SHORT COMMUNICATION

Activation of Dopamine D₁ Receptors in Dermal Fibroblasts Restores Vascular Endothelial Growth Factor-A Production by These Cells and Subsequent Angiogenesis in Diabetic Cutaneous Wound Tissues

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In wound beds, fibroblasts are rich sources of vascular endothelial growth factor A, a cytokine necessary for promoting angiogenesis and thereby the healing of wound tissues. However, in diabetes mellitus, these cells are functionally impaired and produce reduced amounts of vascular endothelial growth factor A, resulting in deficient angiogenesis and delayed wound healing. We here for the first time demonstrate that stimulation of D₁ dopamine receptors present in dermal fibroblasts restores vascular endothelial growth factor A production by these cells, resulting in adequate angiogenesis and subsequent healing of cutaneous wounds in both type 1 and type 2 diabetic mice. This action of D₁ dopamine receptors was mediated through the protein kinase A pathway. As delayed wound healing or chronic wounds are one of the major health problems in diabetic patients, D₁ dopamine receptor agonists, which are already in clinical use for the treatment of other disorders, may be of translational value in the treatment of chronic, nonhealing diabetic wounds. (*Am J Pathol* 2016, 186: 2262–2270; <http://dx.doi.org/10.1016/j.ajpath.2016.05.008>)

In addition to its role as one of the major central catecholamine neurotransmitters controlling cognition, emotion, and movement, dopamine (DA) has many important functions in the periphery.^{1–6} DA regulates cardiovascular, renal, endocrine, gastrointestinal, and immune functions. These actions of DA are mediated through its D₁ and D₂ classes of receptors present in the target cells.^{1–6} Furthermore, results from our laboratory, which have been further confirmed by others, both in the benches and in clinics, indicate that DA can also inhibit vascular endothelial growth factor (VEGF)-A–mediated angiogenesis by acting specifically through its D₂ receptors present in the endothelial cells (ECs) and endothelial progenitor cells.^{7–17} DA acts through its D₂ receptors to suppress VEGF-A–induced VEGF receptor 2 phosphorylation.^{7–11}

Angiogenesis or new blood vessel formation is required for normal physiologic conditions such as wound healing,

and it also plays a crucial role in the pathogenesis of several diseases, including malignant tumors.^{8–22} Among the different proangiogenic factors that mediate angiogenesis, VEGF-A is the most predominant cytokine stimulating new blood vessel formation.^{21,23} VEGF-A mainly acts through its VEGF receptor 2, present in the EC and endothelial progenitor cells, to stimulate migration and proliferation of these cells, the essential steps of the angiogenic process.^{11,21,23}

We have previously reported that stimulation of DA D₂ receptors can significantly inhibit VEGF-A–induced

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angiogenesis in experimental malignant tumors.^{7–9,11} Because stimulation of different classes of DA receptors demonstrates opposite effects in the central nervous system,^{24–27} we thus investigated the actions of DA D₁ receptors on VEGF-A–mediated angiogenesis. Since very low expression of VEGF-A results in deficient and inadequate angiogenesis and it is an important cause of delayed wound healing in diabetes mellitus,^{22,28} we selected murine cutaneous diabetic wound models for our present study to decipher the role of DA D₁ receptors in VEGF-A–induced angiogenesis.

Materials and Methods

Cells and Reagents

Human umbilical vein endothelial cells (HUVECs) were maintained in endothelial cell growth medium (Lonza, Walkersville, MD) supplemented with growth factors and 2% fetal calf serum (Lonza). Normal and diabetic (isolated from the skin of type 2 diabetic patients) human dermal fibroblasts (HDFs; Zen-Bio, Research Triangle Park, NC) were maintained in dermal fibroblast culture medium (Zen-Bio). Diabetic fibroblast media were supplemented with 25 mmol/L glucose. For *in vitro* experiments, HUVECs were serum- and growth factor–starved for 24 hours; then the effects of DA D₁ receptor agonists SKF38393 and SKF81297 (Tocris; Bio-Techne, Minneapolis, MN) or antagonist SCH-23390 (Sigma-Aldrich Corporation, St. Louis, MO), cAMP-stimulator forskolin (Sigma), or protein kinase A (PKA) blocker H89 (Sigma) were assessed.

Animals

Mice experiments were undertaken after approval by the Ohio State University Animal Care and Use Committee. Four- to six-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA); diabetic mice, BKS.Cg-Dock7m *+/+* Lepr^{db}/J (db/db), and DA D₁ receptor knockout mice were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, ME).

Induction of Diabetes and Creation of Wound

Diabetes was induced in C57BL/6 and DA D₁ receptor knockout mice, 4 to 6 weeks of age, of either sex, by injecting i.p. with streptozotocin (Sigma) dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5, 45 mg/kg; injection volume, <0.2 mL) for 5 consecutive days. Blood (10 to 50 µL) was collected from the saphenous vein of the mouse hind foot, and whole-blood glucose levels were measured by a glucose analyzer (OneTouch UltraMini blood glucose monitoring system; LifeScan, Milpitas, CA). Mice with a glucose concentration >300 mg/dL were considered diabetic.²⁹

For the creation of cutaneous wound, diabetic C57BL/6, db/db mice and DA D₁ receptor knockout mice were

anesthetized by i.p. injection of ketamine/xylazine mixture (100/10 mg). The dorsal hair of the mouse was then shaved and disinfected with alcohol (70% ethanol) swab, and excisional wounds were generated at the cranial–caudal level on the dorsomedial back of each animal using a 6-mm dermal punch biopsy. Wound-bearing diabetic mice were divided into three groups: control; and treatment with 10 mg/kg SKF38393 or SKF81297 for 4 consecutive days starting on the day the wounds were generated. The control group received a similar volume of normal saline only.³⁰

IHC Analysis

Murine skin tissues were fixed in 10% formaldehyde, and paraffin blocks were prepared. Sections 5 µm thick were cut. The sections were incubated with primary antibodies against CD31 (Abcam, Cambridge, UK) and VEGF-A (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight and then with respective secondary antibodies. Images were taken and compared using an Axio Scope upright light microscope (Carl Zeiss, Oberkochen, Germany). Microvessel density was determined by a count of the number of CD31-positive blood vessels in random high-power microscopic fields.^{8,20}

Confocal Microscopy

Human skin tissue sections (cryosections) were obtained from Zen-Bio, following the Institutional Review Board rules of The Ohio State University. Both human and mouse cryosections (8 µm) were incubated with unconjugated primary antibodies, DA D₁ receptor (Santa Cruz Biotechnology) and α smooth muscle actin (Abcam) overnight at 4°C. The next day, sections were incubated with respective secondary antibodies conjugated to fluorochrome for 30 minutes at room temperature, visualized under a confocal scanning microscope (FBV-1000; Olympus Corporation, Center Valley, PA), analyzed, and compared between different groups.¹³

Western Blot

Western blot analyses were performed to determine the expression of DA D₁ receptors (Santa Cruz Biotechnology), cyclic AMP–responsive element binding protein (CREB) (Cell Signaling Technology, Beverly, MA), and phospho CREB (Cell Signaling Technology).¹³

RNA Isolation and PCR

Total RNA was isolated from HDFs after 24 hours of treatment using RNeasy Mini Kit (Qiagen, Valencia, CA), reverse-transcribed using OneStep RT-PCR Kit (Qiagen), and amplified using Mastercycler (Eppendorf, Hamburg, Germany). The sequences of primers used were: VEGF-A, forward: 5'-GAGATGAGCTTCCTACAGCAC-3', and reverse: 5'-TCACCGCCTCGGCTTGTCACAT-3'³¹; glyceraldehyde-3-phosphate dehydrogenase, forward:

5'-TGTGGTCATGAGTCCTTCCA-3', and reverse: 5'-CGAGATCCCTCCAAATCAA-3'.³²

Enzyme-Linked Immunosorbent Assay

Human diabetic fibroblasts in culture were treated with either 1 $\mu\text{mol/L}$ SKF38393 or SKF81297 or forskolin alone or along with SCH23390 or H89. Cells were maintained in culture for 48 hours, and the media were collected. Enzyme-linked immunosorbent assay for the determination of VEGF-A was performed using a Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), and absorbance was measured at 450 nm using a PowerWave XS Microplate reader (BioTek Instruments, Winooski, VT).⁸

Scratch Wound Cell Migration Assay

HUVECs were cultured near to confluence in 24-well tissue culture plates in endothelial basal medium containing 2% fetal calf serum. Cells were serum- and growth factor-starved overnight, and a unidirectional scratch was generated in the cell monolayer using a p200 pipette tip. Thereafter, 1 $\mu\text{mol/L}$ SKF38393, 1 $\mu\text{mol/L}$ SKF81297, or 100 ng VEGF-A, which served as a positive control, was added to the plates as needed. Wound closure was calculated as the distance covered by cells in relation to the initial distance between two wound edges and was expressed as percent wound healing.^{13,33,34}

MTT *in Vitro* Cell Proliferation Assay

An MTT cell proliferation assay kit (Roche Life Science, Indianapolis, IN) was used to determine the effect DA D₁ receptor agonists on EC (HUVEC) proliferation. HUVECs were plated according to the manufacturer's instructions, serum-starved overnight, and then 1 $\mu\text{mol/L}$ SKF38393 or SKF81297 or VEGF-A was added. Cells were incubated for 24 hours, and then the absorbance was measured at 575 nm according to the manufacturer's protocol.³⁵

Statistical Analysis

Experimental data are expressed as means $\pm$ SEM. Differences among groups were evaluated by analysis of variance and the unpaired *t*-test or Dunn's multiple comparison test. $P < 0.05$ was considered significant.¹³

Results

Stimulation of DA D₁ Receptor Accelerates Dermal Wound Healing in Type 1 Diabetic Mice

We initially investigated the effects of DA D₁ receptor agonists on the healing of full-thickness cutaneous wounds generated in streptozotocin-induced type 1 diabetic mice (STZ-Diab).²⁹ Our results indicated that in contrast to

nontreated diabetic controls, in mice treated with a specific DA D₁ receptor agonist—SKF38393 or SKF81297 (i.p. 10 mg/kg per day for 5 days)—significantly improved the rate of wound healing in these animals ($P < 0.05$), with intact surface new skin occurring by day 11 (Figure 1A). In addition, the total surface area of the wound also significantly decreased after the completion of treatment in treated mice in comparison to that in nontreated controls (Figure 1B). This percent wound closure changed to approximately 100 in both the SKF38393- and SKF81297-treated groups versus approximately 42 in the nontreated control group by day 11 (Figure 1B). The mean wound sizes of the treated groups were also significantly smaller at all time points than those in the nontreated groups from day 3 until the day of complete healing in treated groups ($P < 0.05$) (Figure 1B). These results thus indicated the involvement of DA D₁ receptors in stimulating healing of wounds in animals with type 1 diabetes mellitus.

Thereafter, to further confirm the role of DA D₁ receptors on diabetic wound healing, experiments were undertaken in DA D₁ receptor knockout C57BL/6 mice (KOD₁ STZ-Diab).²⁹ As expected, in contrast to those in the wild-type STZ-Diab mice, the specific DA D₁ receptor agonists had no effects on promoting wound healing in these KOD₁ STZ-Diab mice (Figure 1A). On day 11, the percent healing values in these animals were approximately 60 in the SKF38393-treated group and approximately 66 in the SKF81297-treated group (Figure 1B), thereby further confirming the role of DA D₁ receptors in regulating diabetic wound healing (Figure 1, A and B).

DA D₁ Receptor Stimulation Increases VEGF-A Expression and Angiogenesis in Wound Beds of STZ-Diab Mice

One of the crucial causes of delayed wound healing in diabetes mellitus is poor VEGF-A expression leading to reduced angiogenesis in wound tissues.²⁸ Reports indicate that activations of DA D₁ and DA D₂ receptors usually have opposite effects in the central nervous system.^{24–27} Since DA has been reported to inhibit VEGF-A–induced angiogenesis by acting through its D₂ receptors present in the ECs,^{7–10} and because treatment with DA D₁ receptor agonists significantly accelerates healing of type 1 diabetic wounds (Figure 1, A and B), we investigated the role of DA D₁ receptors on VEGF-A expression and angiogenesis in wound tissues of STZ-Diab mice (Figure 1C). Our results indicated considerably increased VEGF-A expression and, thereby, angiogenesis (ie, increased microvessel density or CD31 staining) in the wound tissues of both SKF38393- and SKF81297-treated animals (Figure 1, C and D). We also observed that there were no changes in VEGF-A expression and CD31 staining in the KOD₁ STZ-Diab mice (Figure 1, C and D) and that treatment with DA D₁ receptor agonists had no effects (data not shown). These results thus indicated that stimulation of D₁ receptors could improve wound

Figure 1 Effect of D₁ dopamine receptor agonist treatment on full-thickness skin wound closure in streptozotocin (STZ)-induced diabetic mice. **A:** Treatment with a specific D₁ dopamine receptor agonist, SKF38393 or SKF81297, significantly accelerates the rate of wound healing compared to that in untreated controls, with complete closure of wounds by day 11. On the contrary, SKF38393 or SKF81297 treatment does not accelerate wound closure in diabetic D₁ dopamine receptor knockout mice (KOD1). **B:** Percentage wound closure, calculated as [(Area of original wound) – (Area of actual wound)] × 100 / (Area of original wound), was measured by analyzing images using ImageJ software package version 1.49V (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). **C:** Significant increase in vascular endothelial growth factor (VEGF)-A and CD31 expression levels in wound tissues after treatment with SKF38393 or SKF81297. VEGF-A and CD31 expression levels in wound tissues from KOD1STZ diabetic mice were similar to those in nontreated diabetic control mice. **Arrows** indicate CD31⁺ vessels. **D:** Numbers of CD31⁺ microvessels in individual high-power microscopic fields (HPF). Data are expressed as means ± SEM. *n* = 11. **P* < 0.05 versus control. Scale bars = 100 μm.

healing by increasing VEGF-A expression and subsequent angiogenesis in diabetic wound tissues.

Treatment with Specific DA D₁ Receptor Agonists Promotes Wound Healing in Type 2 Diabetic Mice

We next investigated the effects of specific DA D₁ receptor agonists on the healing of full-thickness dermal wounds in type 2 diabetic (db/db) mice. Our results demonstrated that treatment with a specific DA D₁ receptor agonist—SKF38393 or SKF81297 (i.p. 10 mg/kg per day for 5 days)—significantly increased percent wound closure in these animals (*P* < 0.05), with surfacing of intact new skin occurring by day 11 (Figure 2A). In addition, the total surface area of the wound also decreased significantly after the completion of treatment in treated animals in comparison to nontreated db/db controls (Figure 2B). The percent wound closure values on day 11 were approximately 100 in both the SKF38393- and SKF81297-treated groups versus

approximately 80 in the control group (Figure 2B). The mean wound sizes of the treated groups were also significantly smaller at all time points in the treated groups than in the nontreated groups from day 3 until the day of complete healing in treated groups (*P* < 0.05) (Figure 2B). These results, therefore, indicated that treatment with DA D₁ receptor agonists could also significantly accelerate the healing of wounds in animals with type 2 diabetes.

Increased VEGF-A Expression and Angiogenesis in the Wound Tissues of db/db Mice Treated with Specific DA D₁ Receptor Agonists

Considerably increased expression of VEGF-A and angiogenesis, as determined by microvessel density or CD31 staining, similar to that in the STZ-Diab mice was observed in the wound tissues of db/db mice treated with a specific DA D₁ receptor agonist, SKF38393 or SKF81297 (Figure 2, C and D). Taken together, results in both the models of

Figure 2 Effect of D₁ dopamine receptor agonist treatment on full-thickness skin wound closure in genetically diabetic mice. **A:** Treatment with a specific D₁ dopamine receptor agonist, SKF38393 or SKF81297, significantly accelerates the rate of wound healing compared to that in nontreated controls (Con), with complete closure of wounds by day 11. **B:** Percentage wound closure. **C:** Significant increase in vascular endothelial growth factor (VEGF)-A and CD31 expressions in wound tissues after treatment with SKF38393 or SKF81297. **Arrows** indicate CD31⁺ vessels. **D:** Numbers of CD31⁺ microvessels in individual microscopic high-power fields (HPF). Data are expressed as means $\pm$ SEM. $n = 11$. * $P < 0.05$ versus control. Scale bars = 100 μ m.

diabetes thus suggest that activation of DA D₁ receptors could significantly promote diabetic wound healing by stimulating VEGF-A–induced angiogenesis in wound tissues (Figures 1 and 2).

DA D₁ Receptor Agonists Have No Direct Effects on the Migration and Proliferation of ECs

As ECs are one of the major cellular components of angiogenesis, and because our previous results indicated that stimulation of DA D₁ receptors can promote angiogenesis in diabetic wound tissues (Figures 1 and 2), we determined whether DA D₁ receptor agonists have any direct effects on the migration and proliferation of these cells, which are required to promote angiogenesis.^{7,21} Results indicated that treatment with 1 μ mol/L of SKF38393 or SKF81297 had no effects on the migration (Figure 3A) and proliferation (Figure 3B) of HUVECs *in vitro*. Similarly, we also did not observe any effects of these agonists on the migration and proliferation of adult human dermal ECs (data not shown).

Activation of DA D₁ Receptors in Dermal Fibroblasts Induces VEGF-A Expression in Wound Tissues through the DA D₁–PKA–CREB Pathway

Since our data suggest that stimulation of DA D₁ receptors can induce VEGF-A expression and, thereby, angiogenesis in diabetic wound tissues (Figures 1, 2, and 3), and because there are reports indicating that dermal fibroblasts can be stimulated to up-regulate VEGF-A expression in diabetic wounds,³⁶ we at first investigated the presence of DA D₁ receptors in adult murine dermal fibroblasts and HDFs. Our Western blot and confocal data demonstrated the presence of DA D₁ receptors in the fibroblasts cultured *in vitro* and in skin tissues collected from normal and diabetic mice and human subjects (Figures 3C and 4A).

Thereafter, we determined the effects of treatment with 1 μ mol/L of a specific DA D₁ receptor agonist, SKF38393 or SKF81297, on VEGF-A production by the HDFs. RT-PCR results indicated a low baseline expression of VEGF-A mRNA in HDFs isolated from the skin of diabetic patients (dHDFs) and cultured in high-glucose condition

Figure 3 D₁ dopamine receptor agonists have no direct effects on endothelial cells *in vitro*. **A:** Exogenous administration of SKF38393 or SKF81297 has no direct effect on human umbilical vein endothelial cell (HUVEC) migration, as determined by scratch wound assay. Eighteen hours after the creation of wound, complete wound closure is observed in vascular endothelial growth factor (VEGF)-A (positive control)—treated well. **B:** Exogenous administration of SKF38393 or SKF81297 also has no effect on HUVEC proliferation, as determined by MTT cell proliferation. **C:** Western blot analysis shows the presence of D₁ dopamine receptor (DA D₁) in HUVECs, normal human fibroblasts, diabetic human fibroblasts, and mouse kidney lysate (positive control). Figures are representative of three separate experiments. Data are expressed as means $\pm$ SEM. $n = 9$. * $P < 0.05$ versus control cells. Scale bars = 200 μ m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDF, human dermal fibroblasts; M, mouse; O.D., optical density.

(25 mmol/L) in comparison to normal HDFs collected from the skin of normal human subjects and cultured under normal conditions. The VEGF-A mRNA expression significantly increased in dHDFs when treated with either SKF38393 or SKF81297 for 24 hours ($P < 0.05$) (Figure 4B), and the expression was comparable to that found in normal HDFs. The specificity of DA D₁ receptor actions was further confirmed when 30-minute prior treatment of these cells with 10 μ mol/L of the specific DA D₁ antagonist SCH23390 significantly abrogated the effects of these agonists ($P < 0.05$) (Figure 4B). Moreover, since DA D₁ receptors classically act through the PKA signaling pathway, we further evaluated the pretreatment effects of 10 μ mol/L PKA inhibitor H89 on the VEGF-A—stimulatory effects of SKF38393 or SKF81297 on dHDFs. Our results indicated that this pretreatment significantly inhibited the stimulatory effect of these agonists on VEGF-A synthesis ($P < 0.05$) (Figure 4B), thereby suggesting that the VEGF-A—stimulatory action of DA D₁ receptors was through the PKA pathway. This notion was further confirmed when treatment with 10 μ mol/L of forskolin, a potent cAMP/PKA activator, for 24 hours also significantly increased VEGF-A expression in dHDFs ($P < 0.05$).

In conformity with our RT-PCR results, the concentration of VEGF-A in the supernatants collected from dHDFs treated with 1 μ mol/L of a specific DA D₁ receptor agonist, SKF38393 or SKF81297, for 48 hours also increased significantly, as determined by enzyme-linked immunosorbent assay ($P < 0.05$) (Figure 4C). Similarly, prior treatment with 1 μ mol/L of specific DA D₁ antagonist SCH23390 abrogated these actions of DA D₁ receptor agonists, thereby

confirming the involvement of DA D₁ receptors (Figure 4C) in VEGF-A secretion by dHDFs. Importantly, the increase in the production of VEGF-A by dHDFs after treatment with 10 μ mol/L of forskolin for 48 hours and abrogation of SKF38393 or SKF81297 induced an increase in VEGF-A synthesis in cells pretreated with 10 μ mol/L of H89 ($P < 0.05$), further confirming that stimulation of DA D₁ receptors induces VEGF-A synthesis in dHDFs through the PKA pathway.

Finally, our Western blot data also confirmed that this DA D₁ receptor—mediated stimulation of VEGF-A production by dHDFs was through the PKA pathway and via phosphorylation of CREB (Figure 4D).

Discussion

In summary, our results indicate that activation of DA D₁ receptors present in dermal fibroblasts restores VEGF-A production by these cells, leading to angiogenesis and hence acceleration of diabetic wound healing. Among the different cellular sources in wound beds, fibroblasts play a pivotal role in the synthesis and release of VEGF-A,³⁶ which ultimately acts in the paracrine way to stimulate proliferation and migration of ECs and formation of new blood vessels.³⁰ However, fibroblasts in diabetic wound tissues are dysfunctional, and their ability to synthesize VEGF-A is impaired.^{36–38} These results are therefore significant as they suggest that treatment with specific DA D₁ receptor agonists can correct this anomaly. We had also previously reported that DA inhibits VEGF-A—induced angiogenesis by acting through its D₂ receptors present in

Figure 4 D_1 dopamine receptor agonist treatment increases the synthesis of vascular endothelial growth factor (VEGF)-A in diabetic human dermal fibroblasts through phosphorylation (p) of cAMP response element-binding protein (CREB). **A:** Confocal microscopy images show the presence of D_1 dopamine receptor (D1DR; red) in fibroblasts [smooth muscle actin (α -SMA); green] in normal, streptozotocin-induced type 1 diabetic mice (STZ-Diab) and db/db mouse wound tissues as well as in normal and diabetic human skin. **B:** Semiquantitative RT-PCR analysis shows that synthesis of VEGF-A is significantly decreased in diabetic human fibroblasts (control) when compared to normal human fibroblasts (normal). Treatment of diabetic human fibroblasts with SKF38393, SKF81297, or forskolin significantly increases VEGF-A synthesis after 24 hours. Treatment of diabetic fibroblasts with a specific D_1 dopamine receptor antagonist, SCH23390 or H89, before treatment with SKF38393 or SKF81297, abrogates their effects. **C:** Enzyme-linked immunosorbent assay for human VEGF-A. Treatment with SKF38393, SKF81297, or forskolin increases VEGF-A secretion by diabetic fibroblasts at 48 hours. However, treatment of diabetic fibroblasts with SCH23390 or H89 before treatment with SKF38393 or SKF81297 abrogates their effects. **D:** Western blot analysis shows that treatment of diabetic fibroblasts with SKF38393, SKF81297, or forskolin for 1 hour significantly increases phosphorylation of CREB, but that prior treatment with SCH23390 or H89 abrogates the effect of SKF38393 or SKF81297. Figures are representative of three separate experiments. Data are expressed as means $\pm$ SEM. $n = 11$. * $P < 0.05$ versus control cells; † $P < 0.05$ versus D_1 dopamine receptor agonist- and forskolin-treated groups. Scale bars = 50 μ m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the ECs and endothelial progenitor cells.^{7–11} This study for the first time demonstrates a novel and different action of DA D_1 receptors in stimulating VEGF-A-induced angiogenesis and suggests that DA may have a dual role in regulating VEGF-A-mediated angiogenesis. Interestingly, DA D_1 receptors promote angiogenesis by stimulating VEGF-A production by fibroblasts and on the contrary, DA D_2 receptors inhibit angiogenesis by suppressing VEGF-A-induced VEGF receptor 2 phosphorylation in ECs and endothelial progenitor cells.^{7–11} Therefore DA acts as an important regulator of the angiogenic switch.

CREB is a well-known transcription factor closely associated with the classic intracellular second messenger

cAMP. When intracellular cAMP level increases, PKA is activated, diffuses into the nucleus, and activates gene expression by phosphorylating CREB.^{39,40} Recent reports indicate that VEGF-A expression is also regulated via the PKA/CREB pathway.^{41,42} In the present study, we demonstrate that DA D_1 receptors act specifically through this pathway to phosphorylate CREB, and thereby stimulate VEGF-A production by dHDFs, as the inhibition of PKA by H89 abolishes this effect of DA (Figure 4). This significantly increased VEGF-A production by fibroblasts in the wound microenvironment, in turn, stimulates angiogenesis and subsequently accelerates healing of the diabetic wounds (Figures 1 and 2).

Finally, deficient angiogenesis is an important cause of delayed wound healing in diabetes mellitus, resulting in morbidity and loss of manpower and money.^{22,28,43–45} This underlines the development of newer angiogenesis-based therapeutic approaches to expedite faster healing of diabetic wounds.^{28,46} DA D₁ agonists are already in clinical use for the treatment of other disorders; therefore, in addition to establishing a newer link between DA D₁ receptors and VEGF-A-mediated angiogenesis, our present study indicates that DA D₁ receptor agonists may also be useful for the treatment of diabetic wounds and diseases in which VEGF-A-stimulated angiogenesis plays a beneficial role.^{2,6}

Acknowledgments

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