marker CD34 and increased the susceptibility of expanded EPCs to apoptosis but had no impact on the phenotypic differentiation or migration of EPCs. Ex vivo, GTN exposure increased apoptosis while decreasing phenotypic differentiation, migration, and mitochondrial dehydrogenase activity of EPCs, compared with EPCs expanded in the absence of GTN. Taken together, these results suggest that continuous GTN therapy might impair EPC-mediated processes, an effect that could be detrimental in the setting of ischemic cardiovascular disease.

Bone marrow-derived endothelial progenitor cells (EPCs) are thought to differentiate into functional endothelial cells (ECs) and participate in endothelial repair and the process of adult neovascularization (Asahara et al., 1997; Takahashi et al., 1999; Crosby et al., 2000; Szmitko et al., 2003; Urbich and Dimmeler, 2004). The results of a recent study indicate that EPCs may contribute up to 25% of ECs in newly formed blood vessels (Murayama et al., 2002). In addition, transplantation of EPCs into patients has been shown to induce blood flow recovery in ischemic limbs (Tateishi-Yuyama et al., 2002) and to improve myocardial viability after infarction (Assmus et al., 2002). Thus, EPCs may be integral mediators of the response to chronic ischemia and acute myocardial infarction.

Research has also highlighted the potential of EPCs to serve as diagnostic and prognostic indicators. The number and migratory capacity of circulating EPCs has been shown to inversely correlate with risk factors for coronary artery disease (Vasa et al., 2001). Conversely, levels of circulating EPCs increase acutely after myocardial infarction (Shintani et al., 2001; Massa et al., 2005). Thus, the number and functional capacity of circulating EPCs may serve as a novel biological indicator of vascular health and cumulative cardiovascular risk (Hill et al., 2003; Hunting et al., 2005).

Both animal and human studies have confirmed that con-
tinuous therapy with nitroglycerin (glyceryl trinitrate, GTN), frequently used in the treatment of coronary artery disease and congestive heart failure, increases the vascular levels of reactive oxygen species (ROS), such as superoxide anion (O$_2^-$) and peroxynitrite (ONOO$^-$), and causes endothelial dysfunction (Gori and Parker, 2002a,b). Increased vascular ROS, a risk factor implicated in the pathogenesis and progression of several cardiovascular diseases, is considered to be a principal cause of GTN tolerance and nitrate-induced endothelial dysfunction (Gori and Parker, 2002b). However, the etiology of nitrate tolerance and nitrate-induced endothelial dysfunction is complex and certainly multifactorial, involving biochemical and enzymatic mechanisms within endothelial and vascular smooth muscle cells as well as neurohormonal adaptations and abnormalities in the autonomic nervous system (Gori and Parker, 2002a,b), effects that may also have pathophysiological implications.

When we consider the adverse vascular effects of continuous GTN therapy and the association between EPCs and vascular health, it follows that GTN therapy may negatively affect these cells. Thus, we sought to determine the effect of in vivo GTN exposure on the circulating number, phenotypic differentiation, migration, and apoptosis of human EPCs. We also sought to determine whether ex vivo exposure to clinically relevant concentrations of GTN (simulating high-dose continuous therapy) would affect the phenotypic differentiation, migration, mitochondrial viability, and apoptosis of human EPCs.

Materials and Methods

Study Population. Twenty-eight healthy, nonsmoking male volunteers (18–35 years old) were enrolled. All subjects were instructed to abstain from alcohol or any drugs, including supplemental vitamins, for the duration of the study.

Study Protocol. This randomized, investigator-blind study was approved by the Mount Sinai Hospital and St. Michael’s Hospital ethics review committees. Written informed consent was obtained from all subjects. After consent was obtained, standing heart rate and blood pressure were determined in triplicate. Subsequently, an 80-ml sample of peripheral venous blood was obtained. These pre-randomization (day 0) blood samples were used as a source of control EPCs. Subjects were then randomized in an investigator-blind manner to receive either transdermal administration of 0.6 mg/h GTN (Transderm-Nitro; Novartis, Basel, Switzerland) or no treatment for 7 days (GTN-treated, n = 17; nontreated, n = 11; −3:2 ratio of GTN-treated to -nontreated subjects). A research nurse not involved in experimentation or analysis of the data conducted the randomization, and the investigators remained blinded throughout the study and analysis period. On day 7, standing heart rate and blood pressure were determined, and another 80-ml sample of venous blood was obtained. The number and functional characteristics of EPCs isolated and expanded from day 0 and day 7 blood samples of GTN-treated subjects were compared in an effort to determine the effects of in vivo GTN exposure. EPCs isolated and expanded from the day 0 and day 7 blood samples of GTN-treated subjects were compared in an effort to determine the effects of in vivo GTN exposure.

EPC Apoptosis Assay. EPC apoptosis was quantified with terminal deoxyuridine triphosphate nick end-labeling (TUNEL), a staining method used to detect DNA fragmentation. After 6 days of expansion, EPCs were fixed with 2% paraformaldehyde (Sigma Chemical) in PBS for 10 min, washed three times with PBS, and stained with the mature endothelial cell markers rabbit anti-human vascular endothelial growth factor receptor-2 (VEGFR-2) (Alpha Diagnostic International, San Antonio, TX) and FITC-conjugated EC-lectin (Ulex Europaeus UEA-1; Sigma Chemical). The binding of primary anti-VEGFR-2 antibody was confirmed by exposure of the cells to a FITC-conjugated secondary anti-rabbit antibody. Cells were mounted in VectaShield mounting medium (Vector Laboratories, Burlingame, CA), with propidium iodide as a nuclear marker. Cells stained with only propidium iodide served as negative controls. EPCs were visualized with dual-emission confocal microscopy, and the percentage of cells binding EC-lectin or expressing VEGFR-2 in four random microscopic fields (200× magnification) was determined.

EPC Phenotypic Differentiation. EPC phenotypic differentiation was evaluated with immunohistochemistry. After 6 days of expansion with or without GTN, EPCs were fixed with 2% paraformaldehyde in PBS for 10 min, washed three times with PBS, and stained with the mature endothelial cell markers rabbit anti-human vascular endothelial growth factor receptor-2 (VEGFR-2) (Alpha Diagnostic International, San Antonio, TX) and FITC-conjugated EC-lectin (Ulex Europaeus UEA-1; Sigma Chemical). The binding of primary anti-VEGFR-2 antibody was confirmed by exposure of the cells to a FITC-conjugated secondary anti-rabbit antibody. Cells were mounted in VectaShield mounting medium (Vector Laboratories, Burlingame, CA), with propidium iodide as a nuclear marker. Cells stained with only propidium iodide served as negative controls. EPCs were visualized with dual-emission confocal microscopy, and the percentage of cells binding EC-lectin or expressing VEGFR-2 in four random microscopic fields (200× magnification) was determined.

EPC Isolation and Expansion. EPCs were isolated and expanded by the enriched-medium method as described previously (Ashara et al., 1997). In brief, the total mononuclear cell (MNC) fraction was isolated from peripheral venous blood by Ficol-Paque density gradient (Becton Dickinson, Franklin Lakes, NJ) centrifugation. MNCs were washed twice with sterile phosphate-buffered saline (PBS; Sigma Chemical, St. Louis, MO) and plated at a density of 1.5 × 10^6 cells/cm$^2$ on fibronectin-coated culture slides (Becton Dickinson) in endothelial cell basal medium-2 (Cambrex, Walkersville, MD) supplemented with 5% fetal bovine serum, human vascular endothelial growth factor (VEGF)-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid (EGM-2-MV-SingleQuots; Cambrex). EPCs were expanded for 6 days at 37°C and 5% CO$_2$, with culture medium changes every 24 h.

Ex Vivo GTN Exposure Protocol. EPCs isolated from day 0 blood samples were expanded in medium supplemented with 100 nM, 300 nM, or 1 μM GTN (5 mg/ml GTN solution; Sabex, Boucherville, PQ, Canada). EPCs expanded without GTN served as controls (n = 10). The EPCs were expanded for 6 days, with culture medium changes and GTN supplementation every 24 h. The phenotypic differentiation, apoptosis, migration, and mitochondrial dehydrogenase activity of the EPCs were then determined.

Quantification of Circulating EPCs. Fluorescence-activated cell sorting (FACS) was used to quantify the percentage of cells expressing EPC markers in the MNC fractions isolated from day 0 and day 7 blood samples. In brief, the whole MNC fraction was isolated from peripheral venous blood and resuspended in PBS-staining buffer to obtain a concentration of 1.5 × 10^6 cells/ml. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 (Becton Dickinson) and phycoerythrin-conjugated mouse anti-human CD133 (Sigma) were used separately and in combination to label the cells. Unstained cells acted as controls. CD34 is an antigen expressed by late hematopoietic stem cells (HSCs) (the putative precursors for EPCs) and EPCs and at a relatively low level on mature ECs (Asahara et al., 1997; Szmitko et al., 2003; Urbich and Dimmeler, 2004). CD133, also known as prominin or AC133, is expressed by early hematopoietic progenitor cells but is absent on late EPCs and mature ECs (Szmitko et al., 2003; Urbich and Dimmeler, 2004). Cells were analyzed with a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 20,000 cells was quantified for each sample. The data were reported as the percentage of peripheral blood MNCs that expressed CD34 (MNC$^{+/+}$), CD133 (MNC$^{+/+}$), and both markers (MNC$^{+/+}$).
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EPC Mitochondrial Dehydrogenase Activity Assay. EPC mitochondrial dehydrogenase activity was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which in turn may be interpreted as a measure of cell viability. After being expanded for 6 days, EPCs were detached from the fibronectin-coated slides with 0.25% trypsin and then counted and suspended in serum-free medium at a concentration of 5 × 10^5 cells/ml in a 96-well culture plate (200 μl/well). To each well, 10 μl of MTT (5 mg/ml) was added, and the preparation was incubated for 2 h at 37°C. After the incubation, the EPC preparation was agitated with 200 μl of MTT solubilization solution (10% Triton X-100 with 0.1 N HCl in anhydrous isopropanol) for 10 min before the optical density was measured at 490 and 650 nm with an ultraviolet plate reader. Each experiment was performed in quadruplicate, with serum-free medium acting as a control. The data are presented as the optical density at 490 nm minus the optical density at 650 nm, with the values being directly proportional to the level of mitochondrial dehydrogenase activity.

EPC Migration Assay. EPC migration was evaluated with the modified Boyden’s chamber assay. After 6 days of expansion, EPCs were detached from the fibronectin-coated slides with 0.25% trypsin and then counted and suspended in serum-free M199 culture medium (Sigma Chemical) to obtain a concentration of 5 × 10^5 cells/ml. The EPC suspension (500 μl) then was placed in the upper compartment of a modified Boyden’s chamber (Becton Dickinson), and human VEGF (15 ng/ml) in serum-free M199 media was placed in the lower compartment. A membrane with 8-micron pores separated the upper and lower compartments. After 5 h of incubation at 37°C, the membrane was washed gently with PBS to remove any nonmigratory cells and fixed with 50% methanol. EPCs trapped in the pores while migrating into the lower compartment were stained with Diff-Quick staining solutions (Dade Behring, Deerfield, IL) and visualized with an inverted light microscope. Experiments conducted without VEGF served as controls. After being expanded without vehicle served as controls. After 6 days of expansion with or without vehicle, EPCs were assayed for phenotypic differentiation, as determined by EC-lectin binding and VEGFR-2 expression, and apoptosis, as determined by TUNEL.

Results

In Vivo GTN Exposure. The percentages of circulating MNC133+, MNC34/133+, and MNC34/133- in day 0 and day 7 blood samples were determined with FACS (Fig. 1A; Table 1). The percentage of circulating MNC34+ increased ~2-fold with in vivo GTN exposure (Fig. 1A; Table 1; P = 0.04). The percentages of MNC133+ and MNC34/133+ were not significantly altered because of in vivo GTN exposure (Table 1, P = N.S.). FACS analysis of day 0 and day 7 MNC fractions isolated from nontreated subjects was conducted to establish the number of circulating EPCs and the stability of these numbers over 7 days in normal subjects receiving no treatment. The percentages of MNC34+, MNC133+, and MNC34/133+ at day 0 and day 7 were not significantly different in subjects who received no treatment (Table 1, P = N.S.). To evaluate EPC apoptosis, EPCs expanded from day 0 and day 7 blood samples were subjected to TUNEL (Fig. 1B). The percentage of apoptotic cells was significantly increased in EPCs expanded from subjects who received GTN (day 0 6 ± 1% versus day 7 29 ± 6%). The data are presented as the total number of cells counted in five random microscopic fields (200× magnification).

GTN Metabolism Assay. To determine whether EPCs can metabolize GTN to form the metabolites 1,2- and 1,3-glyceryl dinitrate (GDN), EPCs expanded for 6 days under control conditions were detached from the fibronectin-coated slides with 0.25% trypsin, counted, and suspended in endothelial growth medium at a concentration of 5 × 10^5 cells/ml. The EPC suspension was then incubated with 100 nM GTN for 24 h at 37°C. Media supplemented with 100 nM GTN acted as controls. After the incubation, the preparations were immediately centrifuged to remove the cells, and the medium was collected. 1,2-GDN, 1,3-GDN, and GTN were then extracted from the medium, and a previously described method utilizing megabore capillary column gas-liquid chromatography (McDonald and Bennett, 1990) was used to quantify the amounts of these metabolites present (n = 6). Data are presented as the ratio of the total concentration of GDNs (1,2- and 1,3-GDN) to GTN.

Vehicle Exposure Protocol. To determine the contribution of vehicle to the observed effects of GTN on EPCs, cells isolated from 6-day zero blood samples were expanded in medium supplemented daily with propylene glycol and ethanol at concentrations equal to those encountered in cultures supplemented with 1 μM GTN, the highest level of exposure used. The vehicle solution was 29% propylene glycol and 28.5% ethanol in sterile, deionized water. EPCs expanded without vehicle served as controls. After 6 days of expansion with or without vehicle, EPCs were assayed for phenotypic differentiation, as determined by EC-lectin binding and VEGFR-2 expression, and apoptosis, as determined by TUNEL.

Statistical Analysis. For the in vivo protocol, within-group comparisons were made using Student’s t test for paired data. Comparisons between GTN-treated and control subjects were made using Student’s t tests for unpaired data. Where data were not normally distributed, nonparametric testing was done with the Wilcoxon signed rank test. For the ex vivo protocol, the effect of different concentrations of GTN on EPCs was compared using repeated measures analysis of variance (ANOVA) and Student-Newman-Keuls post hoc test. Data are presented as means ± S.E.M.

Fig. 1. A, percentage of MNC34+ in day 0 and day 7 peripheral blood samples of subjects who received no treatment or GTN. The percentage of MNC34+ was significantly increased due to in vivo GTN exposure. B, percentage of total cells expanded from day 0 and day 7 blood samples of control and GTN-exposed subjects that were TUNEL-positive (% Apoptosis) after 6 days of expansion. EPCs expanded from day 7 blood samples of GTN-exposed subjects displayed a significantly increased level of apoptosis. Each value represents the mean ± S.E.M.
day 7 17 ± 4%; P < 0.05) but was unaltered in EPCs expanded from subjects who received no treatment (day 0 5 ± 1% versus day 7 5 ± 1%; P = N.S.). The modified Boyden’s chamber assay was used to assess the in vitro migratory capacity of EPCs expanded from day 0 and day 7 blood samples (Table 1). The capacity for migration was not significantly different between EPCs expanded from day 0 and day 7 blood samples of subjects who received GTN or no treatment (P = N.S. for both). The phenotypic differentiation of MNCs to EPCs, as indicated by EC-lectin binding and VEGFR-2 expression, was assessed after expansion (Table 1). The percentage of cells binding EC-lectin and expressing VEGFR-2 was not significantly different between cells expanded from day 0 and day 7 blood samples of subjects who received GTN (P = N.S. for all groups) or no treatment (P = N.S. for all groups).

**Ex Vivo GTN Exposure.** After 6 days of expansion under control conditions, 5 ± 1% of adherent cells stained positive for apoptosis with TUNEL (Fig. 2), 92 ± 1% bound EC-lectin (Fig. 3A), and 92 ± 2% expressed VEGFR-2 (Fig. 3B). Apoptosis was significantly increased in cells expanded with 100 nM, 300 nM, and 1 μM GTN (Fig. 2, 12 ± 2, 15 ± 2, and 17 ± 1%, respectively; P < 0.001 ANOVA, Student-Newman-Keuls post hoc results: 100 nM versus 300 nM, P < 0.05, 300 nM versus 1 μM, P = N.S.). Representative photomicrographs of TUNEL staining are presented below the graph in Fig. 2. Note the increased yellow fluorescence resulting from dual staining with the red fluorescing nuclear label propidium

### Table 1

<table>
<thead>
<tr>
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<th>Control Day 0</th>
<th>Control Day 7</th>
<th>GTN Day 0</th>
<th>GTN Day 7</th>
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<td><strong>Circulating EPC quantification</strong></td>
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<tr>
<td>% MNC&lt;sup&gt;34&lt;/sup&gt;</td>
<td>0.48 ± 0.08 [0.46]</td>
<td>0.56 ± 0.13 [0.4]</td>
<td>0.46 ± 0.18 [0.32]</td>
<td>0.86 ± 0.35* [0.4]</td>
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<td>% MNC&lt;sup&gt;133&lt;/sup&gt;</td>
<td>0.24 ± 0.05 [0.26]</td>
<td>0.17 ± 0.02 [0.18]</td>
<td>0.14 ± 0.02 [0.15]</td>
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<tr>
<td>% MNC&lt;sup&gt;54/133&lt;/sup&gt;</td>
<td>0.22 ± 0.03 [0.23]</td>
<td>0.15 ± 0.03 [0.14]</td>
<td>0.12 ± 0.01 [0.11]</td>
<td>0.12 ± 0.02 [0.11]</td>
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<td><strong>EPC migration assay</strong></td>
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<tr>
<td>Total no. of cells migrated</td>
<td>96 ± 1 [78]</td>
<td>88 ± 18 [57]</td>
<td>88 ± 8 [94]</td>
<td>80 ± 8 [76]</td>
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<td><strong>EPC phenotypic differentiation</strong></td>
<td></td>
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<tr>
<td>% binding lectin</td>
<td>92 ± 1 [92]</td>
<td>88 ± 2 [90]</td>
<td>89 ± 1 [90]</td>
<td>84 ± 4 [88]</td>
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<td>% expressing VEGFR-2</td>
<td>88 ± 3 [90]</td>
<td>84 ± 4 [86]</td>
<td>89 ± 2 [92]</td>
<td>83 ± 4 [86]</td>
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* P < 0.05 for GTN day 0 versus GTN day 7.

**Fig. 2.** Percentage of total cells that were TUNEL-positive (% Apoptosis) after 6 days of expansion with 100 nM, 300 nM, 1 μM, or no GTN. Ex vivo GTN exposure significantly increased the percentage of apoptotic EPCs compared with cells expanded without GTN (*, P < 0.05 versus control; †, P < 0.05 versus 300 nM). Each value represents the mean ± S.E.M. (n = 8). Red fluorescence indicates the nucleus. Green fluorescence indicates TUNEL staining. Note the increased dual staining (yellow fluorescence; apoptosis) in GTN-exposed EPCs (200× magnification).

**Fig. 3.** A and B, percentage of total cells binding EC-lectin (A) and expressing VEGFR-2 (B) after 6 days of expansion with 100 nM, 300 nM, 1 μM, or no GTN. Ex vivo GTN exposure dose-dependently decreased the binding of EC-lectin and expression of VEGFR-2 compared with cells expanded without GTN (+, P < 0.05 versus control; †, P < 0.05 versus 300 nM; #, P < 0.05 versus 1000 nM). Each value represents the mean ± S.E.M. (n = 8). Red fluorescence indicates the nucleus. Green fluorescence indicates either EC-lectin binding or VEGFR-2 expression. Note the decreased dual staining (yellow fluorescence) in GTN exposed cells (200× magnification).
iodide and green fluorescing TUNEL, indicating apoptosis, in cells expanded with GTN. EC-lectin binding was significantly decreased in adherent cells expanded with 100 nM, 300 nM, and 1 μM GTN (Fig. 3A, 75 ± 2, 67 ± 1, and 56 ± 3%, respectively, \( P < 0.001 \) ANOVA, Student-Newman-Keuls post hoc results: 100 nM versus 300 nM, \( P < 0.05 \); 300 nM versus 1 μM, \( P < 0.05 \)). VEGFR-2 expression was also significantly decreased with all treatments compared with control cells (Fig. 3B, 77 ± 2, 65 ± 5, and 57 ± 4%, respectively, \( P < 0.001 \) ANOVA, Student-Newman-Keuls post hoc results: 100 nM versus 300 nM, \( P < 0.05 \); 300 nM versus 1 μM, \( P < 0.05 \)). Representative photomicrographs of EC-lectin binding and VEGFR-2 staining are presented below the corresponding graphs in Fig. 3. Note the decreased yellow fluorescence resulting from dual staining with the red fluorescing nuclear marker propidium iodide and green fluorescing FITC, which indicates either EC-lectin binding or VEGFR-2 expression in cells expanded with GTN. EPCs expanded with 100 nM and 1 μM GTN demonstrated a significantly decreased capacity for in vitro migration compared with control EPCs (Fig. 4, control, 92 ± 7 cells migrated; 100 nM GTN, 73 ± 9; 1 μM GTN, 56 ± 10; \( P < 0.001 \) ANOVA, Student-Newman-Keuls post hoc results: 100 nM versus 1 μM, \( P < 0.05 \)). The effect of continuous ex vivo GTN exposure on EPC mitochondrial dehydrogenase activity was determined with the MTT cell proliferation assay (Fig. 5). Mitochondrial dehydrogenase activity was significantly reduced in EPCs expanded with 100 nM and 1 μM GTN compared with controls (control, 0.086 ± 0.005; 100 nM, 0.075 ± 0.003; 1 μM, 0.066 ± 0.006; \( P = 0.002 \) ANOVA, Student-Newman-Keuls post hoc results: 100 nM versus 1 μM, \( P < 0.05 \)). To exclude a contribution of the vehicle for GTN to the observed effects, we expanded EPCs with propylene glycol and ethanol at the concentrations encountered in cultures supplemented with 1 μM GTN, the highest level of exposure used. Exposure to the vehicle of GTN during the 6-day expansion did not significantly alter EPC apoptosis, as determined by TUNEL (control 4 ± 1% versus vehicle 4 ± 1%; \( P = \) N.S.) or phenotypic differentiation, as determined by EC-lectin binding (control 88 ± 2% versus vehicle 86 ± 3%, \( P = \) N.S.) and VEGFR-2 expression (control 87 ± 2% versus vehicle 86 ± 4%, \( P = \) N.S.).

EPCs and GTN Biotransformation. Although we observed low-level, noncellular biotransformation of GTN due to serum components in the control incubations, 1,2- and 1,3-GDN formation doubled in the presence of EPCs (ratio of GDNs to GTN after incubation: control 1.2 ± 0.07, EPCs 2.2 ± 0.09, \( P < 0.05 \)), indicating that these cells can metabolize GTN.

Discussion

In the present study, we demonstrated that both in vivo and ex vivo exposure to GTN had effects on human EPCs. Continuous ex vivo exposure dose-dependently inhibited the phenotypic differentiation of MNCs to EPCs, as indicated by decreased binding of EC-lectin and decreased expression of the vital growth factor receptor VEGFR-2 after 6 days of expansion. We also observed a significant increase in the level of apoptosis in EPCs expanded with GTN. This result is in accord with previous studies demonstrating GTN-induced apoptosis of Jurkat leukemic cells (Ushmorov et al., 1999) and several human colon cancer cells lines (Millet et al., 2002), although millimolar concentrations of GTN were used in these studies. EPCs expanded with GTN also displayed decreased migration in response to a concentration gradient of VEGF, a potent mediator of EPC mobilization and migration to sites of vascular damage and/or neovascularization (Szmikto et al., 2003). The decreased migration of EPCs expanded with GTN may be directly attributable to the observed decrease in VEGFR-2 expression, as this receptor is required for VEGF-mediated migration (Szmikto et al., 2003; Urbich and Dimmeler, 2004). Finally, we observed decreased mitochondrial dehydrogenase activity in EPCs expanded with GTN, a finding that would suggest a GTN-induced mitochondrial insult and decreased cellular viability. Exposure to the vehicle of GTN caused no significant change in the extent of phenotypic differentiation or level of apoptosis compared with cells expanded under control conditions, suggesting that GTN was the mediator of the observed effects. We also demonstrated that these cells metabolize GTN, as EPCs produced roughly twice the 1,2- and 1,3-GDN from 100 nM GTN compared with control incubations with growth medium alone. This result further implicates GTN as the mediator of the effects we observed, as the metabolism of GTN is thought to be integral to the development of tolerance, ni-
trate-induced endothelial dysfunction, and other documented detrimental biochemical effects of this compound (Sydow et al., 2004).

The concentrations of GTN we used in the ex vivo GTN exposure experiments are approaching the upper limit of what would be achieved during therapy, as administration of GTN in humans via sublingual, transdermal, or i.v. routes generally yields plasma concentrations in the low nanomolar range (Armstrong et al., 1979, 1980; Imhof et al., 1982; Curry et al., 1984; Jewell et al., 1992; Hashimoto and Kobayashi, 2003). Nevertheless, we believe that the concentrations used have clinical relevance. Transdermal administration of 4 mg/h GTN yielded a maximal venous plasma concentration of 45 nM in 119 healthy volunteers (Curry et al., 1984), and studies have documented that plasma concentrations exceeding 100 nM can be achieved with moderate intravenous infusion rates of GTN (Imhof et al., 1982; Curry et al., 1993; Booth et al., 1994). Thus, plasma concentrations approaching or exceeding 100 nM are achieved during GTN therapy. Furthermore, reported plasma concentrations of GTN must be interpreted with care, as the pharmacokinetics of this compound are characterized by prominent interindividual variability and a marked arteriovenous concentration gradient, with higher concentrations of GTN in arterial plasma (Armstrong et al., 1982). The majority of human studies reporting plasma concentrations of GTN either did not specify the sampling site or used venous plasma, in which case the reported plasma concentrations would have been substantially lower than those found in arterial plasma. Given this background, the concentrations of GTN we used are clinically relevant.

To establish whether these same effects would result from in vivo exposure to GTN, we exposed healthy, male subjects to 0.6 mg/h GTN for 7 days. In contrast to the results from our ex vivo GTN exposure protocol, in vivo exposure to GTN did not convey the same effects on EPC phenotypic differentiation or migration. However, EPCs isolated from subjects who received GTN displayed a significantly increased level of apoptosis after 6 days of expansion. Despite the observed increase in apoptosis of EPCs, there was a significant increase in the percentage of circulating MNC$^{34+}$, a finding suggesting that HSC/EPC production/mobilization may have increased in subjects receiving GTN to compensate for an increased rate of EPC turnover. Importantly, studies have documented an increase of similar magnitude in the number of circulating MNC$^{34+}$ in humans after acute myocardial infarction (Shintani et al., 2001; Massa et al., 2005) and in the early phase of congestive heart failure (Valgimigli et al., 2004). Conversely, the number of circulating MNC$^{34+}$ has been shown to be reduced in patients with risk factors for coronary artery disease (Vasa et al., 2001). Therefore, it is clear that a number of factors are involved in the regulation of both the production and utilization of these precursors and that any change in the circulating number of these cells can represent a change in their production rate or utilization rate or both. Importantly, CD34-positive ECs circulate within peripheral blood for a short time after being shed from the vessel wall (Urbich and Dimmel, 2004), and thus we cannot exclude the potential contribution of these cells to the observed increase in circulating MNC$^{34+}$. Overall, the results of the in vivo exposure protocol indicate that continuous exposure to GTN modifies the number of circulating MNC$^{34+}$ and increases the susceptibility of EPCs to apoptosis ex vivo. However, the clinical significance of these effects remains to be determined. Regardless, the observed increase in EPC apoptosis is worrisome and augments the growing body of evidence that long-term, continuous GTN therapy may be harmful.

This was an observational study, and we did not attempt to elucidate the underlying mechanisms of the observed effects. However, it may be that a GTN-induced increase in the production of O$_2^-$ and ONOO$^-$ (Munzel et al., 1995) and/or inhibition of the mitochondrial electron transport chain (Boime and Hunter, 1971; Ushmorov et al., 1999; Chen et al., 2002; Millet et al., 2002; Sydow et al., 2004) may have contributed to the observed effects. Endothelial nitric-oxide synthase activity is thought to be integral to HSC/EPC function (Murohara et al., 1998; Guthrie et al., 2005), and thus, uncoupling/inhibition of endothelial nitric-oxide synthase, an effect documented to occur with prolonged GTN exposure (Kaesemeyer et al., 2000; Munzel et al., 2000) may have played a role in the observed effects. Finally, GTN-induced impairments of endothelial NO signal transduction pathways (Mulsch et al., 2001; Warnholtz et al., 2002) may have also contributed to the observed effects of GTN on EPCs. The mechanisms described above are thought to contribute significantly to the development of nitrate tolerance and nitrate-induced endothelial dysfunction, both documented to occur within 24 to 48 h of continuous exposure to GTN (Gori and Parker, 2002a,b). Further investigation will be necessary to establish whether these mechanisms played a role in the observed effects.

In summary, in vivo exposure to 0.6 mg/h GTN for 7 days significantly increased the percentage of circulating MNC$^{34+}$ and the percentage of apoptotic EPCs after 6 days of expansion under control conditions. Ex vivo exposure of human EPCs to GTN dose-dependently attenuated phenotypic differentiation, migration, and mitochondrial dehydrogenase activity and was associated with increased EPC apoptosis. Given that no study has unequivocally proven that EPC dysfunction as detected in the laboratory correlates with impairments of EPC-mediated endothelial repair and neovascularization in humans, we cannot conclude yet whether the observed effects would be harmful. Furthermore, this study was conducted in healthy volunteers; thus, these effects may not be observed in cardiovascular disease states. Nonetheless, these data augment the growing body of evidence that continuous GTN therapy has systemic effects unrelated to its mode of action and illuminate a novel mechanism by which this compound may exert negative cardiovascular effects. With accumulating evidence of nitrate-induced cardiovascular toxicity, we believe that it is time to organize large-scale clinical trials to establish the effects of continuous nitrate therapy on long-term clinical outcome.

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References


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