

Research Article

Suppression of Receptor for Advanced Glycation End Products Improves Angiogenic Responses to Ischemia in Diabetic Mouse Hindlimb Ischemia Model

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Received 16 June 2013; Accepted 8 July 2013

Academic Editors: P. Schoenhagen, V. K. Sharma, S. Takebayashi, and Y. Tohno

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Background. The role of the receptor for advanced glycation end products (RAGE) for the impaired angiogenic response in diabetic patients is not well known. We investigated the impact of RAGE suppression by soluble RAGE (sRAGE) on the angiogenic response in a diabetic hindlimb ischemia mouse model. **Materials and Methods.** Hindlimb ischemia model was prepared by ligation of femoral artery in diabetic and nondiabetic mice. Ischemia-induced angiogenic response was evaluated by laser-Doppler perfusion imaging, muscle capillary density, and protein expression of vascular endothelial growth factor (VEGF) and high-mobility group box (HMGB)-1. **Results.** Diabetic mice showed attenuated recovery of ischemic limb perfusion on laser-Doppler perfusion imaging compared with nondiabetic mice. The treatment with sRAGE significantly improved blood flow in the ischemic limbs of diabetic mice. The expression levels of VEGF and HMGB-1 in the limb muscle tissues of diabetic mice were lower than in those of nondiabetic mice. The treatment with sRAGE significantly increased the VEGF and HMGB-1 protein expression in the ischemic limb muscle tissues in the diabetic mice. **Conclusion.** The suppression of RAGE by sRAGE administration improved angiogenic response to ischemia in diabetic mice and was associated with increased HMGB-1 and VEGF levels in muscle tissues.

1. Introduction

Diabetic patients with peripheral artery disease are known to be at a higher risk of developing critical limb ischemia and undergoing limb amputation than nondiabetic patients [1]. In vivo animal experiments with hindlimb ischemia model showed that recovery of blood flow in ischemia-induced limb was slower in diabetic animals than nondiabetic animals [2, 3]. Angiogenic response to ischemia is generally considered to be impaired in diabetic patients [4–8]. Various pathomechanisms have been suggested for the impaired angiogenic response in diabetic patients such as endothelial dysfunction, decreased endothelial nitric oxide synthetase activity, attenuated function of endothelial progenitor cells

and bone marrow cells [4], chronic inflammation due to increased reactive oxygen species and advanced glycation end products (AGE), resistance to vascular endothelial growth factor (VEGF), and reduced growth factor levels in the ischemic limb [5, 6]. Chronic hyperglycemia in diabetes mellitus is known to result in formation of AGEs [7]. The accumulation of AGEs in the vessel wall has been implicated in the pathogenesis of diabetes complications [8]. The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules present on epithelial, neuronal, vascular, and inflammatory cells, usually expressed at low levels in homeostasis and to increased degrees at sites of stress or injury [9, 10]. Binding of AGE to RAGE in endothelial cells activates the

transcription factor nuclear factor- κ B (NF- κ B), subsequently leading to increased expression of proatherogenic mediators, such as monocyte chemoattractant protein-1 or vascular cell adhesion molecule-1 [8, 11, 12]. Whether activation of RAGE is pro- or anti-angiogenic is still controversial. Several studies demonstrated that the blockade of the AGE/RAGE system restored impaired angiogenic response to ischemia in diabetic animals [8, 13]. However, other studies showed that the activation of RAGE by AGE or HMGB-1, another ligand of RAGE, induced angiogenesis via increased VEGF production [14, 15]. Therefore, the purpose of the present study is to investigate the impact of RAGE suppression on the ischemia-induced angiogenesis in a diabetes mouse hindlimb ischemia model.

2. Materials and Methods

2.1. Experiment Design and Groups. All investigations were approved by Yonsei University College of Medicine institutional animal care and use committee. Male C57BL/6J mice (ORIENT Laboratory, Seongnam, Republic of Korea) aged 8 weeks old were used for experiments. To confirm the impaired angiogenic response to ischemia, diabetic ($n = 20$) and nondiabetic mice ($n = 20$) were used. To investigate the effect of RAGE suppression, diabetic and nondiabetic mice were treated either with sRAGE (diabetes $n = 20$; nondiabetes $n = 20$) or control (diabetes $n = 20$; nondiabetes $n = 20$).

2.2. Mouse Model of Diabetes. Diabetes was induced by intraperitoneal injections of 50 mg/kg body weight streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) in citrate buffer (0.1M, pH 4.5) during the fasting state for 5 days as previously described [16]. Diabetes was confirmed at 7 days after streptozotocin injection by an Accu-Chek Active glucometer (Roche, High Wycombe, Germany) using tail vein blood when blood glucose was over 16 mmol/L [17].

2.3. Hindlimb Ischemia Model. Induction of unilateral hindlimb ischemia was performed in both diabetic and nondiabetic mice at 2 weeks after the injection of streptozotocin as previously described [18]. Briefly, all animals were anesthetized with an intraperitoneal injection of tiletamine/zolazepam 0.05 mg/kg (Zoletile, Virbac, Carros Cedex, France) and xylazine 0.15 mL/kg (Rompen, Bayer, Leverkusen, Germany). The proximal and distal portions of the femoral artery and the distal portion of the saphenous artery were ligated. The arteries and all side branches were dissected free and excised.

2.4. Exogenous sRAGE Administration. To suppress RAGE system, a daily dose of sRAGE protein (A&R Therapeutics, Daejeon, Republic of Korea) 1 μ g/mL at a concentration of 0.1 μ g per mouse in 0.1 mL of saline was administered after femoral artery ligation for 14 days by intramuscular injection, directly into the ischemic and nonischemic limbs of diabetic and nondiabetic mice. A control group of diabetic mice received an intramuscular injection of 100 μ l of saline in the ischemic area. The sRAGE used for

our experiment was a purified mouse sRAGE-Fc fusion protein. Expression and purification of sRAGE-Fc fusion protein has been performed as previously reported [19]. For the sRAGE-Fc construction, a leader sequence (gene ID: K02149; protein: AAA51633), mouse IgG H chain (primer set: 5'-ATAGGCTAGCGCCACCATGGGATGG-3', 5'-TGTGTGAGTTTTGTCCGAGTGGACATCTGT-3'), a.a 23–341 of mouse sRAGE (primer set: 5'-GGTCAGAACATCACAGCCCGGATTG-3', 5'-TGTGTGAGTTTTGTCCCCCATGGGTGCAAGA-3'), and the human IgG1 Fc region (primer set: 5'-GGCTAGCGTACCCAGCCAGACTC-3', 5'-CCAGCTCGAGCTATTTACCCGGAGACAG-3') were amplified, and the overlap extension PCR was performed. To express the desired domain, PCR product was treated with SfiI and ligated into pYK 602-His vector (vector constructed by KRIBB). To express the mRAGE-Fc, mouse sRAGE-Fc was transfected into HEK293E cells and supernatants collected every other day. To purify, a protein A-Sepharose column (Amersham Biosciences, Piscataway, NJ, USA) was used according to the manufacturer's instructions. The purified recombinant sRAGE was dialyzed with PBS, analyzed by SDS-PAGE. After quantification, mRAGE was aliquoted and stored at -70°C for experiment. And analysis with the Limulus amoebocyte lysate test kit (Cape Cod, East Falmouth, MA, USA) was performed to examine the endotoxin level.

2.5. Laser-Doppler Perfusion Imaging. A laser-Doppler image system (Moor LDI2, Moor Instruments, Axminster, UK) was used to measure blood flow perfusion before and at 3, 7, and 14 days after femoral artery ligation. Before imaging, excess hairs were removed from the limbs using depilatory cream, and mice were placed on a temperature controller at 37°C . The results were expressed as the ratio between perfusion in the left (ischemic) and right (nonischemic) limb.

2.6. Measurement of Capillary Density. Anterior tibial muscle was harvested 14 days after induction of ischemia and frozen at -80°C in OCT compound. Cryosections (10 μ m) were prepared and incubated with anti-mouse CD31 rat antibody (dilution 1:100; polyclonal; Abcam, Cambridge, MA, USA) to identify endothelial cells. Capillary density was expressed as the ratio of CD31+ cells to myofibers. This measurement was determined in five randomly selected low-power (original magnification $\times 200$) fields from each animal, and the average value was used as a single data point for each mouse.

2.7. Western Blotting. For immunoblotting, homogenates of limb muscle tissues were analyzed. Equal amounts of proteins were loaded and separated in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against VEGF (1:1000; Abcam), HMGB-1 (1:1000; Abcam), and RAGE (1:1000; Abcam). VEGF, HMGB-1, and RAGE protein expression was normalized using a mouse monoclonal anti-GAPDH antibody (Santa Cruz biotechnology, Santa Cruz, CA, USA).

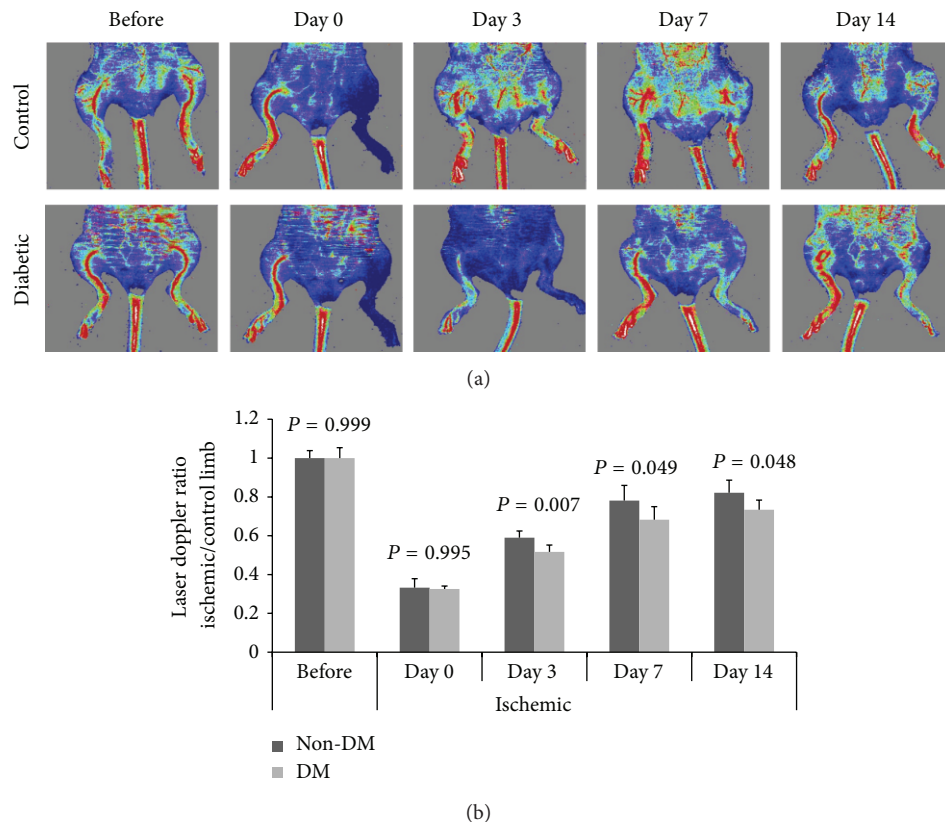


FIGURE 1: Limb perfusion in diabetic versus nondiabetic mice by laser-Doppler perfusion imaging. (a) Femoral artery blood flow by laser-Doppler perfusion imaging in sRAGE-treated versus nontreated diabetic mice before surgery and on days 3, 7, and 14 after surgery. Representative images of the ischemic (left) and nonischemic (right) hindlimbs. (b) Laser-Doppler ischemic/nonischemic limb perfusion ratios.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). Equal amounts of muscle homogenates were used for quantitative analysis of VEGF (R&D Systems Inc., Minneapolis, MN, USA), HMGB-1 (USCNK Life Sciences, Inc., Wuhan, China), and RAGE (USCNK) proteins using commercially available ELISA kits according to the manufacturer's instructions.

2.9. Statistical Analysis. Statistical analysis was performed using SPSS (version 18.0, SPSS, Inc., Chicago, IL, USA). All results are expressed as means \pm standard deviation. Comparisons of continuous variables among the groups were performed using Student's *t* test or ANOVA. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Recovery of Blood Flow by Laser-Doppler Perfusion Imaging: Diabetic versus Nondiabetic Mice. Immediately after the femoral artery ligation, the blood flow was equally reduced in the ischemic limbs of both diabetic and nondiabetic mice (Figure 1). The blood flow ratio ischemic/control limb by laser-Doppler was gradually recovered from the postoperative day 3 on in both groups. However, the blood flow recovery was significantly attenuated in diabetic mice

compared with nondiabetic mice on postoperative days 3, 7, and 14.

3.2. Recovery of Blood Flow by Laser-Doppler Perfusion Imaging: sRAGE versus Saline. Immediately after the femoral artery ligation, the blood flow was equally reduced in both sRAGE-treated and control groups (Figure 2). The blood flow ratio ischemic/control limb by laser-Doppler showed gradual recovery in the ischemic limbs from the postoperative day 3 on in both groups. However, the blood flow ratio was significantly higher in the sRAGE-treated mouse group than in control group on postoperative days 3, 7, and 14.

3.3. Capillary Density in the Ischemic Muscle Tissues of sRAGE-Treated versus Control Diabetic Mice. Compared to nondiabetic mice, diabetic mice showed a significantly decreased capillary density in the ischemic limb muscle tissue (Figure 3). The diabetic mice treated with sRAGE showed a significantly higher capillary density compared with the diabetic mice treated with saline.

3.4. Protein Expression of VEGF, RAGE, and HMGB-1 in Hindlimb Muscle. Western blotting shows decreased protein expression level of VEGF in diabetic ischemic limb

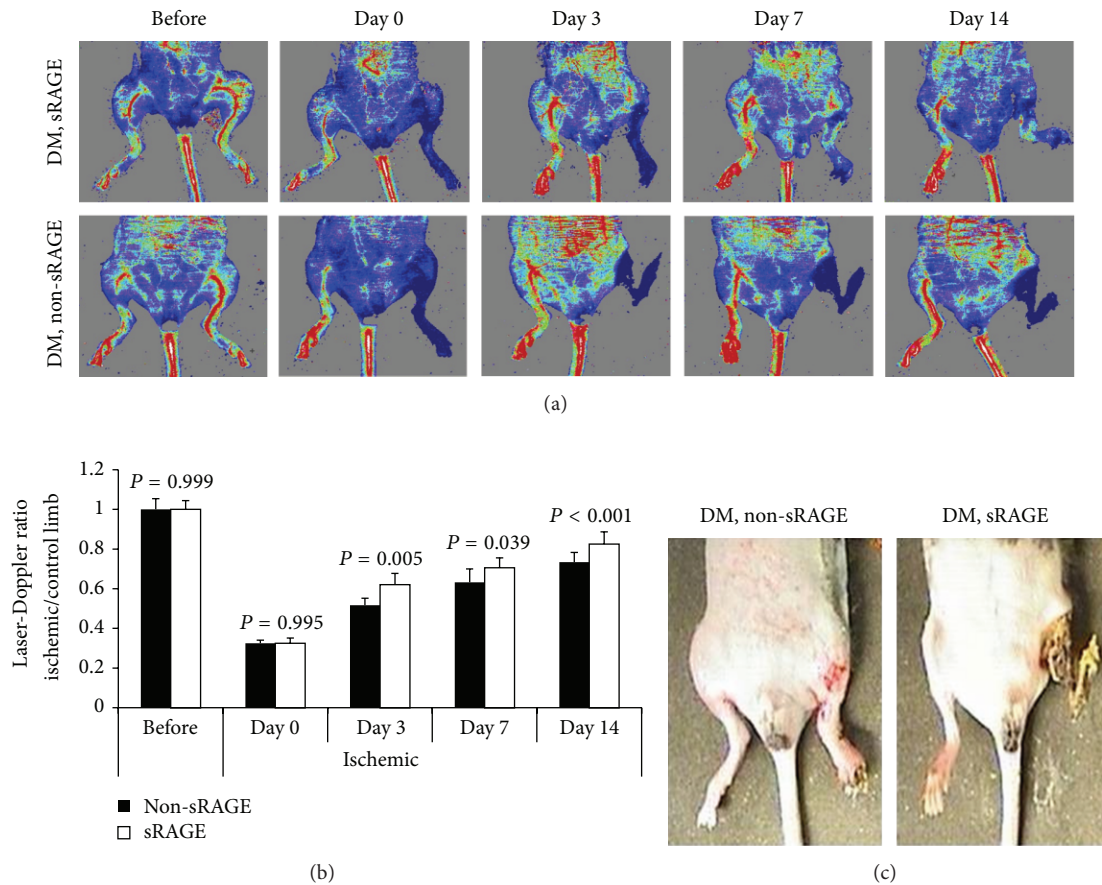


FIGURE 2: Perfusion in the ischemic limbs of the sRAGE-treated versus nontreated diabetic mice by laser-Doppler perfusion imaging. (a) Femoral artery blood flow by laser-Doppler perfusion imaging in sRAGE-treated versus nontreated diabetic mice before surgery and on days 3, 7, and 14 after surgery. Representative images of the ischemic (left) and nonischemic (right) hindlimbs. (b) Laser-Doppler ischemic/nonischemic limb perfusion ratios. (c) Representative photos of sRAGE-treated (left) and control diabetic mice (right).

compared to nonischemic limbs of nondiabetic or diabetic mice (Figure 4(a)). The treatment with sRAGE significantly increased VEGF protein expression in the ischemic limb of diabetic mice. Similar findings were also observed in ELISA data for muscle tissue VEGF protein expression (Figure 4(b)). Western blotting and ELISA data demonstrated that the RAGE protein expression level was significantly increased in limb muscle tissue of diabetic mice compared to those of nondiabetic mice (Figures 4(c) and 4(d)). Compared with nonischemic limbs, ischemic limbs of diabetic mice showed significantly higher RAGE protein expression. The treatment with sRAGE significantly decreased RAGE expression in the ischemic limb of diabetic mice. Western blotting and ELISA data showed that the HMGB-1 protein expression level was significantly increased in limb muscle tissues of diabetic mice compared with those of nondiabetic mice (Figures 4(e) and 4(f)). Compared with nonischemic limbs, ischemic limbs of diabetic mice showed significantly higher HMGB-1 protein expression. The treatment with sRAGE significantly increased HMGB-1 expression in the ischemic limb of diabetic mice.

4. Discussion

The main findings of the present study are summarized as follows. (1) The recovery of ischemic limb perfusion was attenuated in diabetic mice compared with nondiabetic mice. (2) The treatment with sRAGE significantly improved blood flow in the ischemic limbs of diabetic mice. (3) The VEGF and HMGB-1 expression levels in the limb muscle tissues of diabetic mice were lower than in those of nondiabetic mice. (4) The treatment with sRAGE increased the VEGF and HMGB-1 protein expression in the ischemic limb muscle tissues of the diabetic mice. (5) The treatment with sRAGE decreased RAGE protein expression in the ischemic limb of diabetic mice. Impaired angiogenic response to ischemia is generally considered as an important factor contributing to increased risk of critical limb ischemia and amputations in diabetic patients [20]. Various studies suggested that activation of RAGE by AGE is involved in the pathomechanisms of impaired angiogenesis in diabetic subjects [8, 13, 21]. Tamarat et al. [22] demonstrated that blockade of AGE by aminoguanidine normalized ischemia-induced angiogenesis

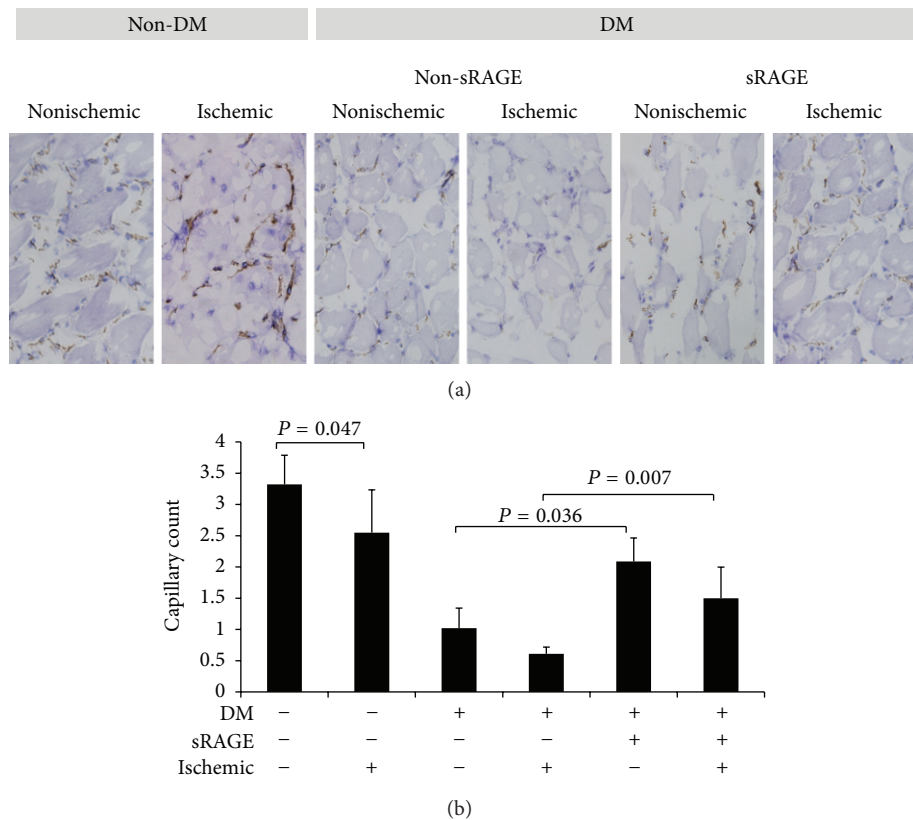


FIGURE 3: Capillary density in the muscle tissue. (a) Representative microscopic images of muscle tissues stained with antibody directed against CD31 14 days after surgery. Magnification $\times 200$. (b) Capillary density in the muscle tissue from ischemic and nonischemic limbs of nondiabetic, diabetic sRAGE-treated, and diabetic control-treated mice.

in diabetic mouse hindlimb ischemia model. Shoji et al. [8] showed that adenovirus-induced overexpression of sRAGE which acts as a decoy receptor for RAGE and thereby inhibits RAGE activation improved angiogenic response to matrigel implantation in diabetic mice. By contrast, Yamagishi et al. [14] demonstrated in an in vitro study that AGE can elicit angiogenesis through the induction of autocrine vascular VEGF. Biscetti et al. [15] reported that the expression level of HMGB-1, another ligand of RAGE, was decreased in a hindlimb ischemia diabetes mouse model and that administration of HMGB-1 improved angiogenesis in the ischemic limb. Therefore, it is uncertain whether the activation of RAGE in diabetes patients suppresses or activates the angiogenic response to ischemia. In our opinion, the results of our study are compatible with the findings of Shoji et al. [8] The method of RAGE suppression and the model of angiogenesis were different in our study. However, the suppression of RAGE system by administration of sRAGE also significantly improved blood flow in ischemic limbs of diabetic mice. The treatment of sRAGE was also associated with increased expression of VEGF and HMGB-1 in muscle tissues of the ischemic limbs. This observation also at least partially supports the study results of Biscetti et al. [15] that increment of HMGB-1 expression leads to increased angiogenic response by VEGF-dependent pathway. However, the precise molecular signal pathway from RAGE suppression

to increased VEGF expression still remains unknown. An important limitation of the present study is that sRAGE did not specifically inhibit RAGE pathway alone, but had also suppressive effects on toll-like receptor and other receptor signalings to capture various bioactive molecules of RAGE ligands including HMGB1, S100 proteins, lipopolysaccharides (LPS), advanced oxidation protein products (AOPP), lysophosphatidic acid (LPA), and advanced glycation end products (AGE). Therefore, RAGE knockout model would have been more desirable. Further studies are required to investigate the molecular mechanism of enhanced angiogenesis by RAGE suppression.

5. Conclusion

The suppression of RAGE by administration of sRAGE improved angiogenic response to ischemia in diabetic mice and was associated with increased HMGB-1 and VEGF levels in muscle tissues. However, the precise mechanisms of improved angiogenesis by RAGE suppression need to be investigated in further studies.

Conflict of Interests

There are no financial or other dealings that could lead to a conflict of interest.

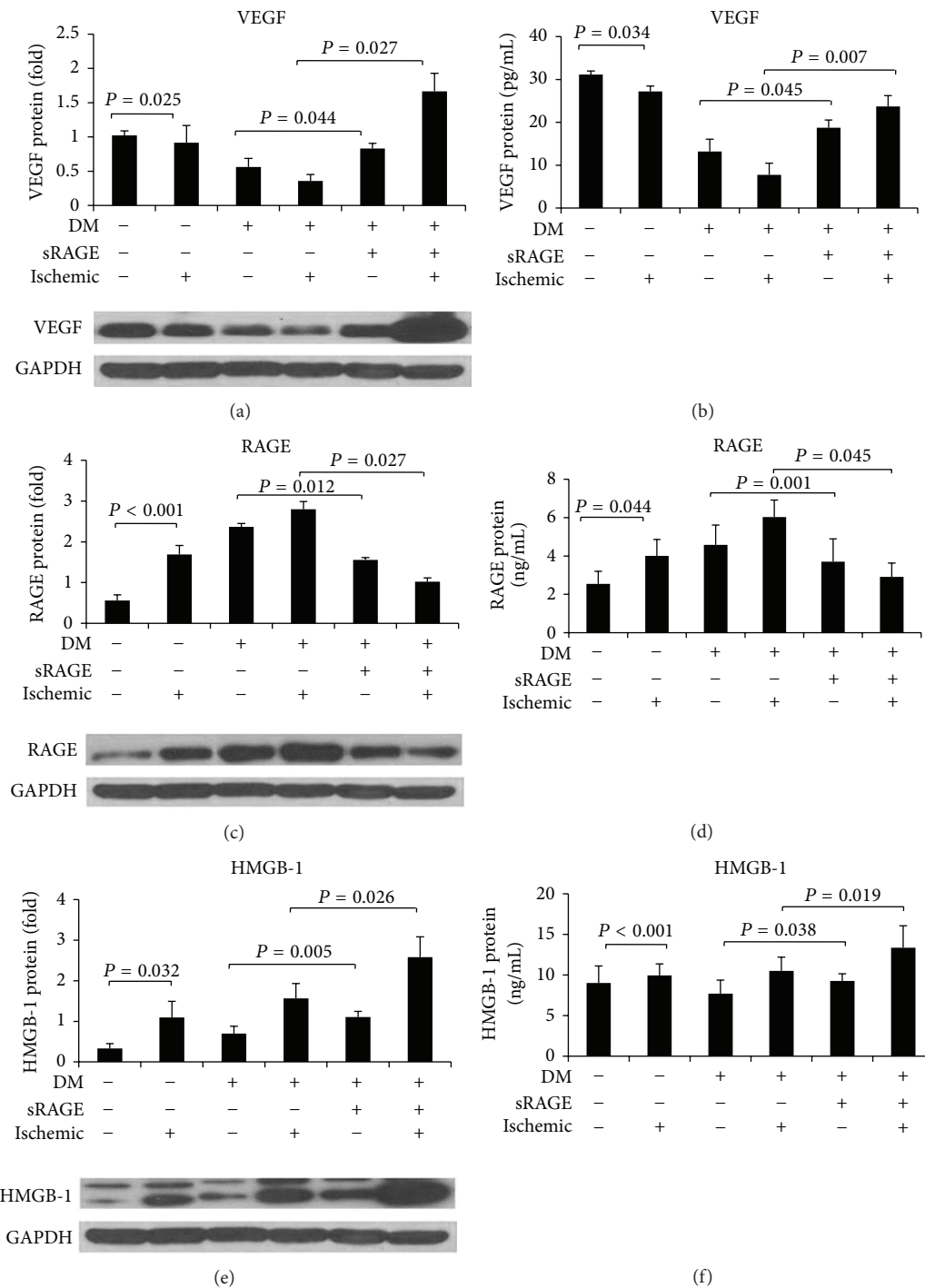


FIGURE 4: The protein expression levels of VEGF, RAGE, and HMGB-1 in hindlimb muscle tissues. (a) Representative Western blot of VEGF protein level in the ischemic gastrocnemius muscle tissues of control, diabetic mice and sRAGE-treated diabetic mice on postoperative day 14. (b) VEGF protein level in the ischemic gastrocnemius muscle tissues by ELISA. (c) Representative Western blot of RAGE protein level in the ischemic gastrocnemius muscle tissues of control, diabetic mice and sRAGE-treated diabetic mice on postoperative day 14. (d) RAGE protein level in the ischemic gastrocnemius muscle tissues by ELISA. (e) Representative Western blot of HMGB-1 protein level in the ischemic gastrocnemius muscle tissues of control, diabetic mice and sRAGE-treated diabetic mice on postoperative day 14. (f) HMGB-1 protein level in the ischemic gastrocnemius muscle tissues by ELISA.

Acknowledgment

This study was supported partly by Grants from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare, & Family Affairs, Republic of Korea (nos. A110879, A120478, and A085136).

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