Intracavernous Delivery of a Designed Angiopoietin-1 Variant Rescues Erectile Function by Enhancing Endothelial Regeneration in the Streptozotocin-Induced Diabetic Mouse

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OBJECTIVE—Patients with diabetic erectile dysfunction often have severe endothelial dysfunction and respond poorly to oral phosphodiesterase-5 inhibitors. We examined the effectiveness of the potent angiopoietin-1 (Ang1) variant, cartilage oligomeric matrix protein (COMP)-Ang1, in promoting cavernous endothelial regeneration and restoring erectile function in diabetic animals.

RESEARCH DESIGN AND METHODS—Four groups of mice were used: controls; streptozotocin (STZ)-induced diabetic mice; STZ-induced diabetic mice treated with repeated intracavernous injections of PBS; and STZ-induced diabetic mice treated with COMP-Ang1 protein (days −3 and 0). Two and 4 weeks after treatment, we measured erectile function by electrical stimulation of the cavernous nerve. The penis was harvested for histologic examinations, Western blot analysis, and cGMP quantification. We also performed a vascular permeability test.

RESULTS—Local delivery of the COMP-Ang1 protein significantly increased cavernous endothelial proliferation, endothelial nitric oxide (NO) synthase (NOS) phosphorylation, and cGMP expression compared with that in the untreated or PBS-treated STZ-induced diabetic group. The changes in the group that received COMP-Ang1 restored erectile function up to 4 weeks after treatment. Endothelial protective effects, such as marked decreases in the expression of $p47^{phox}$ and inducible NOS, in the generation of superoxide anion and nitrotyrosine, and in the number of apoptotic cells in the corpus cavernosum tissue, were noted in COMP-Ang1–treated STZ-induced diabetic mice. An intracavernous injection of COMP-Ang1 completely restored endothelial cell-cell junction proteins and decreased cavernous endothelial permeability. COMP-Ang1–induced promotion of cavernous angiogenesis and erectile function was abolished by the NOS inhibitor, *N*-nitro-L-arginine methyl ester, but not by the NADPH oxidase inhibitor, apocynin.

CONCLUSIONS—These findings support the concept of cavernous endothelial regeneration by use of the recombinant Ang1 protein as a curative therapy for diabetic erectile dysfunction. ***Diabetes*** **60:969–980, 2011**

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Received 10 March 2010 and accepted 24 December 2010.

DOI: 10.2337/db10-0354

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0354/-/DC1.

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Erectile dysfunction affects up to 75% of all men with diabetes and occurs earlier in such patients than in the general population (1,2). The Massachusetts Male Aging Study revealed that erectile dysfunction is three times as prevalent in diabetic men as in men without diabetes (3). Moreover, men with diabetic erectile dysfunction tend to respond less positively to the currently available oral phosphodiesterase-5 (PDE5) inhibitors than do nondiabetic men (4–6). The reduced responsiveness to PDE5 inhibitors in patients with diabetes may be related to the severity of endothelial dysfunction (7). Because the effects of PDE5 inhibitors rely on endogenous nitric oxide (NO) formation, PDE5 inhibitors may fail to increase the level of cyclic guanosine monophosphate (cGMP) above the necessary threshold if the bioavailable NO is insufficient as a result of severe endothelial dysfunction. Furthermore, PDE5 inhibitors must be used on demand, thus limiting the spontaneity of the sexual act. The use of PDE5 inhibitors is absolutely contraindicated in men who take nitrate compounds because of the risk of extreme hypotension or even death (8). Therefore, new therapeutic strategies are needed.

Augmented generation of reactive oxygen species (ROS) is one of the major causes of decreased NO bioavailability in diabetes (9–12). We recently reported in streptozotocin (STZ)-induced type 1 diabetic mice that increased superoxide anion production and peroxynitrite formation in the corpus cavernosum tissue play an important role in the diabetes-induced functional and structural impairments in the cavernous endothelium, such as the decrease in endothelial NO synthase (eNOS) enzyme activity, endothelial cell apoptosis, and subsequent loss of endothelial cell content (12). Therefore, restoring NO bioavailability by inhibiting ROS-mediated endothelial cell damage, regenerating cavernous endothelial cells, or both may serve as promising therapeutic strategies for treating patients with diabetic erectile dysfunction.

Various antioxidants have been shown to improve endothelial function and erectile ability in animal models of diabetic erectile dysfunction (10,13,14). Adenovirus-mediated gene transfer of extracellular superoxide dismutase into the corpus cavernosum tissue was shown to reduce superoxide anion levels and raise cavernous cGMP levels, which resulted in partial recovery of erectile function in STZ-induced diabetic rats 2 days after transfection (14). Despite encouraging results in preclinical studies,

however, no antioxidants have so far been approved for the treatment of patients with erectile dysfunction. A possible reason is that antioxidant therapy may not be effective if structural damage to cavernous endothelial cells has already progressed. In this regard, regeneration of damaged endothelial cells using angiogenic factors is a more logical approach to treating erectile dysfunction than is the prevention of endothelial cell damage with antioxidants.

Local delivery of the vascular endothelial growth factor-A (VEGF-A) gene or protein into the penis has been shown to induce partial or complete recovery of erectile function in STZ-induced type 1 diabetic rats (15–17). However, these studies did not demonstrate whether VEGF-A therapy induces endothelial regeneration. Furthermore, VEGF-A often leads to the formation of disorganized vessels that are leaky, hyperpermeable, and inflamed in experimental systems (18,19), thus greatly compromising the therapeutic utility of VEGF-A. In comparison, angiopoietin-1 (Ang1), the ligand of the Tie2 receptor tyrosine kinase, is a specific growth factor that functions to generate a nonleaky, stable, and functional vasculature (19–22). Transgenic overexpression or gene transfer of Ang1 not only enhances vessel formation but also protects the adult vasculature against vascular leakage (19,23–25). Thus, Ang1 has potential therapeutic applications in angiogenesis and the prevention of vascular leakage. However, our previous study (26) revealed that a single intracavernous delivery of the adenovirus-mediated *Ang1* gene failed to induce an angiogenic response in the penis of a hypercholesterolemic rat. Recently, Cho et al. (27) developed a soluble and potent Ang1 variant, cartilage oligomeric matrix protein (COMP)-Ang1, that is more potent than native Ang1 in phosphorylating Tie2 in primary cultured endothelial cells. COMP-Ang1 stimulates angiogenesis with nonleaky neovessel formation in the mouse corneal micropocket assay, whereas VEGF-A stimulates angiogenesis with leaky neovessel formation (27). One mechanism for inducing nonleaky and healthy angiogenesis is the specific activation of Tie2 in endothelial cell-cell or cell-matrix contacts by COMP-Ang1 (28,29).

In the current study, we determined the effectiveness of COMP-Ang1 in promoting cavernous endothelial regeneration and restoring erectile function in a mouse model of diabetic erectile dysfunction. In addition, because increased vascular permeability by loss of endothelial cell-cell junction proteins is an important pathophysiological mechanism involved in diabetic retinopathy (30–33), we investigated whether COMP-Ang1 induces nonleaky angiogenesis in the penis by restoring endothelial cell-cell junction proteins.

RESEARCH DESIGN AND METHODS

Generation of COMP-Ang1 adenovirus and COMP-Ang1 recombinant protein. Recombinant adenovirus-expressing FLAG-tagged COMP-Ang1 or bacterial β-gal was constructed and COMP-Ang1 recombinant protein was prepared as previously described (22).

Animals and treatments. Eight-week-old C57BL/6J mice were used in this study. The experiments were approved by the institutional animal care and use subcommittee of our university. Diabetes was induced by intraperitoneal injections of multiple low doses of STZ (50 mg/kg body wt in 0.1 mol/L citrate buffer, pH 4.5) consecutively for 5 days, as previously described (12). Eight weeks after diabetes was induced, the animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) intramuscularly and were placed supine on a thermoregulated surgical table. The penis was exposed by use of a sterile technique. A 30-gauge insulin syringe was used to administer a single injection of ad-LacZ (2×10^8 parts/20 μL) or ad–COMP-Ang1 (2×10^8 parts/20 μL) and repeated injections of PBS (days −3 and 0; 20 μL) or COMP-Ang1 recombinant protein (days −3 and 0; 5.8 μg/20 μL) into the midportion of the corpus cavernosum. The incision was closed with 6-O Vicryl (polyglactin 910) sutures. We evaluated erectile function (n = 6 per group) by electrical stimulation of the cavernous nerve 2 and 4 weeks after treatment. We administered ad–COMP-Ang1 at a concentration of 2×10^8 parts because we could achieve the highest erectile response at this concentration (J.-K. Ryu, W.J. Kim, S. Piao, H.-R. Jin, and J.-K. Suh, unpublished observation). STZ-induced diabetic mice that received two successive intracavernous injections of COMP-Ang1 protein showed significant recovery of erectile function 2 and 4 weeks after treatment, which was comparable to the function in the mice that received a single intracavernous injection of ad–COMP-Ang1 (Fig. 1 and Supplementary Fig. 2). On the basis of these functional results, the mice were divided into the following four groups for histologic examination, biochemical study, and vascular permeability testing: age-matched controls, STZ-induced diabetic mice without treatment, STZ-induced diabetic mice receiving repeated intracavernous injections of PBS (days −3 and 0; 20 μL), and STZ-induced diabetic mice receiving repeated intracavernous injections of COMP-Ang1 protein (days −3 and 0; 5.8 μg/20 μL). Fasting and postprandial blood glucose levels were determined with an Accu-Check blood glucose meter (Roche Diagnostics, Mannheim, Germany) before the mice were killed.

Physiological erection and inhibition studies. Erectile function was evaluated by electrical stimulation of the cavernous nerve. Stimulation parameters were 1–5 V at a frequency of 12 Hz, a pulse width of 1 ms, and a duration of 1 min. The ratios of maximal intracavernous pressure (ICP) and total ICP (area under the curve) to mean systolic blood pressure (MSBP) were calculated to adjust for variations in systemic blood pressure, as previously described (12).

For inhibition studies, a separate group of STZ-induced diabetic mice was given *N*-nitro-L-arginine methyl ester (L-NAME) (a NOS inhibitor, 75 μmol/L; Sigma-Aldrich, St. Louis, MO) or apocynin (an inhibitor of NADPH oxidase activation, 180 μmol/L; Sigma-Aldrich) in their drinking water beginning 1 day before the intracavernous injection of the COMP-Ang1 protein.

Immunohistochemistry, in situ detection of superoxide anion, and the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunohistochemistry, in situ detection of superoxide anion, and the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay were performed as described in the Supplementary Methods.

Western blot and cGMP determination. Western blot for COMP-Ang1, eNOS, phospho-eNOS, $p47^{phox}$, inducible NOS (iNOS), and endothelial cell-cell junction proteins and cGMP determination were performed as described in the Supplementary Methods.

Measurement of nitrite/nitrate (NO_x) levels. Plasma NO_x concentrations were determined as described in the Supplementary Methods.

Measurement of permeability across the endothelium of cavernous sinusoids. The mice from each STZ-induced diabetic group and their age-matched controls were anesthetized, and the fluorescent tracer rhodamine-dextran (40 kDa, 25 mg/mL in saline; Molecular Probes, Eugene, OR) was injected into the jugular vein. After 10 min, electrical stimulation of the cavernous nerve was performed for 1 min to induce penile erection and to increase blood flow into the penis. Ten minutes after cavernous nerve stimulation, corpus cavernosum tissues were harvested and fixed in 4% paraformaldehyde for 24 h at 4°C. Frozen tissue sections (n = 4 per group) were incubated with antibody to platelet–endothelial cell adhesion molecule (PECAM)-1 (1:50; Chemicon, Temecula, CA). The intensity of fluorescence in the nonvascular area of the corpus cavernosum tissue was measured by image analysis (NIH Image J).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed by using one-way ANOVA followed by Student-Newman-Keuls post hoc tests. $P < 0.05$ was considered to be statistically significant.

RESULTS

Metabolic variables. The fasting and postprandial blood glucose concentrations of the STZ-induced diabetic mice were significantly higher than those of the control mice. Body weight was significantly lower in the STZ-induced diabetic mice than in the controls. The body weight and blood glucose levels of the STZ-induced diabetic mice did not differ significantly, regardless of the treatment given (Table 1 and Supplementary Table 1).

In vivo COMP-Ang1 or β-gal expression in diabetic corpus cavernosum tissue. Exogenous COMP-Ang1 protein expression was detected by Western blot in the corpus cavernosum tissue of STZ-induced diabetic mice 3, 7, 14, and 21 days after injection of ad–COMP-Ang1. The level of

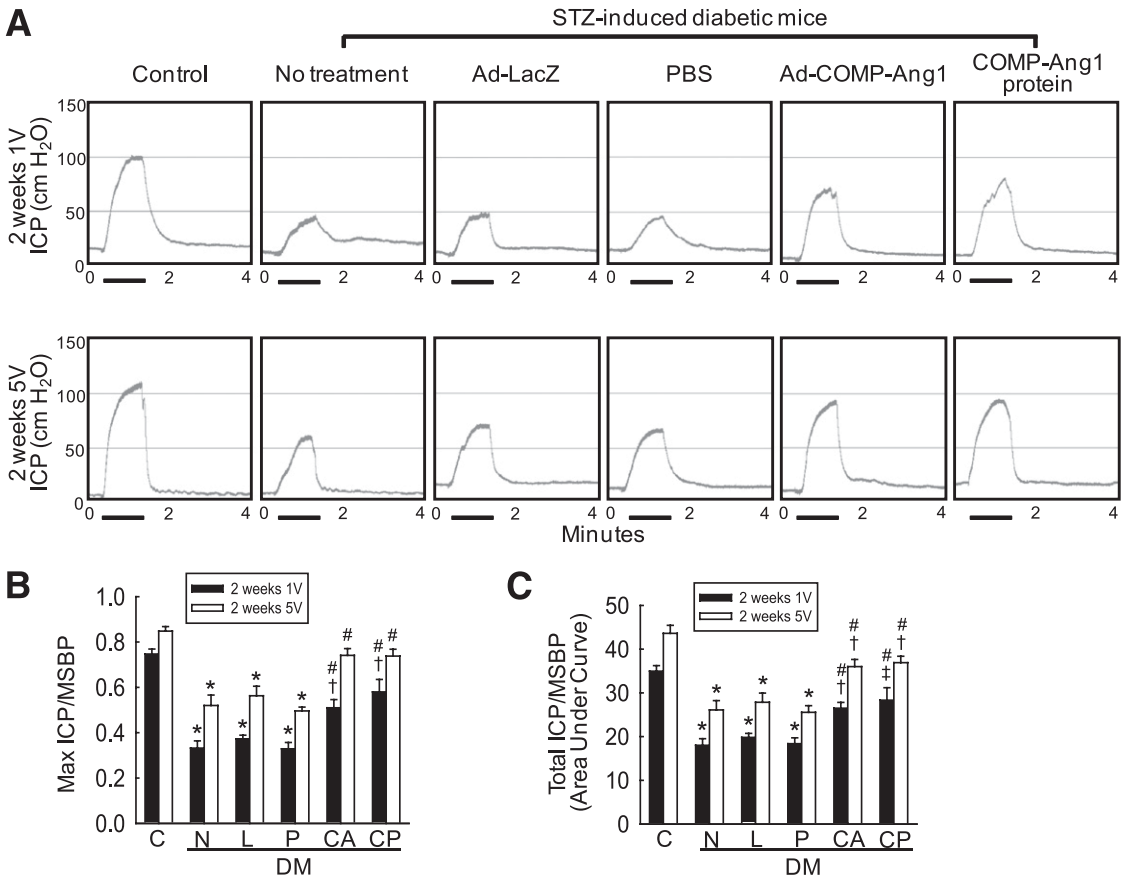

FIG. 1. Adenoviral COMP-*Ang1* gene or COMP-Ang1 protein transfer restores ICP elicited by electrical stimulation of the cavernous nerve. *A*: Representative ICP responses for age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice stimulated at 2 weeks after intracavernous injection of ad-LacZ (L; 2×10^8 parts/20 μL), PBS (P; 20 μL), ad–COMP-Ang1 (CA; 2×10^8 parts/20 μL), or COMP-Ang1 protein (CP; 5.8 μg/20 μL). A single injection of ad-LacZ or ad–COMP-Ang1 (day 0) and repeated injections of PBS or COMP-Ang1 protein (days −3 and 0) were done into the midportion of the corpus cavernosum. The cavernous nerve was stimulated at 1 and 5 V. The stimulus interval is indicated by a solid bar. *B* and *C*: Ratios of mean maximal ICP and total ICP (area under the curve) to MSBP were calculated for each group. Each bar depicts the means ± SE from *n* = 6 animals per group. **P* < 0.01 vs. the C, CA, and CP groups; #*P* < 0.01 vs. the N, L, and P groups; †*P* < 0.01 vs. the C group; ‡*P* < 0.05 vs. the C group. DM, diabetes.

exogenous COMP-Ang1 protein expression was highest 7 days after injection, and expression still was detectable at 21 days (Supplementary Fig. 1*A*). We also evaluated the expression of COMP-Ang1 protein in the corpus cavernosum at 1, 6, and 24 h and 3, 7, and 14 days after injection of COMP-Ang1 recombinant protein. The level of COMP-Ang1 protein peaked at the earliest time point assayed (1 h) and was detectable up to 3 days after injection (Supplementary Fig. 1*A*).

To localize the COMP-Ang1 protein, either expressed from ad–COMP-Ang1 or injected as a recombinant protein, immunohistochemical staining for FLAG-tagged COMP-Ang1 was performed 7 days after intracavernous injection of ad-LacZ or ad–COMP-Ang1 and 1 h after intracavernous injection of PBS or COMP-Ang1 protein.

The FLAG-tagged protein was strongly expressed in endothelial cells in both the ad–COMP-Ang1– and COMP-Ang1 protein–treated animals (Supplementary Fig. 1*B*).

TABLE 1
Physiological and metabolic parameters: 2 weeks after treatment

		STZ-induced diabetic group				
	Control	No treatment	Ad-LacZ	PBS	Ad–COMP-Ang1	COMP-Ang1 protein
Body weight (g)	30.0 ± 1.4	23.3 ± 1.9*	22.9 ± 1.3*	23.1 ± 2.4*	22.4 ± 1.2*	23.0 ± 2.7*
Fasting glucose (mg/dL)	121.4 ± 14.6	383.3 ± 84.9*	413.4 ± 66.8*	370.8 ± 37.6*	377.8 ± 62.3*	369.9 ± 104.2*
Postprandial glucose (mg/dL)	171.9 ± 14.7	498.1 ± 23.7*	572.4 ± 32.8*	565.4 ± 24.4*	518.3 ± 46.2*	459.9 ± 71.9*
Blood pressure (mm Hg)						
SBP	97.3 ± 4.9	97.4 ± 6.2	99.3 ± 5.8	102.3 ± 5.8	97.5 ± 4.9	98.6 ± 5.7
MBP	71.8 ± 5.5	73.3 ± 7.3	72.3 ± 5.7	77.1 ± 6.2	74.4 ± 5.6	74.1 ± 4.3
DBP	59.0 ± 7.9	62.1 ± 8.0	59.4 ± 6.7	64.5 ± 7.0	62.9 ± 7.8	62.0 ± 4.9

Data are means ± SE for $n = 6$ animals per group. *$P < 0.01$ vs. control group.

X-gal histochemistry in fixed sagittal blocks of the whole penis 7 days after injection of ad-LacZ showed uniform staining along the penile shaft, whereas virtually no endogenous β-gal activity was observed in animals injected with the virus vehicle alone. Frozen transverse sections from the penises of diabetic mice 7 days after injection of ad-LacZ were used to determine its cellular distribution. The X-gal staining was mainly observed in endothelial cells lining the cavernous space (Supplementary Fig. 1*C*).

COMP-Ang1 gene or protein transfer restores erectile function in STZ-induced diabetic mice. A representative intracavernous tracing after stimulation of the cavernous nerve (1–5 V, 12 Hz, 1 ms) for 1 min in age-matched control or STZ-induced diabetic mice 2 and 4 weeks after treatment is shown in Fig. 1*A* and Supplementary Fig. 2*A*, respectively. During electrical stimulation of the cavernous nerve, the ratios of maximal ICP and total ICP to MSBP were significantly lower in untreated STZ-induced diabetic mice and STZ-induced diabetic mice treated with ad-LacZ or PBS than in age-matched controls. A single intracavernous injection of the ad–COMP-*Ang1* gene or repeated intracavernous injections of the COMP-Ang1 protein restored erection parameters, which reached up to 83–89% of control values, and the physiological improvement lasted up to 4 weeks after treatment (Fig. 1*B* and *C*, and Supplementary Fig. 2*B* and *C*). No detectable differences were found in MSBP among the six experimental groups (Table 1 and Supplementary Table 1). At 6 and 8 weeks after administration of either the ad–COMP-Ang1 or COMP-Ang1 protein, however, erectile function returned to baseline levels (Supplementary Fig. 3).

COMP-Ang1 protein transfer increases cavernous endothelial content through enhanced endothelial cell proliferation in STZ-induced diabetic mice. Immunohistochemical staining of cavernous tissue with an antibody to PECAM-1 was performed in age-matched control and STZ-induced diabetic mice 2 weeks after treatment. We found significantly lower cavernous endothelial cell content in the untreated and PBS-treated STZ-induced diabetic mice than in the control mice. Repeated intracavernous injections of the COMP-Ang1 protein completely restored cavernous endothelial content (Fig. 2*A* and *C*).

To test whether the COMP-Ang1 protein–induced increase in cavernous endothelial content resulted from endothelial cell proliferation, we assessed the number of endothelial cells staining positive for phosphohistone H3 (a nuclear protein indicative of cell proliferation). Because only a few or virtually no phosphohistone H3–positive endothelial cells were noted in diabetic mice 2 weeks after treatment with the COMP-Ang1 protein (data not shown), we further investigated whether the COMP-Ang1 protein induced endothelial cell proliferation at earlier time points. We noted significant increases in phosphohistone H3–positive endothelial cells 1, 3, and 6 h after injection of the COMP-Ang1 protein (Fig. 2*B* and *D*).

COMP-Ang1 protein transfer induces cavernous eNOS phosphorylation and increases cGMP concentrations in STZ-induced diabetic mice. Cavernous phospho-eNOS (Ser1177) expression, as assessed by immunohistochemical staining, was significantly lower in the untreated and PBS-treated STZ-induced diabetic mice than in the age-matched controls at 2 weeks after treatment. Repeated intracavernous injections of the COMP-Ang1 protein significantly increased endogenous eNOS phosphorylation in the STZ-induced diabetic mice, which was comparable to that in age-matched controls (Fig. 3*A* and *B*). Similar to the results of immunohistochemical staining, immunoblot analysis of the corpus cavernosum tissues revealed an increase in cavernous phospho-eNOS expression in STZ-induced diabetic mice 2 weeks after treatment with the COMP-Ang1 protein, whereas no changes were noted in the total amount of eNOS in control or STZ-induced diabetic mice regardless of treatment (Fig. 3*C*).

Cavernous cGMP decreased significantly in untreated and PBS-treated STZ-induced diabetic mice compared with that in the age-matched controls at 2 weeks after treatment. Intracavernous administration of the COMP-Ang1 protein significantly increased cGMP concentrations compared with those in untreated or PBS-treated STZ-induced diabetic mice (Fig. 3*D*).

COMP-Ang1 protein transfer decreases cavernous ROS production in STZ-induced diabetic mice. We performed Western blots to evaluate the cavernous tissue expression of $p47^{phox}$ and iNOS in age-matched control and STZ-induced diabetic mice 2 weeks after treatment. Cavernous $p47^{phox}$ and iNOS protein expression was significantly higher in the untreated and PBS-treated STZ-induced diabetic mice than in the age-matched controls. Repeated intracavernous injections of the COMP-Ang1 protein significantly decreased cavernous $p47^{phox}$ and iNOS expression in the STZ-induced diabetic mice (Supplementary Fig. 4).

In the in situ analysis of superoxide anion production, the fluorescent products of oxidized hydroethidine in both the cavernous endothelium and the smooth muscle were significantly higher in the untreated and PBS-treated STZ-induced diabetic groups than in the control group. Intracavernous administration of the COMP-Ang1 protein significantly decreased superoxide anion production in the cavernous endothelial cells and smooth-muscle cells of STZ-induced diabetic mice but not to the level found in the age-matched controls (Fig. 4).

We also performed immunohistochemical localization of nitrotyrosine to determine peroxynitrite generation, which is derived from NO and superoxide anion. Nitrotyrosine expression in endothelial cells and smooth-muscle cells of the corpus cavernosum was higher in untreated and PBS-treated STZ-induced diabetic groups than in the control group. Repeated intracavernous injections of the COMP-Ang1 protein significantly decreased nitrotyrosine generation in the cavernous endothelial cells and smooth-muscle cells of the STZ-induced diabetic mice, which was comparable to that in age-matched controls (Fig. 5).

COMP-Ang1 protein transfer decreases apoptosis in the cavernous endothelial cells and smooth-muscle cells of STZ-induced diabetic mice. Double labeling of cavernous tissue with TUNEL and the antibody to PECAM-1 or smooth-muscle α-actin showed that the number of apoptotic cells in cavernous endothelial cells and smooth-muscle cells was significantly greater in the untreated and PBS-treated STZ-induced diabetic groups than in the control group. Repeated intracavernous injections of the COMP-Ang1 protein significantly decreased apoptosis in the cavernous endothelial cells and smooth-muscle cells of the STZ-induced diabetic mice, although not to the level found in the age-matched controls (Fig. 6).

COMP-Ang1 protein transfer restores endothelial cell-cell junction proteins and decreases cavernous endothelial permeability in STZ-induced diabetic mice. We performed Western blots to evaluate the cavernous tissue expression of the adherens junction protein (vascular endothelial [VE]-cadherin) and tight junction

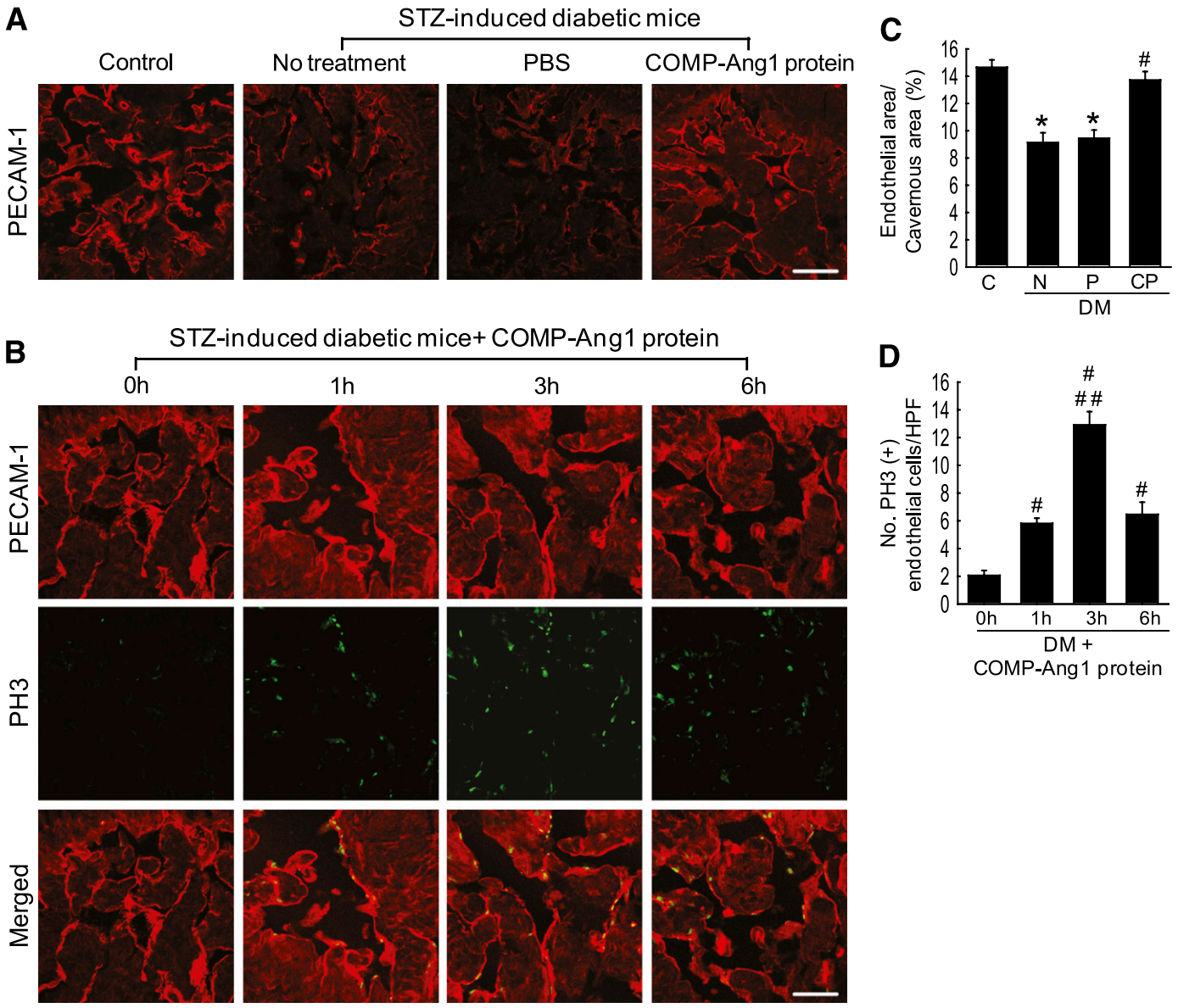

FIG. 2. COMP-Ang1 protein transfer increases cavernous endothelial content through enhanced endothelial cell proliferation. *A*: Anti–PECAM-1 staining of cavernous tissue from age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving repeated intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). Scale bar = 100 μm. *B*: Immunohistochemical staining of cavernous tissue using antibodies to PECAM-1 (red) and phosphohistone H3 (PH3; green) in untreated STZ-induced diabetic mice (0 h) or STZ-induced diabetic mice 1, 3, and 6 h after intracavernous injection of the COMP-Ang1 protein (5.8 μg/20 μL). Scale bar = 50 μm. *C*: Quantitative analysis of endothelial cell content in the cavernous tissue was performed by using an image analyzer. Each bar depicts the means ± SE from n = 6 animals per group. *P < 0.01 vs. the C and CP groups; #P < 0.01 vs. the N and P groups. *D*: Number of PH3-immunopositive endothelial cells per high-power field (screen magnification ×400). Each bar depicts the means ± SE from n = 6 animals per group. #P < 0.01 vs. the 0-h group; ##P < 0.01 vs. the 1- and 6-h groups. DM, diabetes. (A high-quality color representation of this figure is available in the online issue.)

proteins (zonular occludens-1 [ZO-1], occludin, and claudin-5) in age-matched control and STZ-induced diabetic mice 2 weeks after treatment. The cavernous expression of VE-cadherin, ZO-1, occludin, and claudin-5 protein was significantly lower in the untreated and PBS-treated STZ-induced diabetic mice than in the age-matched controls. Repeated intracavernous injections of COMP-Ang1 protein completely restored the expression of endothelial cell-cell junction proteins in the diabetic mice (Fig. 7*A*–*E*).

Endothelial cell-cell junctions serve as a barrier by regulating paracellular permeability (30–33). Thus, we examined cavernous endothelial permeability in age-matched control and STZ-induced diabetic mice 2 weeks after treatment. We used 40 kDa rhodamine-dextran because we found that significant leakage of the 40-kDa fluorescent tracer occurred across the cavernous endothelium in STZ-induced diabetic mice, whereas control mice showed minimal leakage (J.-K. Ryu, W.J. Kim, S. Piao, H.-R. Jin, and J.-K. Suh, unpublished observation). We noted in a significant increase in rhodamine-dextran fluorescence in the nonvascular area of the corpus cavernosum tissue in untreated and PBS-treated mice compared with that in control mice. Repeated intracavernous injections of COMP-Ang1 protein significantly decreased cavernous endothelial permeability in the STZ-induced diabetic mice, which was similar to the level found in age-matched controls (Fig. 7*F* and *G*).

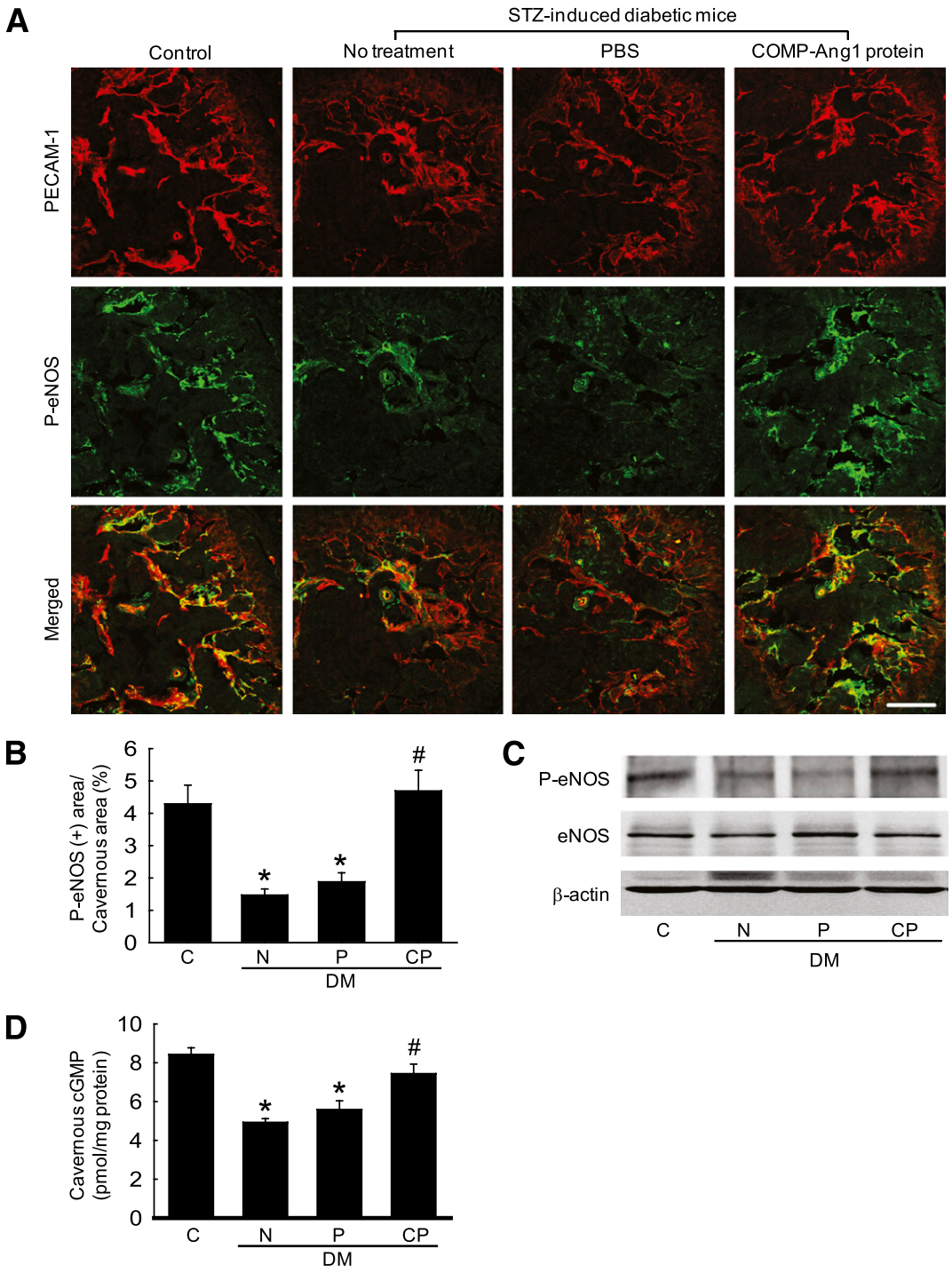

FIG. 3. COMP-Ang1 protein transfer induces cavernous eNOS phosphorylation and increases cGMP concentration. *A*: Immunofluorescent double staining of cavernous tissue performed with antibodies to PECAM-1 (red) and phospho-eNOS (Ser1177; green) in age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). Scale bar = 100 μm. *B*: Quantitative analysis of phospho-eNOS–positive area in the cavernous tissue was performed by using an image analyzer. Each bar depicts the means ± SE from $n = 6$ animals per group. *$P < 0.01$ vs. the C and CP groups; #$P < 0.01$ vs. the N and P groups. *C*: Western blot analysis demonstrating the relative abundance of phospho-eNOS (Ser1177) in each group. Corpus cavernosum tissues were harvested after electrical stimulation of the cavernous nerve for 1 min. Results were similar for four independent experiments. *D*: Cavernous cGMP concentrations. Each bar depicts the means ± SE from $n = 4$ animals per group. *$P < 0.01$ vs. the C and CP groups; #$P < 0.01$ vs. the N and P groups. DM, diabetes; P-eNOS, phosphorylated eNOS. (A high-quality color representation of this figure is available in the online issue.)

COMP-Ang1 protein–induced recovery of cavernous endothelial content and erectile function is NOS dependent but not NADPH oxidase dependent. We determined whether NOS or NADPH oxidase participated in COMP-Ang1–induced cavernous angiogenesis and subsequent restoration of the erectile response in L-NAME– or apocynin-treated STZ-induced diabetic mice. Histologic and physiological erection studies indicated that L-NAME–treated

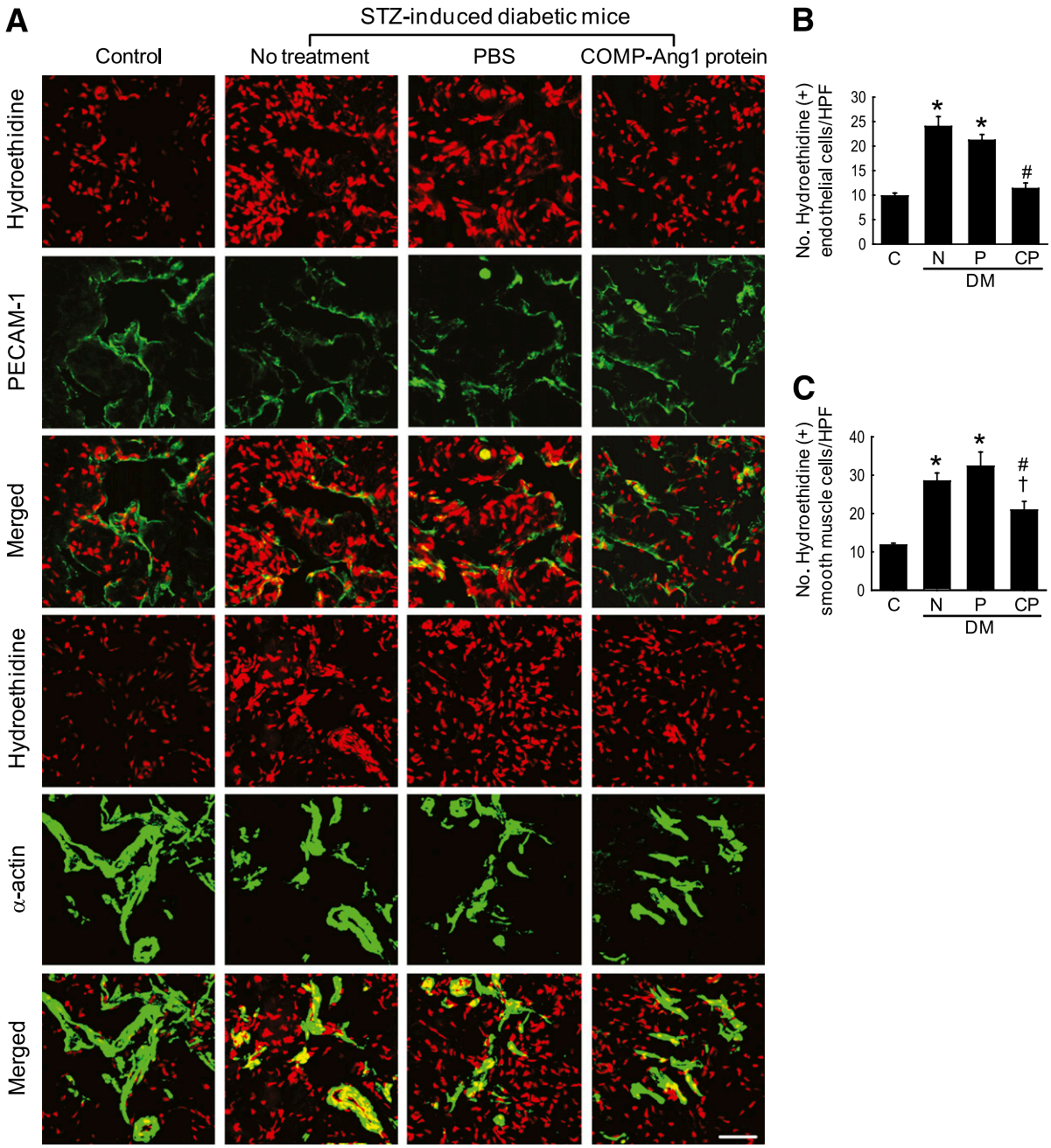

FIG. 4. COMP-Ang1 protein transfer decreases cavernous superoxide anion production. *A*: In situ detection of superoxide anion in endothelial cells or smooth-muscle cells in age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). Corpus cavernosum tissue was incubated with hydroethidine, an oxidative fluorescent dye used to detect superoxide anion, and the antibody to PECAM-1 or with hydroethidine and antibody to smooth-muscle α-actin. Scale bar = 50 μm. *B* and *C*: Number of ethidium bromide fluorescence–positive endothelial cells or smooth-muscle cells per high-power field (screen magnification ×400). Each bar depicts the means ± SE from $n = 6$ animals per group. *$P < 0.05$ vs. the C and CP groups; #$P < 0.05$ vs. the N and P groups; †$P < 0.05$ vs. the C group. DM, diabetes. (A high-quality color representation of this figure is available in the online issue.)

STZ-induced diabetic mice that received COMP-Ang1 protein failed to restore cavernous endothelial content or recover erectile function (Supplementary Fig. 5). In contrast, treatment with apocynin did not block the COMP-Ang1 protein–induced enhancement of cavernous angiogenesis and restoration of erectile function. Instead, a partial increase in cavernous endothelial content or improvement in erectile function was noted in the apocynin-treated STZ-induced diabetic mice, but it did not reach the level of the COMP-Ang1 treatment group. Apocynin significantly decreased superoxide anion production in the diabetic corpus cavernosum tissue (Supplementary Fig. 5). We observed a significant reduction in plasma NO_x levels in L-NAME–treated diabetic animals (Supplementary Fig. 6). The administration of L-NAME or apocynin did not influence the body weight and blood glucose levels in the STZ-induced diabetic mice (data not shown). These findings suggest that the recovery of erectile function promoted by COMP-Ang1–induced cavernous angiogenesis depends on NOS but not on NADPH oxidase.

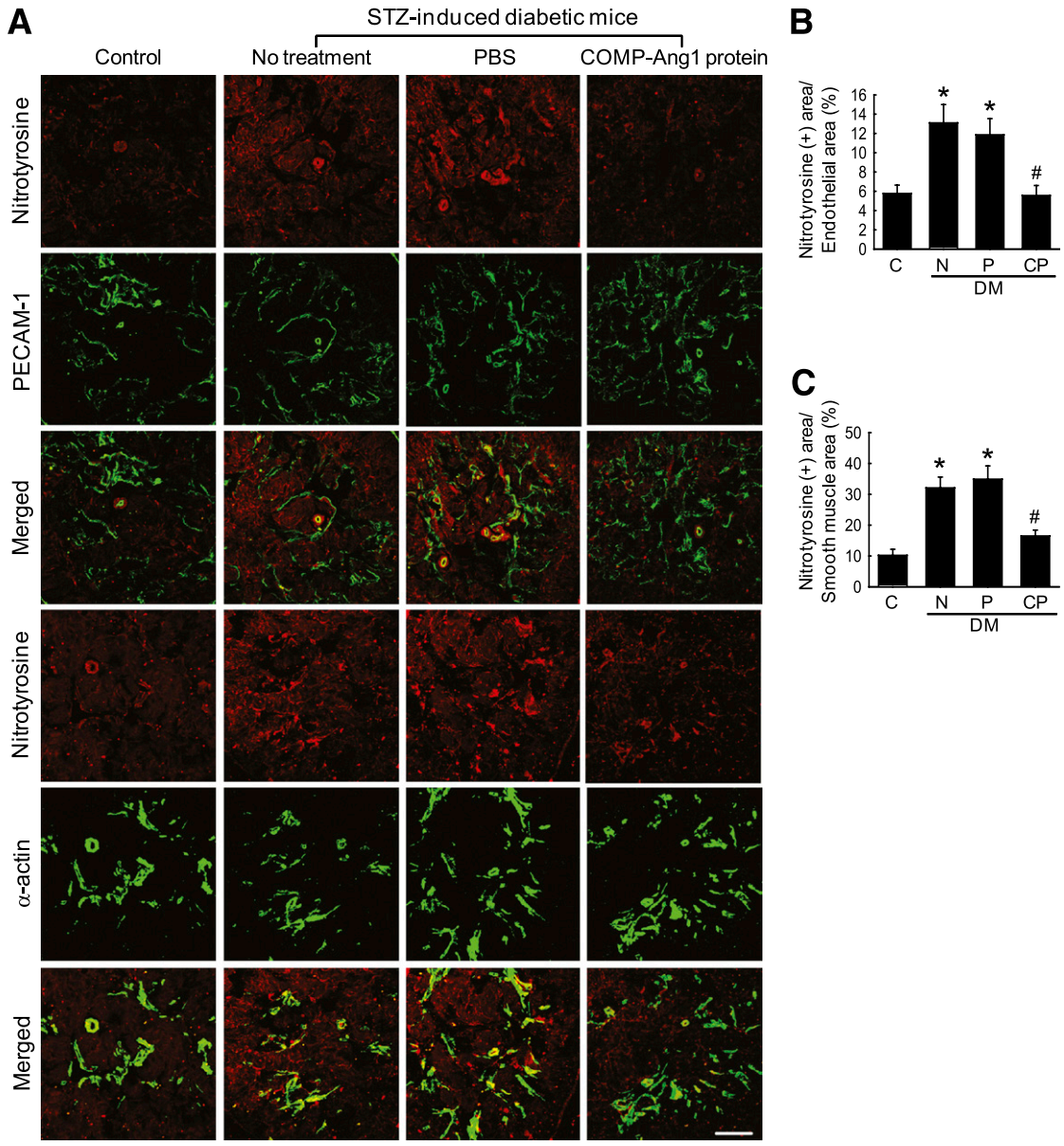

FIG. 5. COMP-Ang1 protein transfer decreases cavernous nitrotyrosine production. *A*: Immunofluorescent double staining of cavernous tissue performed with the antibody to nitrotyrosine, a marker of peroxynitrite formation, and the antibody to PECAM-1 or with antibodies to nitrotyrosine and smooth-muscle α-actin in age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). Scale bar = 100 μm. *B* and *C*: An image analyzer was used to quantitate nitrotyrosine-immunopositive endothelial or smooth-muscle area. Each bar depicts the means ± SE from *n* = 6 animals per group. **P* < 0.05 vs. the C and CP groups; #*P* < 0.05 vs. the N and P groups. DM, diabetes. (A high-quality color representation of this figure is available in the online issue.)

DISCUSSION

We showed here that two successive injections of COMP-Ang1 recombinant protein into the corpus cavernosum of STZ-induced diabetic mice significantly increased cavernous endothelial cell proliferation, eNOS phosphorylation (Ser1177), and cGMP expression and decreased the production of ROS, such as superoxide anion and peroxynitrite. These changes restored erectile function in STZ-induced diabetic mice. Intracavernous injection of COMP-Ang1 protein also induced complete recovery of endothelial cell-cell junction proteins and decreased cavernous endothelial permeability in STZ-induced diabetic mice (Supplementary Fig. 7).

To determine whether the improved erectile response was related to an increase in the number of endothelial cells, we assessed the expression of PECAM-1 by immunohistochemical analysis. In agreement with the findings of previous studies in STZ-induced diabetic rats (12,34,35), we found a significant decrease in the cavernous endothelial area in untreated and PBS-treated STZ-induced diabetic mice compared with that in control mice. COMP-Ang1 protein induced complete recovery of the PECAM-1–positive endothelial area in the corpus cavernosum. Immunohistochemical examination of phosphohistone H3 expression revealed that the COMP-Ang1–induced increase in cavernous endothelial content was the result of endothelial proliferation, which is consistent with a recent report (22).

In this study, COMP-Ang1 failed to induce cavernous angiogenesis and restore erectile function in L-NAME–treated

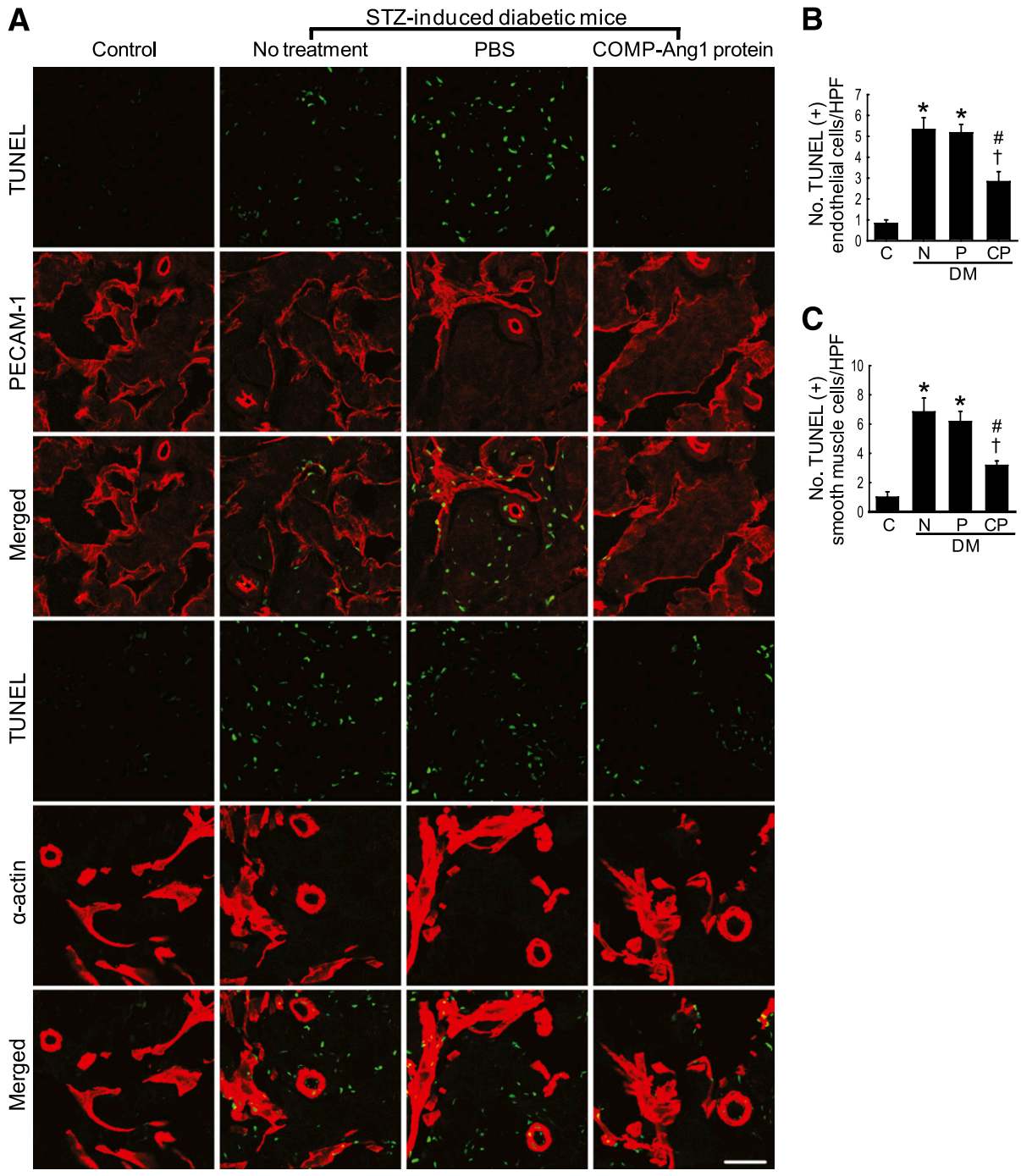

FIG. 6. COMP-Ang1 protein transfer decreases apoptosis in cavernous endothelial cells and smooth-muscle cells. *A*: Double labeling of cavernous tissue with TUNEL and the antibody to PECAM-1 or with TUNEL and the antibody to smooth-muscle α-actin in age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). Scale bar = 50 μm. *B* and *C*: Number of apoptotic cells in endothelial cells or smooth-muscle cells per high-power field (screen magnification ×400). Each bar depicts the means ± SE from $n = 6$ animals per group. *$P < 0.01$ vs. the C and CP groups; #$P < 0.01$ vs. the N and P groups; †$P < 0.05$ vs. the C group. DM, diabetes. (A high-quality color representation of this figure is available in the online issue.)

diabetic mice, suggesting that the recovery of erectile function mediated by COMP-Ang1–induced cavernous angiogenesis depends on NOS, similar to results of a previous study (36) showing that Ang1-induced angiogenesis was abolished or reduced in the presence of the NOS inhibitor L-NAME or in *Nos3*$^{-/-}$ mice. The NO/cGMP system is the principal mediator of penile erection (37). In the current study, eNOS phosphorylation (Ser1177) was significantly decreased in both untreated and PBS-treated STZ-induced diabetic mice compared with the control group. A previous study reported that eNOS is inactivated in the penis of STZ-induced diabetic animals by *O*-linked *N*-acetylglucosamine modification, specifically at Ser1177 (38), and by increased ROS generation (12). In the current study, endogenous eNOS phosphorylation was completely restored in the STZ-induced diabetic mice by the intracavernous injection of

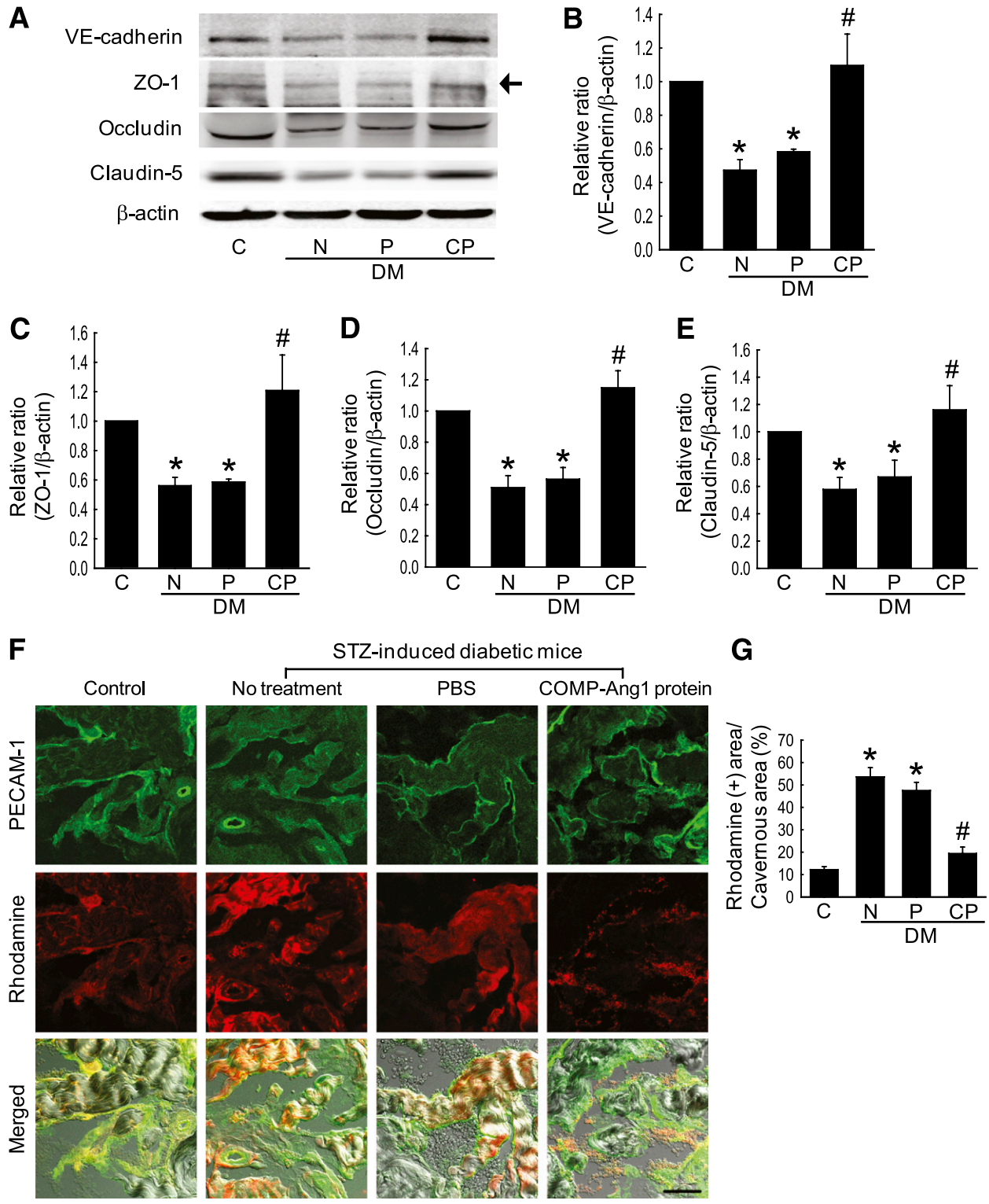

FIG. 7. COMP-Ang1 protein transfer restores endothelial cell-cell junction proteins and decreases cavernous endothelial permeability. *A*: Representative Western blot for VE-cadherin, ZO-1, occludin, and claudin-5 in age-matched control mice (C), untreated STZ-induced diabetic mice (N), or STZ-induced diabetic mice 2 weeks after receiving intracavernous injections of PBS (P; days −3 and 0; 20 μL) or COMP-Ang1 protein (CP; days −3 and 0; 5.8 μg/20 μL). *B–E*: Data are presented as the relative density of each protein compared with that of β-actin. The relative ratio of the control group was arbitrarily set equivalent to 1. Data are representative of four independent experiments. **P* < 0.05 vs. the C and CP groups; #*P* < 0.05 vs. the N and P groups. *F*: Diffusion of fluorescent tracer in the corpus cavernosum tissue. The fluorescent tracer rhodamine-dextran (red, 40 kDa; 25 mg/mL in saline) was injected into the jugular vein of each group of mice. After 10 min, corpus cavernosum tissues were harvested after electrical stimulation of the cavernous nerve for 1 min to enhance blood flow into the penis and were then stained with the antibody to PECAM-1 (green). Scale bar = 50 μm. *G*: The intensity of fluorescence in the nonvascular area of the corpus cavernosum tissue was measured by image analysis. Each bar depicts the means ± SE from *n* = 4 animals per group. **P* < 0.01 vs. the C and CP groups; #*P* < 0.01 vs. the N and P groups. DM, diabetes. (A high-quality color representation of this figure is available in the online issue.)

COMP-Ang1 protein. Thus, regeneration of cavernous endothelial cells and a decrease in the generation of superoxide anion and peroxynitrite in endothelial cells by COMP-Ang1 may account for the restoration of eNOS activity. Intracavernous administration of COMP-Ang1 protein also significantly increased the concentration of cGMP, although not to the level found in the age-matched controls. The increased formation of cGMP resulting from recovery of NO bioavailability is clear evidence of improved erectile response to cavernous nerve stimulation in the diabetic mice.

The penises from STZ-induced diabetic mice had higher superoxide anion and peroxynitrite levels than did those from the controls. Endothelial cells generate ROS, which plays a crucial role in physiological and pathological conditions. Low levels of ROS function as signaling molecules to mediate endothelial cell proliferation and migration in response to hypoxia, ischemia, and angiogenic factors, such as VEGF-A and Ang1 (39,40). A previous study (41) in human umbilical vein endothelial cells reported that Ang1 triggers the production of superoxide anion by activating NADPH oxidase and that this generation of superoxide anions promotes endothelial cell migration. Furthermore, endothelial NADPH oxidase–derived ROS plays a critical role in Ang1-induced angiogenesis in porcine coronary artery endothelial cells, and targeted deletion of the NADPH oxidase subunit $p47^{phox}$ gene suppresses Ang1-induced angiogenesis (41), which suggests that ROS plays an important role in promoting Ang1-mediated angiogenesis in physiological conditions. In contrast, high levels of ROS in pathological conditions induce apoptosis and cell death (42). In pathological conditions in which ROS is already generated at high levels, as in the current study, COMP-Ang1 profoundly reduces the generation of superoxide anion and peroxynitrite in endothelial and smooth-muscle cells, possibly by inhibiting the expression of cavernous $p47^{phox}$ and iNOS, respectively (Supplementary Fig. 7). In agreement with these findings, intracavernous administration of COMP-Ang1 protein significantly reduced the number of apoptotic cells in the cavernous endothelium and smooth muscle in STZ-induced diabetic mice. In contrast to previous findings in physiological conditions in vitro (41), inhibition of NADPH oxidase by apocynin did not block COMP-Ang1–mediated cavernous angiogenesis in STZ-induced diabetic mice in vivo. Instead, we observed partial recovery of cavernous endothelial content and erectile function in STZ-induced diabetic mice treated with apocynin, although not to the level found in the COMP-Ang1–treated group.

In the current study, cavernous endothelial permeability to a 40-kDa fluorescent tracer was increased in STZ-induced diabetic mice concurrently with decreased expression of endothelial cell-cell junction proteins, including VE-cadherin, ZO-1, occludin, and claudin-5. Similar to our findings, several investigators (30–33,43,44) also reported an increase in vascular permeability in STZ-induced diabetic retinal or cerebral microvasculature coincident with a decrease in endothelial junction proteins. These findings suggest that alterations in endothelial junction proteins in diabetes may be a mechanism by which cavernous endothelial permeability is increased. Intracavernous injection of the COMP-Ang1 protein significantly decreased cavernous endothelial permeability in STZ-induced diabetic mice by restoring endothelial cell-cell junction proteins. Specific activation of Tie2 in cell-cell contact in the quiescent vasculature also is known to be a mechanism by which Ang1 reduces endothelial permeability and promotes vessel stabilization (29).

Because of concerns about the utility of viral vector–mediated gene therapy for non–life-threatening disease, such as erectile dysfunction, it is necessary to evaluate whether an angiogenic factor can restore erectile function when it is given as a recombinant protein. Compared with that in STZ-induced diabetic animals that received a single intracavernous injection of ad–COMP-Ang1, the similar restoration of erectile function after two successive injections of COMP-Ang1 protein is noteworthy. From a clinical standpoint, therapeutic proteins are safer and more feasible for treating humans than is gene therapy.

In summary, intracavernous delivery of the recombinant COMP-Ang1 protein successfully restored the endogenous NO-cGMP pathway through the regeneration of healthy and nonleaky cavernous endothelium and inhibition of ROS-mediated cavernous endothelial cell apoptosis. Furthermore, these changes resulted in physiologically relevant changes in erectile function. These findings support the concept of angiogenic factor therapy as a curative therapy for erectile dysfunction. Cavernous endothelial regeneration by use of angiogenic factor protein is a novel therapeutic strategy for treating erectile dysfunction resulting from diabetes.

ACKNOWLEDGMENTS

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (R0A-2007-000-20018-0 to J.-K.S.) and by a grant from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084511 to J.-K.S.).

H.-R.J. wrote the manuscript and researched data. W.J.K. researched data and contributed to discussion. J.S.S., S.P., M.J.C., M.T., S.H.S., and G.N.Y. researched data. G.Y.K. contributed to discussion. J.-K.R. wrote the manuscript and reviewed and edited the manuscript. J.-K.S. reviewed and edited the manuscript.

The authors thank Jennifer Holmes, Medical Editing Services Co., for help in preparing the manuscript.

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