Differential Effects of Organic Nitrates on Endothelial Progenitor Cells Are Determined by Oxidative Stress

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**Objective**—Reduced levels and impaired function of endothelial progenitor cells (EPCs) foster development and progression of atherosclerotic lesions. Endothelial nitric oxide synthase (eNOS)–derived NO regulates EPC mobilization and function. Organic nitrates release NO, and therefore may favorably affect EPC biology.

**Methods and Results**—We compared the effects of 2 different nitrates on circulating EPC numbers and function. Treatment of rats with pentaerythritol-trinitrate (PETriN) or isosorbide dinitrate (ISDN) increased circulating EPC levels. EPC from ISDN- but not PETriN-treated animals displayed impaired migratory capacity and increased reactive oxygen species formation in EPCs. In vitro treatment with ISDN reduced migration and incorporation of human EPCs into vascular structures on matrigel, whereas PETriN improved EPC function. ISDN, but not PETriN, increased NADPH oxidase–mediated oxidative stress in cultured human EPCs. Addition of polyethylene-glycolated superoxide dismutase or diphenyliodonium normalized both ISDN-induced superoxide anion production and impaired migratory capacity of EPCs.

**Conclusions**—Long-acting nitrates increase levels of circulating EPCs, but differ in their effects on EPC function dependent on the induction of intracellular oxidative stress. Organic nitrates that improve EPC function may confer long-term cardiovascular protection based on their beneficial effects on EPC biology. (Arterioscler Thromb Vasc Biol. 2007;27:748-754.)

**Key Words:** endothelial progenitor cells [nitrate] [nitric oxide] [reactive oxygen species] [atherosclerosis] [free radicals]
In Vivo Studies

Osmotic mini-pumps for continuous drug infusion were implanted into rats to investigate effects of nitrate administration (ISDN, PETriN). Adult healthy male Wistar rats (250 to 300 g; Charles River, Sulzfeld, Germany) were treated continuously for 4 days with equimolar doses of ISDN (450 mmol/L, solved in ethanol, 1 μL/h; n=5), or PETriN (450 mmol/L, solved in DMSO, 1 μL/h; n=5) or the respective control solvents (ethanol/DMSO; each n=5). Dosage was estimated based on a previous study.20

In addition, left coronary artery ligations were performed in adult male Wistar rats (250 to 300 g) as described.20–22 Starting 3 hours after ligation, sham-operated rats received placebo treatment (Sham, n=6) and surviving rats with myocardial infarction (MI) were randomly allocated to 3 days treatment by gavage twice daily (in the morning and afternoon) with placebo (PLA, n=9), ISDN (50 mg/kg, n=5), or PETN (100 mg/kg, n=8). Mean infarct sizes of rats were similar among the experimental groups (3 days: MI placebo 43±2%, MI ISDN 43±6%, MI PETN 45±3%).

Isolation of Bone Marrow and Peripheral Blood Mononuclear Cells

Blood samples were collected from the right carotid artery into EDTA vials. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation.20 Hollow bone of rat legs were prepared by standard surgical procedures, and whole bone marrow was harvested by flushing marrow with 500 μL PBS using a syringe with a 20-gauge needle. Bone marrow extracts was shock-frozen before further analysis. For in vitro assays human PBMCs were harvested by leukapheresis (Cobe Spectra device, Gambro) of healthy volunteers (n=5).

Determination of Endothelial Progenitor Cell Numbers and Cellular Characterization

PBMCs (3×10^6) were cultured on fibronectin-precoated 6-wells in EBM-2 culture medium supplemented with EBM SingleQuots (Clonetix) and 20% FCS for 4 days. To exclude contamination with mature circulating endothelial cells, we carefully removed nonadherent cells 8 hours after initial seeding and placed them on new fibronectin-precoated chamber slides. After dilution of 1,1′-dioctadecyl-3,3′,3′-tetramethyl-indocarbocyanine perchlorate labeled acetylated LDL (dil-acLDL; Molecular Probes) and fluorescein isothiocyanate (FITC)-conjugated lectin from Ulex europeus (UEA-1, Sigma) in serum-free EBM2 media, cells were washed twice and incubated for 4 hours at 37°C in EBM2 medium containing 10 μg/mL dil-acLDL and 20 μg/mL UEA-1. After washing, cells were observed by appropriate flow cytometric analyses as described.9,20 Only double positive (dil-acLDL and UEA-1) cells were counted. Expression of VEGFR-2 and eNOS in dil-ac-LDL–labeled EPCs in formed endothelial tubes were examined under a fluorescence microscope. The amount of incorporated dil-ac-LDL–labeled EPCs in formed endothelial tubes was determined. Two investigators in blinded experiments examined at least 4 randomly selected high-power fields. At least 5 experiments were done per study group.

Detection of Intracellular ROS in EPCs

This assay was done essentially as described.24 The redox-sensitive, cell-permeable fluorophore dihydroethidium (DHE) becomes oxidized in the presence of O2− to yield fluorescent ethidium. Thus, dye oxidation is an indirect measure of the presence of reactive oxygen intermediates. To confirm the specific detection of O2−, several cell dishes were incubated with 350 μM polyclonos-3-fluorescein isothiocyanate (FITC)-conjugated lectin from Ulex europeus (UEA-1, Sigma) in serum-free EBM2 media. After washing, EPCs were immediately analyzed with a computer-based digitizing image system (AxioVision Rel. 4.5, Zeiss) using a fluorescence microscope (AxioVert 135, Zeiss) connected to a camera (AxioCam MRm, Zeiss). Fluorescence was detected with a 515 to 560 nm excitation and a 590 nm emission long-pass filter both within EPCs and in areas without EPCs (background). After background subtraction fluorescence was measured from at least 40 different EPCs in 4 different visual fields per sample by the AxioVision (Rel. 4.5, Zeiss) software and was then given in mean±SEM.

Determination of Superoxide Anion Formation by Lucigenin-Enhanced Chemiluminescence

Basal superoxide formation was measured by lucigenin-enhanced chemiluminescence as described.25 In brief, bone marrow extracts were transferred into scintillation vials containing lucigenin and Krebs/HIES buffer (final composition mmol/L: lucigenin 0.005, NaCl 99.01, KCl 4.69, CaCl2 2.5, MgSO4 1.2, KH,PO4 1.03, NaHCO3 25, Na-HIES 20, glucose 5.6, pH 7.4). Signals were assessed over 20 minutes at 37°C in a luminometer (Wallac) at 30-second intervals under basal conditions in absence of additional NADPH. The chemiluminescence signal was adjusted for the amount of protein of bone marrow extract.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed by one-way ANOVA followed by multiple comparisons using Fisher protected least-significant difference test. Statistical analysis was performed using StatView 5.0 statistic program (Abacus Concepts). Statistical significance was assumed at P<0.05.

Results

Characterization of Endothelial Progenitor Cells

Characterization of EPCs is still controversial, and it is more than likely that different types of EPCs exist (reviewed in26,27). In our present study we rely on a monocytic early

membrane were stained with dil-acLDL and counted by fluorescence based microscopic evaluation of the bottom side of the membrane (n=4).

Incorporation Into Endothelial Tube-Like Structures

We measured incorporation of EPCs during endothelial tube formation as previously described.19 Studies were performed after a 24-hour incubation of EPCs with ISDN (100 μmol/L) or PETriN (100 μmol/L). Cells were also concomitantly treated with polyethylene glycol (PEG)-SOD (350U/mL), or the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; 100 μmol/L).

Briefly, dil-ac-LDL-pre-labeled EPC (2×10^4 cells) were mixed with human umbilical vein endothelial cells (HUVECs; 4×10^5) on an 8-well glass slide precoated with 200 μL Matrigel (BD Bioscience) in 500 μL EBM-2 medium with supplements (Cambrex). After 24 hours of incubation in 5% CO2 humidified atmosphere at 37°C, cells were examined under a fluorescence microscope. The amount of incorporated dil-ac-LDL–labeled EPCs in formed endothelial tubes was determined. Two investigators in blinded experiments examined at least 4 randomly selected high-power fields. At least 5 experiments were done per study group.
type of EPCs, which has been previously characterized by others and us in detail. In brief, after 4 days of culture in the presence of endothelial growth media, >90% of cells were capable for cellular uptake of acLDL and UEA-1 lectin binding and demonstrated expression of VEGFR-2 and eNOS (see also Figure 3B). Functionally, cells were able for migration, when VEGF and SDF-1 was added to the lower compartment of a modified Boyden chamber, and EPCs integrated into forming vascular networks after coculturing with mature endothelial cells on Matrigel. This type of monocytic EPC also has profound angiogenic effects when transplanted to ischemic tissues. In conclusion, the term "EPC" was used for monocytic-like cells with adherence on fibronectin-coated dishes, the ability for dil-acLDL uptake, binding to UEA-1, expression of the VEGFR2 and eNOS, the ability to migrate and to incorporate into tube-like structures on matrigel.

**In vivo Effects of Nitrates on Circulating EPC Levels, EPC Function, and Intracellular ROS Production in EPCs and Bone Marrow**

Continous treatment of healthy rats with ISDN or PETriN via implanted osmotic mini-pumps for 4 days led to 51±13% and 58±17% increases in circulating EPC numbers when compared with the respective controls (Figure 1A). There was no difference between the different solvents (ethanol versus DMSO) on circulating EPC levels (209±35 versus 228±37 EPC/µL blood \(P=NS\)). As we previously have shown reduced levels of circulating EPCs 3 days after extensive myocardial infarction in rats, we investigated the ability of nitrate treatment to enhance circulating EPC levels after experimental myocardial infarction. Indeed, both treatment with ISDN or PETN completely normalized reduced EPC levels 3 days after myocardial infarction (see Figure 1B).

Ex vivo we tested function and intracellular ROS levels of EPCs derived from healthy rats treated with either ISDN or PETriN. In EPCs from ISDN-treated rats we found an inverse correlation between EPC function and intracellular ROS levels: migratory capacity of EPCs was significantly impaired by ISDN treatment, but intracellular ROS production was increased. In contrast, PETriN treatment improved migratory function of EPCs compared with ISDN treatment (\(P<0.05\)), whereas intracellular ROS levels were not altered (see Figure 2A and 2B). We therefore performed further in vitro studies to analyze whether nitrate treatment effects directly ROS production and/or functional parameters of EPCs.

**In Vitro Effects of Nitrates on EPC Function and Oxidative Stress**

Migratory capacity of EPCs was assessed after 24 hours of treatment with different nitrates. PETriN treatment resulted in
a 26.6±6.9% (P<0.05) increase of migrated cells, whereas ISDN-treatment reduced migration by 20.7±3.1% (P<0.05). Addition of the NO scavenger PTIO abolished the stimulatory effects of PETriN (P<0.01) but had no effect on ISDN treatment. In contrast, concomitant treatment of ISDN-treated EPCs with polyethylene-glycolated SOD (350 IU/mL) or DPI (10 μmol/L) completely normalized migratory capacity, but had no effect on PETriN-treated EPCs (Figure 3A). In the absence of promigratory stimuli (VEGF/SDF-1) the PETriN-mediated improvement of EPC migration was less pronounced when compared with a respective control (without VEGF/SDF-1) and no longer statistically significant (23.0%±14.8% increase, P=0.19).

Treatment with PETriN increased incorporation of EPCs into forming capillaries on matrigel by 62.3±23.1% (P<0.05), but addition of the NO scavenger PTIO completely abolished the PETriN effects, whereas polyethylene-glycolated SOD further improved incorporation of EPCs (see Figure 4). In contrast, ISDN treatment decreased incorporation of EPCs by 42.3±5.4% (P<0.05). This was reversed by an additive treatment with polyethylene-glycolated SOD, whereas PTIO had no further effect (P<0.05; Figure 4).

To detect intracellular ROS production, EPCs were stained with dihydroethidium (DHE) 24 hours after treatment with PETriN or ISDN. Whereas intracellular ROS production was not affected by PETriN treatment, ISDN treatment increased the fluorescence signal by 35±6% (P<0.01), demonstrating enhanced intracellular concentrations of O$_2^-$ (Figure 5A). Addition of polyethylene-glycolated SOD or DPI to ISDN-treated EPCs attenuated fluorescence intensity (Figure 5A).

We recently described increased oxidative stress in bone marrow after myocardial infarction, which also may impact EPC functionality. Here, we assessed bone marrow O$_2^-$ formation 3 days after myocardial infarction with and without nitrate treatment. O$_2^-$ formation was enhanced by daily ISDN treatment. In contrast, PETN treatment reduced O$_2^-$ levels in bone marrow to that of Sham-operated controls (see Figure 5B).

**Discussion**

The results of the current study demonstrate important influences of different nitrates on circulating levels and function of EPCs. In vivo, we demonstrated that both ISDN and PETN/PETriN increase circulating EPC levels. However, ISDN impaired EPC function and increased intracellular ROS levels in EPCs and bone marrow. In vitro we demonstrated favorable effects of PETriN on EPC function, whereas ISDN...
induced EPC dysfunction. This could be explained at least in part by altered cellular superoxide anion production, which was only increased by ISDN and could be attenuated by polyethylene-glycolated SOD or the NADPH oxidase inhibitor DPI.

Nitrates are potent NO donors but may also induce ROS formation, which is partly involved in the development of nitrate tolerance (reviewed in 32). This has been observed especially for the short-acting NTG1,3,5 and was mediated at least in part by activation of NADPH oxidases.33–35 Further, considerable increases of oxidative stress were seen after treatment with certain long-acting nitrates, such as ISDN.2,4 In contrast, treatment with PETN did not cause tolerance and was not associated with evidence of increased ROS levels in patients.2 These differences may be explained by the ability of PETN to induce antioxidative defense proteins. Indeed, in cultured human endothelial cells the active PETN metabolite PETriN increased expression and activity of the antioxidative enzyme heme oxygenase-1, and pretreatment with PETriN protected cells from hydrogen-peroxide–mediated toxicity.36 This effect was not seen with other long-acting nitrates, such as ISDN. Chemically, PETN undergoes reductive metabolism, leading to the formation of a PETN-trinitrate radical, which does not result in a rise in ROS concentration compared with other nitrates.13,37,38 PETriN is a denitrated phase I metabolite of PETN and also a highly potent donor of NO.23 The obvious differences between the both nitrates ISDN and PETriN in terms of induction of oxidative stress may also turn into functional effects in the long term. Indeed, in vivo treatment with PETN but not ISDN prevented plaque forma-

![Figure 4.](image)

**Figure 4.** Incorporation capacity of EPCs. Incorporation capacity of human endothelial progenitor cells into vascular networks of HUVECs was assessed after 24 hours of in vitro treatment of EPCs with ISDN (100 μmol/L), ISDN (100 μmol/L) + polyethylene-glycolated SOD (350U/mL), ISDN (100 μmol/L) + PTIO (100 μmol/L), PETriN (100 μmol/L), PETriN (100 μmol/L) + polyethylene-glycolated SOD (350U/mL), and PETriN (100 μmol/L) + PTIO (100 μmol/L). Data represent mean ± SEM, n=4 to 6 per group.

![Figure 5.](image)

**Figure 5.** Detection of superoxide anion production in EPCs and bone marrow. A, Detection of fluorescent ethidium after staining of cultured EPCs with the redox-sensitive, cell-permeable fluorophore dihydoroethidium. EPCs were treated for 24 hours with either ISDN (100 μmol/L), ISDN (100 μmol/L) + polyethylene-glycolated SOD (350U/mL), ISDN (100 μmol/L) + PTIO (100 μmol/L), PETriN (100 μmol/L), PETriN (100 μmol/L) + PTIO (100 μmol/L), and PETriN (100 μmol/L) + polyethylene-glycolated SOD (350U/mL). Data represent mean ± SEM, n=4 to 6 per group. B, Superoxide anion production in bone marrow extracts of sham-operated controls, and rats after myocardial infarction treated with placebo (MIP), ISDN (MI ISDN), or PETN (MI PETN) for 3 days as assessed by lucigenin (5 μmol/L)-enhanced chemiluminescence. Data represent mean ± SEM, n=5 to 9 per group.
tion and endothelial dysfunction in animal models of atherosclerosis.11,14 Recently, bone marrow–derived EPCs have been identified that circulate in the blood and contribute to the formation of new blood vessels and homeostasis of the vasculature including repair of vascular lesions.15,16 Patients with reduced EPC levels are at increased risk for cardiovascular events and death.19,20 Augmentation of circulating EPCs or other cells with proangiogenic properties results in improved coronary collateral development in coronary artery disease.41 Next to alterations of circulating progenitor numbers, their function likely is important for vascular homeostasis, as patients with advanced coronary artery disease experience impaired EPC function.19,42

Characterization of EPCs is rather controversial and it is obvious that different types of EPC exist (reviewed in26,27). Here we analyzed the effects of nitrates on monocyctic early EPC.19,20,27,28 We previously characterized this type of EPC in a similar manner with Dil-acLDL uptake, UEA-1 staining, the ability to migrate and to incorporate into vascular structures, as well as detectable eNOS expression (see also Figure 3b) and activity.19,20 This type of monocyctic EPC has found angiogenic effects when transplanted to ischemic tissues.26,27 However, so far it is not entirely clear whether this effect is mediated by the secretion of angiogenic cytokines,43 immunomodulatory effects,44 or direct incorporation and production of new blood vessels.26,27

Nevertheless, our findings of partly opposing effects of different nitrates on EPC biology may therefore have important clinical implications. In a small clinical trial NTG treatment of healthy volunteers increased circulating CD34 progenitor cells, but also enhanced susceptibility of expanded EPCs to apoptosis.45 Ex vivo NTG exposure increased apoptosis and decreased phenotypic differentiation of EPCs.45 In the present study, we demonstrate therapy with two different long-acting nitrates to increase levels of circulating EPCs. However, functionally there were strong differences between the tested nitrates. This was mainly related to their different capacity to induce ROS formation in EPCs. Indeed, an important role for NO in regulation of progenitor cell mobilization and function has been described previously; Landmesser and coworkers demonstrated that the 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitor atorvastatin increased EPC mobilization in NOS3+/− but not NOS3−/− mice, which underlines the pivotal role of endothelial-derived NO in regulation of the transit of EPCs from bone marrow to circulation.18 Next to NO, increased oxidative stress may play a further role for the development of EPC dysfunction in diabetes and cardiovascular diseases.46 Likewise, impaired angiogenesis in glutathione peroxidase-1–deficient mice with enhanced oxidative stress is associated with EPC dysfunction.47 Here, we demonstrate exaggerated ROS production to impair migratory capacity of EPCs, which could be rescued by ex vivo treatment with polyethylene-glycolated SOD. Increased ISDN-mediated ROS formation in EPCs was at least in part mediated by NADPH oxidases. Recently, it was shown that NO is able to reverse cytoskeletal defects that lead to an impaired migratory potential in EPC from diabetic subjects, which also experience increased oxidative stress.48 Migratory capacity EPC appears to be tightly controlled by the intracellular balance of ROS and NO.

In conclusion, the organic nitrates ISDN and PETN/PETriN increase levels of circulating EPCs. However, there are significant differences of the tested nitrates on EPC function mediated by increased intracellular ROS production; ISDN increased ROS formation and impaired EPC function, whereas PETriN had favorable effects. Further prospective studies are needed that determine the long-term effects of organic nitrates on number and function of EPCs and in turn the development and/or prevention of atherosclerosis.

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