Original Article

Endothelial Nitric Oxide Synthase Uncoupling Impairs Endothelial Progenitor Cell Mobilization and Function in Diabetes

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Uncoupling of the endothelial nitric oxide synthase (eNOS) resulting in superoxide anion (O_2^-) formation instead of nitric oxide (NO) causes diabetic endothelial dysfunction. eNOS regulates mobilization and function of endothelial progenitor cells (EPCs), key regulators of vascular repair. We postulate a role of eNOS uncoupling for reduced number and function of EPC in diabetes. EPC levels in diabetic patients were significantly reduced compared with those of control subjects. EPCs from diabetic patients produced excessive O_2^- and showed impaired migratory capacity compared with nondiabetic control subjects. NOS inhibition with N^G-nitro-L-arginine attenuated O_2^- production and normalized functional capacity of EPCs from diabetic patients. Glucose-mediated EPC dysfunction was protein kinase C dependent, associated with reduced intracellular BH_4 (tetrahydrobiopterin) concentrations, and reversible after exogenous BH_4 treatment. Activation of NADPH oxidases played an additional but minor role in glucose-mediated EPC dysfunction. In rats with streptozotocin-induced diabetes, circulating EPCs were reduced to 39 ± 5% of controls and associated with uncoupled eNOS in bone marrow. Our results identify uncoupling of eNOS in diabetic bone marrow, glucose-treated EPCs, and EPCs from diabetic patients resulting in eNOS-mediated O_2^- production. Subsequent reduction of EPC levels and impairment of EPC function likely contributes to the pathogenesis of vascular disease in diabetes. *Diabetes* 56: 666–674, 2007

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BH_4, tetrahydrobiopterin; CFU, colony forming unit; EBM, endothelial basal medium; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; HPLC, high-performance liquid chromatography; L-NNA, N^G-nitro-L-arginine; PBMC, peripheral blood mononuclear cell; PKC, protein kinase C; ROS, reactive oxygen species.

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Cardiovascular disease is a common complication of diabetes (1,2). Endothelial dysfunction as a first step in the pathogenesis of diabetes promotes arteriosclerosis (3). Mechanistically, uncoupling of the endothelial nitric oxide synthase (eNOS) in blood vessels of diabetic patients leads to excessive superoxide anion (O_2^-) production and diminishes nitric oxide (NO) availability (4–6). The underlying molecular events are not completely clear, but reduction of the essential eNOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) and involvement of the protein kinase C (PKC) in exaggerated O_2^- production have been proposed (4,7).

Endothelial progenitor cells (EPCs) are bone marrow–born cells with the potential to differentiate into functional mature endothelial cells, which can substitute diseased endothelium (8). Decreased levels of circulating EPCs are correlated with increased risk for coronary artery disease and myocardial infarction (9–12). Reduced levels of EPCs have been described in both type 1 and type 2 diabetic patients (13,14). EPC recruitment for re-endothelialization after vascular injury is impaired in diabetes (15). These alterations are likely to be involved in the pathogenesis of vascular disease in diabetes (16). Although eNOS is of paramount importance for the regulation of mobilization and function of EPCs (17,18), data concerning the role of eNOS uncoupling in diabetic EPCs are not available.

EPCs are embedded in a microenvironment of bone marrow stromal and endothelial cells and can be translocated to the circulation. NO-mediated signaling pathways have been previously proposed to be essential for EPC mobilization (17,19). Whether these pathways are altered in diabetic bone marrow is not known.

We tested our hypothesis that eNOS uncoupling occurs in EPCs from diabetic patients and is involved in impaired cellular function. We also investigated a potential mechanistic role of BH_4 and PKC signaling in glucose-mediated eNOS uncoupling in EPCs. Finally, we analyzed whether low circulating EPC levels observed in diabetes may result from alterations in bone marrow EPC mobilizing pathways.

RESEARCH DESIGN AND METHODS

The study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (publ. no. 85-23, revised 1996). Clinical study approval from the ethics committee of the University of Würzburg was obtained, as was informed written consent from patients.

TABLE 1
Patient characteristics

	Type 2 diabetic patients	Nondiabetic control subjects
n	5	5
Sex (male/female)	2/3	2/3
Age (years)	70.6 ± 1.7	70.0 ± 2.9
Glucose (mg/dl)	294 ± 32.0	100 ± 6.4
A1C (%)	8.8 ± 0.59	5.4 ± 0.06
Hypertension	3	3
Coronary artery disease	1	1
medication		
Aspirin	1	2
β-Blocker	3	3
ACE inhibitor	2	2
Statin	2	2

Data are means ± SEM or *n*.

Isolation of bone marrow and peripheral blood mononuclear cells. We isolated peripheral blood mononuclear cells (PBMCs) derived from leucophoresis material of healthy volunteers or from whole blood of type 2 diabetic ($n = 5$, aged 70.6 ± 1.7 years) and nondiabetic ($n = 5$, aged 70.0 ± 2.9 years) patients, as described (11). Glucose concentrations and A1C values were determined in the morning after an overnight fasting period. Detailed patient characteristics are shown in Table 1. As diabetes had not been diagnosed previously in these patients, they were not on insulin or any oral antidiabetes medication at the time of blood withdrawal. Rat PBMCs were isolated by Ficoll density centrifugation (20). Hollow bones 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 as described (20). Bone marrow was pelleted and shock frozen before further analysis.

Determination of endothelial progenitor cell numbers and cellular characterization. We used an adhesion-related selection method for isolation of monocytic EPCs, as described (11,15,20,21). PBMCs (2×10^5) were cultured on fibronectin-precoated eight-well chamber slides (Lab-Tek; Nunc, Wiesbaden, Germany) in endothelial basal medium (EBM)-2 culture medium supplemented with EBM SingleQuots (Cambrex, Verviers, Belgium) and 20% fetal calf serum for 3 days. To exclude contamination with mature circulating endothelial cells, we carefully removed culture supernatant 2 h after initial seeding and placed it on new fibronectin-precoated chamber slides. EPCs were characterized by cellular uptake of acetylated LDL (dil-acLDL; Molecular Probes, Eugene, Oregon), binding of fluorescein isothiocyanate–conjugated lectin from Ulex europeus (UEA-1; Sigma, Deisenhofen, Germany), expression of vascular endothelial growth factor receptor-2 and eNOS, capacity for integration during endothelial tube formation, and cellular migration and colony forming unit (CFU) capacity, as described (20). Additionally, EPCs were stained with an anti–von Willebrand factor antibody (Sigma).

Animal study protocol. Diabetes was induced by a single intravenous injection of streptozotocin (50 mg/kg) in male Wistars rats (180–200 g) obtained from Harlan-Winkelmann (Borchen, Germany). After 12 weeks hyperglycemia was confirmed by a blood glucose monitoring system in the morning after an overnight fasting period (Ascensia Elite; Bayer, Leverkusen, Germany). Only streptozotocin-induced diabetic rats with blood glucose levels >300 mg/dl were included in the study.

Western blot analyses and enzyme-linked immunosorbent assay. Westen blotting was performed as described (22). In brief, cell extracts were mixed with sample loading buffer and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electro-transferred onto polyvinylidine fluoride membranes (Immun-Blot 0.2 μm; Bio-Rad), incubated for 2 h in Tris-buffered saline with Tween (TBS-T) with 5% blocking agent (Amersham), and then incubated overnight at 4°C with primary antibodies. The bands were detected using a chemiluminescence assay (ECL+Plus; Amersham). Primary antibodies used included mouse anti-eNOS and anti–phospho-eNOS (Transduction Laboratories, BD Biosciences, Heidelberg, Germany) and mouse anti-GAPDH (Abcam, Acris, Germany).

Low-temperature SDS-PAGE. Cell extracts were mixed with 3× SDS sample buffer (187.5 mmol/l Tris-HCl [pH 6.8], 6% wt/vol SDS, 30% glycerol, 0.03% wt/vol bromophenol blue, and 15% vol/vol 2-mercaptoethanol) at 0°C. Samples were loaded on 7.5% polyacrylamide gels and subjected to electrophoresis. Gels and buffers were cooled to 4°C before electrophoresis and the buffer tank placed in an ice bath during electrophoresis. eNOS dimer/monomer protein was detected by Western blot analysis.

Detection of reactive oxygen species in total bone marrow and EPCs. A variety of different techniques were used to measure reactive oxygen species (ROS) production, as described below.

Detection of malondialdehyde in bone marrow. Malondialdehyde-thiobarbituric acid adducts were measured by high-performance liquid chromatography (HPLC) as described (23). Bone marrow supernatants were separated, and fractionation of the protein-free extract was performed using a C18 column (Micro Bondapak; Waters, Milford, MA) in an HPLC system (Pharmacia LKB, Freiburg, Germany). We used a flow rate of 1 ml/min at a pressure of 1,800 psi at 35°C. Exact quantification was achieved using 1,13,3-tetraethoxypropane standards.

Determination of superoxide anions by lucigenin-enhanced chemiluminescence. Superoxide anion formation was measured by lucigenin-enhanced chemiluminescence. Cell extracts were transferred into scintillation vials containing lucigenin and Krebs/HEPES buffer (final composition: 0.005 mmol/l lucigenin, 99.01 mmol/l NaCl, 4.69 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.03 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 20 mmol/l Na-HEPES, and 5.6 mmol/l glucose [pH 7.4]). Signals were assessed over 20 min in a luminometer (Wallac, Freiburg, Germany) at 30-s intervals. To address the effects of NOS inhibition on superoxide production, aliquots of bone marrow extracts were incubated with N^G-nitro-L-arginine (L-NNA, 1 mmol/l) for 20 min.

Dihydroethidium assay. The redox-sensitive, cell-permeable fluorophore dihydroethidium becomes oxidized in the presence of O_2^- to yield fluorescent ethidium. Thus, dye oxidation is an indirect measure of the presence of reactive oxygen intermediates. Cultured EPCs were incubated with dihydroethidium (2.5 μmol/l) for 30 min. After washing mean channel fluorescence, each sample was measured during fluorescence-microscopic evaluation of at least four different visual fields. Intracellular ROS in human EPCs were additionally detected using a FACSCalibur flow cytometer (Becton Dickinson, San Juan, CA). Fluorescence was measured using an FL-3 filter (670 nm), and histograms of 10,000 events were analyzed per experiment. The degree of fluorescence in EPCs was then evaluated using Cell Quest software (Becton Dickinson).

Detection of reactive nitrogen species in EPCs

3-Nitrotyrosine assay. Peroxynitrite reacts quickly with a variety of different biomolecules to produce 3-nitrotyrosine, which therefore is a good biomarker for reactive nitrogen species (24). Human EPCs were treated with glucose (30 mmol/l, 24 h), and then cell lysates were prepared on ice using cell lysis buffer (1 mol/l Tris-HCl [pH 7.6], 0.5 mol/l EDTA, and 1.25 mol/l dithiothreitol). 3-Nitrotyrosine from cell lysates was then isolated by HPLC and subjected to a sensitive gas chromatography–tandem mass spectrometry method, as recently described by us (24), in the electron capture negative-ion chemical ionization mode on a triple-stage quadrupole mass spectrometer model ThermoQuest TSQ 7,000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX) (24).

Measurements of biopterin content in EPCs. Measurements of biopterin content in EPCs were performed using HPLC analysis and a differential oxidation method as previously described (25). The amount of BH_4 was determined from the difference between total (BH_4 plus BH_2 plus biopterin) and alkaline-stable oxidized (BH_2 plus biopterin) biopterin. A Nucleosil C-18 column (4.6 × 250 mm, 5 μm) was used with 5% methanol/95% water as a solvent at a flow rate of 1.0 ml/min. The fluorescence detector was set at 350 nm for excitation and 450 nm for emission.

Functional analysis of EPCs

CFU assay. Endothelial CFUs from isolated PBMCs of diabetic and nondiabetic patients were determined using the EndoCult system (StemCell Technologies) as described (11).

Migration assay. Migratory capacity of EPCs was investigated using the modified Boyden chamber assay as previously described (11,20). After treatment, 5×10^4 EPCs were cultured in inlets (Falcon HTS Fluoro Blok insert, 8-μm pore size), which were placed in 24-well culture dishes containing EBM (Clonetics) and 50 ng/ml vascular endothelial growth factor, and 100 ng/ml stromal cell–derived factor-1. After 24 h, migrated cells were stained with dil-acLDL and manually counted by fluorescence-based microscopic evaluation of the bottom side of the membrane.

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

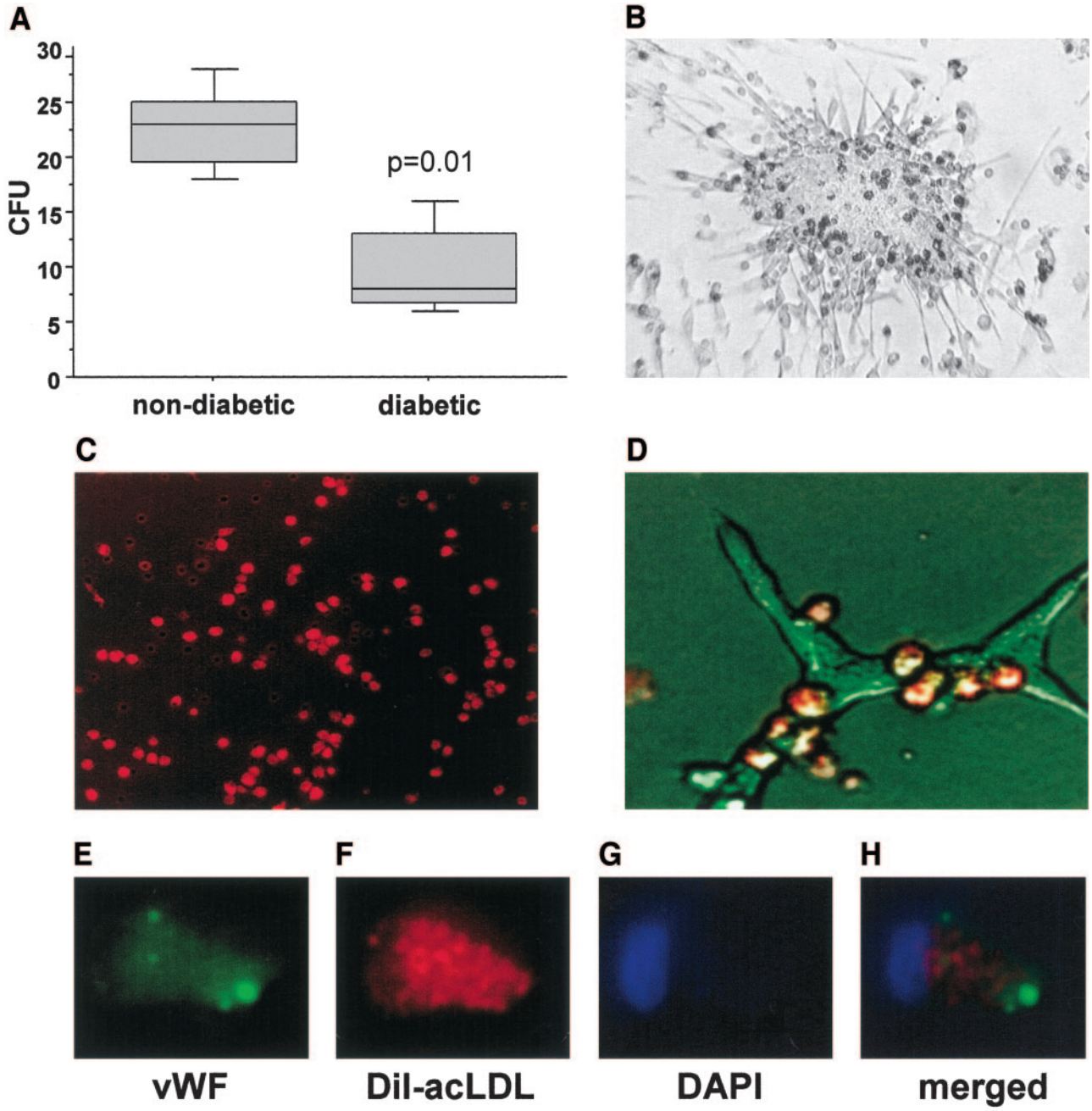

FIG. 1. Circulating endothelial progenitor cells are reduced in diabetic patients. *A* and *B*: Number of endothelial CFUs in diabetic patients and nondiabetic control subjects. *C* and *D*: Characterization of monocytic endothelial progenitor cells (labeled in red) based on their ability to migrate toward an vascular endothelial growth factor/stromal cell–derived factor-1 gradient (*C*) and to incorporate/adhere during vascular network formation on matrigel when cocultured with human umbilical vein endothelial cells (labeled in greeen, *D*) (see ref. 11 for details). *E*: Expression of the von Willebrand (vWF) factor in EPCs, including formation of typical Weibel-Palade Bodies (intense green spots). Figures *E–H* show vWF expression (*E*), DiI-acLDL uptake (*F*), nuclear staining by DAPI (*G*), and merged pictures (*H*). Data of endothelial CFUs represent means ± SEM. $n = 5$ measurements per group.

RESULTS

Reduced numbers and impaired function of EPCs from diabetic patients: evidence for eNOS uncoupling. Diabetic patients were characterized by increased plasma glucose concentration (294.0 ± 32.0 vs. 100.0 ± 6.4 mg/dl) and increased A1C values (8.8 ± 0.6 vs 5.4 ± 0.1%) compared with nondiabetic age-matched subjects. Patient characteristics are shown in Table 1.

Diabetic patients had reduced formation of endothelial CFUs compared with nondiabetic subjects (9.8 ± 1.9 vs. 22.6 ± 1.7 CFUs, $P = 0.001$; Fig. 1*A* and *B*). A detailed description of human EPC characterization has been previously published (11) (Fig. 1). EPCs from diabetic patients displayed increased ROS formation (11.7 ± 0.7 vs. 6.2 ± 1.1 arbitrary units, $P < 0.001$; Fig. 2*A* and *B*) but impaired migratory capacity compared with EPCs from nondiabetic age-matched subjects (40.4 ± 4.6 vs. 72.7 ± 6.2 migrated cells, $P < 0.0001$; Fig. 2*C*). After NOS inhibition with L-NNA in EPCs from diabetic patients, ROS production was attenuated and cellular migration improved by 34.6 ± 5.6% ($P < 0.05$; Fig. 2*A–C*). Incubation of diabetic EPCs with pegylated superoxide dismutase com-

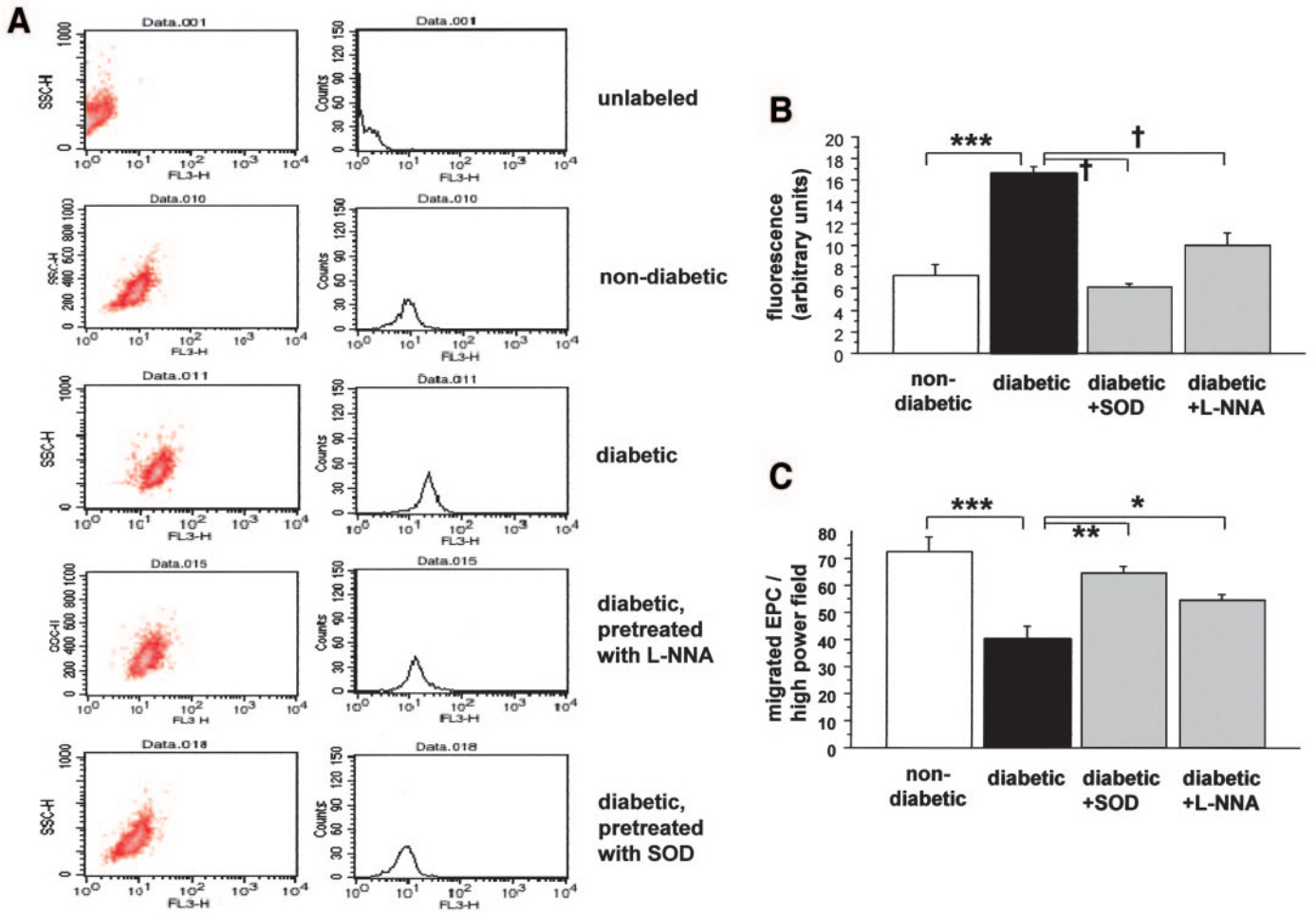

FIG. 2. Uncoupling of eNOS leads to ROS-mediated EPC dysfunction in diabetic patients. FACS analysis demonstrating intracellular ROS levels (*A* and *B*) and migratory capacity (*C*) of isolated EPCs from healthy control subjects and diabetic patients. A subgroup of EPCs from diabetic patients was additionally pretreated with superoxide dismutase (SOD) or L-NNA. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$; †$P < 0.0001$ vs. diabetic EPCs. Data represent means ± SEM. $n = 5$ measurements per group.

pletely inhibited ROS formation and improved migration by 60.1 ± 11.3% ($P < 0.001$; Fig. 2*A–C*).

Uncoupling of eNOS and ROS-mediated EPC dysfunction in cultured EPCs by glucose treatment: effects of PKC inhibition and tetrahydrobiopterin (BH_4) treatment. To test whether observations from the clinical study can be related to the increased glucose concentration in patients, we carried out further in vitro studies. Glucose treatment increased ROS production up to threefold and impaired migratory capacity of cultured EPCs by 55.7 ± 8.2% ($P < 0.001$), whereas treatment with mannitol (30 mmol/l) as osmotic control had no effect (Fig. 3*A* and *B*). Increased concentrations of O_2^- react with NO in a reaction forming peroxynitrite, the latter being a powerful toxicant to a wide range of cells (26). Peroxynitrite is difficult to measure and reacts quickly with a variety of different biomolecules to produce 3-nitrotyrosine. In glucose-challenged EPCs, 3-nitrotyrosine concentration was substantially higher than in control subjects, whereas NOS and to a lesser extent NADPH oxidase inhibition attenuated exaggerated 3-nitrotyrosine production (Fig. 3*C*). Combined NOS and NADPH oxidase inhibition completely inhibited 3-nitrotyrosine production in glucose-challenged EPCs (Fig. 3*C*).

The glucose-mediated impairment of EPC migration was diminished when cells were concomitantly treated with pegylated supoxide dismutase (Fig. 3*B*). Inhibition of NOS with L-NNA attenuated the detrimental effects of glucose (Fig. 3*A* and *B*).

Because PKC is involved in vascular O_2^- in diabetic vessels (4), we analyzed its importance in glucose-mediated ROS production in EPCs. Inhibition of PKC by chelerythrine attenuated O_2^- production after treatment of EPCs with glucose. In parallel, EPC function was improved (Fig. 3*A* and *B*). PKC may also activate NADPH oxidases (27); therefore, we additionally tested the contribution of NADPH oxidases in glucose-mediated impairment of EPCs. Inhibition of NADPH oxidases resulted in a slight reduction of intracellular O_2^- levels after glucose challenge, whereas combined inhibition of NOS and NADPH oxidases strongly lowered O_2^- production. A trend for improved migratory capacity was also observed after concomitant treatment with glucose and DPI, although this was not statistically significant. However, synergistic effects on the improvement of EPC migration after glucose challenge were observed after combined treatment with L-NNA and DPI (Fig. 3).

In addition, a reduction of the essential eNOS cofactor BH_4 has been described to be mechanistically involved in eNOS uncoupling (4,7). Intracellular levels of BH_4 and total and oxidized biopterin levels were measured in lysates from glucose-challenged and control EPCs. Glu-

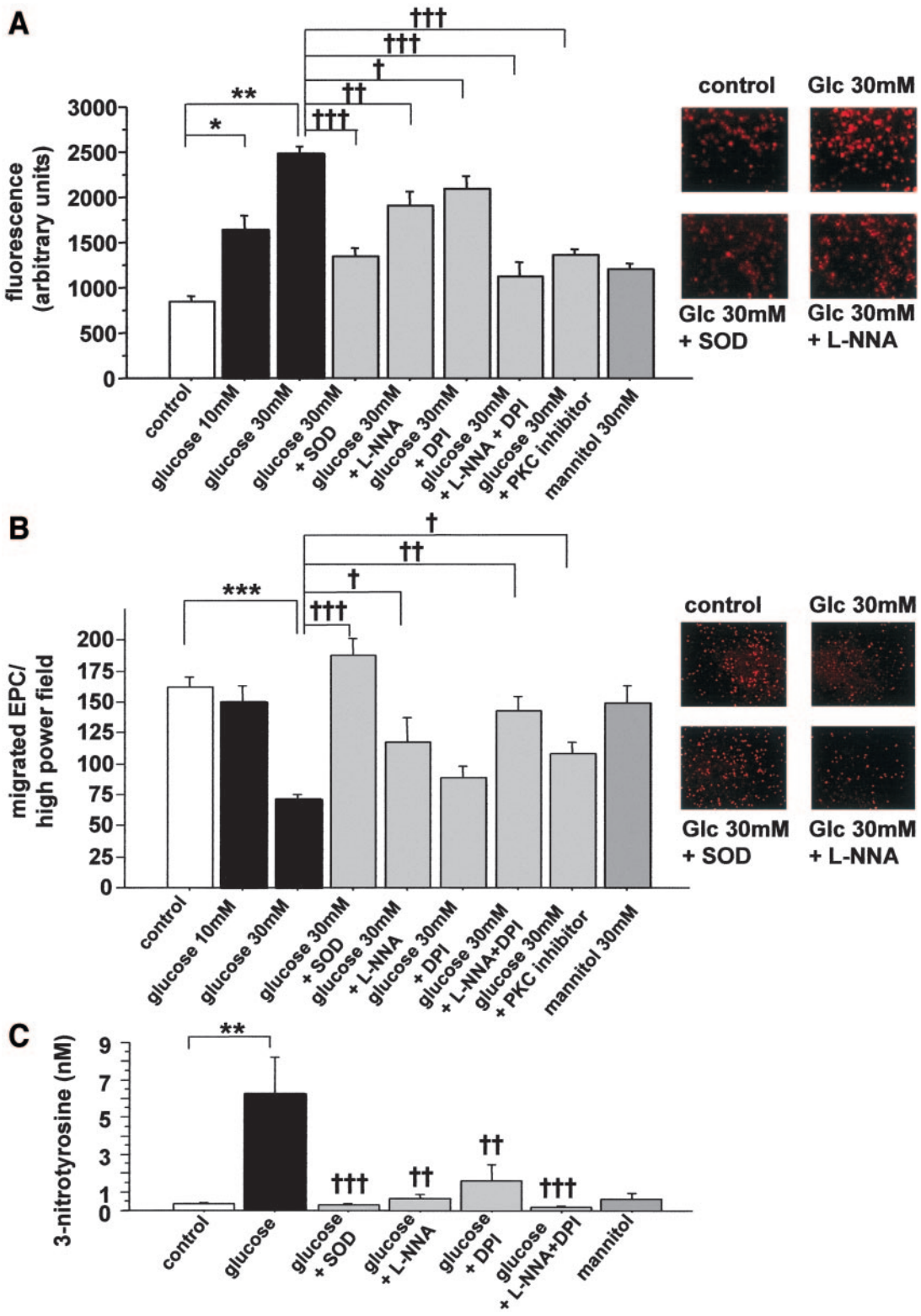

FIG. 3. Glucose treatment leads to eNOS uncoupling and impaired migratory capacity of EPC. *A*: Detection of fluorescent ethidium after staining of cultured EPCs with the redox-sensitive, cell-permeable fluorophore dihydroethidium. *B*: Migratory capacity of EPCs. EPCs were treated with ascending doses of glucose, glucose + superoxide dismutase (SOD), glucose + L-NNA, glucose + the PKC inhibitor chelerythrine, glucose + the NADPH oxidase inhibitor DPI or mannitol. *C*: Free 3-nitrotyrosine levels in EPC lysates after treatment with glucose (24 h, 30 mmol/l). *$P <$ 0.001, **$P <$ 0.0001, †$P <$ 0.05 vs. 30 mmol/l glucose, ††$P <$ 0.01 vs. 30 mmol/l glucose, †††$P <$ 0.0001 vs. 30 mmol/l glucose. Data represent means ± SEM. n = 4–6 measurements per study group.

cose treatment significantly reduced the concentration of intracellular BH_4 by 59%, whereas oxidized biopterin levels raised by 36% ($P < 0.05$). Total biopterin levels were basically unchanged (Fig. 4*A* and *B*). To test whether reversal of the glucose-mediated reduction in intracellular BH_4 levels would rescue EPC function, we performed further studies. Exogenous treatment of glucose-challenged EPCs with BH_4 increased their intracellular avail-

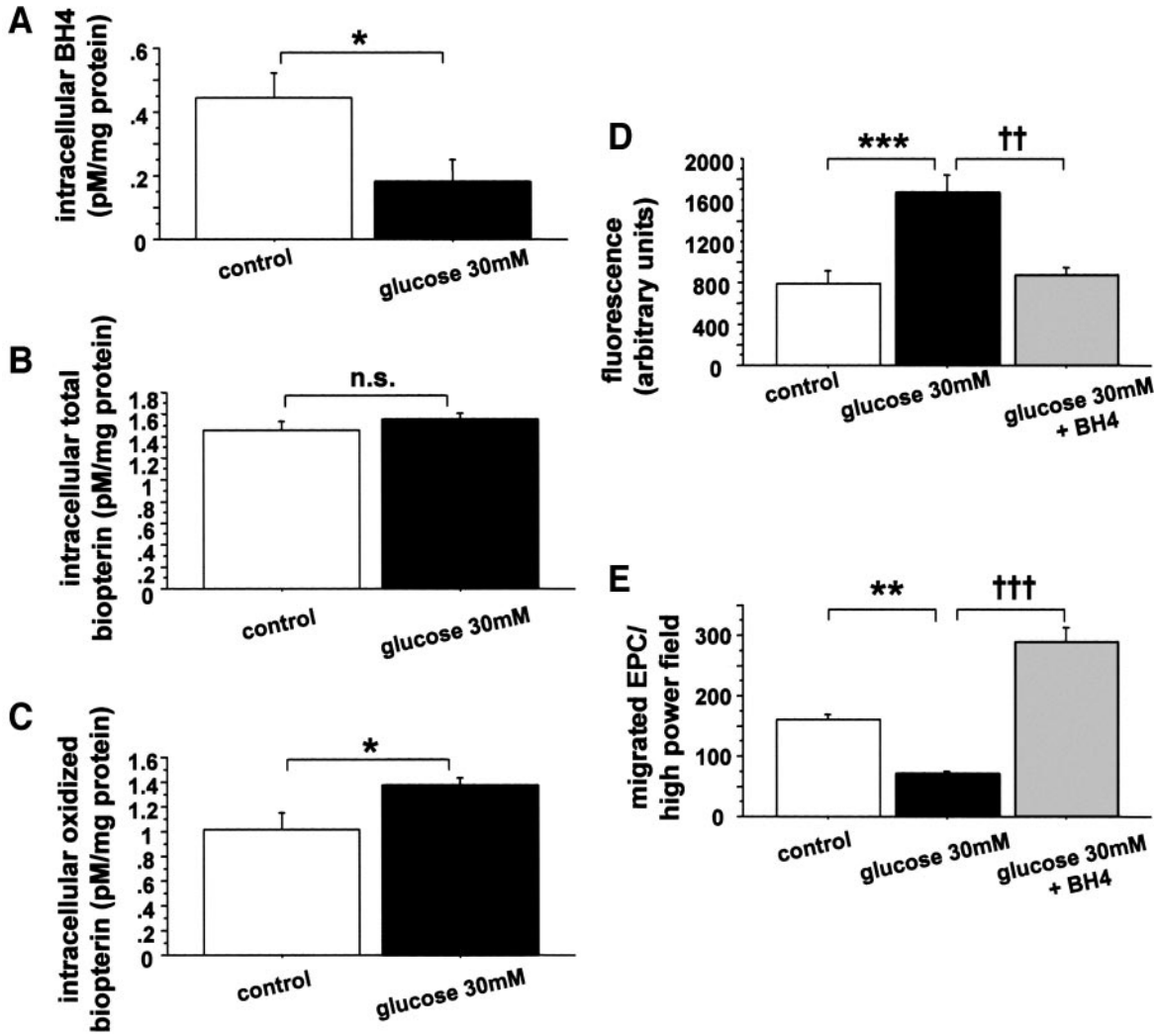

FIG. 4. BH_4-dependent rescue of glucose-mediated EPC dysfunction. Detection of intracellular BH_4 levels (*A*), total biopterin (*B*), alkaline-stable oxidized biopterin (BH_2 plus biopterin) (*C*), superoxide anions (*D*), and measurement of migratory capacity (*E*) of EPCs. EPCs were treated with glucose or glucose + BH_4 (10 μmol/l). Each $n = 4–6$ experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ††$P < 0.01$ vs. glucose, †††$P < 0.001$ vs. glucose. Data represent means ± SEM.

ability by fivefold compared with untreated controls. As a result, glucose-mediated exaggerated O_2^- production was attenuated and EPC migratory capacity significantly improved (Fig. 4*C* and *D*).

eNOS uncoupling in bone marrow in experimental diabetes: consequence for circulating EPCs. To understand whether reduced levels of EPCs in diabetes are potentially mediated by changes of EPC-mobilizing pathways in bone marrow, we used rats with streptozotocin-induced diabetes. Diabetic animals had higher serum glucose levels compared with control animals (459 ± 39 vs. 151 ± 15 mg/dl, $P < 0.0001$). In contrast, levels of circulating EPCs were reduced to 39 ± 5% compared with nondiabetic animals ($P < 0.05$) (Fig. 5*A*). A detailed rat EPC characterization has been previously published (20).

Diabetes was associated with 4.9-fold increased malondialdehyde-thiobarbituric acid adduct formation, an index of ROS generation (data not shown), as well as with increased O_2^- production in bone marrow (Fig. 5*B* and *C*). eNOS protein expression was increased in bone marrow extracts of diabetic rats (Fig. 5*D*), whereas eNOS phosphorylation (Fig. 5*E*), as well as eNOS dimer-to-monomer ratio, was reduced (Fig. 5*F*). NOS inhibition by L-NNA increased O_2^--mediated chemiluminescence in bone marrow extracts from control subjects but decreased O_2^- levels in diabetic bone marrow, indicating uncoupling of eNOS to be involved in exaggerated O_2^- production (Fig. 5*C*).

DISCUSSION

Diabetic patients are at high risk for endothelial lesions and arteriosclerosis. EPCs essentially contribute to vascular lesion repair (8). In the present study, we demonstrate for the first time uncoupling of the eNOS in EPCs from diabetic patients, in glucose-treated EPCs, and in bone marrow from diabetic rats. eNOS uncoupling explains at least in part reduced levels and impaired function of EPCs observed in diabetes and provides a valuable pharmacological target.

Under physiological conditions, eNOS confers anti-arteriosclerotic vascular protection (28). Indeed, eNOS-deficient mice display enhanced onset and rapid progression of arteriosclerosis (29). Exogenous NO may reverse the migratory defect of EPCs associated with diabetes (30). In general, increased eNOS expression is considered to be

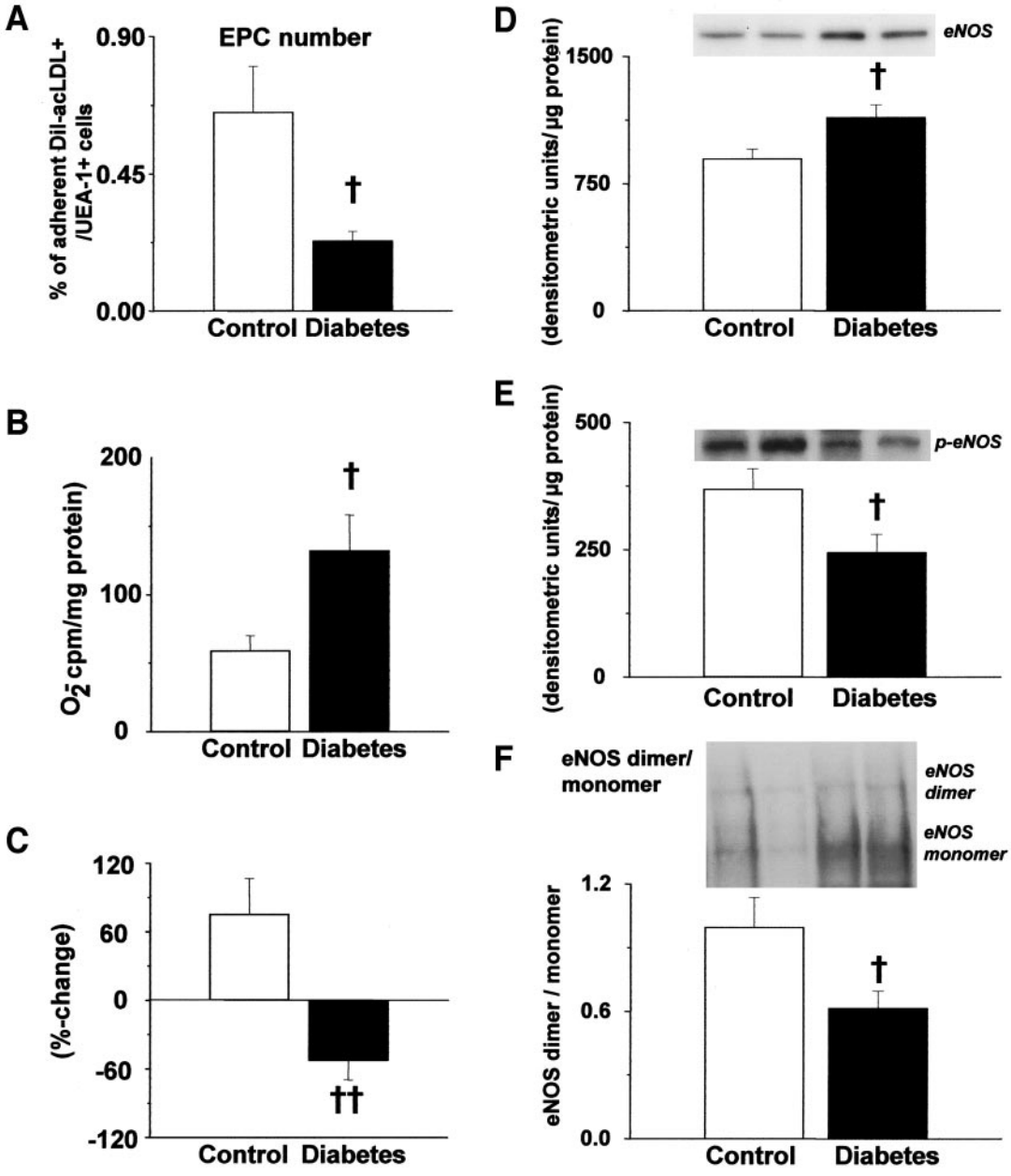

FIG. 5. Reduced circulating EPCs and eNOS uncoupling in bone marrow in rats with streptozotocin-induced diabetes. *A*: Circulating endothelial progenitor cells (n = 6 controls, n = 7 diabetic animals). *B* and *C*: Superoxide anion production in bone marrow extracts assessed by lucigenin (5 µmol/l)-enhanced chemiluminescence (*B*) and percentage of change after addition of L-NNA (1 mmol/l) (*C*). *D* and *E*: eNOS protein expression (*D*) and eNOS phosphorylation as revealed by Western blot analysis (*E*). *F*: eNOS dimer-to-monomer ratio. Control = control rats, n = 7; diabetes = rats with streptozotocin-induced diabetes, n = 7. †P < 0.05, ††P < 0.001 vs. control.

beneficial. However, under certain pathophysiological conditions, upregulation of eNOS expression is associated with reduced endothelium-dependent vasodilatation (31–34) explained by the so-called "eNOS uncoupling" (rev. in 7,33,34). Under this condition eNOS itself can be a source of O_2^-, instead of NO (7,31–34). eNOS uncoupling was shown in a variety of experimental and clinical vascular disease states, especially in diabetes (6,7,33,35). Diabetes-associated eNOS uncoupling has been described in the heart, vessels, and the kidney (4,34–39), resulting in decreased NO bioavailibility, increased superoxide production, and disrupted eNOS dimer formation within the vascular wall, while eNOS mRNA and/or protein levels are maintained or even increased (36–38). A reduction of the essential eNOS cofactor BH_4 has been described to be mechanistically involved in eNOS uncoupling (7,31,35, 37,40).

In our present study, EPCs from diabetic patients had increased intracellular ROS levels, reduced endothelial CFU capacity, and impaired migratory function, which were improved after NOS inhibition. In vitro, high glucose concentration led to increased ROS production and 3-nitrotyrosine levels and impairment of EPC function in EPCs. While EPCs are equipped with powerful antioxidative enzyme systems that may reduce the damaging effects of ROS (41), a direct correlation between functional capacity of EPCs and intracellular ROS production recently has been proposed (42,43). Glutathione peroxidase-1–deficient mice had increased ROS production and suffered from impaired migratory capacity of EPCs and

reduced angiogenesis in vitro (42). The protective role of eNOS may only be maintained under a normoglycemic environment, as under hyperglycemic conditions eNOS has been shown to become uncoupled to produce O_2^- (4–7). Addition of superoxide dismutase, which highly efficiently detoxifies O_2^-, completely normalized ROS production and EPC function. As NOS inhibition only partly attenuated O_2^- production, other systems may also be involved in ROS production and EPC dysfunction in a diabetic environment, such as excessive mitochondrial production of ROS (42) or increased activation of the p38 MAP kinase in EPCs (44). However, eNOS uncoupling in EPCs as shown in the present study seems to play an important and dominant role in glucose-mediated EPC dysfunction.

The underlying molecular mechanisms by which increased ROS may disturb EPC function are unclear, but reduced bioavailability of NO or the eNOS cofactor BH_4 may be involved (7,17,20,29,40). Indeed, intracellular BH_4 levels depend by large on their degradation due to excessive oxidation (rev. in 7). Reduced BH_4 levels have been reported in diabetic eNOS uncoupling (7,31,35), and restoration of BH_4 levels can "recouple" eNOS and enhance its regular enzymatic activity (7,40). In the present study we demonstrate for the first time a critical role for intracellular BH_4 levels for coupling and uncoupling of eNOS in EPCs. Indeed, we observed a significant reduction of intracellular BH_4 levels after challenging EPCs with high concentrations of glucose, whereas oxidized biopterin levels were significantly increased. This switch from BH_4 levels to oxidized biopterin strengthens our results that during glucose challenge eNOS in EPCs becomes uncoupled. Strikingly, addition of exogenous BH_4 resulted in reduction of intracellular O_2^- levels and complete rescue of EPC function during high-dose glucose treatment.

PKC is involved in the vascular O_2^- production in diabetic vessels (4). Its inhibition restores vascular NO bioavailability and endothelial function (4), but a role in EPCs has not been investigated so far. We demonstrate PKC to be essential in translation of the detrimental effects of high levels of glucose to EPCs. PKC inhibition resulted in reduced glucose-mediated O_2^- production and improvement of EPC function, suggesting a potential therapeutic role of PKC inhibitors in dysfunctional EPCs.

While reduced circulating EPC levels in diabetes have been observed in several human studies (13–14,45,46), alterations in EPC mobilizing pathways within diabetic bone marrow have not been identified so far. A pivotal role for eNOS in the regulation of EPC mobilization was shown with eNOS-deficient mice, which show impaired capacity to mobilize EPCs and impaired function of isolated EPCs (17). In the bone marrow, eNOS is the uniquely expressed NOS isoform, whereas iNOS or neuronal NOS are not detected (47). We observed enhanced eNOS expression in the bone marrow of diabetic rats, whereas eNOS monomerization and superoxide anion production were increased. O_2^- production was blocked upon NOS inhibition. Our data therefore provide evidence for eNOS uncoupling within bone marrow in diabetes. The uncoupling of eNOS has previously been linked to its monomerization upon treatment of endothelial cells with peroxynitrite or glucose (37,48). In diabetic bone marrow, eNOS dimer-to-monomer ratio was shifted to monomerization. Changes in the dimer-to-monomer ratio are not directly linked to functional uncoupling of eNOS, as only the dimeric form appears to be biochemically active and is able to generate either NO or O_2^-. However, the eNOS monomer can be viewed as a marker for eNOS uncoupling (5). eNOS uncoupling in bone marrow lowers NO bioavailability and thus likely contributed to the observed reduced levels of circulating EPCs.

We cannot rule out that other O_2^--producing systems play an additional role in the observed effects. For instance, we also observed a minor contribution of NADPH oxidases in glucose-mediated increase in ROS formation and subsequent reduction in EPC function. However, a stronger attenuation of O_2^- formation was observed after eNOS inhibition, indicating a major role for uncoupled eNOS in superoxide production in diabetes.

In conclusion, eNOS uncoupling impairs EPC number and function in diabetes, thus contributing to the pathogenesis of vascular disease.

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