Vascular Endothelial Growth Factor Inhibition by dRK6 Causes Endothelial Apoptosis, Fibrosis, and Inflammation in the Heart via the Akt/eNOS Axis in db/db Mice

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OBJECTIVE—Vascular endothelial growth factor (VEGF), which is associated with the stimulation of angiogenesis and collateral vessel synthase, is one of the crucial factors involved in cardiac remodeling in type 2 diabetes.

RESEARCH DESIGN AND METHODS—We investigated VEGF inhibition by dRK6 on the heart in an animal model of type 2 diabetes. Male db/db and db/m mice either were treated with dRK6 starting at 7 weeks of age for 12 weeks (db/db-dRK6 and db/m-dRK6) or were untreated.

RESULTS—Cardiac dysfunction and hypertrophy were noted by echocardiogram and molecular markers in the db/db-dRK6 mice. The presence of diabetes significantly suppressed the expression of VEGF receptor (VEGFR)-1 and VEGFR-2, phospho-Akt, and phospho-endothelial nitric oxide synthase (eNOS) in the heart. In db/db-dRK6 mice, dRK6 completely inhibited VEGFR-2, phospho-Akt, and phospho-eNOS expression, whereas no effect on VEGFR-1 was observed. Cardiac fibrosis, microvascular scarcity associated with an increase in apoptotic endothelial cells, and inflammation were prominent, as well as increase in angiogenic growth factors. Cardiac 8-hydroxy-deoxyguanine and hypoxia-inducible factor-1 expression were significantly increased and unaltered serum or plasma VEGF levels were observed in type 1 and 2 diabetic patients versus control subjects (17,18). On the other hand, the serum VEGF level was increased in patients with diabetic symptomatic poly-
neuropathy, overt diabetic nephropathy, and coronary artery disease, particularly to the extent of myocardial damage (19–21).

dRK6 is an arginine-rich anti-VEGF hexapeptide and a d-amino acid derivative of RK6 (Arg-Arg-Lys-Arg-Arg-Arg). dRK6 binds with VEGF-A and can thereby block the interaction between VEGF-A (mainly VEGF₄₅ and VEGF₁₆₅) and the VEGF-Rs. In a previous study, dRK6 showed significant inhibition of VEGF-induced angiogenesis and retarded the growth and metastasis of colon carcinoma cells without direct cytotoxicity (22). dRK6 is stable, and its half-life at 37°C in the serum is 13.5 h (22). Previous results demonstrated that a subcutaneous injection of dRK6 (25–50 µg) every other day for 3 weeks reduced the incidence and severity of collagen-induced arthritis in a dose-dependent manner without any toxicity (22,23).

Therefore, we determined whether systemic VEGF blockade, using dRK6, could lead to structural and functional deterioration of the heart in db/db mice in a murine model of type 2 diabetes.

**RESEARCH DESIGN AND METHODS**

**Experimental methods.** All experiments were performed according to the institutional animal care guidelines, and the investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. Institutes of Health (National Institutes of Health Publication No. 85–23, revised 1996). Six-week-old male C57BLKS/J db/db and db/m mice were purchased from Jackson Laboratories (Bar Harbor, ME); db/m mice were used as controls in all of the experiments. Fifty micrograms of dRK6 was administered three times per week by intraperitoneal injection to db/m or db/db (db/db-dRK6) mice (n = 8) beginning at 7 weeks of age for 12 weeks (n = 8). The diabetic db/db (db/db) and nondiabetic db/m control groups (db/m) received only PBS.

After 12 weeks of treatment, the systolic blood pressure was determined by the noninvasive tail cuff system in conscious mice (ITC Life Science, Woodland Hill, CA) after a 5-day accommodation. At the end of the study, the animals were anesthetized and killed.

**Measurement of serum parameters.** Blood was collected from the left ventricle and centrifuged; the plasma was stored at –70°C for subsequent analyses. The AIC was measured by high-pressure liquid chromatography (BioRad, Richmond, CA). The total cholesterol, triglycerides, free fatty acids (FFAs), and insulin concentrations were measured by an autoanalyzer (Wako, Osaka, Japan), and phospho-Akt, Akt, total eNOS, and phospho-eNOS conjugates were measured by the ELISA kit (Kamiotitech, Seoul, South Korea).

**Assessment of heart function.** The blood samples were collected after systemic perfusion with PBS and then fixed in 4% paraformaldehyde. To examine the effect of dRK6 on cardiac fibrosis, the analysis was performed on trichrome-stained sections. The heart samples were collected after systemic perfusion (20 views, ×200 magnification), randomly located in the middle portion of the myocardium of each slide. We also examined (Scion Image Beta 4.0.2).

**Western blot and semi-quantitative RT-PCR for total- and phospho-ENOS (Ser1177), total- and phospho-Akt (Ser473), VEGFR-1, VEGFR-2, CTGF, and HIF-1α.** Western blot analysis was performed using the following antibodies: total Akt, phospho-Akt (Ser473), total eNOS, and phospho-eNOS (Ser1177), all from Cell Signaling Technology, Danvers, MA; HIF-1α, CTGF, and β-actin (all from Abcam, Cambridge, U.K.). Protein for HIF-1α from nuclear fractions of the hearts was isolated using a nuclear extraction kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's protocol. We also performed RT-PCR to assess the levels of gene expression of VEGFR-1 and -2, HIF-1α, CTGF, and 18S rRNA in an internal control. Primers for amplification of VEGFR-1 and -2, HIF-1α, CTGF, and 18S rRNA are listed in the supplemental data available in an online version at http://diabetes.diabetesjournals.org/cgi/content/full/09-0130/DC1.

**Quantiative PCR for atrial natriuretic factor, B-type natriuretic peptide, α-myosin heavy chain, and β-myosin heavy chain.** Total reactive nitrogen species was performed using an ABI PRISM Sequence Detector System 7500 (Applied Biosystems, Foster City, CA). The PCR primers for atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), α-myosin heavy chain (MHC), and β-MHC are listed in the supplemental data available in an online version at http://diabetes.diabetesjournals.org/cgi/content/full/09-0130/DC1.

**Immunofluorescent staining for the hypoxia-inducible factor-1α.** To evaluate the hypoxic injury to the heart, we performed hypoxia-inducible factor (HIF)-1α immunofluorescent staining of the heart (1:50; Novus Biological, Littleton, CO). For quantification of the proportion of the area stained, >20 views (×200 magnification), randomly located in the middle portion of the myocardium of each slide, were also examined (Scion Image Beta 4.0.2).
the serum VEGF concentrations in the db/m-dRK6 and the db/db-dRK6 mice compared with the db/m and db/db control mice (P < 0.001 and P < 0.05, respectively). These findings suggest that VEGF blockade with dRK6 significantly increased the circulating VEGF concentrations.

**Assessment of heart function.** To assess heart function, we performed echocardiograms on the mice in the study groups (Fig. 1A). There were no significant differences in the diastolic interventricular wall thickness (IVSTd), left ventricular posterior wall thickness (LVPWTd), and left ventricular mass (LVM) among the db/m, db/m-dRK6, and db/db mice. By contrast, these parameters in the db/db-dRK6 mice were markedly increased compared with the other groups (P < 0.05, Fig. 1B). Molecular markers of cardiac hypertrophy (ANF, BNP, α-MHC, and β-MHC) were also elevated in the db/db-dRK6 mice (P < 0.05, Fig. 1D). These results suggest that dRK6 treatment caused development of cardiac hypertrophy. Diabetic db/db mice also exhibited a persistent decrease in fractional shortening (P < 0.05, Fig. 1C) compared with the db/m mice. There was a more prominent decrease in the fractional shortening, mean velocity of circumferential fiber shortening (Vcf), and peak E/A ratio in the db/db-dRK6 mice. These findings demonstrate that treatment with dRK6 had a negative effect on the cardiac function in the presence of diabetes.

**Effect of dRK6 on VEGFR-1 and -2, Akt, and eNOS on the heart.** As shown in Fig. 2A–C, there were marked decreases in the expression of VEGFR-1 and -2 mRNA in the db/db control mice compared with the db/m mice. In addition, dRK6 treatment completely abolished the expression of VEGFR-2 mRNA and protein in db/db-dRK6 mice. By contrast, there was no change in the expression of VEGFR-1 mRNA in db/db-dRK6 mice; no such changes were detected in db/m-dRK6 mice. There was significantly decreased expression of phospho-Akt protein, eNOS mRNA, and phospho-eNOS protein in db/db mice compared with db/m mice (Fig. 2D–G). Consistent with the VEGFR-2 findings, dRK6 treatment completely inhibited expression of phospho-Akt/eNOS protein in db/db-dRK6 mice.

**Histologic examination of the heart and effects on connective tissue growth factor.** In the db/m and db/db control mice, there was no apparent cardiac fibrosis observed, which was likely because of the short period of diabetes exposure (Fig. 3A and B). However, dRK6 treatment markedly increased the cardiac fibrosis observed in the db/db-dRK6 mice (P < 0.05, Fig. 3D and E). By contrast, there was no such change observed in the db/m-dRK6 mice (Fig. 3B). We also performed immunostaining, Western blot, and RT-PCR for CTGF to evaluate the expression of profibrotic and inflammatory growth factors in the heart. Compared with the increased myocardial fibrosis in the heart, the positive staining for CTGF was significantly increased in the db/db-dRK6 mice compared with the db/db and db/m-dRK6 mice (P < 0.05, Fig. 4A–E). Consistent with the immunostaining results, the expression of CTGF mRNA and protein levels was also significantly increased in the db/db-dRK6 mice (Fig. 4F and G). These findings suggest that CTGF is one of the major profibrotic growth factors in dRK6-induced cardiac fibrosis.

**Immunohistochemistry for PECAM-1, TUNEL, thrombospondin-1, and F4/80.** To evaluate vascular homeostasis, we performed immunohistochemistry staining for PECAM-1, PECAM-1+TUNEL, and thrombospondin-1. There were no changes in the expression of PECAM-1 in db/db mice. By contrast, dRK6 treatment in db/db-dRK6 mice was associated with a decrease in expression of PECAM-1 in the myocardium, reflecting a decrease in endothelial cells (Fig. 5A–E). It is well known that thrombospondin-1 is an antiangiogenic peptide; therefore, we evaluated the expression of thrombospondin-1 in the heart. The expression of thrombospondin-1 was significantly increased in the db/db-dRK6 mice compared with the db/m, db/m-dRK6, and db/db mice (P < 0.05, Fig. 5F–J). Further evaluation of the effects of endothelial cell apoptosis on the rare vessels in the heart was assessed by double immunostaining with PECAM-1+TUNEL in the heart. It was difficult to find TUNEL-positive stained endothelial cells in the db/m, db/m-dRK6, and db/db mice. By contrast, an increased number of TUNEL-positive cells were found in the db/db-dRK6 mice (P < 0.001, Fig. 5K–O).

Only mild macrophage infiltration, as assessed by F4/80-positive staining, was observed in the myocardium of db/m-dRK6 and db/db mice. By contrast, F4/80 immunostaining was markedly increased in the myocardium of db/db-dRK6 mice (P < 0.001, Fig. 4P–T).

**Effect of dRK6 on HIF-1α and 8-OH-dG.** There was no difference in expression of HIF-1α in the hearts of db/m, db/m-dRK6, and db/db mice. However, in db/db-dRK6 mice, expression of HIF-1α was significantly increased compared with the other groups (P < 0.001, Fig. 6A–E). The expression of HIF-1α protein and mRNA levels in the heart significantly increased in db/db-dRK6 mice (Fig. 6F–H). Diabetic db/db mice and db/m-dRK6 mice had increased immunostaining of 8-OH-dG in the myocardial cells compared with db/m mice (Fig. 6J). In the myocardium, increased expression of 8-OH-dG (dark brown nucleus) in db/db mice was markedly accentuated by treatment with dRK6 (Fig. 6L and M). The 8-OH-dG levels

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**TABLE 1**

|                  | db/m   | db/m-dRK6 | db/db   | db/db-dRK6 |
|------------------|--------|-----------|---------|------------|
| Body weight (g)  | 31.6 ± 1.2 | 31.2 ± 1.3 | 50.3 ± 5.8* | 42.4 ± 5.9* |
| Heart weight (g) | 0.12 ± 0.04 | 0.11 ± 0.05 | 0.12 ± 0.04 | 0.14 ± 0.05 |
| Mean SBP (mmHg)  | 95.6 ± 5.4 | 97.6 ± 3.5 | 99.1 ± 6.2 | 102.3 ± 5.0 |
| Hematocrit (%)   | 34.0 ± 2.9 | 35.6 ± 3.4 | 39.8 ± 5.2 | 38.6 ± 6.4 |
| Glucose (mmol/l) | 11.2 ± 1.1 | 11.8 ± 1.9 | 58.0 ± 5.9† | 57.6 ± 7.8† |
| A1C (%)          | 4.3 ± 0.2 | 4.1 ± 0.3 | 12.7 ± 1.2† | 13.6 ± 1.0† |
| Total cholesterol (mmol/l) | 1.66 ± 0.38 | 1.53 ± 0.34 | 2.08 ± 0.37 | 1.92 ± 0.31 |
| Triglycerides (mg/dl) | 60 ± 16 | 66 ± 20 | 103 ± 27‡ | 114 ± 21‡ |
| FFAs (mEq/l)     | 1.45 ± 0.34 | 1.57 ± 0.55 | 3.18 ± 1.03‡ | 3.76 ± 1.19‡ |
| Insulin (pg/ml)  | 0.17 ± 0.13 | 0.19 ± 0.14 | 0.50 ± 0.14‡ | 0.33 ± 0.21‡ |
| Serum VEGF (pg/ml) | 1.25 ± 1.73 | 23.5 ± 7.7§ | 6.6 ± 3.9† | 29.6 ± 8.5§ |

Data are means ± SD. *P < 0.01, †P < 0.001, ‡P < 0.05 vs. dm and dm-dRK6. §P < 0.001 vs. dm and P < 0.05 vs. db/db.
in the DNA from the hearts were significantly increased compared with db/db mice (Fig. 6M), the levels of which were also increased compared with db/m mice. These findings suggest that dRK6 treatment increased oxidative stress in the hearts, especially in mice with diabetes.

**Effect of dRK6 on HUVEC apoptosis.** To examine the direct role of dRK6 on HUVECs, we investigated whether dRK6 induced apoptosis of the HUVECs using TUNEL assay with different doses of dRK6 (10⁻⁶, 10⁻⁸, and 10⁻¹⁰ mmol/l) and various concentrations of glucose in the

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FIG. 1. Representative M-mode echocardiograms in diabetic db/db and nondiabetic db/m mice with or without dRK6 treatment (A). Echocardiographic changes in IVSTd and LVPWTd, LVM (B), fractional shortening, mean Vcf, and peak E/A ratio (C). Quantitative RT-PCR analysis for ANF, BNP, α-MHC, and β-MHC transcript levels in hearts (D) from diabetic db/db and nondiabetic db/m mice without (db/db, db/m) or with dRK6 treatment (db/db-dRK6, db/m-dRK6) for 12 weeks beginning at 7 weeks of age. *P < 0.05, **P < 0.01 compared with other groups.
media (5 and 30 mmol/l of D-glucose, and 5 mmol/l D-glucose + 25 mmol/l mannitol) for 48 h. Only at a concentration of 30 mmol/l of D-glucose were the HUVEC TUNEL-positive cells significantly increased in a dose-dependent manner (P < 0.01, Fig. 7). Treatment with dRK6 completely inhibited the expression of phospho-Akt and phospho-eNOS protein with high glucose concentrations in the media (Fig. 7B). However, there was no significant difference in phospho-Akt and phospho-eNOS protein expression for concentrations of 5 mmol/l D-glucose or 5 mmol/l D-glucose + 25 mmol/l mannitol treated with or without dRK6. As shown in Fig. 6F and G, the 8-iso-PGF$_{2\alpha}$ and 8-OH-dG concentrations were increased with high glucose concentrations, which were further increased by dRK6 treatment (3.0- and 3.5-fold, respectively). However, these changes were not observed at concentrations of 5 mmol/l D-glucose (low glucose) or 5 mmol/l D-glucose + 25 mmol/l mannitol.

**DISCUSSION**

A growing number of drugs that inhibit VEGF signaling are being considered for the treatment of cancer and diabetic microvascular complications. These agents are generally well tolerated but sometimes may be accompanied by serious side effects. Most of the side effects are associated with downstream effects of suppression of VEGF-NO signaling through the inhibition of VEGF-A and VEGFR-2 signals in the endothelial cells of normal organs (4). Therefore, we evaluated the role of VEGF inhibition using dRK6 in the development and progression of cardiac adverse effects in a model of type 2 diabetes (db/db mice). In db/db mice, systemic dRK6 administration completely inhibited the cardiac VEGFR-2–Akt-eNOS axis, which subsequently caused the development of systolic dysfunction, cardiac fibrosis, and hypertrophy. From one perspective, the deterioration caused by dRK6 might be related to the regression of blood vessels, which was associated with an increase in antiangiogenic growth factors, such as thrombospondin-1, transforming growth factor (TGF)-β, and CTGF. These events were accompanied by an increase in endothelial cell apoptosis, inflammatory cell infiltration, and myocardial fibrosis. In addition, these pathologic alterations led to the aggravation of hypoxic and oxidative stress in the myocardium accompanied by metabolic ab-

**FIG. 2.** RT-PCR for VEGF-R1, VEGF-R2 (A), eNOS (D) mRNA, and 18s rRNA expression in the hearts of diabetic db/db and nondiabetic db/m mice with or without dRK6 treatment. Quantitative assessment of the expression of VEGF-R1 (B), VEGF-R2 mRNA (C), and eNOS mRNA (F) to 18s rRNA ratios in the hearts of the study groups. Western blot analysis of the total and phospho-Akt and phospho-eNOS (E) and quantitative assessment of the expression of phospho-Akt (E) and phospho-eNOS protein (G). *P < 0.05 compared with other groups, **P < 0.01 compared with other groups, #P < 0.05 compared with the db/db group, +P < 0.05 compared with the db/m group; n = 4.
normalities. However, no such changes were observed in the diabetic control or db/m-dRK6 mice. By using in vitro HUVECs, we have shown that dRK6 had potent apoptotic effects on endothelial cells, which were associated with inhibition of the phospho–Akt-eNOS axis and enhancement of oxidative stress.

To date, the effects of systemic inhibition of VEGF on the heart remain uncertain. Previous findings from studies of the structural and functional changes in the organs of the normal adult mouse, after inhibition of VEGF signaling, revealed little or no capillary regression was detected in the normal adult mouse, after inhibition of VEGF signaling and increases in the production of NO. Therefore, downregulation of the Akt-eNOS axis by dRK6 might relate to endothelial cell apoptosis in diabetic db/db mice.

Under hypoxic conditions, HIF-1α is stabilized, translocates into the nucleus, dimerizes with HIF-1β, and upregulates the genes involved in angiogenesis, glycolytic energy metabolism, cell proliferation, and survival (40,41). In the present study, the markedly upregulated expression of nuclear HIF-1α after treatment with dRK6 suggests that dRK6-induced hypoxic conditions in the heart and HIF-1α function to prevent excessive ROS production under conditions of chronic hypoxia. These results indicate that continuous VEGF blockade by dRK6 under diabetic conditions ultimately leads to the accumulation of reactive oxygen species and oxidative stress leading to increased expression of 8-OH-dG in the heart.

Previous reports have demonstrated that db/db mice usually have no gross cardiac hypertrophy at 3 months (42,43); isolated working hearts as well as studies using magnetic resonance imaging, however, indicate that significant left ventricular dysfunction develops in the hearts of diabetic conditions ultimately leads to the accumulation of reactive oxygen species and oxidative stress leading to increased expression of 8-OH-dG in the heart.

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FIG. 3. Cardiac histopathology in diabetic db/db and nondiabetic db/m mice with or without dRK6 treatment (Trichrome stain, ×100). The histopathology shows marked cardiac fibrosis in diabetic db/db-dRK6 (D) compared with nondiabetic db/m and db/m-dRK6 mice (A and B) and db/db control mice (C). Original magnification ×200. Quantitative assessment of the areas of fibrosis in the myocardial tissue of the study groups (E). *P < 0.01 compared with the other groups; n = 4. (A high-quality color digital representation of this figure is available in the online issue.)
of db/db mice at much earlier ages. In this study, in contrast to the db/db control mice, systemic inhibition with dRK6 resulted in more severe systolic dysfunction and gross cardiac hypertrophy and fibrosis in the db/db mice at 5 months of age. The data from our study showed that the direct effects of VEGF blockade, as well as the metabolic abnormalities associated with diabetes such as hyperglycemia, dyslipidemia, and hyperinsulinemia, might be important to the development of pathologic conditions, including severe cardiac hypertrophy, fibrosis, and inflammation.

Data regarding circulating VEGF levels in diabetes are highly discrepant. One study reported that plasma VEGF levels were higher in type 2 diabetics than in controls (20). In another study, plasma VEGF levels were elevated only in type 2 diabetic patients with characteristics of atherosclerosis (21). In our study, there were sixfold higher serum VEGF levels in db/db mice compared with db/m mice. dRK6 treatment also increased the serum VEGF level independently from diabetic conditions. It is well known that as VEGF is a paracrine mediator, systemic levels may not adequately reflect changes in the local VEGF system (44). Therefore, elevated plasma VEGF levels after dRK6 treatment might be a consequence of feedback of systemic VEGF-VEGFR inhibition.

In summary, our results indicate that the systemic dRK6 completely inhibited endogenous cardioprotective mechanisms through suppression of the VEGF-R2 and Akt-eNOS axis in the heart of mice with type 2 diabetes and subsequently led to cardiac systolic dysfunction resulting from cardiac fibrosis and hypertrophy, which were related to the regression of the microvasculature in the heart. These changes were accompanied by intracardiac hypoxia and oxidative stress. Therefore, the protective role of VEGF appears to be predominantly dependent on its ability to stimulate activation of Akt-eNOS in the endothelial cells in tissues. Further clinical investigations are needed to evaluate whether VEGF inhibition using anti-VEGF agents for the treatment of age-related macular diseases, various cancers, and diabetic microvascular complications has robust effects on the heart, especially in patients with type 2 diabetes.

FIG. 4. Immunohistochemical staining for CTGF in the myocardial tissue of nondiabetic db/m and diabetic db/db mice (B and D, respectively) with or without dRK6 treatment (A and C, respectively; ×200). Original magnification ×200. E: Quantitative assessment of CTGF immunostaining in the myocardial tissue of the study groups. F: Expression of CTGF protein, mRNA, and 18s rRNA in the hearts of the study groups. Quantitative assessment of the expression of CTGF mRNA (G) and protein (H). *P < 0.05, **P < 0.01 compared with the other groups, *P < 0.01 compared with the db/db group; n = 4. (A high-quality color digital representation of this figure is available in the online issue.)
FIG. 5. Myocardial morphology and immunohistochemical staining for PECAM-1, thrombospondin, TUNEL++, and F4/80 in the hearts of diabetic db/db and nondiabetic db/m mice with or without dRK6 treatment. A representative photomicrograph of myocardial immunostaining for PECAM-1 in nondiabetic db/m (A), db/m-dRK6 (B), diabetic db/db (C), and db/db-dRK6 mice (D, ×200). Original magnification ×200. Representative immunostains for thrombospondin-1 (F–I, ×200), PECAM-1++TUNEL (a dark-brown nuclear for TUNEL-positive and a red cytoplasm for PECAM-1-positive; open arrow, K–N, ×400) and F4/80 (open arrow, P–S, ×400) in diabetic and nondiabetic db/db and db/m mice with or without dRK6 treatment. Quantitative assessment of PECAM-1 (E), thrombospondin-1 (J), PECAM-1++TUNEL (O), and F4/80 (T) immunoreactivity in diabetic db/db and nondiabetic db/m mice without or with dRK6 treatment. *P < 0.05, **P < 0.01 compared with the other groups. (A high-quality color digital representation of this figure is available in the online issue.)
FIG. 6. Immunofluorescent staining for HIF-1α in the heart. Representative pictures illustrating expression in nondiabetic db/m (A), db/m-dRK6 (B), diabetic db/db (C), and db/db-dRK6 mice (D) in myocytes. Original magnification ×200. E: Quantitative assessment of HIF-1α immunoreactivity in the myocardial cells in diabetic db/db and nondiabetic db/m mice with or without dRK6 treatment. **P < 0.001 compared with the other groups. F: Expression of HIF-1α protein, mRNA, and 18s rRNA in the hearts of the study groups. Quantitative assessment of the expression of HIF-1α mRNA (G) and protein (H). *P < 0.05, **P < 0.01 compared with the other groups, #P < 0.01 compared with the db/db group; n = 4. Immunohistochemical expression of 8-OH-dG protein in myocardial cells. In diabetic db/db mice (K), 8-OH-dG protein is markedly accentuated in the myocardium (dark nucleus) compared with nondiabetic db/m and db/db-dRK6 mice (I and J, respectively). More prominent 8-OH-dG immunostaining is seen in the db/db-dRK6 mice compared with the diabetic db/db mice (L). Original magnification ×200. M: Quantitative assessment of 8-OH-dG levels in the DNA from the hearts. *P < 0.05, compared with db/m, **P < 0.01 compared with db/m and P < 0.05 compared with db/m-dRK6 and db/db; n = 4. (A high-quality color digital representation of this figure is available in the online issue.)
FIG. 7. The effect of dRK6 on apoptosis in the HUVECs, determined by in situ TUNEL assay. Representative pictures of TUNEL-positive HUVECs (A, original magnification ×200). The apoptosis of HUVECs treated with different doses of dRK6 (10⁻⁶, 10⁻⁸, and 10⁻¹⁰ mmol/l) and concentrations of glucose in the media (5 and 30 mmol/l of d-glucose, and 5 mmol/l d-glucose + 25 mmol/l mannitol) for 48 h (B). Representative Western blots for total and phospho-Akt and phospho-eNOS (C–E). 8-ISO-PGF₂α (F) and 8-OH-dG (G) concentrations of cell culture media in each group. *P < 0.05, **P < 0.01 compared with HG or HG + dRK6. NG, 5 mmol/l of d-glucose; HG, 30 mmol/l d-glucose; MN, 5 mmol/l d-glucose + 25 mmol/l mannitol; dRK6, treated with dRK6. *P < 0.05, **P < 0.01 compared with the other groups; n = 4. HG, high glucose; NG, normal glucose. (A high-quality color digital representation of this figure is available in the online issue.)
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No potential conflicts of interest relevant to this article were reported.

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