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Organic Nitrates Differentially Modulate Circulating Endothelial Progenitor Cells and Endothelial Function in Patients with Symptomatic Coronary Artery Disease

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Abstract

Symptomatic coronary artery disease (CAD) is usually treated with organic nitrates. Endothelial progenitor cells (EPCs) are a circulating cell population participating in vascular homeostasis in a nitric oxide-dependent manner. We investigated the effects of the nitric oxide donors isosorbide dinitrate (ISDN) and pentaerythritol tetranitrate (PETN) on EPC and endothelial function in patients with symptomatic CAD. We randomized 36 patients with angiographically proven CAD to treatment with either ISDN (40 mg retarded release orally two times per day; $n = 18$) or PETN (80 mg orally two times per day; $n = 18$) for 14 days (clinical trial number: NCT01030367). PETN treatment substantially increased numbers of circulating $CD34^+/KDR^+$ EPCs ($p = 0.02$), whereas no effects were observed in patients treated with ISDN. EPC function assessed by formation of endothelial colonies was enhanced by twofold ($p = 0.04$) in patients treated with PETN. No changes were observed after ISDN treatment. Endothelial function, assessed by peripheral arterial tonometry, remained unchanged during PETN treatment, but was significantly impaired in patients treated with ISDN. Treatment of symptomatic CAD patients with PETN for 14 days significantly increased levels of circulating EPC and improved markers for EPC function, whereas ISDN was without effects on EPCs and worsened endothelial function. *Antioxid. Redox Signal.* 15, 925–931.

Introduction

Organic nitrates are among the most commonly used drugs in patients with symptomatic coronary artery disease (CAD) (1). Despite their long-lasting use, an understanding of their mechanism of action particularly on vascular biology remains incomplete. Short-term treatment with organic nitrates results in vasodilatation by liberation of nitric oxide (NO) or related compounds. However, most organic nitrates during long-term treatment increase oxidative stress in the vasculature associated with endothelial dysfunction and development of nitrate tolerance (8, 24).

Endothelial progenitor cells (EPCs) are a circulating cell population participating in angiogenesis and vascular homeostasis. These cells are of fundamental importance as (re)vascularization is essential for the survival of growing, injured, or ischemic tissues. Indeed, EPC contribute to formation of new blood vessels and subset diseased endothelium by an NO-dependent mechanism (2, 3). EPC numbers and function are impaired in patients with CAD (12, 26, 28). The concentration of circulating EPC also has prognostic importance: patients with reduced EPC levels are at increased risk for cardiovascular events and death (19, 31), and circulating EPC correlate with enhanced coronary collateral development (14).

We previously compared the effects of two different organic nitrates, isosorbide dinitrate (ISDN) and pentaerythritol tetranitrate (PETN), on EPC number and function in an animal model and found beneficial effects of PETN on EPC function (24). To translate our previous animal findings into a clinical scenario, we now compared the effects of ISDN and PETN treatment on EPC number and function as well as endothelial function in patients with symptomatic CAD.

Materials and Methods

Patients

Approval from the ethics committee from the University of Wurzburg was obtained, as was informed written consent from patients. We recruited patients with stable angina admitted to the Division of Cardiology (University of Wuerzburg, Germany) for coronary angiography. The clinical study has been registered under www.clinicaltrials.gov (clinical

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trial number: NCT01030367). We included patients with symptomatic angiographically proven CAD without need for coronary intervention. We excluded patients with acute infections, known cancer, age <18 years, or acute or previous (4 weeks) treatment with organic nitrates. Baseline characteristics of patients are shown in Table 1. We investigated EPC number and function, as well as endothelial function at a baseline visit (day 0) and after 14 days of treatment with ISDN (40 mg retarded release two times per day) or PETN (80 mg two times per day). A total of 36 patients were included. All measurements (baseline and follow-up) were performed in the late afternoon before administration of the evening medication.

Determination of EPC number and function

An established flow-cytometric assay was used to quantify the amount of $CD34^+$, $CD133^+$, $CD133^+/CD34^+/KDR^+$ ($VEGFR2^+$) (early EPC), and $CD34^+/KDR^+(VEGFR2^+)$ (late EPC) cells in total blood as described (25, 26). To determine the amount of a further EPC subtype, monocytic EPC, an adhesion-related cell culture assay was used with subse-

Table 1. Baseline Patient Characteristics

	PETN		*ISDN*		
	Mean	*SEM*	*Mean*	*SEM*	p-*Value*
Basic characteristics					
n	18		18		
Age (years)	65.33	2.47	65.89	2.47	0.88
BMI	26.43	0.85	28.66	1.29	0.19
Gender (% male)	72.22		72.22		1.00
Cardiovascular data					
Mean art. pressure (mmHg)	91.72	3.78	99.62	3.01	0.08
EF (%)	65.69	3.65	68.31	2.07	0.54
Coronary vessel disease (1–3)	2.17	0.25	1.67	0.20	0.12
Basic blood parameters					
Hemoglobin (g/dl)	14.01	0.28	14.69	0.21	0.06
Leucocytes ($n\times^{\circ}10^3/\mu$l)	7.43	0.48	7.42	0.44	0.99
CRP (mg/dl)	0.64	0.30	0.36	0.09	0.38
HbA1c (%)	6.12	0.33	6.22	0.26	0.83
Total cholesterol (mg/dl)	166.75	8.63	164.73	6.75	0.86
LDL (mg/dl)	89.94	6.69	86.60	5.48	0.71
HDL (mg/dl)	44.94	3.25	46.73	2.97	0.69
Uric acid (mg/dl)	6.78	0.38	6.00	0.21	0.08
MDRD (ml/min/1.73 m^2)	85.44	6.77	95.28	6.07	0.29
TSH (mIU/l)	0.98	0.15	1.59	0.44	0.17
Serum creatinine (mg/dl)	0.97	0.08	0.84	0.05	0.17
Urea (mg/dl)	39.10	4.14	34.20	1.45	0.27
Cardiovascular risk factors					
Hypertension	18	0	18	0	1.00
Obesity	9	5	15	2	0.10
Diabetes	3	14	4	13	0.91
Medication					
ASS/Clopidogrel	18	0	18	0	1.00
ACE-inh./AT1-antag.	16	2	15	3	0.63
Statins	18	0	18	0	1.00
Antidiabetics	1	17	3	15	0.29
Insulin	1	17	1	17	1.00
Beta-blockers	18	0	18	0	1.00
Diuretics	16	2	14	4	0.37
Digitalis	3	15	1	17	0.29
Calcium-antagonists	5	13	7	11	0.48
Dose level of selected medication	*Mean*	*SEM*	*Mean*	*SEM*	p-*Value*
Simvastatin (total mg/day)	30.63	1.26	34.12	1.14	0.64
Ramipril (total mg/day)	5.21	0.66	4.69	0.60	0.67

All patients except one (PETN-group; atorvastatin) received simvastatin as HMG-CoA reductase inhibitor therapy. Most patients (PETN group: 12; ISDN group: 8) received ramipril as ACE inhibitor, whereas others received enalapril or an AngII-receptor blocker. We therefore only report dose levels of the most prescribed statin (simvastatin) and ACE inhibitor (ramipril). Both drugs were administered at least 6 weeks before study inclusion.

BMI, body mass index; ISDN, isosorbide dinitrate; PETN, pentaerythritol tetranitrate; SEM, standard error of the mean; ACE, angiotensin converting enzyme; ASS, acetylsalicylic acid; CRP, C-reactive protein; EF, ejection fraction; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoprotein; MDRD, Modification of Diet in Renal Disease; TSH, thyroid stimulating hormone; art., arterial; inh., inhibitor; antag., antagomir.

quent determination of DiI-acLDL/Ulex europeus-1 (UEA-1)-positive cells. To analyze EPC function the capability of cells to form endothelial colony-forming units (CFU) and their migratory capacity toward an VEGF/SDF-1 gradient were determined as described (23).

Determination of endothelial function

Endothelial function was determined by an established peripheral arterial tonometry (PAT) method based on a plethysmographic device (EndoPAT2000; Itamar-Medical) (17). Details about the method have been previously described (13, 17). In brief, PAT represents a noninvasive technology that determines a beat-to-beat plethysmographic recording of the finger arterial pulse wave amplitude with pneumatic probes (4). PAT probes were placed on the index finger of each hand for continuous recording of the PAT signal. After a 5 min equilibration period, which was used as baseline, the blood pressure cuff was inflated to suprasystolic pressure for 5 min. Thereafter, the cuff was deflated and PAT was recorded for further 8 min. The reactive hyperemia index (RHI) was calculated as the ratio of the digital pulse volume during reactive hyperemia over a 1 min time interval starting 1 min after cuff deflation to that at baseline (13).

Statistical analysis

Data are presented as mean and standard error of the mean unless otherwise stated. Statistical analysis was performed using the StatView (SAS Institute) package. For statistical comparison of two groups, we employed paired or unpaired, two-tailed Student's *t*-test as appropriate. Differences were considered significant when $p < 0.05$.

Results

Patient characteristics at baseline

All study objects had an angiographically proven symptomatic CAD. At baseline there were no significant differences among the investigated treatment groups (PETN *vs.* ISDN) with respect to age, body mass index, gender, blood pressure, ejection fraction, severity of CAD, basic blood parameters, cardiovascular risk factors (hypertension, adipositas, or type II diabetes), or current medication (see Table 1). In addition, dose levels between statins and angiotensin converting enzyme (ACE) inhibitors that also may interfere with EPC number and function, as well as with endothelial function, were similar between the two groups (see Table 1).

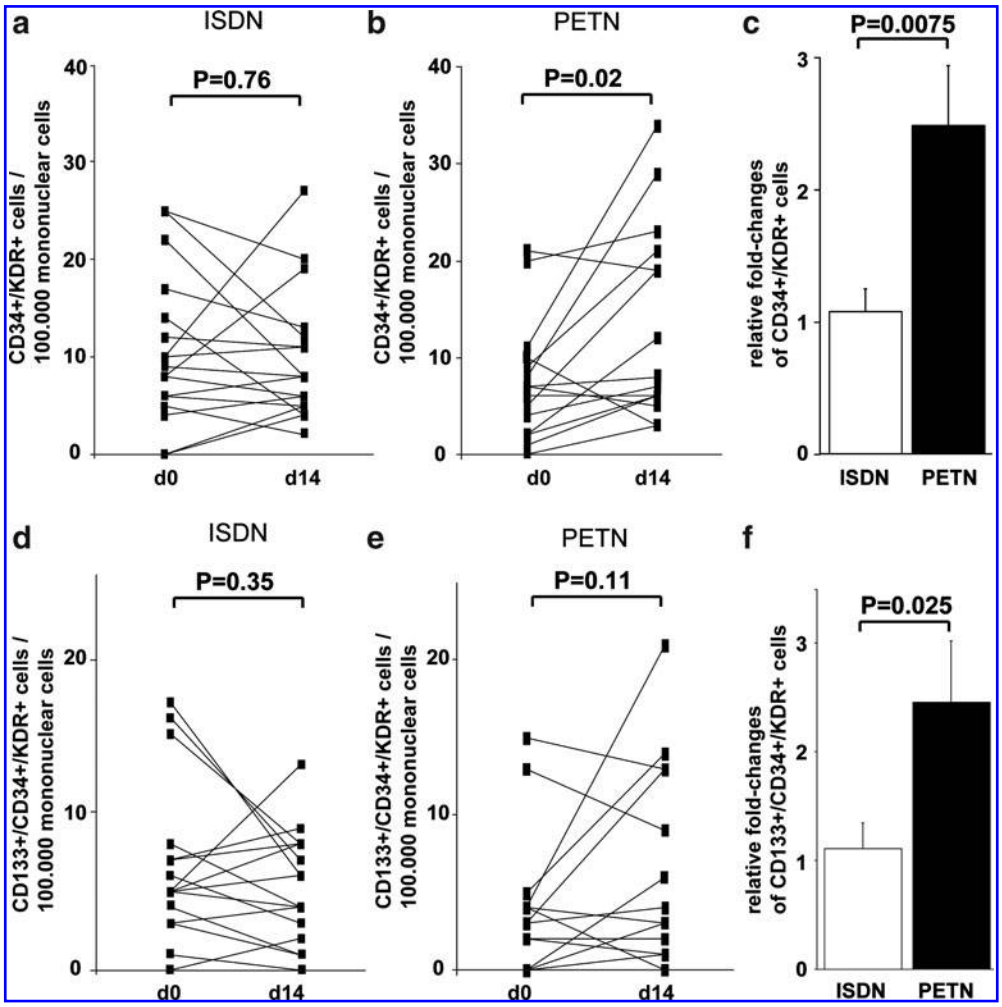

FIG. 1. Effects of organic nitrate treatment on circulating endothelial progenitor cell (EPC) levels in patients with coronary artery disease. $CD34^+/KDR^+$ cells in patients before and after twice daily treatment with isosorbide dinitrate (ISDN) **(a)** or pentaerythritol tetranitrate (PETN) **(b)** for 14 days. **(c)** Relative increase in $CD34^+/KDR^+$ cells after treatment with ISDN or PETN. $CD133^+CD34^+/KDR^+$ cells in patients before and after twice daily treatment with ISDN **(d)** or PETN **(e)** for 14 days. **(f)** Relative increase in $CD133^+/CD34^+/KDR^+$ cells after treatment with ISDN or PETN. Fluorescence-activated cell sorting analysis was performed in $n = 17$ patients from the ISDN treatment group and $n = 16$ of the PETN treatment group.

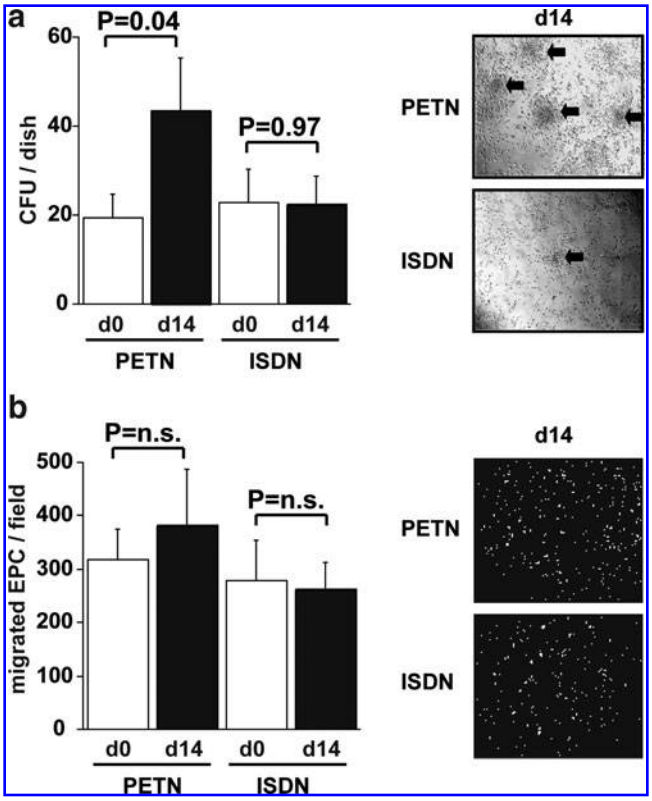

FIG. 2. Effects of organic nitrate treatment on EPC function in patients with coronary artery disease. Changes in endothelial colony-forming units **(a;** *black arrows*) and migratory capacity **(b)** of EPC are shown before and after 14 days of treatment of patients with coronary artery disease with ISDN or PETN. n.s., non significant.

Effects of organic nitrates on number of circulating EPCs

We determined three different subtypes of EPC in patients before and after treatment with ISDN or PETN for 14 days. Using a flow-cytometric method we identified the amount of $CD133^+/CD34^+/KDR^+$ cells/100,000 mononuclear blood cells (early EPC) and $CD34^+/KDR^+$ cells/100,000 mononuclear blood cells (late EPC). Treatment with ISDN did not change the number of circulating early or late EPC (Fig. 1a, c, d, f). In contrast, treatment of patients with PETN for 14 days significantly increased levels of circulating late EPC (CD34+/KDR+ cells; $p = 0.02$) and showed a trend for increased levels of circulating early EPC ($p = 0.11$) (Fig. 1b, c, e, f). We also compared the relative increase in circulating EPC levels between both treatment groups and show PETN to significantly increase early and late EPC levels compared with the ISDN treatment group (Fig. 1c, f). In contrast no significant effects of ISDN or PETN on the levels of circulating hematopoietic progenitor cells ($CD34^+$ cells, $CD133^+$ cells) were observed (data not shown). We also determined treatment effects on monocytic EPC levels using an adhesion-based method. Monocytic EPC were characterized by Dil-acLDL/UEA-1 staining. We did not find any effects of either treatment with ISDN (587.7 ± 79.2 cells before and 678.4 ± 82.3 cells after treatment, $p =$ non significant) or PETN (556.4 ± 45.6 cells before and 533.7 ± 37.2 cells after treatment, $p =$ non significant) suggesting effects only on specific EPC subsets.

Effects of organic nitrates on function of circulating EPCs

To determine EPC function we measured ability of EPC to form endothelial colony units (CFU assay) and migratory capacity using a modified Boyden chamber. Treatment of patients with PETN led to a twofold increase ($p = 0.04$) of CFU formation after 14 days of treatment, whereas ISDN had no effects (Fig. 2a). Migratory capacity toward an SDF-1/VEGF gradient was not significantly changed either by PETN or ISDN treatment (Fig. 2b).

Effects of organic nitrates on endothelial function

We measured effects of organic nitrate treatment on endothelial function by testing for changes of the RHI induced by forearm cuff occlusion in patients with CAD. Measurements were not possible in 2/18 patients in the ISDN treatment group

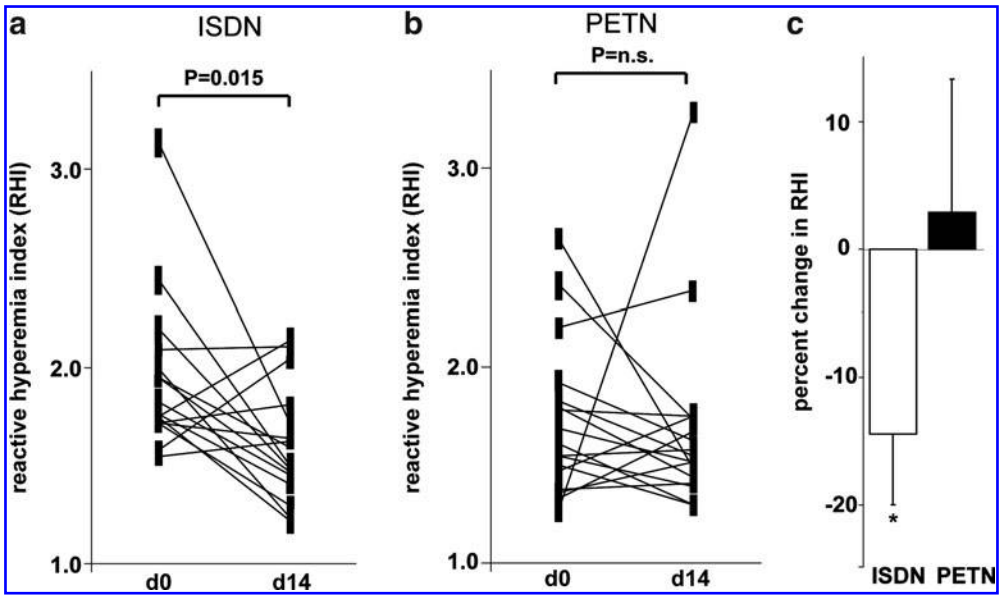

FIG. 3. Effects of organic nitrate treatment on endothelial function in patients with coronary artery disease. Changes in the reactive hyperemia index (RHI) before and after treatment of patients with coronary artery disease with ISDN **(a)** or PETN **(b)**. **(c)** Relative changes of RHI after treatment with ISDN or PETN. $^*p < 0.05$.

and 1/18 in the PETN treatment group due to discomfort during arterial occlosure phase. Treatment with PETN for 14 days did not significantly change RHI ($+2.7\% \pm 10.5\%$). In contrast, treatment with ISDN led to a significant decrease of the reactive hyperemia response ($-14.6\% \pm 5.6\%$; Fig. 3).

Discussion

The present study demonstrates that organic nitrates differentially modulate number and function of EPCs in patients with symptomatic CAD. Treatment with PETN increased EPC levels, whereas ISDN had no effects.

In general, therapies that increase NO bioavailability usually increase EPC levels (22, 27), whereas NO lowering substances result in a decrease of circulating EPC (26). Therapeutic modulation of EPC may be of clinical importance as patients with reduced EPC levels have a significantly higher risk to develop coronary events (19, 31). We previously found ISDN and PETN to increase EPC levels in rats after oral administration or by infusion with implanted mini-pumps. However, only PETN improved EPC function, whereas ISDN increased intracellular superoxide anion formation resulting in impaired EPC function. Treatment with the short-acting nitrate nitroglycerin increased circulating EPC levels *in vivo* but decreased survival *ex vivo* (6).

In the present study we extend our previous findings to patients with symptomatic CAD, and show PETN treatment to result in a robust increase in $CD34^+/KDR^+$ EPCs, whereas no effects were seen after ISDN. Likewise, PETN but not ISDN improved EPC function. A possible underlying reason could be the different effects of the two organic nitrates on NO bioavailability and production of oxidative stress as shown in animal studies (24). Clinically, Schnorbus and colleagues recently compared markers of oxidative stress and NO in blood samples of coronary artery diseased patients before and after an 8-week treatment with PETN (3×80 mg/day) but did not find differences in bilirubin, ferritin, TBARS, or CRP (20). In addition, Keimer *et al.* compared the effects of ISDN (3×30 mg/day for 5 days) and PETN (3×80 mg/day for 5 days) on the oxidative stress markers 8-iso-PGF2alpha and 3-nitrotyrosine but did not find significant differences between the groups (11). Obviously, in patients the estimated changes in markers for oxidative stress and/or NO after treatment with organic nitrates appear rather modest. In future studies the impact of different organic nitrates on intracellular markers for oxidative stress and NO, including expression of antioxidative enzyme systems such as the heme oxygenase 1 (16, 30), should be also investigated in EPC from patients.

The increase of functional EPC by PETN may result in stabilization of endothelial function in the context of an ongoing CAD process as EPC are of importance for vascular homeostasis and correlate with endothelial function in patients with CAD (32). Indeed, systemic transfusion of vascular progenitor cells improves endothelial function underlining the close relationship between EPC and vascular function (29). Impaired flow-mediated and/or acetylcholine-mediated vasodilatation is a hallmark of endothelial dysfunction. Continuous treatment with short acting nitrates such as glyceroltrinitrate leads to oxidative stress-induced worsening of endothelial function in patients with CAD (5, 21) or to induction of endothelial dysfunction in healthy subjects (9). Indeed, endothelial dysfunction may be an important sequela of nitrate therapy as it is a major predictor of cardiovascular events in patients with CAD (10, 18). A meta-analysis of clinical studies suggested that development of nitrate tolerance may underlie the increases in cardiovascular events associated with long-term nitrate use (15). Unfortunately large-scale controlled studies regarding the efficacy and safety of chronic nitrate therapy in CAD are lacking at present. Our study demonstrating for the first time the different effects of two different organic nitrates may therefore be of ample clinical relevance. Whereas PETN did not change endothelial function, ISDN treatment within 14 days resulted in a reduction of the RHI suggesting a development of endothelial dysfunction. Our data about endothelial function are in good agreement with the recent PENTA study that also did not identify detrimental effects of PETN treatment in CAD patients (20). In the absence of any larger study demonstrating prevention of cardiovascular events by long-term treatment with organic nitrates, our data may point to a preferential use of PETN in symptomatic CAD patients, although larger double-blinded studies are needed to validate our findings.

An explanation for the observed differences relates to the different molecular structure of PETN and ISDN, which was reported to result in different biological effects; PETN increases antioxidative enzyme systems in endothelial cells, thus preventing an excessive production of reactive oxygen species, resulting in oxidative stress, whereas most other nitrate compounds, including ISDN, lack antioxidative capacity (7, 16, 30).

Our study has several limitations. First, the number of included patients is small and larger trials comparing the effects of different organic nitrates on EPC and endothelial function are warranted. In addition, the present study was not conducted in a double-blinded fashion and was not placebo-controlled. However, the assessment of endothelial function and the analyses of EPC numbers and function were blinded and were performed without knowledge of the actual treatment of the patients. Finally, patients were cotreated with drugs (*e.g.*, statins and ACE inhibitors) that may have impacted on development of nitrate tolerance under organic nitrate therapy and thus to changes in EPC number and function as well as endothelial function. However, there were no differences in dosing between statins and ACE inhibitors in both treatment groups.

In conclusion, PETN treatment increased EPC number and function, whereas ISDN had no effects on EPC but worsened markers of endothelial function in patients with CAD.

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Author Disclosure Statement

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Abbreviations Used

CAD = coronary artery disease
CFU = colony-forming units
EPCs = endothelial progenitor cells
ISDN = isosorbide dinitrate
NO = nitric oxide
PAT = peripheral arterial tonometry
PETN = pentaerythritol tetranitrate
RHI = reactive hyperemia index

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1. Roberta R. Holt, Christian Heiss, Malte Kelm, Carl L. Keen. 2012. The Potential of Flavanol and Procyanidin Intake to Influence Age-Related Vascular Disease. *Journal of Nutrition in Gerontology and Geriatrics* **31**:3, 290-323. [CrossRef]
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3. Felix Fleissner , Thomas Thum . Critical Role of the Nitric Oxide/Reactive Oxygen Species Balance in Endothelial Progenitor Dysfunction. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]