Oxidant Stress Impairs In Vivo Reendothelialization Capacity of Endothelial Progenitor Cells From Patients With Type 2 Diabetes Mellitus

Restoration by the Peroxisome Proliferator-Activated Receptor-γ Agonist Rosiglitazone

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Background—Endothelial progenitor cells (EPCs) are thought to contribute to endothelial recovery after arterial injury. We therefore compared in vivo reendothelialization capacity of EPCs derived from patients with diabetes mellitus and healthy subjects. Moreover, we examined the effect of treatment with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone on oxidant stress, nitric oxide (NO) bioavailability, and the in vivo reendothelialization capacity of EPCs from diabetic individuals.

Methods and Results—In vivo reendothelialization capacity of EPCs from diabetic patients (n=30) and healthy subjects (n=10) was examined in a nude mouse carotid injury model. Superoxide and NO production of EPCs was determined by electron spin resonance spectroscopy. Thirty patients with diabetes mellitus were randomized to 2 weeks of rosiglitazone (4 mg BID PO) or placebo treatment. In vivo reendothelialization capacity of EPCs derived from diabetic subjects was severely reduced compared with EPCs from healthy subjects (reendothelialized area: 8±3% versus 37±10%; $P<0.001$). EPCs from diabetic individuals had a substantially increased superoxide production and impaired NO bioavailability. Small-interfering RNA silencing of NAD(P)H oxidase subunit $p47^{phox}$ reduced superoxide production and restored NO bioavailability and in vivo reendothelialization capacity of EPCs from diabetic patients. Importantly, rosiglitazone therapy normalized NAD(P)H oxidase activity, restored NO bioavailability, and improved in vivo reendothelialization capacity of EPCs from diabetic patients (reendothelialized area: placebo versus rosiglitazone, 8±1% versus 38±5%; $P<0.001$).

Conclusions—In vivo reendothelialization capacity of EPCs derived from individuals with diabetes mellitus is severely impaired at least partially as a result of increased NAD(P)H oxidase–dependent superoxide production and subsequently reduced NO bioavailability. Rosiglitazone therapy reduces NAD(P)H oxidase activity and improves reendothelialization capacity of EPCs from diabetic individuals, representing a potential novel mechanism whereby peroxisome proliferator-activated receptor-γ agonism promotes vascular repair. **(*Circulation*. 2007;116:163-173.)**

Key Words: endothelium ■ nitric oxide synthase ■ oxidative stress ■ PPAR gamma ■ progenitor cells

Accelerated vascular disease is the principal cause of death and disability in patients with diabetes mellitus. Endothelial injury is thought to represent a crucial step in initiation and progression of atherosclerotic vascular disease.[1,2] This concept has recently been supported by the close association of endothelial dysfunction, as observed in diabetic individuals,[3] with cardiovascular events.[2] Furthermore, insufficient numbers of endothelial progenitor cells (EPCs) have been related to endothelial dysfunction[4] and an adverse clinical outcome,[5] further suggesting that endothelial injury in the absence of sufficient circulating progenitor cells promotes progression of vascular disease. Moreover, 2 recent studies have observed an

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association of reduced EPC numbers with peripheral artery disease and its severity in diabetic patients.[6,7]

Experimental studies have demonstrated that EPCs promote endothelial repair after injury.[8–10] In diabetic patients, however, an impaired migration capacity and tube formation of EPCs have been observed in vitro,[11,12] and a diabetes-induced delay in reendothelialization by EPCs has been described for diabetic mice,[13] raising the question of whether the in vivo reendothelialization capacity of human EPCs from patients with diabetes mellitus is altered. Moreover, mechanisms underlying EPC dysfunction in diabetic individuals remain largely unknown. In the present study, we therefore compared the in vivo reendothelialization capacity of EPCs derived from diabetic and healthy subjects and analyzed mechanisms of EPC dysfunction.

Increased oxidant stress has been proposed as a molecular mechanism for vascular complications in diabetes mellitus, in part by reducing nitric oxide (NO) availability.[14] In this respect, we and others have observed a role of endothelial NO synthase (eNOS) for EPC mobilization and function in studies using eNOS-deficient mice.[15–17] We therefore examined the role of oxidant stress and NO bioavailability for the in vivo reendothelialization capacity of EPCs.

Notably, peroxisome proliferator-activated receptor (PPAR)-γ stimulation, a promising treatment for diabetes mellitus, has recently been shown to stimulate NO production in mature endothelial cells in vitro.[18] Moreover, experimental studies have suggested that PPAR-γ stimulation may exert antioxidant effects.[19] In a small, uncontrolled clinical study, it has been proposed that rosiglitazone improves EPC in vitro function (ie, migratory activity).[20] In the present study, diabetic individuals were therefore randomized to 2-week treatment with the PPAR-γ agonist rosiglitazone or placebo, and effects on superoxide and NO production and the in vivo reendothelialization capacity of EPCs were analyzed.

Methods

Patient Characteristics and Study Protocol

Written informed consent was obtained from all participants, and the study protocol was approved by the local ethics committee. Patients were included into the study between March 2004 and August 2005. Peripheral venous blood samples (60 mL) were obtained for EPC isolation from individuals with type 2 diabetes mellitus (n=30) and healthy subjects (n=10). Those with diabetes were randomized (3:2) to 2-week rosiglitazone (4 mg BID PO) or placebo therapy. Patient characteristics are shown in Tables 1, 2, and 3.

Isolation and Cultivation of EPCs

EPCs were isolated and cultured as described in detail previously.[17,21,22] In brief, peripheral blood mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrome, Berlin, Germany), and 10^7 cells were cultured on fibronectin-coated 6-well plates in endothelial cell basal medium-2 (containing 5 mmol/L glucose) supplemented with endothelial growth medium–SingleQuots exactly as indicated by the manufacturer except for hydrocortisone (Clonetics, Inc). After 4-day culture, nonadherent cells were removed by washing plates with PBS. Remaining cells were trypsinized and used for in vitro and in vivo functional analysis.

TABLE 1. Characteristics of Healthy and Diabetic Subjects

	Healthy (n=10)	Diabetic (n=30)	*P*
Age, y	65±3	65±2	NS
Gender, M/F	7/3	28/2	NS
Body mass index, kg/m^2	27±1	30±1	NS
Mean arterial pressure, mm Hg	99±5	110±2	NS
HbA1c, %	5.4±0.1	6.6±0.2	<0.01
Fasting glucose, mg/dL	92±4	142±6	<0.01
LDL cholesterol, mg/dL	131±12	120±8	NS
HDL cholesterol, mg/dL	50±4	43±4	NS
C-reactive protein, mg/dL	1.37±0.5	1.41±0.43	NS
Creatinine, μmol/L	82±4	80±2	NS

Values are mean±SEM. HbA1c indicates glycohemoglobin; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

EPC Characterization

Adherent EPCs were characterized by dual staining for acetylated low-density lipoprotein and lectin as described previously[17,21,22] and by flow cytometry analysis for expression of endothelial marker proteins (CD31, von Willebrand factor [vWF], and kinase-insert domain receptor [KDR]) and the monocytic lineage marker CD14 (see the online-only Data Supplement for more details). The vast majority of cells cultured for 4 or 7 days from healthy subjects and diabetic subjects were double positive for acetylated low-density lipoprotein–lectin staining and expressed both endothelial marker proteins and the monocytic marker CD14 at comparable levels (online-only Data Supplement Figure I). EPC quantification and acetylated low-density lipoprotein–lectin staining were performed as described previously[17,21,22] and in the online-only Data Supplement.

TABLE 2. Characteristics of Diabetic Subjects Receiving Placebo or Rosiglitazone Treatment

	Placebo (n=12)	Rosiglitazone (n=18)	*P* (Between Groups After Treatment)
Age, y	64±2	65±2	>0.9*
Gender, M/F	11/1	17/1	0.65†
Body mass index, kg/m^2	30±1	30±1	>0.9*
Medication, n/N			
ACE/ARB	10/12	14/18	0.88†
ASA	6/12	11/18	0.99†
Diuretic	4/12	11/18	0.58†
Calcium antagonist	5/12	11/18	0.79†
Nitrates	2/12	5/18	0.90†
β-Blocker	4/12	9/18	0.82†
Statin	5/12	11/18	0.79†

Values are expressed as mean±SEM where appropriate. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; and ASA, acetylsalicylic acid.

*Bonferroni corrected.

†χ^2 Analysis.

Animals and In Vivo Reendothelialization Assay

Male NRMI$^{nu/nu}$ athymic nude mice, aged 7 to 10 weeks, were used to allow injection of human EPCs. Animals were anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Carotid artery electric injury was performed as described previously.[23,24] In brief, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE-Elektromedizin GmbH, Tuebingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimeter of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery.

EPCs (5×10^5 cells) were resuspended in 100 μL of prewarmed PBS (37°C) and transplanted 3 hours after carotid injury via tail vein injection with a 27-gauge needle. The same volume of PBS was injected into placebo mice. Three days after carotid injury, endothelial regeneration was evaluated by staining denuded areas with 50 μL of solution containing 5% Evans blue dye via tail vein injection as described previously.[25] The reendothelialized area was calculated as difference between the blue-stained area and the injured area by computer-assisted morphometric analysis. Of note, this model has been shown to allow accurate quantification of reendothelialization.[23] EPCs from each diabetic/healthy subject (before and after treatment) were injected into 2 nude mice, and mean values of reendothelialized area were used for analysis (more details are available in the online-only Data Supplement).

Confocal laser scanning microscopy (Leica DM-IRB microscope with a TCS-SP2-AOBS scan head) was performed to detect homing of transplanted EPCs to the site of vascular injury in separate experiments (n=5) with the use of PKH26 (Sigma-Aldrich)-labeled EPCs. The local committee on animal research approved all procedures involving animals.

EPC Superoxide Production and NAD(P)H and Xanthine Oxidase Activity

EPC superoxide ($O_2^{\cdot-}$) production and NAD(P)H and xanthine oxidase activity were determined by electron spin resonance (ESR) spectroscopy analysis with the use of the spin-trap 1-hydroxy-3-carboxy-pyrrolidine (CP-H; Alexis Corporation) in 250 000 resuspended or homogenized EPCs as described previously[26,27] and in the online-only Data Supplement.

NO Production in EPCs

NO production was measured by ESR spectroscopy analysis with the use of the spin-trap colloid $Fe(DETC)_2$ as described previously[28] and in the online-only Data Supplement.

EPC Adhesion to Endothelial Cells In Vitro

A monolayer of human umbilical vein endothelial cells (Cambrex, Taufkirchen, Germany) was prepared 48 hours before the assay by plating 2×10^5 cells (passage 1 to 5) in each well of a 4-well plate. Human umbilical vein endothelial cells were pretreated with tumor necrosis factor-α (BD Biosciences; 1 ng/mL; 12 hours) or media. Then 1×10^5 diI-labeled EPCs were added to each well and incubated for 3 hours at 37°C. Nonattached cells were gently removed with PBS, and adherent EPCs were fixed with 4% paraformaldehyde and counted in 4 random fields.

EPC Migration In Vitro

EPC migration was evaluated with the use of a modified Boyden's chamber assay as described previously.[29] Briefly, cell suspensions (1×10^5 cells per well) were placed in the upper chamber, and the lower chamber was filled with medium containing human recombinant vascular endothelial growth factor (50 ng/mL; R&D Systems). The chamber was incubated for 16 hours (37°C). Migration activity was evaluated as mean number of migrated cells in 3 high-power fields per chamber.

Small-Interfering RNA Transfection

NAD(P)H oxidase subunit p47^{phox}, eNOS, and PPAR-γ expression were silenced with the use of Validated Stealth RNAi (Invitrogen, Carlsbad, Calif), adapting the manufacturer's protocol after determining optimal transfection conditions (data not shown). The small-interfering RNA (siRNA) sequences (sense strands) used for targeting human p47^{phox}, eNOS, and PPAR-γ were 5′-CGGAGAGACGAGUAUAACCCAGUUU-3′ (Invitrogen primer number: 104380E05), 5′-UGUGUUACUGGACUCCUUCCUCUUC-3′ (Invitrogen primer number: 95777G10), and 5′-UCAGCUCCGUGGAUCUCUCCGUAAU-3′ (Invitrogen primer number: 111556H08). A Stealth RNAi Negative Control Duplex (Invitrogen) was used as a negative control. In preliminary experiments, transfection efficiency was >90% of EPCs as determined by transfection with fluorescence-labeled siRNA. All siRNA transfections were performed for 24 hours preceding subsequent EPC measurements.

Western Blot Analysis

Protein extracts were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes, and probed with anti-human antibodies for NAD(P)H oxidase subunits p22^{phox}, p47^{phox}, p67^{phox}, or eNOS (Santa Cruz Biotechnology, Santa Cruz, Calif), followed by enhanced chemiluminescence detection.

Statistical Analysis

Data in the text and figures are expressed as mean±SD. Differences between means of groups were compared with the use of Wilks λ 1-way MANOVA testing, followed by Student or Welch t test. Comparisons between frequencies were performed by χ^2 analysis. To account for inflation of experiment type-1 error due to multiple testing, Bonferroni correction was used for multiple comparisons of results presented in Tables 2 and 3 and the figures. The rationale of the Bonferroni adjustments for the results presented in Tables 2 and 3 and the figures was as follows: A primary and secondary weighting of hypotheses (primary: placebo versus rosiglitazone; secondary: in vitro experiments) was performed, and Bonferroni corrections were applied separately to each hypothesis. A Bonferroni-corrected probability value of <0.05 was considered statistically significant. A nonparametric analysis using the Wilcoxon rank sum test was performed when appropriate. All statistical analyses used SPSS statistical software (SPSS version 14).

The primary end point of the present study was the in vivo reendothelialization capacity of EPCs, which was therefore used to determine the study size. For the randomized substudy (effect of rosiglitazone versus placebo therapy in diabetic patients), the relevant alternative was a difference of 20% of reendothelialized area between the treatment groups. With the assumption of a common SD of 15%, a sample size of 30 patients randomized 2:1 was needed to have a power of 90% to reject the null hypothesis in favor of the alternative hypothesis with a 0.025 type I error.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

In Vivo Reendothelialization Capacity of EPCs From Diabetic Subjects Is Severely Reduced

Transplantation of EPCs from healthy subjects (n=10) markedly accelerated reendothelialization of denuded carotid arteries in nude mice (Figure 1A and 1B). Confocal laser scanning microscopy analysis of a subgroup of nude mice (n=5) revealed that transplanted EPCs were attached at the site of vascular injury (Figure 1C). Notably, in vivo reendothelialization capacity of EPCs derived from diabetic subjects (n=30) was markedly reduced (Figure 1A and 1B). To further determine whether EPCs from diabetic subjects had a delayed or diminished response, reendothelialization was examined later (ie, 7 days after EPC transplantation in a subgroup of healthy and diabetic subjects [n=5]). Seven days after transplantation of EPCs from healthy subjects, the endothelial layer was almost completely restored, whereas

TABLE 3. Characteristics of Diabetic Subjects Before and After Placebo or Rosiglitazone Treatment

	Placebo (n=12)			Rosiglitazone (n=18)			
	Before	After	P*	Before	After	P*	P* (Between Groups After Treatment)
Mean arterial pressure, mm Hg	112±3	109±2	>0.9	110±3	103±4	0.340	>0.9
HbA1c, %	6.9±0.4	6.9±0.4	>0.9	6.4±0.3	6.4±0.2	>0.9	>0.9
Fasting glucose, mg/dL	149±12	144±10	>0.9	135±6	124±5	0.250	>0.9
Insulin levels, μU/mL	18.3±2.9	25.3±9.0	>0.9	35.7±5.3	38.5±6.7	>0.9	0.28
HOMA index	6.8±1.6	9.1±1.2	>0.9	11.9±1.3	11.8±1.7	>0.9	>0.9
LDL cholesterol, mg/dL	137±9	123±9	>0.9	108±7	112±7	>0.9	0.15
HDL cholesterol , mg/dL	47±5	46±4	>0.9	40±2	41±2	>0.9	>0.9
Free fatty acids, mg/dL	11.6±1.33	11.9±1.7	>0.9	11.8±1.6	8.4±1.2	>0.9	0.95
C-reactive protein, mg/dL	1.0±0.3	0.6±0.3	>0.9	1.5±0.8	0.70±0.2	0.690	>0.9
Creatinine, μmol/L	77±2	80±3	>0.9	83±4	82±4	0.640	>0.9

Values are expressed as mean±SEM where appropriate. Hba1c indicates glycohemoglobin; HOMA, Homeostasis Model Assessment; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

*Bonferroni-corrected.

EPCs from diabetic individuals had no significant effect on reendothelialization (Figure IIA and IIB in the online-only Data Supplement). To evaluate whether a potential contamination of EPCs with endothelial cells may have contributed to reendothelialization, human endothelial cells (5×10^5 cells) were transplanted into 5 nude mice but had no effect on reendothelialization (Figure IIC and IID in the online-only Data Supplement).

Fluorescence-activated cell-sorting analyses revealed a similar endothelial marker protein (vWF, CD31, and KDR) and monocytic lineage marker (CD14) expression on EPCs from healthy and diabetic subjects, suggesting that EPCs

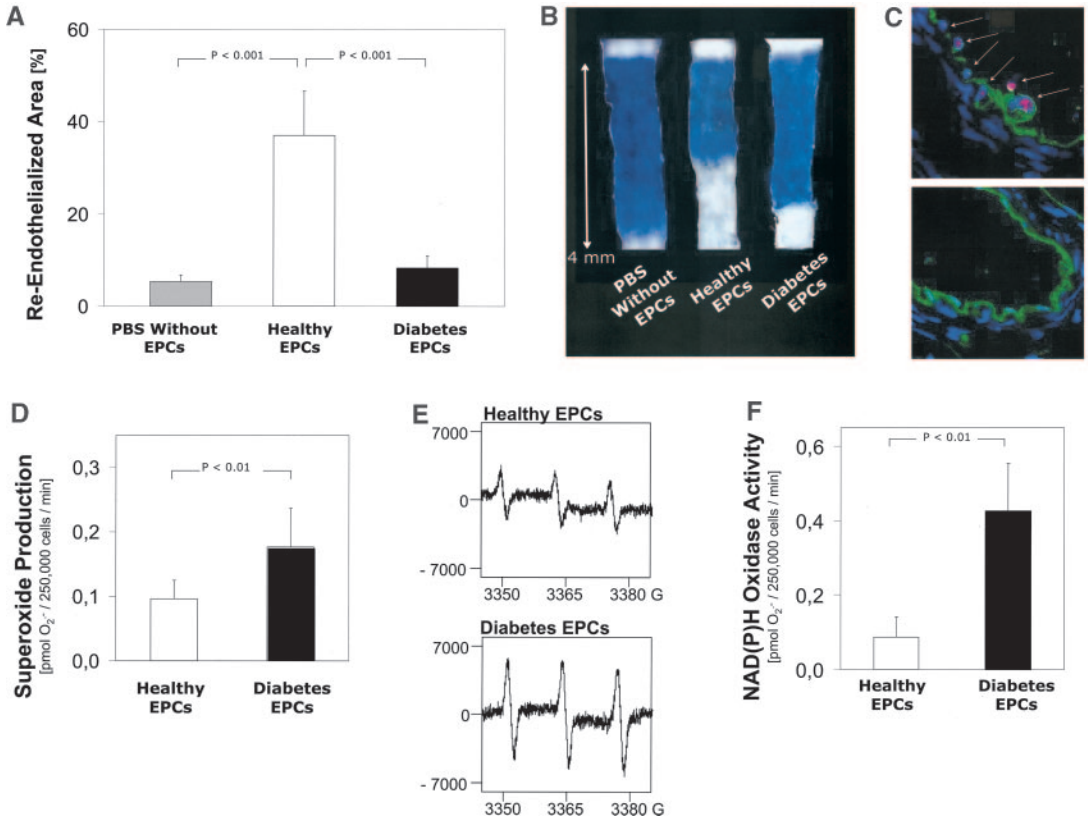

Figure 1. A, Reendothelialized area at day 3 after carotid injury in nude mice with placebo injection (n=15), transplantation of EPCs from healthy subjects (n=10), or diabetic subjects (n=30; each 5×10^5 EPCs). B, Representative photographs. EPCs from each patient or healthy subject were injected into 2 nude mice; mean values of reendothelialized area are shown. C, Confocal laser scanning microscopy (magnification ×80): top, carotid artery 3 days after injury showing PKH26-labled EPCs (red) attached to isolectin-B4–stained endothelium (green); bottom, contralateral uninjured carotid artery (nuclei stained blue; TO-Pro3). Data are representative of 5 separate experiments. D through F, ESR spectroscopy analyses of superoxide production (D) and NAD(P)H oxidase activity (F) of EPCs from diabetic subjects (n=30) and healthy subjects (n=10) are shown, as well as representative ESR spectra of superoxide production (E).

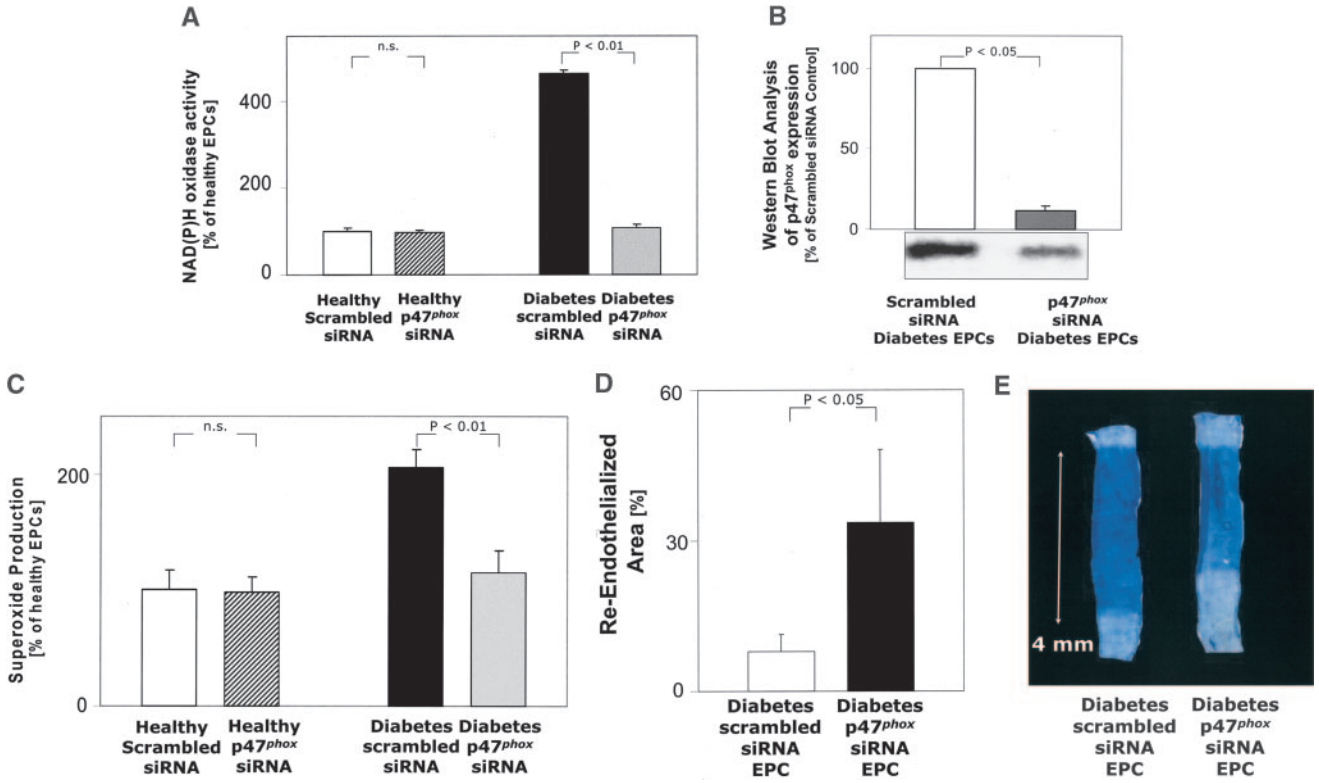

Figure 2. Effect of siRNA silencing of NAD(P)H oxidase subunit p47^{phox} on EPC NAD(P)H oxidase activity (A), p47^{phox} protein expression (B), superoxide production (C), and in vivo reendothelialization capacity (D; n=6). E, Representative photographs showing reendothelialization of carotid artery on day 3 after injury after transplantation of diabetic EPCs treated with p47^{phox}-specific siRNA or scrambled siRNA.

from diabetic subjects did not lose endothelial marker proteins (Figure I in the online-only Data Supplement).

Superoxide Production and NAD(P)H Oxidase Activity Are Increased in EPCs From Diabetic Subjects

EPCs from diabetic subjects had a markedly increased superoxide production compared with EPCs from healthy subjects (Figure 1D and 1E). Notably, activity of NAD(P)H oxidase, a major oxidant enzyme, was substantially increased in EPCs from those with diabetes mellitus (Figure 1F).

Of note, xanthine oxidase (XO) activation dependent on NAD(P)H oxidase has been observed in endothelial cells.[30] In EPCs from diabetic subjects, XO activity was increased compared with EPCs from healthy subjects (1.52±0.14 versus 0.83±0.04 pmol $O_2^{\cdot-}$)/250 000 EPCs per minute; $P<0.05$; n=6 to 12). XO activity in diabetic EPCs was normalized after NAD(P)H oxidase inhibition by apocynin (data not shown), suggesting NAD(P)H oxidase–dependent XO activation.

NAD(P)H Oxidase Inhibition Restores In Vivo Reendothelialization Capacity of EPCs From Diabetic Subjects

Both siRNA silencing of NAD(P)H oxidase subunit p47^{phox} and NADPH oxidase inhibition by apocynin (100 μmol/L; 24 hours; data not shown) resulted in a markedly reduced NAD(P)H oxidase activity and superoxide production of EPCs from diabetic individuals (Figure 2A to 2C). Importantly, p47^{phox} siRNA silencing (n=6) restored in vivo reendothelialization capacity of diabetic EPCs (Figure 2D and 2E), suggesting a critical role of NAD(P)H oxidase activation for impaired reendothelialization capacity.

A potential mechanism whereby NAD(P)H oxidase activation may impair EPC in vivo reendothelialization capacity relates to a subsequently reduced NO bioavailability. Notably, EPCs from diabetic subjects had a markedly reduced NO bioavailability (Figure 3A and 3B).

EPC NO Bioavailability Is Critical for In Vivo Reendothelialization Capacity

eNOS-specific siRNA silencing substantially reduced eNOS protein expression (Figure 3C; n=8) and NO production of EPCs from healthy subjects (data not shown). Importantly, eNOS-specific siRNA silencing markedly impaired in vivo reendothelialization capacity of EPCs from healthy subjects (Figure 3D and 3E; n=8), suggesting a crucial role of EPC NO bioavailability for in vivo reendothelialization capacity. In contrast, eNOS-specific siRNA transfection of diabetic EPCs did not reduce in vivo reendothelialization capacity (n=8; data not shown).

Importantly, siRNA silencing of p47^{phox} restored NO bioavailability of EPCs from diabetic subjects (Figure 3F), whereas no effect was observed in EPCs from healthy subjects (data not shown).

EPC Protein Expression of NAD(P)H Oxidase Subunits and eNOS

Avogaro et al[31] recently observed increased expression of NAD(P)H oxidase subunit p22^{phox} in monocytes from individu-

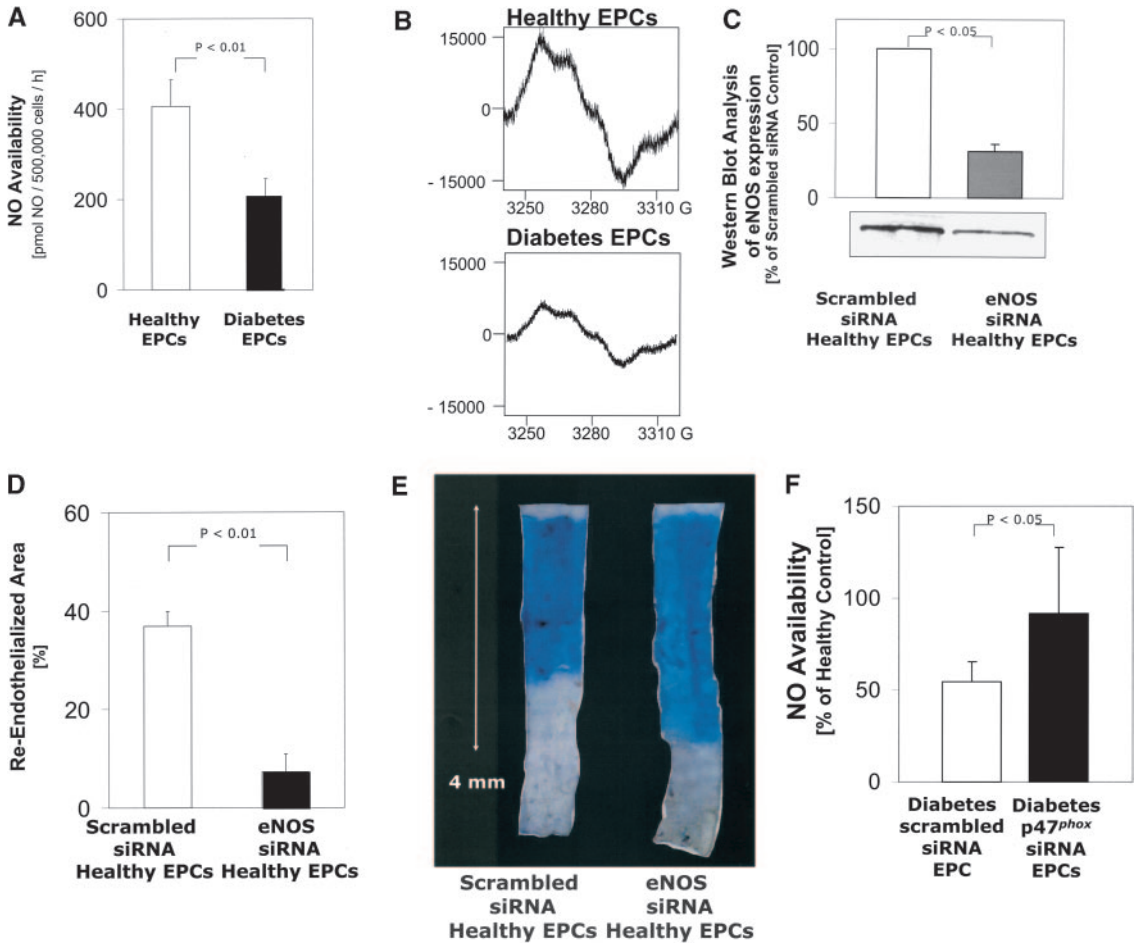

Figure 3. A, ESR spectroscopy analysis of NO production in EPCs from healthy subjects (n=10) and diabetic subjects (n=30). B, Representative ESR spectra. C, eNOS protein expression after eNOS-specific or scrambled siRNA transfection of EPCs from healthy subjects. D, Reendothelialized area at day 3 after carotid injury after transplantation of EPCs from healthy subjects transfected with eNOS-specific or scrambled siRNA (n=8); representative photograph (E). F, Effect of siRNA silencing of $p47^{phox}$ on NO bioavailability of EPCs from diabetic subjects (n=8).

als with diabetes mellitus. In the present study, protein levels of NAD(P)H oxidase subunits $p22^{phox}$, $p67^{phox}$, and $p47^{phox}$ in EPCs from diabetic subjects compared with healthy subjects were as follows: $p22^{phox}$: 260±126%; P=NS; $p67^{phox}$: 86±5%; P=NS; $p47^{phox}$: 159±49%; P=NS; n=5). eNOS protein levels were not significantly different in EPCs from diabetic subjects compared with healthy subjects (data not shown).

Effects of PPAR-γ Agonist Rosiglitazone on EPCs From Diabetic Subjects In Vitro

Treatment of diabetic EPCs with rosiglitazone in vitro reduced superoxide production and NAD(P)H oxidase activity and increased NO bioavailability, which was prevented by PPAR-γ–specific siRNA transfection (Figure 4A to 4D). Furthermore, rosiglitazone treatment prevented XO activation in diabetic EPCs (data not shown). Moreover, 24-hour in vitro treatment of diabetic EPCs with rosiglitazone improved their in vivo reendothelialization capacity (n=6; data not shown).

Endothelial Adhesion and Migration Capacity of EPCs

EPCs from diabetic subjects had a markedly reduced capacity to adhere to tumor necrosis factor-α–activated endothelial cells and an impaired migratory response to vascular endothelial growth factor compared with EPCs from healthy subjects (Figure 5A to 5C). Importantly, $p47^{phox}$ siRNA silencing and treatment with polyethylene glycol–superoxide dismutase (50 U) restored both adhesion capacity to activated endothelial cells and migratory response of EPCs from diabetics (Figure 5A to 5C). Furthermore, both the NO donor 2,2'-(hydroxynitrosohydrazino)*bis*-ethanamine (DETA-NO; 500 μmol/L) and rosiglitazone (10 μmol/L) restored adhesion and migration capacity of EPCs from diabetic subjects, whereas no significant effect was observed in EPCs from healthy subjects (Figure 5A to 5C).

Effects of Oral Treatment With the PPAR-γ Agonist Rosiglitazone on EPCs From Diabetic Subjects: A Randomized, Placebo-Controlled Clinical Study

Rosiglitazone, but not placebo therapy, substantially reduced superoxide production and NAD(P)H oxidase activity in EPCs from diabetic subjects (Figure 6A and 6B). Moreover, rosiglitazone therapy increased NO bioavailability (Figure 6C) and, importantly, restored in vivo reendothelialization capacity of EPCs from diabetic subjects (Figure 6D and 6E).

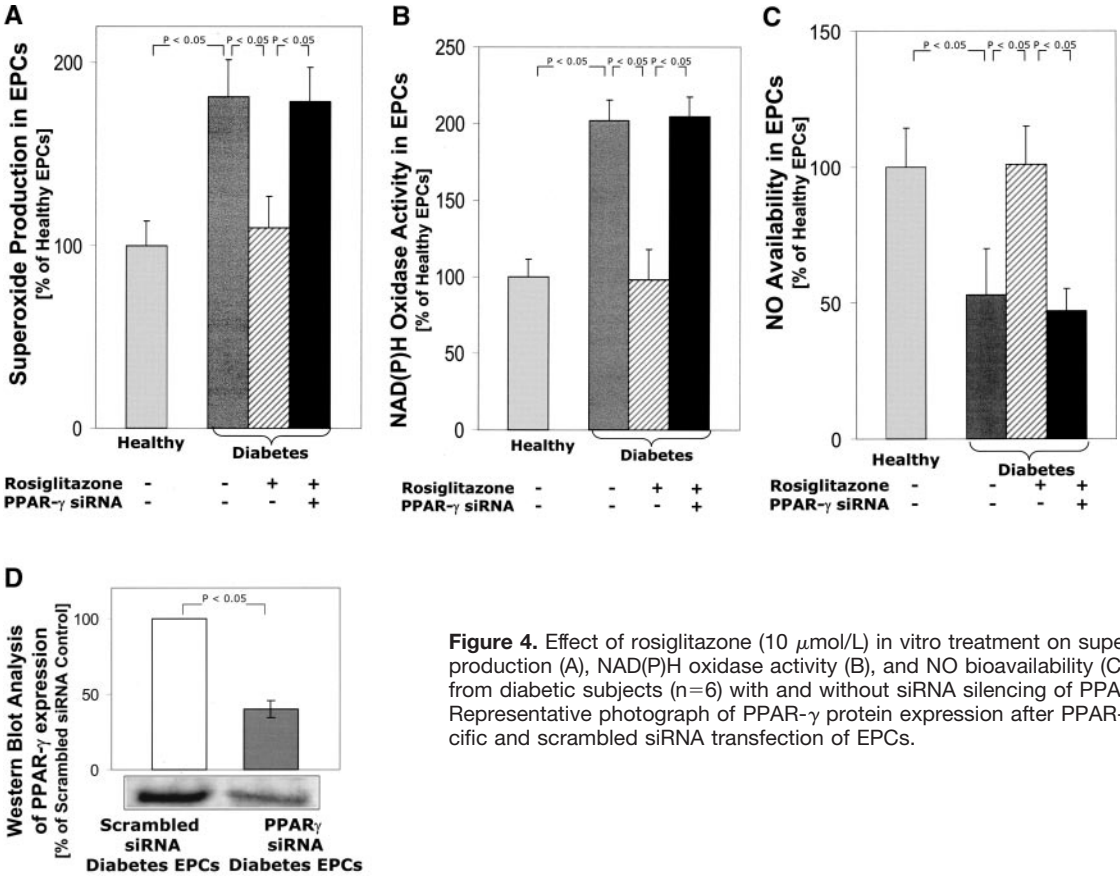

Figure 4. Effect of rosiglitazone (10 μmol/L) in vitro treatment on superoxide production (A), NAD(P)H oxidase activity (B), and NO bioavailability (C) of EPCs from diabetic subjects (n=6) with and without siRNA silencing of PPAR-γ. D, Representative photograph of PPAR-γ protein expression after PPAR-γ–specific and scrambled siRNA transfection of EPCs.

EPC numbers were reduced in diabetic subjects compared with healthy subjects (175±81 versus 299.5±162 EPCs per high-power field; $P<0.05$). Two-week rosiglitazone but not placebo therapy increased EPC numbers in diabetic subjects as assessed by acetylated low-density lipoprotein–lectin staining (Figure IIIA and IIIB in the online-only Data Supplement). No significant changes of metabolic parameters were observed after 2-week rosiglitazone therapy (Tables 2 and 3). No significant differences existed with respect to the patient characteristics shown in Table 1 between patients randomized to rosiglitazone or placebo therapy at baseline (ie, before therapy).

Discussion

The present study demonstrates a severe impairment of in vivo reendothelialization capacity of EPCs derived from diabetic subjects compared with healthy subjects. Furthermore, our findings suggest that increased oxidant stress, in particular NAD(P)H oxidase activation, and a subsequently reduced NO bioavailability of diabetic EPCs represent major mechanisms leading to impaired in vivo reendothelialization capacity and in vitro function. siRNA silencing of $p47^{phox}$, a critical NAD(P)H oxidase subunit, normalized superoxide production and restored NO bioavailability and in vivo reendothelialization capacity of EPCs from diabetic subjects.

Notably, both in vitro and 2-week oral therapy with the PPAR-γ agonist rosiglitazone inhibited NAD(P)H oxidase, reduced superoxide production, and restored NO availability and in vivo reendothelialization capacity of EPCs derived from diabetic subjects.

EPCs have been shown to promote endothelial repair after injury in recent experimental studies.[8–10] The present study, however, demonstrates that in vivo reendothelialization capacity is largely lost in EPCs from diabetic subjects, suggesting a profound alteration of the endogenous endothelial repair system mediated by EPCs. EPCs from diabetic and healthy subjects had a similar expression of CD31, vWF, and KDR, suggesting that impaired reendothelialization capacity was not associated with loss of endothelial marker proteins. A substantial portion of EPCs expressed the monocytic marker CD14, as has been observed previously.[32,33] Notably, it has been shown recently that transfusion of $CD14^+/KDR^+$ but not $CD14^+/KDR^-$ cells accelerated reendothelialization in nude mice, suggesting that only monocytic cells with endothelial markers promote reendothelialization.[34] Furthermore, Romagnani et al[35] have suggested that blood-derived EPCs are to a significant extent derived from $CD14^+/CD34^{low}$ cells. Production of growth factors has been suggested to contribute to EPC function but is likely not sufficient to promote reendothelialization. Whereas monocytes and macrophages are known to produce growth factors,[36] they have not been shown to stimulate reendothelialization.[34]

Two recent studies have suggested that despite a phenotypic overlap of EPCs with macrophages and dendritic cells, EPCs display unique eNOS expression that likely is a reliable marker of endothelial phenotype.[37,38] Impor-

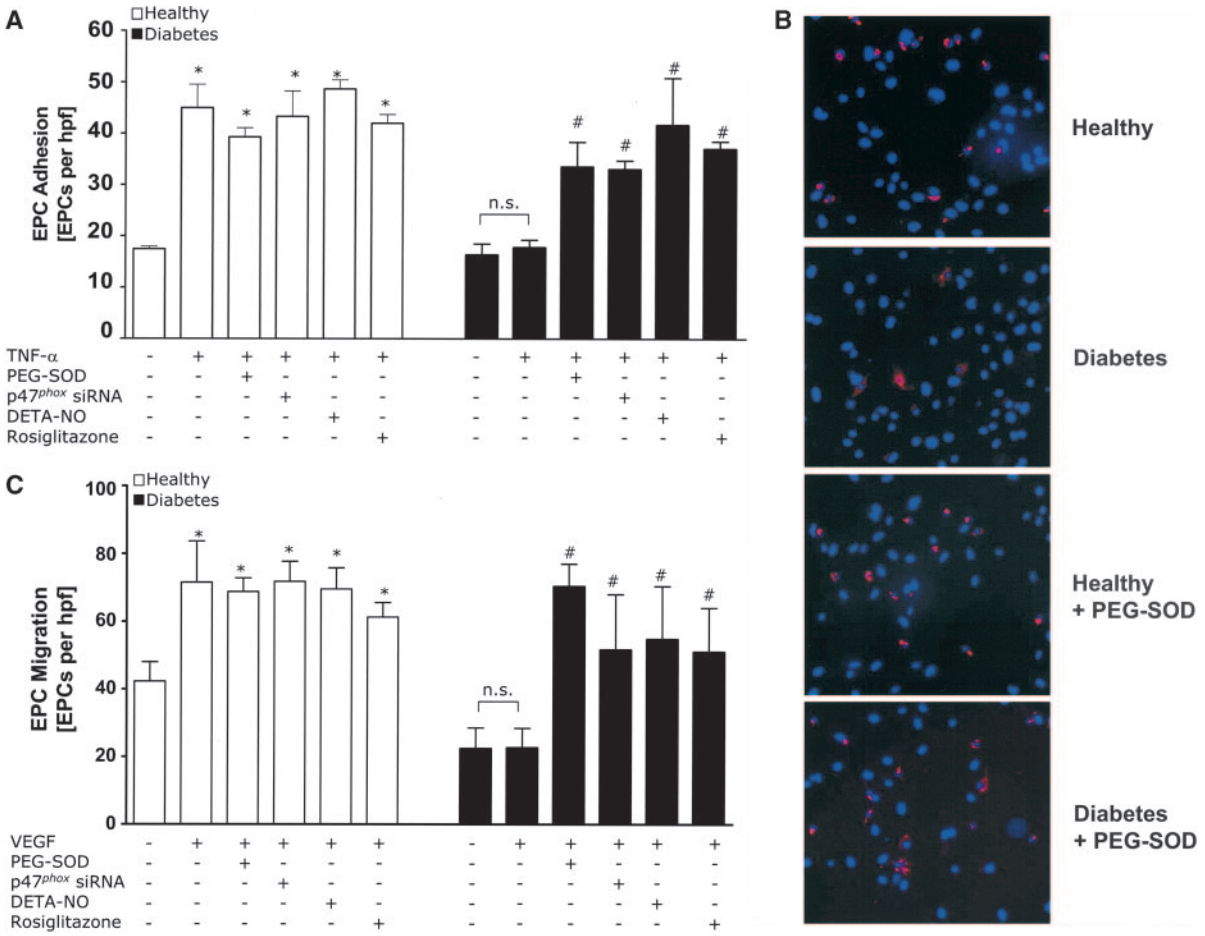

Figure 5. A, Adhesion of EPCs to human umbilical vein endothelial cells after 24-hour in vitro incubation of EPCs with polyethylene glycol–superoxide dismutase (PEG-SOD; 50 U), p47^{phox} siRNA, DETA-NO (500 μmol/L), or rosiglitazone (10 μmol/L; n=6). B, Representative photographs. C, Migratory capacity of EPCs after 24-hour in vitro incubation of EPCs with polyethylene glycol–superoxide dismutase p47^{phox} siRNA, DETA-NO, or rosiglitazone; n=6; *$P<0.01$ vs healthy baseline; #$P<0.01$ vs diabetes baseline. TNF indicates tumor necrosis factor; VEGF, vascular endothelial growth factor; and hpf, high-power field.

tantly, in the present study EPC-mediated reendothelialization was eNOS dependent (ie, was abolished after eNOS siRNA silencing), strongly suggesting that the observed reendothelialization response depends on eNOS-containing EPCs.

In previous studies, we and others have observed a role of eNOS for mobilization and EPC function by using eNOS-deficient mice.[15–17] Furthermore, Ii et al[16] have suggested that EPCs represent repositories of eNOS activity in experiments using bone marrow cells from eNOS-deficient mice. In eNOS-deficient mice, however, impaired maturation of EPCs in the bone marrow may contribute to impaired EPC function. The present study provides direct evidence that NO bioavailability in EPCs is critical for in vivo reendothelialization capacity.

Importantly, NO bioavailability was restored in EPCs from diabetic subjects after NAD(P)H oxidase inhibition, associated with a restored reendothelialization response, suggesting that increased NAD(P)H oxidase activity impairs reendothelialization capacity of diabetic EPCs by reducing NO availability. The concept that increased superoxide production from NAD(P)H oxidase is critical for impaired EPC functionality is further supported by our observation that both NAD(P)H oxidase inhibition and superoxide dismutase treatment improved endothelial adhesion and migratory capacity of EPCs from diabetic subjects.

Of note, 2 previous studies have suggested that EPCs from healthy subjects have a reduced superoxide production compared with mature endothelial cells,[39,40] suggesting that well-controlled superoxide production is important for EPC function. Furthermore, EPCs isolated from glutathione peroxidase-1–deficient mice have an impaired ability to promote angiogenesis, suggesting that reduced antioxidant capacity impairs EPC in vivo function.[41] The present study suggests that EPC function may be highly dependent on a well-controlled oxidant stress because EPC NO availability (which is highly sensitive to oxidant stress) is critical for their in vivo function.

In addition, increased p38 mitogen-activated protein kinase phosphorylation has been reported in EPCs from patients with coronary disease[42] and in mononuclear cells from diabetic mice.[43] Because NAD(P)H oxidase activation promotes p38 mitogen-activated protein kinase phosphorylation,[42] it is tempting to speculate that increased p38 mitogen-

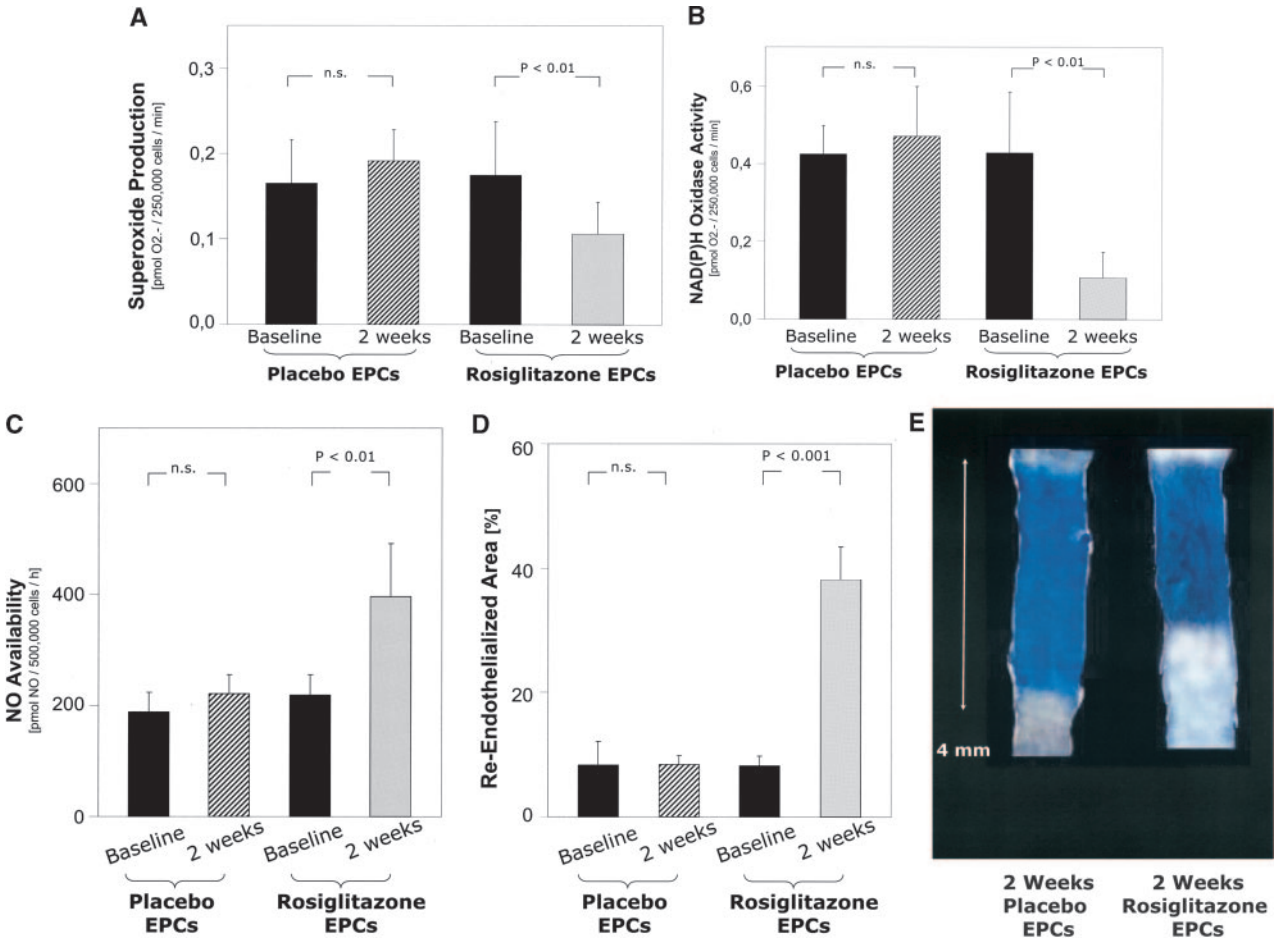

Figure 6. ESR spectroscopy analysis of superoxide production (A), NAD(P)H oxidase activity (B), and NO bioavailability (C) of EPCs from diabetic patients before and after 2 weeks of oral rosiglitazone (n=18) or placebo (n=12) therapy. D, Reendothelialized area at day 3 after carotid injury in nude mice after transplantation of EPCs from diabetic patients before and after 2 weeks of rosiglitazone (n=18) or placebo (n=12) therapy; representative photographs (E). EPCs from each patient after placebo or rosiglitazone therapy were injected into 2 nude mice; mean values of REA are shown.

activated protein kinase phosphorylation may represent an additional potential pathway whereby NAD(P)H oxidase may alter EPC function in diabetics.

Furthermore, in EPCs from diabetic mice, an increased expression of thrombospondin-1 has recently been shown, which may further contribute to impaired EPC adhesion and migration activity and a reduced reendothelialization response in diabetes mellitus.[13]

Short-term in vitro rosiglitazone treatment of EPCs from diabetic subjects reduced NAD(P)H oxidase activity and restored NO availability in the present study. PPAR-γ siRNA silencing prevented these effects of rosiglitazone, suggesting that PPAR-γ agonism exerts a direct effect on NAD(P)H oxidase in diabetic EPCs. Of note, in vitro treatment with the PPAR-γ agonist pioglitazone prevented oxidant stress–induced apoptosis in human EPCs, further supporting a role of PPAR-γ for EPC function.[44]

Importantly, 2-week oral rosiglitazone therapy restored in vivo reendothelialization capacity of EPCs from diabetic subjects in the present study. PPAR-γ agonism had no significant effect on glucose or insulin levels, insulin resistance (as indicated by the Homeostasis Model Assessment index), or nonesterified fatty acids within this time, suggesting that effects on EPC functionality were likely independent of metabolic changes. This concept is further supported by the observation that short-term in vitro rosiglitazone treatment of EPCs from diabetics improved both in vitro and in vivo functionality. Interestingly, a previous study has suggested that nonesterified fatty acids stimulate NAD(P)H oxidase in endothelial cells[45]; however, high concentrations of nonesterified fatty acids were necessary to stimulate the enzyme, suggesting that minor changes of plasma nonesterified fatty acid levels are unlikely to change EPC NAD(P)H oxidase activity. In agreement with this consideration, we did not observe a correlation between changes in nonesterified fatty acid levels and EPC NAD(P)H oxidase activity, suggesting that this may not be a major mechanism whereby rosiglitazone therapy exerts an effect on EPCs, at least after short-term treatment.

Of note, a reduced capacity for endothelial repair is thought to contribute to impaired long-term outcome after coronary intervention in diabetics.[46] Beneficial effects of PPAR-γ agonism on EPC reendothelialization capacity provide a potential explanation for antirestenotic effects observed after PPAR-γ agonist treatment after coronary intervention.[47] No-

tably, a recent experimental study has suggested that rosiglitazone treatment in mice promoted angiogenic progenitor cell differentiation toward the endothelial lineage associated with attenuated restenosis after angioplasty.[48]

Moreover, the present findings may have implications for treatment strategies using autologous cell transplantation. Given the severe impairment of in vivo reendothelialization potential of EPCs from diabetics, pretreatment of these cells with rosiglitazone represents a promising strategy to improve their regenerative potential.

In the present study, a currently widely applied protocol to obtain blood-derived EPCs was used. Further standardization of EPC definitions and nomenclature will be important for future studies, in particular to be able to better compare results between different groups.

In summary, the present study demonstrates that in vivo reendothelialization capacity of EPCs derived from diabetics is severely impaired, at least in part, as a result of increased NAD(P)H oxidase–dependent superoxide production and subsequently reduced NO bioavailability. Treatment with the PPAR-γ agonist rosiglitazone reduced NAD(P)H oxidase activity, increased NO availability, and restored in vivo reendothelialization capacity of EPCs from diabetics. Improved EPC reendothelialization capacity may represent a novel mechanism contributing to vasculoprotective effects of PPAR-γ agonism likely independent of glycemic control.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Accelerated vascular disease is a principal cause of increased mortality and morbidity in patients with diabetes mellitus. Importantly, endothelial injury is thought to represent a major mechanism whereby diabetes promotes initiation and progression of atherosclerosis and restenosis after vascular intervention. Notably, recent experimental studies have suggested that circulating endothelial progenitor cells contribute to endogenous endothelial repair mechanisms after vascular injury. The present study demonstrates a severe impairment of the in vivo reendothelialization capacity of endothelial progenitor cells derived from subjects with type 2 diabetes mellitus compared with healthy subjects that may contribute to an impaired endogenous endothelial repair capacity and a delayed healing after vascular injury in patients with diabetes mellitus. Furthermore, our findings suggest that increased oxidant stress, in particular NAD(P)H oxidase activation and a subsequently reduced nitric oxide bioavailability, represents important mechanisms underlying impaired in vivo reendothelialization capacity of endothelial progenitor cells from diabetic patients. Of note, both in vitro and 2-week oral therapy with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone restored the in vivo reendothelialization capacity of endothelial progenitor cells derived from diabetic individuals, likely at least in part by inhibiting NAD(P)H oxidase activity and increasing nitric oxide availability. Improved reendothelialization capacity of endothelial progenitor cells may therefore represent a novel mechanism contributing to vasculoprotective effects of peroxisome proliferator-activated receptor-γ agonism likely independent of glycemic control.