Blockade of NADPH Oxidase Restores Vasoreparative Function in Diabetic CD34$^+$ Cells

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Purpose. The vasodegenerative phase of diabetic retinopathy is likely caused by endothelial dysfunction and reduced endothelial repair. Migration of endothelial progenitor cells (EPCs) into areas of vascular injury is critical to vascular repair. This key function, often defective in diabetes, is largely mediated by nitric oxide (NO), which is known to be inactivated by superoxide produced by NADPH oxidase. The authors tested the hypothesis that either increasing eNOS expression or inhibiting NADPH oxidase would restore the reparative function in diabetic EPCs.

Methods. Peripheral blood was obtained from healthy (n = 27) and diabetic (n = 31) persons, and CD34$^+$ cells were isolated. Expression and activation of eNOS and NADPH oxidase and intracellular levels of NO, superoxide, and peroxynitrite were evaluated. cGMP production and migration to SDF-1α were also determined. Reparative function was evaluated in a mouse model of retinal ischemia-reperfusion injury.

Results. Diabetic EPCs demonstrate reduced eNOS expression and decreased NO bioavailability and migration in response to SDF-1α. Increasing eNOS expression in diabetic cells by AVE3085 resulted in increased peroxynitrite levels and, therefore, did not enhance NO-mediated functions in vitro and in vivo. Expression of Nox2, NADPH oxidase activity, and superoxide levels were higher in diabetic than in nondiabetic EPCs. Pretreatment with apocynin or gp91ds-tat increased NO bioavailability without increasing eNOS activity in response to SDF-1α. Ex vivo NADPH oxidase inhibition in diabetic cells restored migratory function in vitro and enhanced their homing to ischemic retinal vasculature in vivo.

Conclusions. The NADPH oxidase system is a promising target for correcting vasoreparative dysfunction in diabetic EPCs. (*Invest Ophthalmol Vis Sci.* 2011;52:5093–5104) DOI: 10.1167/iovs.10-70911

Endothelial progenitor cells (EPCs), a subpopulation of the total mononuclear cells, have both hematopoietic stem cell (HSC) and endothelial cell markers.[1] These vascular reparative cells are mobilized from the bone marrow (BM) after tissue and vascular injury. Systemic or local treatment with autologous EPCs has been shown to stimulate vascular repair and re-endothelialization in animal studies and in clinical trials.[2–6] CD34$^+$ cells are considered the "prototype EPCs" because CD34 was used as a surface marker when EPCs were initially isolated from the monocyte population.[1] Recent clinical studies indicate that CD34 alone represents a good marker for human EPCs.[7]

Accelerated vascular dysfunction caused by endothelial injury increases mortality and morbidity in patients with diabetes mellitus. Proliferative diabetic retinopathy, a major cause of blindness worldwide in adults,[8] is thought to arise as a result of diabetes-induced retinal microvascular endothelial dysfunction leading to decreased retinal perfusion, hypoxia, and subsequent induction of angiogenic factors.[9] EPCs can be recruited to sites requiring vascular repair and can contribute to the repair and viability of the vasculature.[10] However, in diabetes, dysfunctional EPCs cannot repair this injury leading to development of acellular capillaries, the hallmark feature of diabetic retinopathy, and sustained retinal ischemia. Previously, we showed that CD34$^+$ cells from healthy subjects could repopulate degenerate retinal capillaries in chronic (diabetes) and in acute (ischemia/reperfusion [I/R] injury and neonatal oxygen-induced retinopathy [OIR]) animal models of ocular vascular damage, whereas diabetic CD34$^+$ cells could not.[11] These results are in agreement with others that the in vivo re-endothelialization capacity of EPCs derived from diabetic patients is severely impaired.[12,13] Specifically, the migration of EPCs in response to hypoxia-regulated cytokines and growth factors, such as stromal derived factor-1α (SDF-1α) and vascular endothelial growth factor (VEGF), is an essential event in the process of EPC-mediated vascular repair and is severely impaired in diabetic EPCs.[14] Recent studies provided experimental evidence for an essential role of nitric oxide (NO) and cGMP levels, a direct indication of NO bioavailability, in proper migration and reparative function of EPCs.[14–16] Mobilization of EPCs from BM and migration of EPCs into ischemic sites are regulated by NO-mediated signaling pathways involving cGMP and cGMP-dependent protein kinase I.[14–16] The defective migration of diabetic EPCs in response to SDF-1α and VEGF is attributed to the decreased NO levels.[14]

Increased oxidative stress associated with diabetes[17] results in reduced NO bioavailability. NADPH oxidase is a prominent source of reactive oxygen species (ROS) in endothelium.[18,19] Overproduction of superoxide from NADPH oxidase in diabetes inactivates NO, resulting in the generation of peroxynitrite,[20] a highly cytotoxic molecule that causes oxidative damage to proteins, lipids, and DNA.[21–23] Peroxynitrite also causes eNOS uncoupling and further enhances superoxide generation.[24]

The enzyme NADPH oxidase consists of membrane-associated cytochrome b558 comprising the catalytic gp91^{phox} (Nox2) and regulatory p22^{phox} subunit and cytosolic compo-

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nents including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and small GTPase Rac.[25] In physiological conditions, ROS have been shown to be involved in cellular signaling mechanisms that are attributable to the reversible oxidation of redox-sensitive target proteins. Protein tyrosine phosphatases are exquisitely sensitive to oxidative modification leading to increased phosphorylation and activation of many receptor tyrosine kinases.[26] Overproduction of ROS in diabetes because of increased activation of NADPH oxidase has been shown to be involved in the initiation and progression of diabetic vascular complications by decreasing the bioavailability of NO.[19,27,28] Decreasing the expression and activation of this enzyme has been shown to be the major mechanism of protection by "statin" treatment in diabetic retinopathy in rat and mouse models.[29,30]

HSCs, which are precursors of EPCs, express NADPH oxidase isoforms. It has been suggested that low levels of ROS in the BM play an essential role in preserving primitive HSCs in the hypoxic environment. Slightly elevated levels promote mobilization of HSCs in the early stages of postischemic neovascularization; however, excessive ROS production causes senescence and impairs the self-renewal of HSCs.[31–35] Studies in circulating EPCs showed that the expression of antioxidant enzymes, including catalase, glutathione peroxidase, and manganese superoxide dismutase (MnSOD), are higher and that basal ROS levels are lower than those in endothelial cells, suggesting that EPCs are highly resistant to oxidative stress.[36,37] In contrast, Ingram et al.[38] reported that the clonogenic proliferative EPCs derived from adult peripheral blood are more sensitive to oxidative stress and exhibit decreased clonogenic capacity and angiogenic function in the presence of oxidants. These studies were carried out in cells that were derived from circulating EPCs cultured in vitro for 3 or more weeks and have yet to be confirmed in freshly isolated $CD34^+$ cells, which represent the ideal cell population for autologous transplantation for cell therapy.[39,40]

In the present study, we tested the hypothesis that either increasing eNOS expression or decreasing NADPH oxidase activation restores vasoreparative function in diabetic EPCs by increasing NO bioavailability. We used AVE3085 to increase eNOS expression and apocynin and gp91ds-tat to achieve the blockade of NADPH oxidase. AVE3085 is a novel small molecule that enhances eNOS expression by stimulating eNOS promoter activity.[41] Apocynin was identified as an NADPH oxidase inhibitor[42] and has been extensively used in experimental cardiovascular studies.[43] The peptide inhibitor gp91ds has an amino acid sequence complementary to the region of the $gp91^{phox}$ subunit that is necessary for the docking of cytoplasmic $p47^{phox}$.[44] The interaction of $p47^{phox}$ and $gp91^{phox}$ subunits is blocked by gp91ds, and, thus, the formation of a functional enzyme complex is prevented.

METHODS

Characteristics of Subjects and Patients

Peripheral blood was obtained from healthy subjects (n = 27) and diabetic patients (n = 31) 22 to 60 years of age visiting clinics at Shands Teaching Hospital at the University of Florida in accordance with the approved protocol by the Institutional Review Board. Study subjects included both men and women, and diabetic patients included those with type 1 and type 2 diabetes with mild, moderate, or severe nonproliferative diabetic retinopathy. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study The following exclusion criteria were used: evidence of ongoing acute or chronic infection (HIV, hepatitis B or C, tuberculosis), ongoing malignancy, cerebral vascular accident or cerebral vascular procedure, history of organ transplantation, presence of a graft, uremic symptoms, estimated glomerular filtration rate <20 mL/min, and albumin <3.6.

Isolation of $CD34^+$ Cells

Blood was collected in CPT tubes (Vacutainer; BD Biosciences, Franklin Lakes, NJ), and mononuclear cells were separated by centrifugation. Peripheral blood mononuclear cells (PB-MNCs) were enriched for lineage $(Lin)^-$ cells by negative selection (StemCell Technologies, Vancouver, BC, Canada). $Lin^-CD45^{dim}CD34^+$ cells were resolved from lymphocytes by fluorescence-activated cell sorting (FACS), as previously described.[45] In some experiments, Lin^- cells were enriched for $CD34^+$ cells by immunomagnetic selection (StemCell Technologies). Freshly sorted cells were used for most experiments. When needed, sorted cells were plated in media containing cytokine cocktail (StemSpan; StemCell Technologies) in round-bottom, 96-well plates (Nunc) for ≤72 hours.

Real-Time PCR

Total mRNA of human $CD34^+$ cells was obtained by reagent (Trizol; Invitrogen, Carlsbad, CA) extraction, and the purity of RNA was determined with a spectrophotometer (ND-1000 UV-Vis; NanoDrop Technologies, Rockland, DE). RNA was reverse-transcribed with a cDNA synthesis kit (iScript; Bio-Rad, Hercules, CA). Real-time PCR was carried out (TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix; Applied Biosystems, Inc., Carlsbad, CA). The following assay systems were used to amplify different genes of interest: eNOS, Hs00167166_m1; iNOS, Hs00167257_m1; nNOS, Hs00167223_m1; Nox1, Hs00246598_m1; Nox2, Hs00166163_m1; Nox3, Hs00210462_m1; Nox4, Hs01558199_m1; Nox5, Hs00225846_m1; and β-actin, Hs99999903_m1. Real-time PCR was performed on a PCR machine (ABI 7500 Fast; Applied Biosystems) for 50 cycles. Results were analyzed by the $2^{-\Delta\Delta Ct}$ method and were normalized to β-actin expression.

Fluorescence Imaging of Superoxide, NO, and Peroxynitrite

Intracellular levels of superoxide, NO, and peroxynitrite in $CD34^+$ cells were evaluated by fluorescence microscopy using dihydroethidium (DHE), DAF-FM, and DCF (CM-H_2DCF diacetate) (Invitrogen), respectively, as described previously.[46] Images were analyzed, and fluorescence was measured in arbitrary units (TillVision software; TILL Photonics, Munich, Germany). For selected studies, $CD34^+$ cells were treated with apocynin or tat peptides for 60 minutes or with ebselen for 15 minutes, after which cells were loaded with fluorescent dyes. NO or peroxynitrite generation was evaluated after stimulation with 100 nM SDF-1α, and their levels were expressed as a percentage change in fluorescence with respect to cells that were used as a time/vehicle control. The oxidized product of DHE, 2-hydroxyethidium (HEt) intercalates with DNA and emits red fluorescence. The fluorescence of HEt was expressed as arbitrary fluorescence units (AFU). Up to 70 cells were imaged for each treatment, and the results were compared by the nonparametric Mann-Whitney test.

Activity of eNOS was evaluated by measuring L-citrulline synthesis using L-[^{14}C]arginine as a substrate, as described previously,[46] and the activity was expressed as L-NAME–sensitive radioactivity per milligram of protein. Migration (Chemicon International, Inc., Temecula, CA) and cGMP (GE Healthcare Biosciences, Piscataway, NJ) assays were carried out in accordance with the manufacturer's instructions. NADPH oxidase activity was determined in whole cell lysates using DHE in a plate reader. The activation of the enzyme was triggered by NADPH.[47]

Evaluation of Vascular Engraftment Potential of $CD34^+$ Cells in Acute Vascular Injury Model: Mouse Retinal Ischemia/Reperfusion

All animal procedures used were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals

and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Florida Institutional Animal Care and Use Committee. Mice were subjected to retinal I/R injury by hydrostatic pressure under isoflurane anesthesia in the anterior chamber of the eye, as described earlier.[11] Seven days after the insult, at which time retinal capillary damage was appreciable,[11,48] the intravitreal injection of CD34$^+$ cells was carried out. After 48 hours, the animals were euthanatized, the eyes were enucleated, and the neural retinas were isolated. Retinas were processed for immunohistochemistry to stain vasculature with rhodamine-conjugated *Ricinus communis* agglutinin I (Vector Laboratories, Burlingame, CA) and with mouse monoclonal anti-human nuclear antigen (Chemicon) followed by secondary staining with FITC-conjugated goat-anti-mouse IgG antibodies to localize the cells.[11] Digital fluorescence microscopy of retinas was carried using a spinning disc confocal microscope (Olympus, Tokyo, Japan). The vascular incorporation of CD34$^+$ cells was quantified by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).[11]

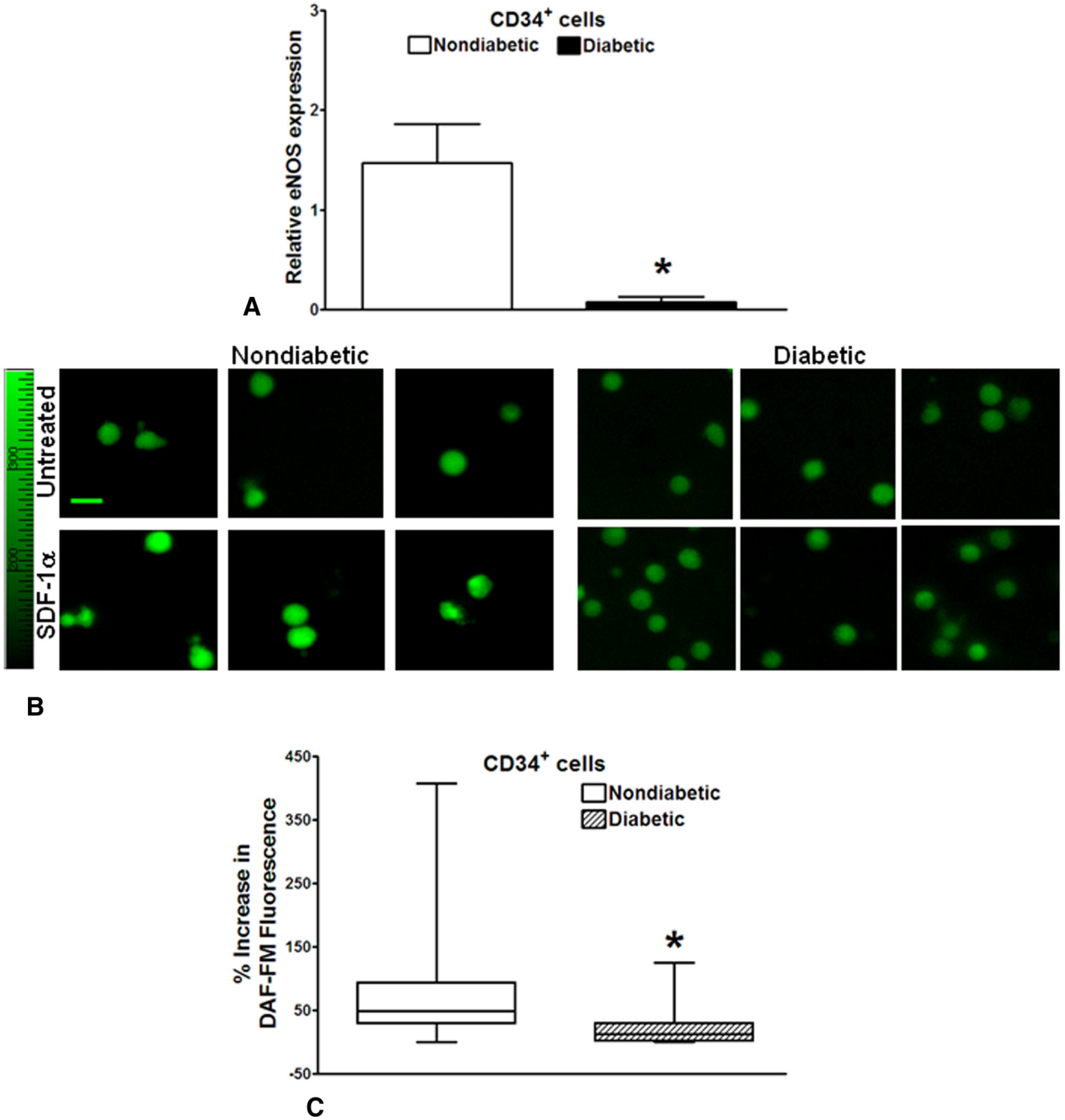


FIGURE 1. eNOS expression and NO bioavailability are reduced in diabetic CD34$^+$ cells. (**A**) Relative expression of eNOS mRNA was lower in diabetic EPCs than in nondiabetic cells ($^*P < 0.02$, $n = 10$). (**B**) Determination of intracellular NO levels by DAF-FM fluorescence. Shown are representative images of DAF-FM fluorescence in nondiabetic and diabetic CD34$^+$ cells with or without treatment (100 nM SDF-1α). (*left*) Color scale for the fluorescence intensity. Scale bar, 20 μm; applicable for all images. (**C**) Box plots of percentage increase in DAF-FM fluorescence. Percentage increase in DAF-FM fluorescence was lower in diabetic cells than in nondiabetic cells. $^*P < 0.0001$, Mann-Whitney test.

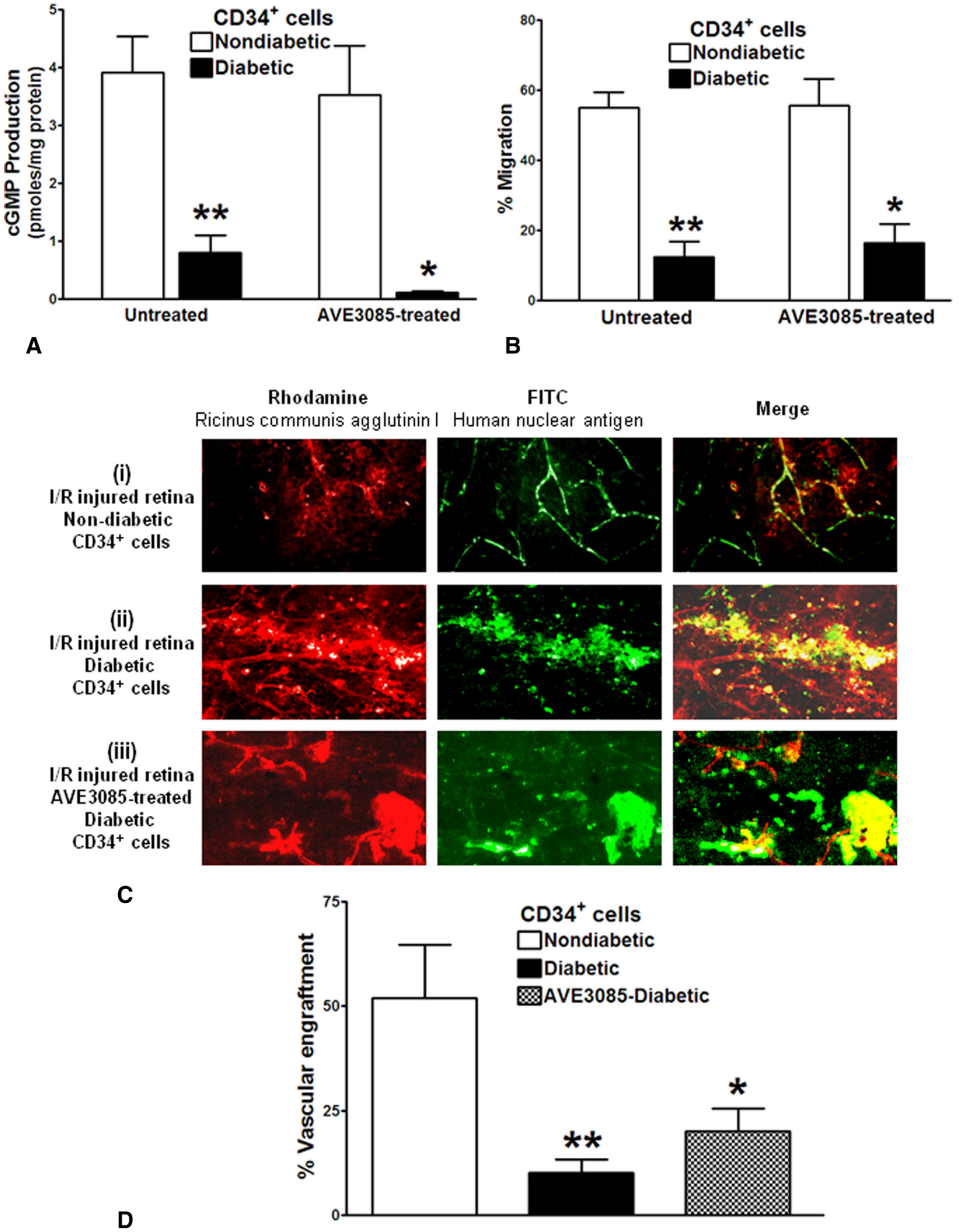


FIGURE 2.

The percentage of colocalization was calculated and compared with the total vascular area for that field.

Materials

SDF-1α was obtained from R&D Systems (Minneapolis, MN), VEGF and apocynin were purchased from Sigma (St Louis, MO) and ebselen was purchased from EMD Biosciences (Gibbstown, NJ). Peptides, gp91scr-tat (scrambled/control) and gp91ds-tat, were obtained from the media culturing HEK293 cells that were transfected with an expression vector that produce and secrete these peptides into the culturing media. Concentrated (~10×) cell-free culture supernatants were used for studies.

Statistical Analysis

Results were expressed as mean ± SEM; n represents the number of donors used. Results were analyzed for statistical significance by the Student's t-test or one-way ANOVA with Newman-Keuls posttest and graphing and statistics software (Prism; GraphPad, Inc., San Diego, CA). In studies involving fluorescence imaging, results were expressed as median/presented in box plots and were compared by the nonparametric test (Mann-Whitney test).

Results

Expression of eNOS and NO Bioavailability Are Decreased in Diabetic CD34$^+$ Cells

Real-time PCR detected eNOS as a major isoform of NOS expressed in CD34$^+$ cells, but the expressions of iNOS and nNOS were not detected. Relative expression of eNOS mRNA was lower in diabetic than in nondiabetic cells ($P < 0.02$; $n = 10$; Fig. 1A). Intracellular NO levels in CD34$^+$ cells in response to SDF-1α, the key cytokine that stimulates their migration, were determined. We have previously shown that the activation of the receptor CXCR4 by SDF-1α causes NO release through the PI3-kinase/Akt pathway in CD34$^+$ cells.[45] SDF-1α (100 nM) increased intracellular NO levels, as determined by the percentage increase in DAF-FM fluorescence, in nondiabetic cells ($n = 4$; Figs. 1B, 1C), and in diabetic cells the effect was lower ($P < 0.0001$; Mann-Whitney test). Consistently, cGMP production, a direct indication of NO bioavailability, in response to SDF-1α was lower in diabetic than in nondiabetic cells ($P < 0.001$; $n = 9$; Fig. 2A). Furthermore, the migratory response to SDF-1α, a functional signature of the vasoreparative ability, was attenuated in CD34$^+$ cells of diabetic origin compared with those of nondiabetic origin ($P < 0.002$; $n = 5$; Fig. 2B).

Increasing eNOS Expression Does Not Correct Vasoreparative Dysfunction in Diabetic CD34$^+$ Cells

We next asked whether increasing eNOS expression by AVE3085 would correct vasoreparative dysfunction in diabetic CD34$^+$ cells using both in vitro and in vivo assays. Treatment of CD34$^+$ cells with 10 μM AVE3085 for approximately 24 hours enhanced eNOS mRNA expression up to 20-fold in both nondiabetic ($n = 3$) and diabetic cells ($n = 3$), consistent with that observed in BM-MNCs previously.[49] Then CD34$^+$ cells pretreated with AVE3085 were evaluated for NO bioavailability and migration in response to SDF-1α in vitro. Generation of cGMP in response to SDF-1α was lower in diabetic than in nondiabetic cells after treatment with AVE3085 ($P < 0.02$; $n = 3$). In addition, the migration of diabetic cells remained impaired after treatment with AVE3085 ($P < 0.01$; $n = 3$). Consistent with in vitro studies, AVE3085-treated diabetic cells did not migrate to areas of vascular injury when injected within the vitreous of mice undergoing the I/R model of retinal injury. As shown in Figure 2C-i, retinas with I/R injury that received nondiabetic CD34$^+$ cells resulted in 51% ± 12% ($n = 5$) incorporation of these cells into retinal vascular structures after intravitreal administration ($n = 5$; Fig. 2D). The retinas of mice that received diabetic CD34$^+$ cells showed lower incorporation (10% ± 3%; $P < 0.004$; $n = 5$) (Figs. 2C-ii, 2D), and cells appeared to form aggregates on the surface of the vitreous and were not associated with vasculature. Treatment with AVE3085 did not improve their homing functions (15% ± 3%; $P < 0.01$; $n = 5$) (Figs. 2C-iii, 2D).

Next we explored the possibility that the NO generated was captured by superoxide-producing peroxynitrite in the diabetic cells treated with AVE3085 after stimulation with SDF-1α. This was carried out by using DCF in nondiabetic and diabetic cells that were pretreated with AVE3085. Basal DCF fluorescence was not significantly different in nondiabetic and diabetic cells (Fig. 3). Basal levels were not increased in nondiabetic cells by AVE3085 treatment, as expressed as a percentage increase in the fluorescence compared with the untreated cells and remained unchanged on stimulation with SDF-1α (Fig. 3). In diabetic cells, the percentage increase in fluorescence was higher than in nondiabetic cells after AVE3085 treatment ($P < 0.001$; Mann-Whitney test) that was further increased after stimulation with SDF-1α (Fig. 3). Pretreatment with ebselen (5 μM), a scavenger of peroxynitrite,[50] almost abolished DCF fluorescence in both nondiabetic and diabetic cells (<100 AFU compared with 1000–1800 AFU in the absence of ebselen), suggesting that the DCF fluorescence was produced by its selective interaction with peroxynitrite. These observations suggest that increasing eNOS expression by pharmacologic treatment without decreasing the production of ROS does not improve vasoreparative function in diabetic CD34$^+$ cells.

NADPH Oxidase Activation and Superoxide Levels Are Higher in Diabetic CD34$^+$ Cells

Expression of the NADPH oxidase catalytic subunit isoforms Nox1, Nox2, Nox3, Nox4, and Nox5 in CD34$^+$ cells from

Figure 2. AVE3085 treatment did not enhance the functions of diabetic CD34$^+$ cells. (**A**) Production of cGMP in response to SDF-1α, a direct indication of NO bioavailability, was lower in diabetic cells than in nondiabetic (**$P < 0.001$, $n = 9$). Treatment with AVE3085 (10 μM) did not alter SDF-1α–mediated cGMP production in either nondiabetic or diabetic cells. Similarly cGMP levels were lower in diabetic than in nondiabetic cells (*$P < 0.02$, $n = 3$). (**B**) Migratory response to SDF-1α was lower in CD34$^+$ diabetic cells than in nondiabetic cells (**$P < 0.002$, $n = 5$). Treatment with AVE3085 (10 μM) did not alter migration to SDF-1α in either nondiabetic or diabetic cells. The response in diabetic cells was lower than in nondiabetic cells (*$P < 0.01$, $n = 3$). (**C**) Vasoreparative function of CD34$^+$ cells was evaluated in mouse retinal I/R injury model. Shown are representative images obtained with a 10× objective. Healthy or injured vasculature is stained with rhodamine-conjugated ricinus communis agglutinin I (*red*). The injected cells were localized with FITC-conjugated human nuclear antigen (*green*). (**i**) Retinas obtained from eyes subjected to I/R injury and injected with nondiabetic CD34$^+$ cells showed high degree of vascular incorporation of CD34$^+$ cells (*green*). (**ii**) I/R-injured retinas injected with diabetic CD34$^+$ cells did not show vascular incorporation. (**iii**) I/R-injured retinas injected with diabetic CD34$^+$ cells pretreated with AVE3085 did not result in vascular incorporation. With or without treatment with AVE3085, clumps of diabetic cells with red/green fluorescence were evident. (**D**) Summary of vascular incorporation of cells observed in nondiabetic and diabetic CD34$^+$ cells with or without treatment with AVE3085. **$P < 0.004$ ($n = 5$) and *$P < 0.05$ ($n = 5$) compared with nondiabetic cells ($n = 5$).

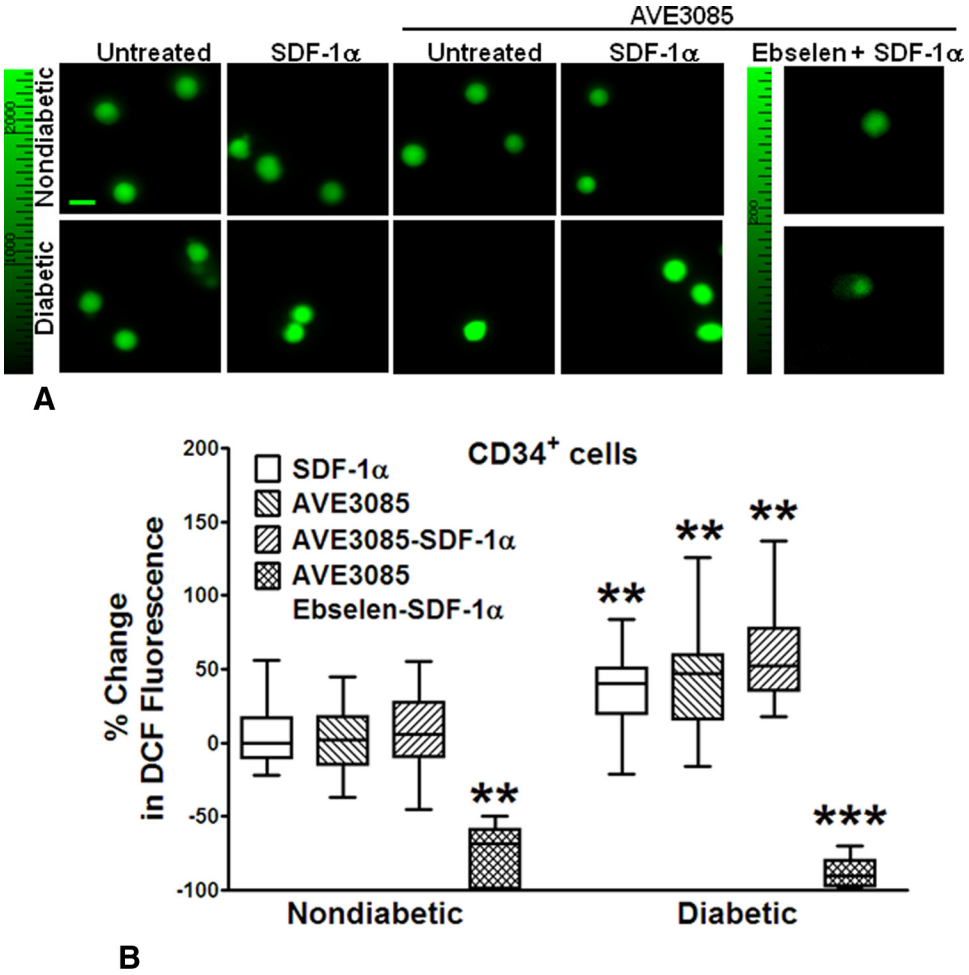


FIGURE 3. Treatment with AVE3085 results in higher peroxynitrite levels in diabetic $CD34^+$ cells. (**A**) Peroxynitrite levels were determined by quantitative fluorescence imaging using DCF. Shown are representative images of DCF fluorescence in nondiabetic and diabetic $CD34^+$ cells with or without treatment. (*left*) Color scale for the fluorescence intensity. Scale bar, 20 μm; applicable for all images. (**B**) Box plots of percentage increase in DCF fluorescence after treatment. DCF fluorescence was unaltered with SDF-1α stimulation in untreated or AVE3085-treated nondiabetic cells. DCF fluorescence was increased in diabetic cells after SDF-1α stimulation compared with nondiabetic cells ($^{**}P < 0.001$, Mann-Whitney test). Treatment with AVE3085 resulted in an increased fluorescence that was further enhanced after SDF-1α stimulation compared with nondiabetic cells ($^{**}P < 0.001$). DCF fluorescence was significantly decreased by pretreatment with ebselen in both nondiabetic ($^{**}P < 0.001$) and diabetic cells ($^{***}P < 0.001$).

healthy subjects was evaluated by real-time PCR. Amplification of Nox1, Nox3, Nox4, and Nox5 isoforms was not observed; however, Nox2 amplification was apparent. In diabetic $CD34^+$ cells, Nox2 expression was found to be higher than it was in nondiabetic cells ($P < 0.01$; $n = 10$; Fig. 4A). This was associated with increased NADPH oxidase activity in the lysates of diabetic $CD34^+$ cells compared with those in nondiabetic cells ($P < 0.01$; $n = 5$, Fig. 4B). We next evaluated intracellular levels of superoxide by quantitative fluorescence imaging using DHE. Fluorescence of HEt was higher in diabetic $CD34^+$ cells than in nondiabetic cells ($P < 0.0001$; $n = 4$; Mann-Whitney test; Figs. 4C, 4D), suggesting higher basal levels of superoxide in diabetes. In the presence of 300 μM L-NAME, HEt fluorescence was not altered (data not shown), suggesting that the superoxide generation was not caused by eNOS uncoupling.[51]

NADPH Oxidase Inhibitors Increase NO Bioavailability in Diabetic $CD34^+$ Cells

To confirm that decreased intracellular levels of NO were caused by the overproduction of ROS, the effects of the NADPH oxidase inhibitors apocynin and gp91ds-tat were evaluated on NO availability in diabetic $CD34^+$ cells. In diabetic cells, NO levels produced by SDF-1α were lower than of nondiabetic cells ($P < 0.0001$; $n = 4$; Mann-Whitney test; Figs. 5A, 5B). Treatment of diabetic $CD34^+$ cells for an hour with 300 μM apocynin resulted in increased DAF-FM fluorescence in response to SDF-1α ($P < 0.0001$; Mann-Whitney test, compared to untreated diabetic cells), which was similar to levels observed in nondiabetic $CD34^+$ cells (Figs. 5A, 5B). Treatment with apocynin did not alter the DAF-FM fluorescence to SDF-1α in nondiabetic $CD34^+$ cells.

Consistent with this, cGMP production in response to SDF-1α was increased in diabetic cells in the presence of gp91ds-tat. SDF-1α–mediated cGMP production was lower in diabetic than in nondiabetic $CD34^+$ cells ($P < 0.01$, one-way ANOVA) and was not altered by the control peptide gp91scr-tat, whereas cGMP levels were increased by treatment with gp91ds-tat ($P < 0.05$, compared with untreated or gp91scr-tat–treated diabetic cells; one-way ANOVA; $n = 6$; Fig. 6A). Activation of eNOS by SDF-1α was lower in diabetic than in nondiabetic $CD34^+$ cells ($P < 0.005$; $n = 6$; Fig. 6B). Treatment with gp91scr-tat or gp91ds-tat did not improve eNOS activation (Fig. 6B), ruling out the nonspecific activation of eNOS by gp91ds-tat.

Then we evaluated whether increasing NO bioavailability/cGMP levels by NADPH oxidase inhibitors in $CD34^+$ cells would enhance their function by determining the migration of diabetic $CD34^+$ cells to hypoxia-regulated factors SDF-1α and VEGF. The migratory response to SDF-1α or VEGF, expressed as percent of untreated control, was lower in diabetic $CD34^+$ cells (100 nM SDF-1α: nondiabetic, 40 ± 2%; diabetic, 5 ± 2%; $n = 4$; $P < 0.0001$) (25 ng/mL VEGF: nondiabetic, 41 ± 8%; diabetic, 7 ± 4%; $n = 4$; $P < 0.0001$) (Fig. 6C). Pretreatment of diabetic $CD34^+$ cells with apocynin enhanced their migration to SDF-1α (51 ± 3%; $n = 3$; $P < 0.0001$) or VEGF (59 ± 13%; $P < 0.05$) (Fig. 6C). Similarly, pretreatment with gp91ds-tat enhanced the migratory response in diabetic $CD34^+$ cells com-

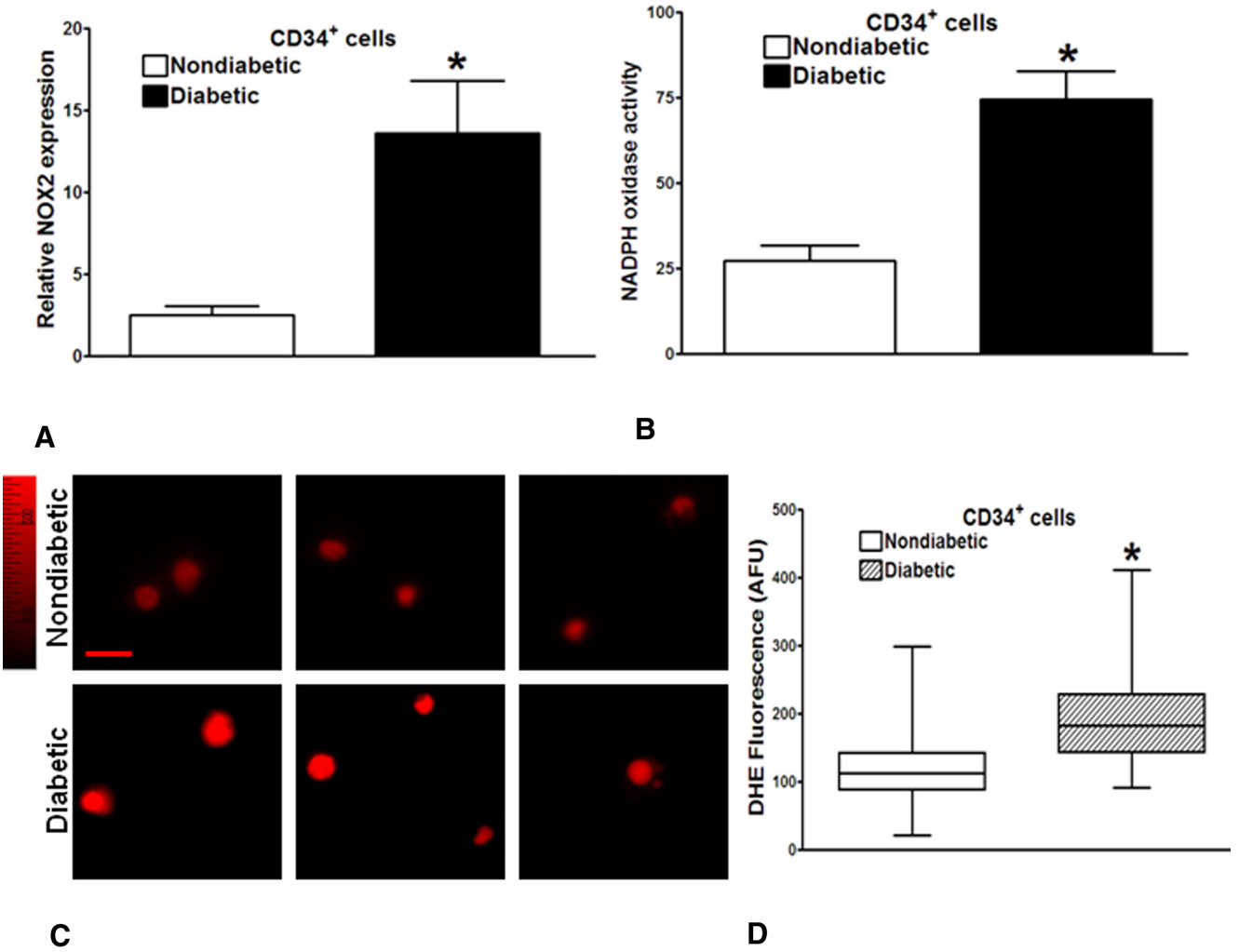


FIGURE 4. NADPH oxidase activation and superoxide levels are higher in diabetic CD34$^+$ cells. (**A**) Expression of Nox2 mRNA relative to β-actin was higher in diabetic CD34$^+$ cells than in nondiabetic cells (*$P < 0.01$, $n = 10$). (**B**) NADPH oxidase activity in the lysates of diabetic CD34$^+$ cells, expressed as AFU per milligram protein, was higher than in nondiabetic cells (*$P < 0.01$, $n = 5$). (**C**) Intracellular levels of superoxide determined by quantitative fluorescence imaging using DHE. Shown are images of red fluorescence emitted by the oxidized product of DHE, 2-hydroxyethidium (HEt) after DNA intercalation. Images were obtained with a Fluor 40×W objective. (*left*) Color scale for the fluorescence intensity. Scale bar, 20 μm; applicable for all images. (**D**) Box plots of HEt fluorescence observed in diabetic and nondiabetic CD34$^+$ cells. HEt fluorescence was higher in diabetic CD34$^+$ cells than in nondiabetic cells (*$P < 0.0001$, $n = 4$, Mann-Whitney test).

pared with that produced by the control peptide (SDF-1α: gp91scr-tat, 2 ± 3%; $n = 3$; gp91ds-tat, 34 ± 5%; $P < 0.001$) (VEGF: gp91scr-tat, 7 ± 5%; $n = 3$; gp91ds-tat, 42 ± 5%; $P < 0.001$).

Ex Vivo Treatment with NADPH Oxidase Inhibitors Enhances In Vivo Vascular Engraftment Potential in Diabetic CD34$^+$ Cells

CD34$^+$ cells treated with NADPH oxidase inhibitors ex vivo were injected into the vitreous of mice undergoing the I/R model, and their vascular engraftment potential was evaluated. Diabetic CD34$^+$ cells were incubated with gp91scr-tat, gp91ds-tat, or apocynin for an hour, and then the blockers were removed by washing before intravitreal injection of these cells. As shown in Figure 7, retinas from I/R-injured eyes showed clear evidence of injured vasculature because labeled vessels were infrequent compared with those of the uninjured retina (Figs. 7A-I, 7B). Retinas with I/R injury that received nondiabetic CD34$^+$ cells resulted in 52% ± 8% ($n = 5$) incorporation of these cells into retinal vascular structures (Figs. 7A-iii, 7B). The retinas of mice that received diabetic CD34$^+$ cells showed lower incorporation (14% ± 4%; $P < 0.01$; $n = 8$) (Figs. 7A-iv, 7B), and cells appeared to form aggregates on the surface of the vitreous that were not associated with vasculature. Diabetic cells that were treated with gp91scr-tat ex vivo also showed lower vascular incorporation (22% ± 3%; $n = 4$), not unlike the untreated diabetic CD34$^+$ cells (Figs. 7A-v, 7B). In contrast, diabetic cells treated with gp91ds-tat showed enhanced vascular engraftment (43% ± 6%; $P < 0.03$; $n = 4$) compared with untreated or gp91scr-tat–treated diabetic cells (Figs. 7A-vi, 7B). Ex vivo treatment with apocynin also dramatically enhanced the vascular incorporation of diabetic CD34$^+$ cells (58% ± 10%; $P < 0.02$; $n = 7$) (Figs. 7A-vii, 7B).

DISCUSSION

In this study, we report several novel observations. eNOS is the predominant isoform of NOS in human CD34$^+$ cells, and the expression of eNOS was decreased in diabetic CD34$^+$ cells. Impaired migration of diabetic cells with SDF-1α was associ-

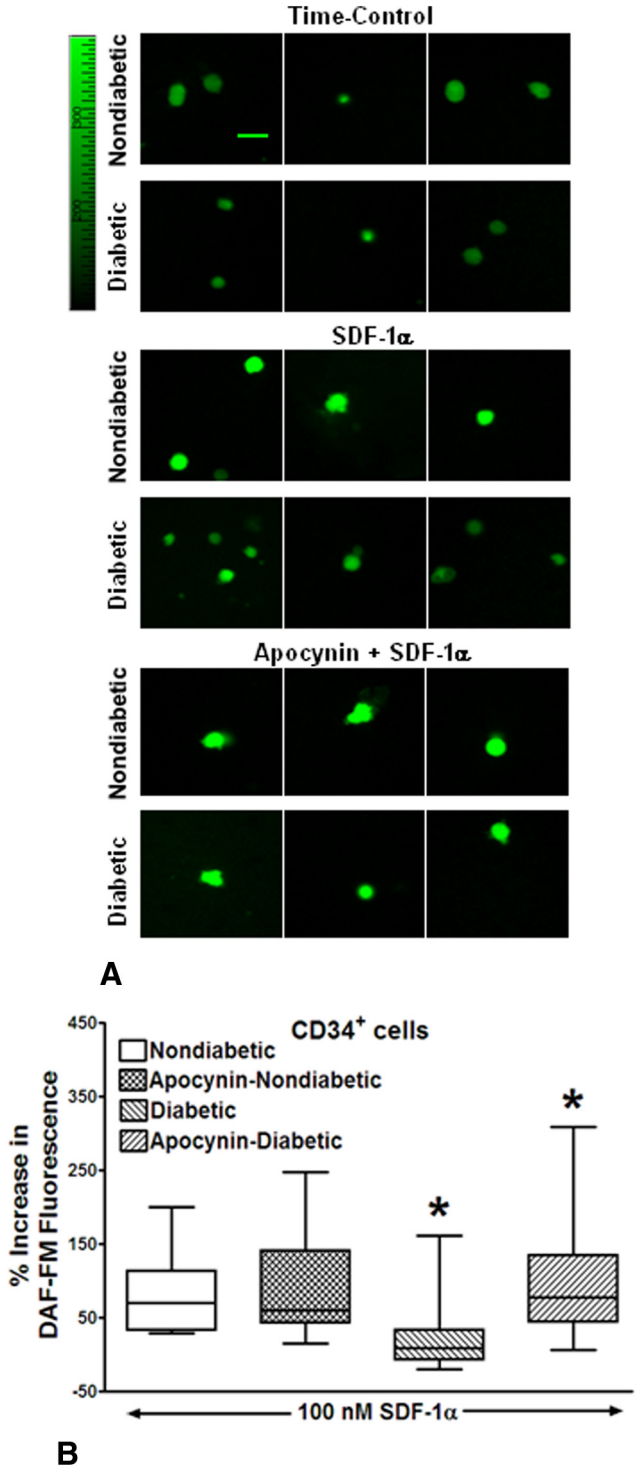


FIGURE 5. NADPH oxidase inhibitor increases intracellular NO levels in diabetic $CD34^+$ cells. (**A**) Shown are representative images of DAF-FM fluorescence in nondiabetic and diabetic $CD34^+$ cells with or without treatment (100 nM SDF-1α). (*left*) Color scale for the fluorescence intensity. Scale bar, 20 μm; applicable for all images. Cells that were not treated served as the time control, and the effect of SDF-1α with and without apocynin pretreatment or combination was expressed as the percentage increase in fluorescence compared with the time control. (**B**) Box plots of percentage increase in DAF-FM fluorescence. Percentage increase in the DAF-FM fluorescence was lower in diabetic cells than in nondiabetic cells (*$P < 0.0001$, Mann-Whitney test). In the presence of apocynin, the percentage increase in DAF-FM fluorescence by SDF-1α was higher than in diabetic cells without apocynin treatment ($P < 0.0001$, Mann-Whitney test).

ated with decreased NO bioavailability/cGMP production. NADPH oxidase system in human $CD34^+$ cells is predominantly Nox2-based, and Nox2 expression was increased in diabetic $CD34^+$ cells, which was associated with increased NADPH oxidase activity and increased basal levels of superoxide. By reducing oxidative stress, NADPH oxidase inhibitors enhanced in vitro and in vivo vasoreparative functions in the dysfunctional diabetic $CD34^+$ cells, resulting in enhancing NO bioavailability. Furthermore, increasing eNOS expression without correcting oxidative stress was not effective.

Several studies demonstrated that EPCs, from either type 1 or type 2 diabetes, do not repair after vascular injury in skin wounds and in retinal or hind limb ischemia.[11–13] In contrast, nondiabetic EPCs are able to revascularize skin wounds and accelerate blood flow restoration in type 1 diabetic mice.[52,53] Diabetic dysfunction of EPCs has been largely attributed to the lack of adequate levels of NO to enable them to migrate and integrate into areas of vascular repair.[14] However, treatment of diabetic $CD34^+$ cells ex vivo with an NO donor, DETA/NO, restored their angiogenic characteristics in vitro[14] but did not enhance their vasoreparative function in vivo (Jarajapu YP, Grant MB, unpublished observations, 2009). We concluded that though diabetic $CD34^+$ cells can exhibit improved in vitro angiogenic function after acute exposure to NO, sustained increases in the bioavailable NO are likely needed for in vivo vasoreparative function. Along similar lines, increasing the eNOS expression at transcriptional and translational levels has been shown to enhance angiogenic function in BM-MNCs.[49] However, in diabetes, because of the presence of an altered metabolic milieu, increasing eNOS expression may increase the risk for the overproduction of ROS.[41,54] This can be overcome by alternative approaches such as decreasing NO degradation or sustained activation of NO-mediated signal transduction in EPCs. The recently identified small molecules AVE9844 and AVE3085 have been shown to be promising in restoring defective NO bioavailability in an apoE knockout mouse model of atherosclerosis.[41] Importantly, ex vivo treatment of BM-MNCs from patients with ischemic cardiomyopathy increased their neovascularization potential in a mouse model of hind limb ischemia.[49] In the present study, ex vivo preconditioning with AVE3085 enhanced eNOS expression but did not result in NO bioavailability/cGMP production in diabetic $CD34^+$ cells and did not enhance vasoreparative function. Our studies further showed that peroxynitrite generation was higher in diabetic cells with and without AVE3085 treatment and was further enhanced by SDF-1α, suggesting that increased NO release by AVE3085 indeed resulted in the overproduction of peroxynitrite. These findings imply that decreasing ROS production or treatment with peroxynitrite scavengers would restore the effectiveness of therapeutic tools that enhance eNOS expression/NO release.

Despite the decreased eNOS mRNA expression, $CD34^+$ cells have sufficient amounts of the enzyme protein that can be activated by SDF-1α, resulting in NO generation. However, it was not bioavailable to produce enough cGMP, which was required to trigger the migratory response. This was most likely the result of the inactivation of NO by superoxide. In agreement with this, the inhibition of NADPH oxidase without increasing eNOS expression/activation resulted in increased NO levels, cGMP production, and migration to SDF-1α, suggesting physiological levels of NO and cGMP could be achieved by reducing the production of ROS in diabetic cells.

In this study, we did not address whether the ex vivo–treated cells would be able to integrate into the retinas of diabetic mice with vascular injury; however, we previously showed that the vasoreparative function of nondiabetic $CD34^+$ cells was identical in different models of retinal vascular injury, including diabetic retinopathy in the streptozotocin-induced

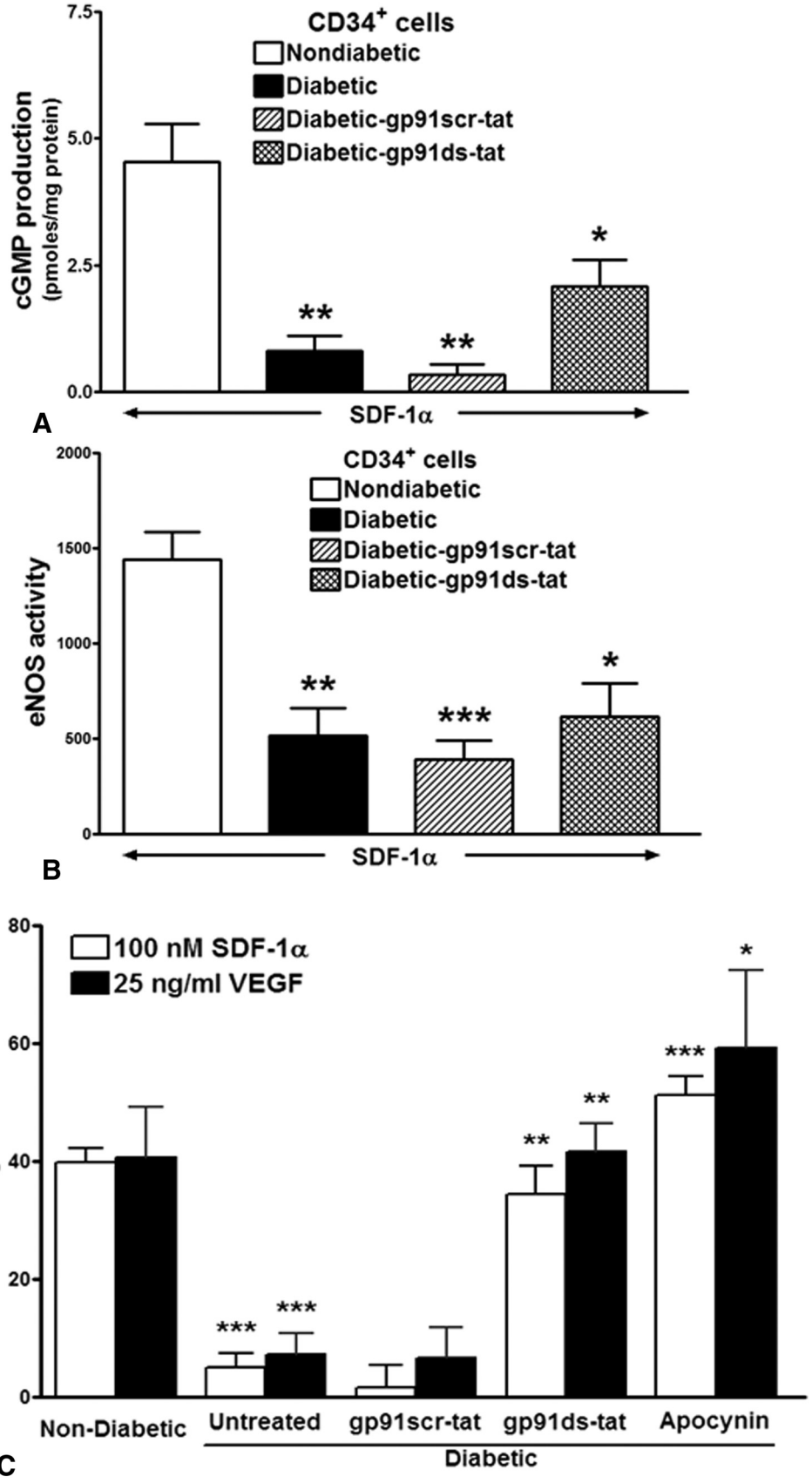
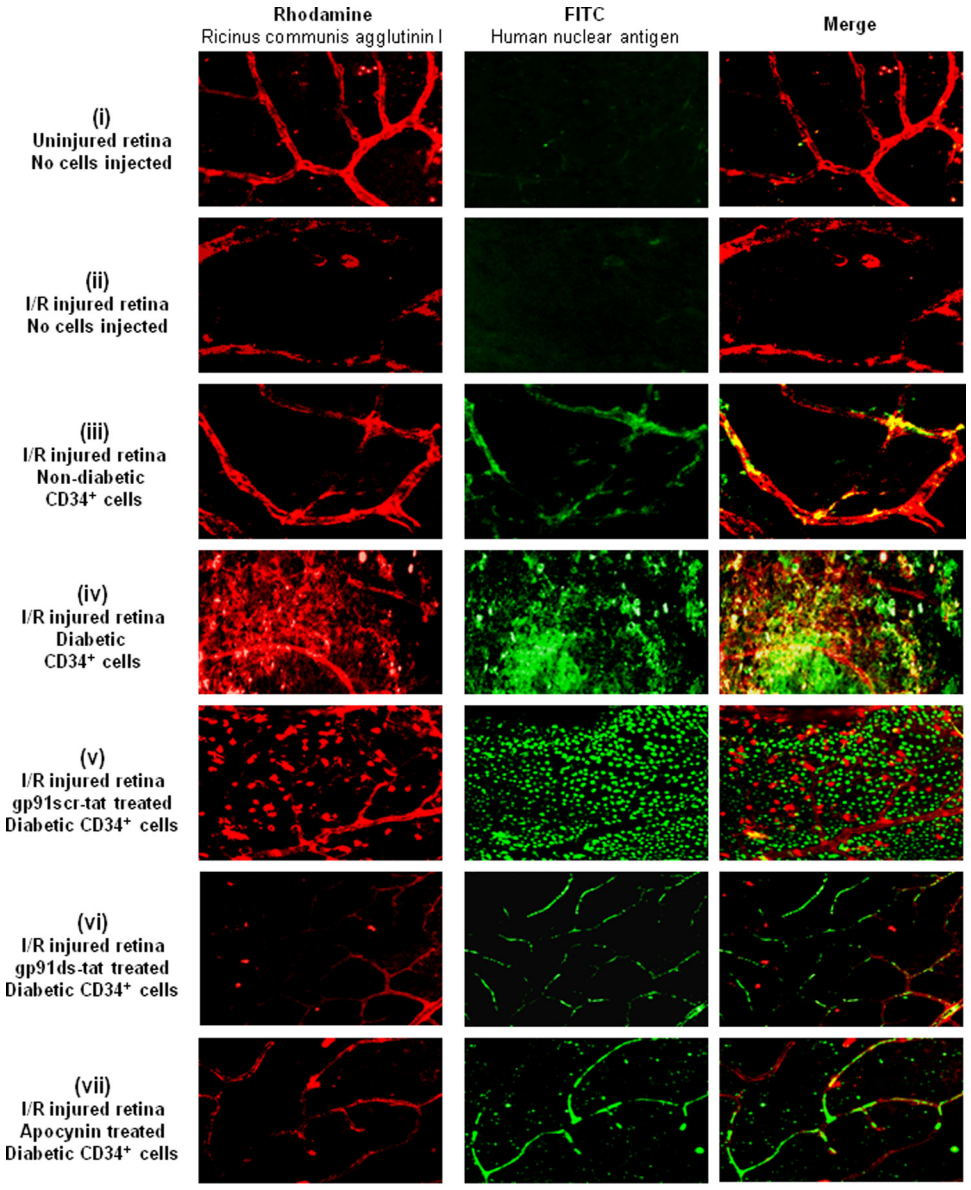


Figure 6. NADPH oxidase inhibitors increase cGMP production and migration by SDF-1α in diabetic CD34$^+$ cells. (**A**) Levels of cGMP produced by SDF-1α were lower in diabetic than in nondiabetic cells (**$P < 0.01$, one-way ANOVA). Treatment with gp91scr-tat had no effect whereas treatment with gp91ds-tat increased cGMP levels in diabetic cells (*$P < 0.05$, compared with untreated or gp91scr-tat–treated diabetic cells; one-way ANOVA, $n = 6$). (**B**) Activity of eNOS in CD34$^+$ cells treated with SDF-1α was expressed as L-NAME–sensitive radioactivity per milligram protein. Activity of eNOS was lower in diabetic ($n = 3$) than in nondiabetic cells (***$P < 0.005$, $n = 6$). The activity remained lower after treatment with gp91scr-tat or gp91ds-tat (***$P < 0.002$ and *$P < 0.01$). (**C**) Inhibitors of NADPH oxidase restored migratory response to SDF-1α or VEGF in diabetic CD34$^+$ cells. Migration of diabetic CD34$^+$ cells in response to SDF-1α or VEGF was lower than in nondiabetic CD34$^+$ cells (***$P < 0.0001$). Pretreatment with gp91scr-tat, control peptide, did not improve migratory function in diabetic CD34$^+$ cells, whereas pretreatment with gp91ds-tat enhanced the response to SDF-1α and VEGF compared with gp91scr-tat (**$P < 0.001$). Pretreatment with apocynin also enhanced the migratory response of diabetic cells compared to untreated cells (*$P < 0.05$; ***$P < 0.0001$).

type 1 diabetic mouse and in the obese BBZDR/WOR type 2 diabetic rat. Incorporated cells in all models were integrated into the vascular wall and formed patent vessels, as evaluated by perfusion with rhodamine-conjugated dextran and differentiation of CD34$^+$ cells into endothelial cells.[11] Therefore, we expect that the enhanced vascular engraftment potential of ex



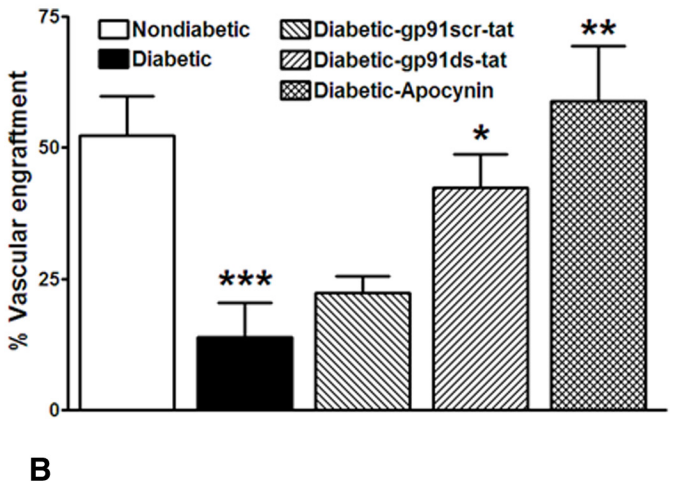


Figure 7. Ex vivo inhibition of NADPH oxidase enhances vasoreparative function in diabetic $CD34^+$ cells in mouse retinal I/R injury model. Shown are representative images of neural retinas. (**A**, **i**) Healthy vessels (*red*) from retinas obtained from untreated control eyes. (**ii**) Retinas obtained from eyes subjected to I/R injury but not injected with cells showing injured/degenerate vessels. (**iii**) Retinas obtained from eyes subjected to I/R injury and injected with nondiabetic $CD34^+$ cells showed a high degree of vascular incorporation (*green*). (**iv**) I/R-injured retinas injected with diabetic $CD34^+$ cells did not show clear vascular incorporation. (**v**) I/R-injured retinas injected with diabetic cells ex vivo treated with gp91scr-tat did not result increase vascular incorporation. Ex vivo treatment of diabetic cells with gp91ds-tat (**vi**) or apocynin (**vii**) resulted in enhanced vascular incorporation in I/R-injured retinas. (**B**) Bar graph summarizing the vascular engraftment by $CD34^+$ cells given different ex vivo treatments. Percentage vascular engraftment by diabetic $CD34^+$ cells was lower than by nondiabetic cells ($^{***}P < 0.01$, $n = 8$) and was enhanced by apocynin compared with no treatment ($^{**}P < 0.02$, $n = 7$) or by gp91ds-tat compared with gp91scr-tat treatment ($^{*}P < 0.03$, $n = 4$).

vivo–treated diabetic CD34$^+$ cells would be similar in diabetic retinal vascular injury.

Sorrentino et al.[55] showed that both in vitro and 2-week oral therapy with the peroxisome proliferator-activated receptor-γ agonist rosiglitazone restored the in vivo re-endothelialization capacity of EPCs derived from diabetic patients, which was partially attributed to the blockade of NADPH oxidase activity and increasing NO availability. Direct evidence, however, was not provided in this study. It is also unknown whether ex vivo preconditioning with this molecule would be a promising approach to enhance the vasoreparative function of dysfunctional diabetic progenitor cells, thereby enhancing the outcome of autologous cell therapies in the treatment of diabetic vascular complications. The present study, however, clearly demonstrates that targeting the NADPH oxidase system represents a promising treatment for increasing the vasoreparative potential in diabetic CD34$^+$ cells. Consistent with this, the clinical benefits of the three most prescribed classes of cardiovascular drugs (ACEI, ARB, and statins- inhibitors of HMG-CoA reductase) are thought to be partially attributed to the inhibition of NADPH oxidase.[43] Available NADPH oxidase inhibitors have potential limitations, including nonspecificity (e.g., diphenyleneiodonium), low affinity (e.g., apocynin), and poor bioavailability. Because of its peptide nature, the use of gp91ds-tat is associated with potential immune response problems with repeated administration in vivo.[43] Therefore, a great need remains for the development of more effective and safer therapeutic agents targeting the NADPH oxidase system.

Acellular capillaries are the driving force of retinal ischemia and subsequent aberrant neovascularization on the surface of the retina. The clinical implication would be that correction of the migration defect seen in CD34$^+$ cells isolated from patients with diabetes would prevent acellular capillaries, subsequent ischemia, and aberrant revascularization. The diabetic vascular complications could be explained in part by defective migration in response to hypoxia-regulated release of SDF-1α or VEGF with subsequent inadequate vascular repair. Administration of autologous CD34$^+$ cells in diabetic patients with restored reparative function by ex vivo pharmacologic inhibition of NADPH oxidase, for example, may represent a safer therapeutic approach to correct the multitude of vascular abnormalities seen in diabetic patients.

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