Low-energy extracorporeal shock wave therapy enhances skin wound healing in diabetic mice: A critical role of endothelial nitric oxide synthase

Denso Hayashi, MD[1]; Kazuyoshi Kawakami, PhD, MD[2]; Kenta Ito, PhD, MD[3]; Keiko Ishii, PhD[2]; Hiromasa Tanno, BN[1]; Yoshimichi Imai, PhD, MD[1]; Emi Kanno, PhD, RN[4]; Ryoko Maruyama, PhD, RN[4]; Hiroaki Shimokawa, PhD, MD[3]; Masahiro Tachi, PhD, MD[1]

1. Department of Plastic and Reconstructive Surgery,
2. Department of Medical Microbiology, Mycology and Immunology,
3. Department of Cardiovascular Medicine, and
4. Department of Science of Nursing Practice, Tohoku University Graduate School of Medicine, Sendai, Japan

Reprint requests:
Dr. Denso Hayashi, Department of Plastic and Reconstructive Surgery, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai-shi, Miyagi 980-8575, Japan.
Tel: +81-22-717-7332;
Fax: +81-22-717-7332;
Email: tonosato@med.tohoku.ac.jp

Manuscript received: February 6, 2012
Accepted in final form: July 19, 2012

DOI:10.1111/j.1524-475X.2012.00851.x

ABSTRACT

Low-energy extracorporeal shock wave (LE-ESW) treatment has been shown to accelerate wound repair; however, the mechanisms of treatment remain unclear. In the present study, we addressed the role of endothelial nitric oxide synthase (eNOS). A single LE-ESW treatment accelerated the healing of wounds in diabetic mice caused by the injection of streptozotocin. This accelerated healing was accompanied by the increased expression of eNOS and vascular endothelial growth factor (VEGF) and the generation of new vessels at the wound tissues. These results raised the possibility that eNOS may be involved in the beneficial effects of LE-ESW treatment. To address this possibility, we compared the effects of this treatment between mice with a genetic disruption of eNOS knockout (eNOS-KO mice) and wild-type (WT) control mice. Interestingly, the LE-ESW-induced acceleration of wound closure and the increase in VEGF expression and neovascularization was significantly attenuated in eNOS-KO mice compared with WT mice. Considered collectively, these results showed that eNOS was induced at the wound tissues by LE-ESW treatment and played a critical role in the therapeutic effects of this treatment by accelerating the wound healing by promoting VEGF expression and neovascularization.

Wound healing plays an important role in protecting the human body from outside threats. Impairment of this process results in the formation of refractory skin ulcers, including diabetic ulcer, decubitus and ulcers in collagen diseases, and vacuities syndrome.[1] These conditions increase the chance of infection and also seriously deteriorate the quality of life.[2] Ischemic ulcers that arise as complications of diabetes mellitus, obliterating arteriosclerosis, and collagen diseases including rheumatoid arthritis are frequently resistant to routine therapy because of poor blood supply to wound tissues and impaired neovascularization.[3] Thus, improvement of the blood supply is important for the improvement of refractory skin ulcers.

Almost 30 years ago, extracorporeal shock wave (ESW) therapy was successfully introduced in urology and gastroenterology as lithotripsy.[4,5] Recently, ESW therapy has been adapted and applied in different clinical fields for the treatment of bone nonunions, plantar fasciitis, epicondylitis of the elbow, and calcifying or noncalcifying tendonitis,[6,7] although it is used in a low-energy (LE) condition compared with lithotripsy. We have reported that LE-ESW treatment effectively improves myocardial ischemia in patients with severe angina pectoris and ameliorates walking ability in those with peripheral artery disease and intermittent claudication.[8,9] The cascades of biological processes elicited by LE-ESW are directly correlated with enhanced blood supply and tissue regeneration.[10,11] In a recent study by Stojadinovic et al., LE-ESW treatment enhanced graft acceptance in murine skin isografts by inducing anti-inflammatory responses and by promoting endothelial cell proliferation and neovascularization.[12] Furthermore, the clinical applications of LE-ESW treatment for the treatment of wounds have been recently described. Schaden et al. demonstrated that LE-ESW treatment is feasible and well-tolerated by patients with acute and chronic wounds.[13] However, the precise mechanisms for the beneficial effects of LE-ESW treatment remain unclear.

Nitric oxide (NO), a free radical that mediates diverse functions in a range of biological responses,[14] is produced by a family of NO synthase (NOS) enzymes.[15] Three NOS isoforms have been distinguished in mammalian species: neuronal NOS, inducible NOS, and endothelial NOS (eNOS).[16] In the vasculature, NO is mostly generated by eNOS.[17] The generated NO plays a crucial role in maintaining vascular homeostasis, including vascular tone, leukocyte adhesion to the endothelium, proliferation of vascular smooth muscle cells, and platelet aggregation and adhesion to the vessel walls.[18,19] Diabetic microangiopathy and macroangiopathy, including foot ulcer and accelerated atherosclerosis, are the main causes of morbidity and mortality in patients with diabetes mellitus.[20] A hallmark of diabetic vascular

complications is endothelial cell dysfunction, which is characterized by the lowered production of NO, including impaired endothelium-dependent vasorelaxation, enhanced leukocyte–endothelial interactions, and thrombosis.[21,22] A large body of evidence has implicated the dysfunctional eNOS and decreased NO availability in endothelium as a major pathogenic mechanism in diabetic vascular complications in humans and diabetic animals.[23]

In the present study, therefore, we induced diabetes mellitus in eNOS knockout (KO) and wild-type (WT) mice with a C57BL/6 background by injecting low-dose streptozotocin (STZ), and we used the dorsal skin defect to evaluate wound healing in diabetic mice. The effects of LE-ESW treatment on the wound healing processes were examined. Here, we attempted to determine whether LE-ESW treatment accelerated wound healing and how eNOS is involved.

MATERIALS AND METHODS

Animals

eNOS-KO mice were established by Huang and coworkers[24] and back-crossed over eight generations with C57BL/6 mice. These mice were bred in a pathogen-free environment in the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine. C57BL/6 mice obtained from Clea Japan, Inc. (Tokyo, Japan) were used as WT control mice. The mice were subjected to experiments at 7 to 8 weeks of age. To minimize possible discomfort to the animals, m-acetamidophenol was mixed with drinking water at 0.25 mg/mL after wounding. The animals were housed in individual cages during the experimental period. All experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Tohoku University.

Induction of diabetes mellitus

Diabetic mice were established by intraperitoneal injection of 150 mg/kg STZ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) dissolved in 5 μM sodium citrate buffer (pH 4.5) after one night starvation. Control mice received injections of equivalent volumes of sodium citrate buffer. Fasting blood glucose levels in diabetic mice were elevated to more than 33.3 mM on day 14 posttreatment, and this condition continued for more than 5 weeks.

Wound creation and tissue collection

The diabetic mice were anesthetized by intraperitoneal injection of 40 mg/kg sodium pentobarbital (Nembutal injection, Dainippon Sumitomo Pharma, Osaka, Japan). The dorsal hairs were shaved to fully expose the skin, which was then rinsed with 70% ethanol. A full-thickness wound was created by a sterile biopsy punch with a diameter of 8 mm (Biopsy Punch, Kai industries Co., Ltd, Gifu, Japan) on the dorsal region, which was 1 cm away from the spinal column in the WT and eNOS-KO mice. The wounds were covered with a polyurethane film (Tegaderm Transparent Dressing, 3 M Health Care, St. Paul, MN), and an elastic adhesive bandage (Hilate, Iwatsuki, Tokyo, Japan) was applied to avoid self-mutilation. At various time points, the mice were sacrificed by intraperitoneal injection of Nembutal, and wound tissues were removed to analyze the histological findings and evaluate the expression of eNOS and vascular endothelial growth factor (VEGF).

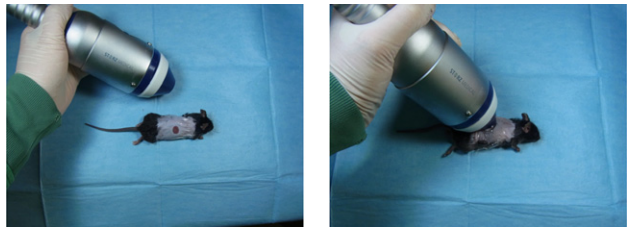

Figure 1. Low-energy extracorporeal shock wave (LE-ESW) treatment. Full-thickness wound was created by a biopsy punch on the dorsal region, which was 1 cm away from the spinal column in wild-type (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) mice. The wounds were covered with a polyurethane film. The shock waves were produced by Duolith SD-1 (Storz Medical, Tagerwilen, Switzerland). We exposed the wounds directly to LE-ESW through the gel.

Application of LE-ESW therapy and device parameters

The shock waves were produced by Duolith SD-1 (Storz Medical, Tagerwilen, Switzerland). LE-ESW treatment was applied according to the following parameters: Duolith SD-1 with a probe corresponding to a depth of entrance of 50 mm and a surface focus of 5 mm × 30 mm, energy flow density per shot set at 0.25 mJ/mm^2 and 4 Hz frequency. The skin wounds were treated with 100 shots onto the surface of 4 cm^2 per side. We exposed the wounds directly to LE-ESW as shown in Figure 1.

Morphometric analysis of wound healing

The process of wound healing was macroscopically monitored by taking digital photographs. When the wounds were created, photographs were taken before dressing. At various time points, the polyurethane film was gently removed in the sacrificed mice, and the wounds were photographed. The wound areas were measured by tracing the margin and calculating the pixel area using AxioVision imaging software Release 4.6 (Carl Zeiss Micro Imaging Japan, Tokyo, Japan). Wound healing was evaluated as percent wound closure, which was calculated by the following formula: % wound closure = (wound area at the indicated time point/wound area on day 0) × 100.

Histology and immunohistochemistry

The resected specimens were fixed with a 4% paraformaldehyde–phosphate buffer solution and embedded in paraffin. Three-micrometer-thick sections from the central portion of each wound were stained with hematoxylin and

eosin (HE) according to the standard method and then subjected to microscopic observation. When the wounds of each group were completely reepithelialized, the height of the dermis (HD) in the center of the wounds and the width of the middle dermis (WD) in the wounds were determined on the HE-stained sections to evaluate wound contraction and scar formation, as previously described.[25]

For immunohistochemistry, the sections were stained with anti-cluster of differentiation (CD)31 Ab (1 : 600 dilution; Santa Cruz Biotechnology, Inc.), CD34 related antigen (1 : 100 dilution; Becton Dickinson and Company, Franklin Lakes, NJ), anti-α-smooth muscle actin (α-SMA) Ab (1 : 200 dilution; Vector Laboratories, Inc., Burlingame, CA), anti-eNOS Ab (1 : 100 dilution; Santa Cruz Biotechnology, Inc.), or anti-VEGF Ab (1 : 1,000 dilution; Santa Cruz Biotechnology, Inc.) after endogenous peroxidase and nonspecific binding were blocked. They were then incubated with peroxidase-conjugated secondary antibodies (Histofine Simple Stain MAX PO, Nichirei Bioscience, Tokyo, Japan). Control sections were treated with nonimmune IgG in place of any of the first antibodies. To evaluate the formation of new blood vessels, the vascular density in the granulation tissues at four random fields was determined by counting the numbers of CD31- and CD34-positive vessels per square millimeter. The eNOS-positive vessels were defined as those in which more than 10% of endothelial cells were stained with Ab against this molecule. The incidence of eNOS expression at wound tissues was expressed as a mean ratio of the eNOS-positive vessels to the total ones detected in four randomly selected fields at 400× magnification under microscopic observation.

Reverse transcription-polymerase chain reaction (PCR)

Total ribonucleic acid (RNA) was extracted from wound tissues using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. The RNA was reverse-transcribed using Oligo (dT) and Superscript RT II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). The obtained complementary deoxyribonucleic acid (cDNA) was then amplified using an automatic DNA thermal cycler (PerkinElmer, GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) using specific primers 5′-AAG ACA AGG CAG CGG TGG AA-3′ (sense) and 5′-GCA GGG GAC AGG AAA TAG TT-3′ (antisense) for eNOS, 5′-TGA ACT TTC TGC TCT CTT GG-3′ (sense), 5′-AAC AAA TGC TTT CTC CGC TC-3′ (antisense) for VEGF, and 5′-GTT GGA TAC AGG CCA AGA CTT TGT TG-3′ (sense), 5′-GAT TCA ACT TGC GCT CAT CTT AGG C-3′ (antisense) for hypoxanthine phosphoribosyl transferase (HPRT). We added 1.0 μL of the sample cDNA solution to 49 μL of the reaction mixture, which was composed of the following constituents: 10 mM Tris–HCl (pH = 8.3), 50 mM KCl, 1.5 mM MgCl2, 10 μg/mL gelatin, deoxyribonucleotide triphosphate (dNTP) (each at a concentration of 200 μM), 1.0 μM sense and antisense primer, and one unit of Takara EX Taq (Takara Bio Inc., Shiga, Japan). PCR was conducted under a condition of 1 minute at 94 °C, and then cycles of 30 seconds at 94 °C, 30 seconds at 61 °C, and 45 seconds at 72 °C, and finally 7 minutes at 72 °C. The number of cycles was 40, 40, and 26 for eNOS, VEGF, and HPRT, respectively. The PCR products were electrophoresed on 1.5% agarose gels, stained with 0.5 μg/mL ethidium bromide, and observed using an ultraviolet transilluminator.

Western blotting

Frozen skin wound tissues were weighed and homogenized at 10 mg/50 μL in a lysis buffer (2,000 μL Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, and 20 μL Protease Inhibitor Cocktail Set III, Calbiochem, Darmstadt, Germany). The protein concentration was determined using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). The lysates added to the 60 μL electrophoresis sample buffer containing 855 μL of laemmli buffer and 45 μL of 5% 2-mercaptoethanol (Bio-Rad Laboratories, Berkeley, CA) were boiled for 4 minutes at 95 °C. Equal sample amounts were electrophoresed on 4 to 15% polyacrylamide gels and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Nonspecific binding was blocked by immersion of the membranes for 60 minutes in 5% skim milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween at room temperature. The membranes were washed with the same buffer and incubated at 1 : 1,000 with anti-VEGF Ab or 1 : 200 with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ab (Santa Cruz Biotechnology, Inc.) for 2 hours at room temperature, followed by washing and incubation at 1 : 3,000 with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Freiburg, Germany) for 60 minutes at room temperature. Then chemiluminescence catalyzed by HRP was detected using a nonradioactive light-emitting system with Western Lightning Plus-ECL (PerkinElmer Life Sciences, Waltham, MA), and the positive signals were quantified using a LAS4000 chemiluminescence detector (Fuji Photo Film, Tokyo, Japan), and the positive signals were quantified. For quantification of Western blots, densitometry was used. Western blot pictures were analyzed with Image J software (National Institutes of Health, Bethesda, MD) in accordance with the Web site tutorial (see http://rsb.info.nih.gov/ij/).

Statistical analysis

Data were expressed as mean ±standard deviation. Statistical analysis between groups was performed using Student's *t*-test. Statistical analysis on the obtained density values was carried out with Microsoft Excel and the Graph Pad Prism program (Graphpad Software, Inc., San Diego, CA). A *p*-value of less than 0.05 was considered to indicate significance.

RESULTS

Role of eNOS in LE-ESW–induced promotion of wound healing

To clarify the effect of LE-ESW treatment on wound healing, we created full-thickness wounds on the dorsal skin of the diabetic WT and eNOS-KO mice, which did or did not undergo LE-ESW treatment. As shown in Figure 2A, there was not much difference in the process of wound closure between these two mouse groups in the absence of

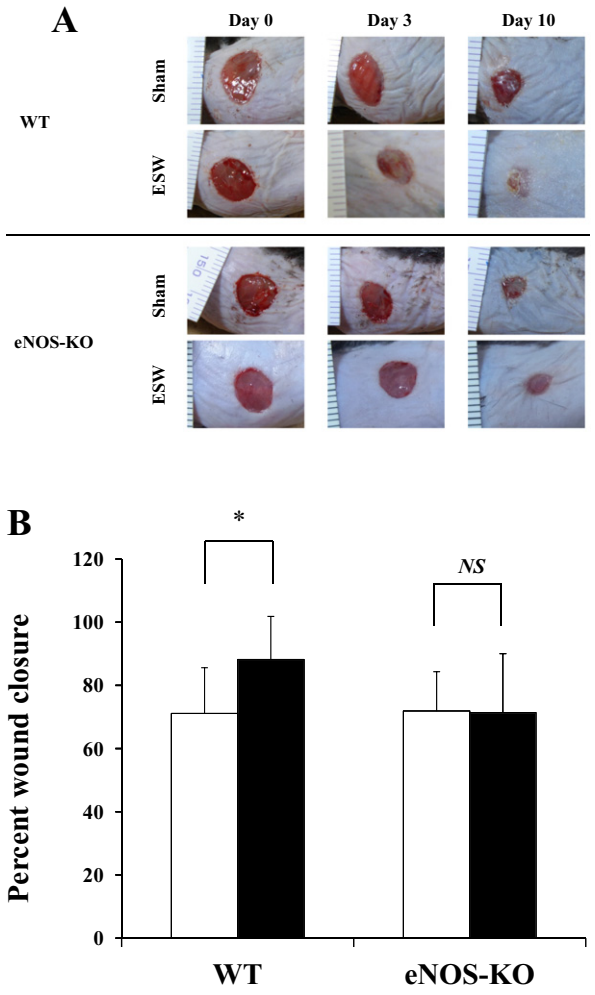

Figure 2. Skin wound healing in wild-type (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) mice. Full-thickness wounds were made with a punch biopsy instrument in WT and eNOS-KO diabetic mice. (A) The wound healing process was monitored on days 0, 3, and 10 after low-energy extracorporeal shock wave (LE-ESW) or sham treatment by taking digital photographs. Pictures are shown for each day. Note the promotion of wound healing in ESW therapy-treated WT mice. (B) The percentages of wound closure are calculated from the photographs ($n = 11$ and 8 in LE-ESW-treated and sham-treated WT mice, respectively; $n = 7$ and 6 for in LE-ESW-treated and sham-treated eNOS-KO mice, respectively). The data are expressed as mean ± standard deviation. Closed column, LE-ESW-treated; ESW, LE-ESW-treated; open column, sham-treated; sham, sham-treated; $*p < 0.05$; *NS*, not significant.

LE-ESW treatment. In contrast, the wound closure was accelerated in the LE-ESW-treated WT mice compared with that in the untreated WT mice, whereas this effect was not clear in the eNOS-KO mice. To quantitatively evaluate these findings, the wound area was measured, and the percent wound closure relative to that on day 0 was shown on day 10 after LE-ESW treatment and after sham treatment. As shown in Figure 2B, this value was significantly increased in the LE-ESW-treated WT mice compared with that in the untreated WT mice (88.2 ± 14.5% vs. 71.1 ± 13.6% in the LE-ESW-treated [$n = 11$] and the untreated WT mice [$n = 8$], respectively), whereas this effect was not observed in the eNOS-KO mice (71.4 ± 12.4% vs. 71.9 ± 18.6% in the LE-ESW-treated [$n = 7$] and the untreated eNOS-KO mice [$n = 6$], respectively). These results indicated that LE-ESW treatment was effective in accelerating wound healing in diabetic mice and that this effect required the expression of eNOS.

Histological analyses of wound granulation and myofibroblast accumulation

To evaluate the effect of LE-ESW treatment on wound contraction and scar formation, we histologically analyzed the HE-stained sections on day 10 after LE-ESW or sham treatment, when the wounds were completely reepithelialized, in the WT and the eNOS-KO mice. When HD and WD were compared (Figure 3A), HD was significantly increased and WD was significantly decreased in the LE-ESW-treated WT mice compared with the untreated WT mice (HD, 310.7 ± 116.4 μm vs. 172.6 ± 85.8 μm; WD, 2639.1 ± 1334.2 μm vs. 4226.9 ± 1225.8 μm in the LE-ESW-treated [$n = 11$] and the untreated WT mice [$n = 8$], respectively) (Figure 3B). In contrast, both HD and WD were not significantly different between the LE-ESW-treated and the untreated eNOS-KO mice (HD, 139.5 ± 30.7 μm vs. 136.4 ± 50.5 μm; WD, 4213.9 ± 765.0 μm vs. 4492.9 ± 730.1 μm in the LE-ESW-treated [$n = 7$] and the untreated eNOS-KO mice [$n = 6$], respectively) (Figure 3B). These results suggested that LE-ESW treatment did not affect wound contraction and scar formation in the eNOS-KO mice.

In alternate experiments, we evaluated wound contraction and scar formation by detecting the α-SMA-expressing myofibroblasts, which play an important role in this process,[26] using immunohistochemical analysis. As shown in Figure 4, α-SMA-positive myofibroblasts formed elongated masses located on the edges of dermal wounds, and the areas with myofibroblast accumulation were much more pronounced in LE-ESW-treated WT mice than those in untreated WT mice. In striking contrast, the α-SMA-positive areas did not differ a great deal between the LE-ESW-treated and the untreated eNOS-KO mice (Figure 4).

Enhanced expression of eNOS in wound tissues by LE-ESW treatment

All of the results thus far have indicated that LE-ESW treatment accelerates the wound healing process in diabetic mice and is totally dependent on eNOS. This suggests that these effects are associated with the induction of eNOS expression in wound tissues. To address this possibility, we examined the expression of eNOS in wound tissues at the messenger RNA (mRNA) and protein levels using reverse transcription (RT)-PCR and immunohistochemical analyses, respectively. As shown in Figure 5A, eNOS mRNA was increased in the LE-ESW-treated WT mice compared with that in the untreated WT mice. In line with these results, in an immunohistochemi-

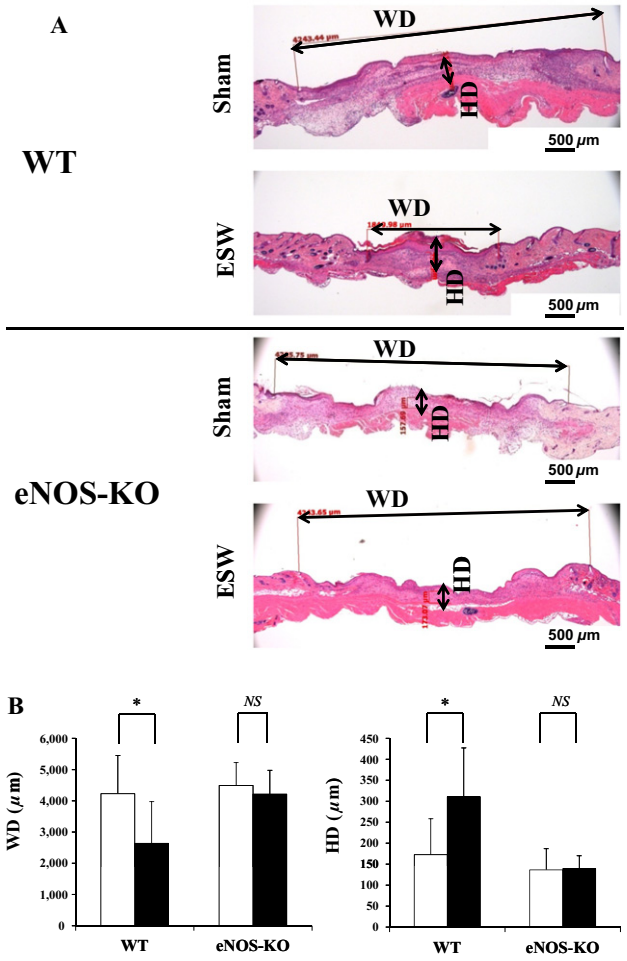

Figure 3. Histological evaluation of skin wounds in wild-type (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) mice. Full-thickness wounds were made with a punch biopsy instrument in WT and eNOS-KO diabetic mice. (A) Sections of skin wounds on day 10 after low-energy extracorporeal shock wave (LE-ESW) or sham treatment were stained with hematoxylin and eosin. Representative pictures are shown with indications of the height of the dermis in the center of the wound (HD) and the width of the middle dermis in the wound (WD). Scale bar represents 500 μm. (B) Evaluation of HD and WD in skin wounds is shown ($n = 11$ and 8 in LE-ESW-treated and sham-treated WT mice, respectively; $n = 7$ and 6 for in LE-ESW-treated and sham-treated eNOS-KO mice, respectively). The data are expressed as mean ± standard deviation. Open column, sham-treated; closed column, LE-ESW-treated. Sham, sham-treated; ESW, LE-ESW-treated. *$p < 0.05$; *NS*, not significant.

cal analysis, eNOS protein was more profoundly expressed in the vascular endothelial cells, identified as $CD31^+$ cells, in the LE-ESW-treated WT mice than in the untreated WT mice (WT; 65.9 ± 10.6 vs. 50.1 ± 11.0 count/mm^2 in the LE-ESW-treated [$n = 4$] and the untreated WT mice [$n = 4$], respectively; eNOS-KO; not tested) (Figure 5B and C).

Angiogenesis in the skin wound tissues

Angiogenesis in the wound tissues in WT and eNOS-KO mice was analyzed by evaluating the vascular density in the sections stained with anti-CD31 or anti-CD34, which was used to localize or confirm the areas of angiogenesis, respectively. The number of blood vessels in the wound tissues was counted on day 7 after LE-ESW or sham treatment in WT or eNOS-KO mice. As shown in Figure 6A and B, the vascular densities were significantly higher in the LE-ESW-treated WT mice than those in the untreated WT mice, whereas this difference was not observed in the eNOS-KO mice in the sections stained with anti-CD31 Ab (18.9 ± 7.4 count/mm^2 vs. 10.5 ± 4.8 count/mm^2 in the LE-ESW-treated [$n = 5$] and the untreated WT mice [$n = 5$], respectively; 14.5 ± 4.5 count/mm^2 vs. 13.6 ± 6.4 count/mm^2 in the LE-ESW-treated [$n = 6$] and the untreated eNOS-KO mice [$n = 5$], respectively). The results with anti-CD34 Ab were consistent with those with anti-CD31 Ab (16.6 ± 1.4 count/mm^2 vs. 9.8 ± 5.2 count/mm^2 in the LE-ESW-treated [$n = 5$] and the untreated WT mice [$n = 5$], respectively; 9.6 ± 3.5 count/mm^2 vs. 10.0 ± 4.0 count/mm^2 in the LE-ESW-treated [$n = 6$] and the untreated eNOS-KO mice [$n = 5$], respectively). These results indicated that eNOS was essential for the angiogenesis in wound tissues caused by LE-ESW treatment and suggested that LE-ESW-induced eNOS expression may be involved in the expression of VEGF, which plays a central role in the generation of new vessels.[27]

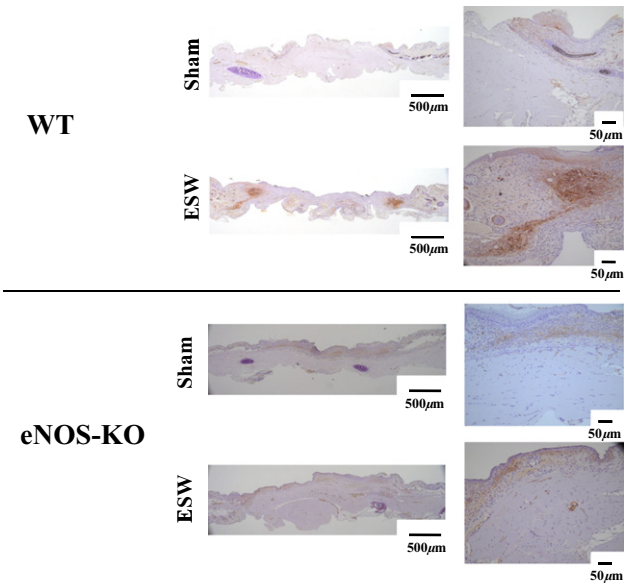

Figure 4. Accumulation of myofibroblasts in dermal area. Full-thickness wounds were made with a punch biopsy instrument in wild-type (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) diabetic mice. Sections of skin wounds on day 7 after low-energy extracorporeal shock wave (LE-ESW) or sham treatment were stained with anti-α-smooth muscle actin Ab for detection of myofibroblasts ($n = 3$ in each group). ESW, LE-ESW-treated; sham, sham-treated.

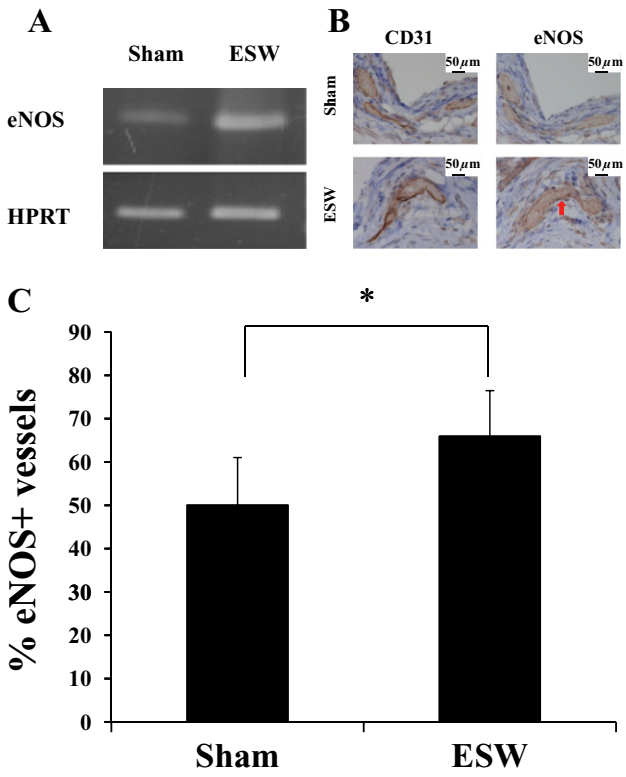

Figure 5. Expression of endothelial nitric oxide synthase (eNOS) in the wound tissues by low-energy extracorporeal shock wave (LE-ESW) treatment. Full-thickness wounds were made with a punch biopsy instrument in diabetic wild-type (WT) mice. (A) Reverse transcription polymerase chain reaction was performed for detection of eNOS messenger ribonucleic acid (mRNA) using total RNA isolated from wound tissues on day 3 after LE-ESW or sham treatment ($n = 3$ per each group). Hypoxanthine phosphoribosyl transferase (HPRT) was used as an internal control. (B) Immunohistochemical analysis was performed to assess the expression of eNOS on the vascular endothelia cells, detected as CD31+ cells, in the wound tissues. Sections of skin wounds on day 5 after LE-ESW or sham treatment were stained with anti-CD31 or anti-eNOS Ab. A red arrow indicates eNOS expressed on the vascular endothelial cells. (C) The ratios of eNOS-positive vessels to the total ones detected in four randomly selected visual fields at 400× magnification under microscopic observation are shown ($n = 4$ in each group). The data are expressed as mean ± standard deviation. Closed column, LE-ESW-treated; ESW, LE-ESW-treated; open column, sham-treated; sham, sham-treated. $^*p < 0.05$.

eNOS-dependent expression of VEGF in skin wound tissues

To address this possibility, we conducted RT-PCR with RNA extracted from wound tissues. As shown in Figure 7A, VEGF mRNA was increased in the LE-ESW-treated WT mice compared with that in the untreated WT mice. In striking contrast, in he eNOS-KO mice, the expression of this molecule was negligible in the absence of LE-ESW treatment, and even after this treatment, no positive bands were detected in VEGF, although HPRT mRNA was almost comparably detected in the LE-ESW-treated and untreated groups. In line with these results, in an immunoblotting analysis (Figure 7B), VEGF expression was significantly increased in the LE-ESW-treated WT mice compared with that in the untreated WT mice as shown by the relative expression of VEGF to GAPDH (2.42 ± 0.41 count/mm^2 vs. 1.58 ± 0.37 count/mm^2 in the LE-ESW-treated [$n = 3$] and the untreated WT mice [$n = 3$], respectively) (Figure 7C). In contrast, in the eNOS-KO mice, the level of VEGF expression was not statistically different between the LE-ESW-treated and the untreated groups (1.01 ± 0.21 count/mm^2 vs. 1.06 ± 0.1 count/mm^2 in the LE-ESW-treated [$n = 3$] and the untreated eNOS-KO mice [$n = 3$], respectively) (Figure 7C).

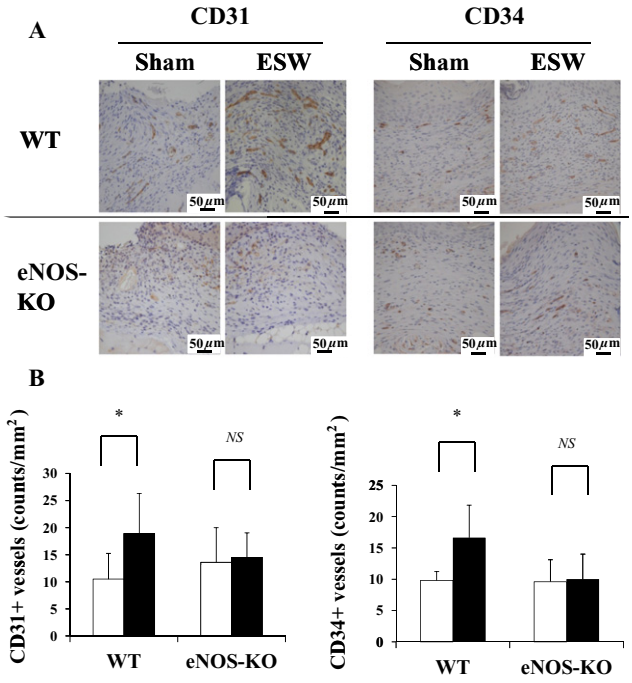

Figure 6. Angiogenesis in the wound tissues. Full-thickness wounds were made with a punch biopsy instrument in wild-type (WT) and endothelial nitric oxide synthase knockout (eNOS-KO) diabetic mice. (A) Immunohistochemical analysis was performed to assess the generation of new vessels in the wound tissues. Sections of the skin wounds on day 7 after low-energy extracorporeal shock wave (LE-ESW) or sham treatment were stained with anti-CD31 or anti-CD34. (B) The vascular density/mm^2 determined by counting the number of CD31$^+$ or CD34$^+$ vessels within four visual fields at 400× magnification under microscopic observation is shown ($n = 5$ in each group). The data are expressed as mean ± standard deviation. Closed column, LE-ESW-treated; ESW, LE-ESW-treated; open column, sham-treated; sham, sham-treated. $^*p < 0.05$.

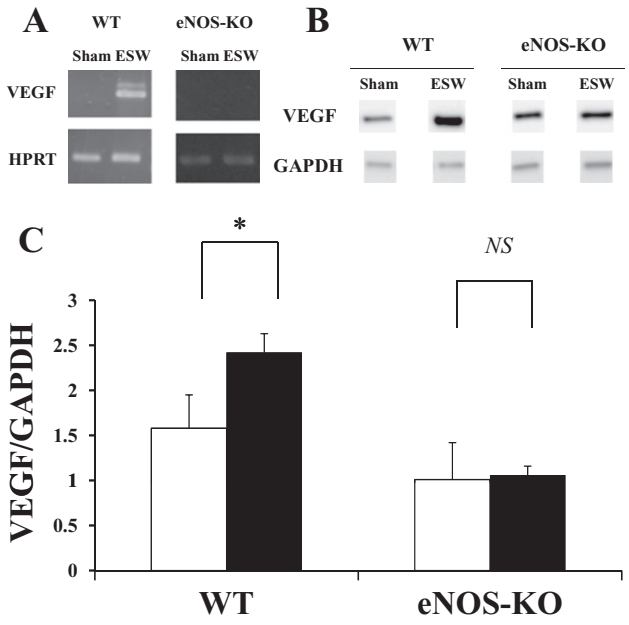

Figure 7. Expression of vascular endothelial growth factor (VEGF) in the wound tissues by low-energy extracorporeal shock wave (LE-ESW) treatment. Full-thickness wounds were made with a punch biopsy instrument in wild-type (WT) mice. (A) Reverse transcription polymerase chain reaction was performed for detection of VEGF messenger ribonucleic acid (mRNA) using total RNA isolated from wound tissues on day 3 after LE-ESW or sham treatment ($n = 3$ per each group). Hypoxanthine phosphoribosyl transferase (HPRT) was used as an internal control. (B) Western blotting analysis was performed to assess the expression of VEGF in the wound tissues obtained on day 7 after LE-ESW or sham treatment. (C) The relative expression of VEGF to anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) obtained by a densitometry analysis are shown ($n = 3$ in each group). The data are expressed as mean ± standard deviation. Closed column, LE-ESW-treated; ESW, LE-ESW-treated; open column, sham-treated; sham, sham-treated. *$p < 0.05$; *NS*, not significant.

DISCUSSION

In the present study, we examined the effect of LE-ESW treatment on the wound healing process using a mouse model of refractory skin wounds under a diabetic condition. We found that this treatment efficiently induced the expression of eNOS and VEGF at the wound sites, promoted the generation of new vessels, and improved wound healing. Furthermore, the beneficial effects of this treatment were significantly attenuated in eNOS-KO mice compared with those in WT mice. These results clearly showed that LE-ESW treatment accelerated the healing process of refractory skin wounds by inducing the expression of VEGF and promoting neovascularization, which was totally dependent on the expression of eNOS.

In previous studies, the expression of eNOS in vascular endothelial cells was reported to be a critical process in the generation of new vessels and wound healing.[28] Brownlee and coworkers demonstrated that activation of eNOS was induced by Akt serine/threonine kinase-dependent phosphorylation of this molecule at Ser1177 in vascular endothelial cells when stimulated with insulin.[29] They also indicated that a high glucose condition led to the introduction of O-linked glycosylation at the same position of the amino acid, which interfered with the phosphorylation and subsequent activation of eNOS.[30] Similarly, Gallagher et al. demonstrated that the decreased expression of eNOS and attenuated vasculogenesis led to refractory skin wounds in diabetic mice.[31] In the present study, diabetic mice established by STZ administration experienced delayed wound healing compared to nondiabetic mice. LE-ESW treatment improved the impaired wound healing in diabetic mice. In accordance with the aforementioned findings, the activation of eNOS is assumed to be involved in the beneficial effects of this treatment. Supporting this idea, we found that LE-ESW treatment induced the expression of eNOS at both the mRNA and protein levels at the wound sites in diabetic mice. Previously, frictional forces along the surface of the endothelium exerted by flowing blood were reported to be potent stimuli for endothelial NO production.[32] These forces are proportional to the product of blood viscosity and blood velocity at the endothelial wall and collectively have been termed "endothelial shear stress." Endothelial surfaces possess mechanoreceptors that detect shear stress, and data concerning the pathways responsible for phosphorylation and activation of eNOS are emerging. Detectable local thermal or chemical effects have also been discussed, but no evidence of clinical relevance could be found.[33] In addition, the energy of LE-ESW is not sufficient to cause a significant warming in focus or even at a distance from it, and therefore, thermal effects can be ruled out in ESW as indicated in the manufacturer's instruction. As suggested by these previous findings, shear stress to the vascular endothelial cells may be generated by LE-ESW treatment, and eNOS may be induced via a PI3K-Akt signaling pathway.

We have previously demonstrated that LE-ESW treatment to the ischemic myocardium increased the expression of VEGF in a pig model of chronic myocardial ischemia.[34] Kuo et al. reported similar findings in a rat model of skin wound healing under a diabetic condition generated by treatment with STZ.[35] In the present study, we also detected the expression of VEGF at both the mRNA and protein levels in diabetic mice after receiving LE-ESW treatment. Interestingly, the LE-ESW-induced expression of VEGF was abrogated or strongly attenuated in eNOS-KO mice. In addition, the beneficial effects of LE-ESW treatment on the vasculogenesis, as evaluated by the density of $CD31^+$ or $CD34^+$ vascular endothelial cells, and in wound healing, as detected by the wound closure and expression of α-SMA, were attenuated in eNOS-KO mice compared with WT mice. Recently, Jing Zheng and coworkers demonstrated that the administration of NG-monomethyl-L-arginine, an inhibitor of eNOS-dependent NO synthesis, leads to a decrease in the expression of VEGF and the generation of new vessels in sheep placenta.[36] Thus, eNOS can be recognized as a critical molecule in the processes of neovasculization and wound healing, although the precise mechanism remains to be clarified.

The wound healing process is composed of the coagulation, inflammation, proliferation, and remodeling phases, in

which a variety of pro-inflammatory cytokines and growth factors, such as interleukin-1β, tumor necrosis factor-α, transforming growth factor-β, epidermal growth factor, platelet-derived growth factor, and basic fibroblast growth factor are produced. In the proliferation phase, vascular endothelial cells play an important role together with fibroblasts. Endothelial progenitor cells migrate into the wound sites while actively proliferating under stimulation from platelets, activated macrophages, and fibroblasts. This leads to the generation of new capillary vessels and the formation of a capillary vessel network, followed by granuloma formation and wound constriction.[37] In chronic wounds, this orderly and intricate biological response to wounding is disrupted. The inability to progress through the inflammatory phase of wound healing to complete epithelialization is characteristic of this type of wound, where the prolonged and aberrant accumulation of matrix metalloproteinases (MMPs), which are released by leukocytes, impedes the endothelial cell migration and down-regulation of tissue inhibitors of MMPs.[38] These pathological conditions interfere with the generation of a new vessel network, a process that is essential for the restoration of local oxygen delivery, and remodeling of the extracellular matrix. Davis and coworkers reported that LE-ESW treatment promoted wound healing by suppressing the production of pro-inflammatory cytokines in the early phase in a rat model of severe burn injury.[39] In recent investigations,[40] it has been considered that NO secreted from the vascular endothelial cells under a homeostatic condition may act as an antiatherosclerotic agent by regulating its own proliferation and platelet aggregation, thrombus formation, and inflammatory responses. Taken together, these previous observations may support the possibility that the shear stress to the vascular endothelial cells caused by LE-ESW treatment activates eNOS and induces NO synthesis, which contribute to the wound healing process by suppressing the pro-inflammatory cytokines and promoting the transition from the inflammatory phase to the proliferation phase. Further investigations are necessary to address this interesting possibility.

In conclusion, we demonstrated that LE-ESW treatment accelerated skin wound healing by inducing the expression of eNOS, which promoted the expression of VEGF and the generation of new vessels in diabetic mice. LE-ESW treatment has many advantages, including the following: neither anesthesia nor invasive manipulation is required; the therapy is repeatable if necessary; and adverse effects are rare. Refractory skin wound healing in diabetic patients is a particularly challenging clinical problem. LE-ESW treatment can be considered a promising therapeutic option in these patients. The present study presents important implications to the progress of this novel therapy.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research (B) (21390476) from the Ministry of Education, Science and Culture of Japan. This study was supported in part by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare, Tokyo, Japan (H21-rinsyokenkyu-ippan-012).

Conflict of Interest: The authors state no financial conflicts of interest.

REFERENCES

1. Broughton G, Janis JE, Attinger CE. The basic science of wound healing. *Plast Reconstr Surg* 2006; 117: 12–34.
2. Ferriera MC, Tuma P Jr, Carvalho VF, Kamamoto F. Complex wounds. *Clinics* 2006; 61: 571–8.
3. Bauer SM, Bauer RJ, Velazquez OC. Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. *Vasc Endovascular Surg* 2005; 39: 293–306.
4. Chaussy C, Brendel W, Schmiedt E. Extracorporeally induced destruction of kidney stones by shock waves. *Lancet* 1980; 2: 1265–8.
5. Sauerbruch T, Delius M, Paumgartner G, Holl J, Wess O, Weber W, Hepp W, Brendel W. Fragmentation of gallstones by extracorporeal shock waves. *N Engl J Med* 1986; 314: 818–22.
6. Gerdesmeyer L, Wagenpfeil S, Haake M, Maier M, Loew M, Wortler K, Lampe R, Seil R, Handle G, Gassel S, Rompe JD. Extracorporeal shock wave therapy for the treatment of chronic calcifying tendonitis of the rotator cuff: a randomized controlled trial. *JAMA* 2003; 290: 2573–80.
7. Thomson CE, Crawford F, Murray GD. The effectiveness of extra corporeal shock wave therapy for plantar heel pain: a systematic review and meta-analysis. *BMC Musculoskelet Disord* 2005; 6: 19–30.
8. Kikuchi Y, Ito K, Ito Y, Shiroto T, Tsuburaya R, Aizawa K, Hao K, Fukumoto Y, Takahashi J, Takeda M, Nakayama M, Yasuda S, Kuriyama S, Tsuji I, Shimokawa H. Double-blind and placebo-controlled study of the effectiveness and safety of extracorporeal cardiac shock wave therapy for severe angina pectoris. *Circ J* 2010; 74: 589–91.
9. Serizawa F, Ito K, Kawamura K, Tsuchida K, Hamada Y, Zukeran T, Shimizu T, Akamatsu D, Hashimoto M, Goto H, Watanabe T, Sato A, Shimokawa H, Satomi S. Extracorporeal shock wave therapy ameliorates walking ability of patients with peripheral artery disease and intermittent claudication. *Circ J* 2012; 76: 1486–93.
10. Ito K, Fukumoto Y, Shimokawa H. Extracorporeal shock wave therapy for ischemic cardiovascular disorders. *Am J Cardiovasc Drugs* 2011; 11: 295–302.
11. Wang CJ, Wang FS, Yang KD, Weng LH, Sun YC, Yang YJ. The effect of shock wave treatment at the tendon-bone interface—an histomorphological and biomechanical study in rabbits. *J Orthop Res* 2005; 23: 274–80.
12. Stojadinovic A, Elster EA, Anam K, Tadaki D, Amare M, Zins S, Davis TA. Angiogenic response to extracorporeal shock wave treatment in murine skin isografts. *Angiogenesis* 2008; 11: 369–80.
13. Schaden W, Thiele R, Kolpl C. *Extracorporeal shock wave therapy (ESWT) in skin lesions*. 9th International Congress of the International Society for Musculoskeletal Shockwave Therapy (ISMST), Rio de Janeiro, Brazil, April 20–24 2006, 1.
14. Farrell JA, Blake RD. Nitric oxide. *Ann Rheum Dis* 1996; 55: 7–20.
15. Wu H, Jin Y, Arias J, Bassuk J, Uryash A, Kurlansky P, Webster K, Adams JA. In vivo upregulation of nitric oxide synthases in healthy rats. *Nitric Oxide* 2009; 21: 63–8.
16. Ni J, McLoughlin MR, Brodovitch A, Moulin P, Brouckaert P, Casadei B, Feron O, Topley N, Balligand J, Devuyst O. Nitric oxide synthase isoforms play distinct roles during acute peritonitis. *Nephrol Dial Transplant* 2010; 25: 86–96.
17. Papapetropoulos A, García-Cardeña G, Madri AJ, Sessa CW. Nitric oxide production contributes to the angiogenic properties

of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; 100: 3131–9.
18. Ziche M, Mashini ME, Granger H, Geppetti P, Ledda F. Nitric oxide promotes DNA synthesis and cyclic GMP formation in endothelial cells from postcapillary venules. *Biochem Biophys Res Commun* 1993; 192: 1198–203.
19. Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bichnell R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 1997; 99: 2625–34.
20. Reiber GE, Vileikyte L, Boyko EJ, del Aguila M, Smith DG, Lavery LA, Boulton AJ. Causal pathways for incident lower-extremity ulcers in patients with diabetes from two settings. *Diabetes Care* 1999; 22: 157–62.
21. Bivalacqua JT, Champion CH, Usta FM, Cellek S, Chitaley K, Webb CR, Lewis LR, Mills MT, Hellstrom GW, Kadowitz JP. RhoA/Rho-kinase suppresses endothelial nitric oxide synthase in the penis: a mechanism for diabetes-associated erectile dysfunction. *Proc Natl Acad Sci U S A* 2004; 101: 9121–6.
22. Du X, Edelstein D, Obici S, Higham N, Zou M, Brownlee M. Insulin resistance reduces arterial prostacyclin synthase and eNOS activities by increasing endothelial fatty acid oxidation. *J Clin Invest* 2006; 116: 1071–80.
23. Wang C, Li F, Hiller S, Kim H, Maeda N, Smithies O, Takahashi N. A modest decrease in endothelial NOS in mice comparable to that associated with human NOS3 variants exacerbates diabetic nephropathy. *Proc Natl Acad Sci U S A* 2011; 108: 2070–5.
24. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995; 377: 239–42.
25. Hattori N, Mochizuki S, Kishi K, Nakajima T, Takaishi H, D'Armiento J, Okada Y. MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol* 2009; 175: 533–46.
26. Goldberg TM, Han Y, Yan C, Shaw CM, Garner LW. TNF-α suppresses α-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing. *J Invest Dermatol* 2007; 127: 2645–55.
27. Benest VA, Stone AO, Miller HW, Glover PC, Uney BJ, Baker HA, Harper JS, Bates OD. Arteriolar genesis and angiogenesis induced by endothelial nitric oxide synthase overexpression results in a mature vasculature. *Arterioscler Thromb Vasc Biol* 2008; 28: 1462–8.
28. Murohara T, Asahara T, Marcy S, Christophe B, Masuda H, Christoph K, Marianne K, Donghui C, Dongfen C, James FS, Mark CF, Paul LH, Jeffrey MI. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998; 101: 2567–78.
29. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; 414: 813–20.
30. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 2001; 7: 947–53.
31. Gallagher KA, Liu ZJ, Xiao M, Chen H, Goldstein LJ, Buerk DG, Nedeau A, Thom SR, Velazquez OC. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1. *J Clin Invest* 2007; 117: 1249–59.
32. Zhen C. Shear stress, SIRT1, and vascular homeostasis. *Proc Natl Acad Sci U S A* 2010; 107: 10268–73.
33. Gerdesmeyer L, Maier M, Haake M, Schmitz C. Physical–technical principles of extracorporeal shockwave therapy (ESWT). *Orthopade* 2002; 31: 610–7.
34. Nishida T, Shimokawa H, Oi K, Tatewaki H, Uwatoku T, Abe K, Matsumoto Y, Kajihara N, Eto M, Matsuda T, Yasui H, Takeshita A, Sunagawa K. Extracorporeal cardiac shock wave therapy markedly ameliorates ishemia-induced myocardial dysfunction in pig in vivo. *Circulation* 2004; 110: 3055–61.
35. Kuo YR, Wang CT, Wang FS, Chiang YC, Wang CJ. Extracorporeal shock-wave therapy enhanced wound healing via increasing topical blood perfusion and tissue regeneration in a rat model of STZ-induced diabetes. *Wound Repair Regen* 2009; 17: 522–30.
36. Zheng J, Wen Y, Song Y, Wang K, Chen DB, Magness RR. Activation of multiple signaling pathways is critical for fibroblast growth factor 2- and vascular endothelial growth factor-stimulated ovine fetoplacental endothelial cell proliferation. *Biol Reprod* 2008; 78: 143–50.
37. Pierce GF, Vande Berg J, Rudolph R. Platelet-derived growth factor-BB and transforming growth factor beta 1 selectively modulate glycosaminoglycans, collagen, and myofibroblasts in excisional wounds. *Am J Pathol* 1991; 138: 629–46.
38. Lai JY, Borson ND, Strausbauch MA, Pittelkow MR. Mitosis increases levels of secretory leukocyte protease inhibitor in keratinocytes. *Biochem Biophys Res Commun* 2004; 316: 407–10.
39. Davis TA, Stojadinovic A, Anam K, Amare M, Naik S, Peoples GE, Tadaki D, Elster EA. Extracorporeal shock wave therapy suppresses the early proinflammatory immune response to a severe cutaneous burn injury. *Int Wound J* 2008; 6: 11–21.
40. Gélinas DS, Bernatchez PN, Rollin S, Bazan NG, Sirois MG. Immediate and delayed VEGF-mediated NO synthesis in endothelial cells: role of PI3K, PKC and PLC pathways. *Br J Pharmacol* 2002; 137: 1021–30.