Sonic Hedgehog Induces Arteriogenesis in Diabetic Vasa Nervorum and Restores Function in Diabetic Neuropathy

Kengo F. Kusano, Karen L. Allendoerfer, William Munger, Roberto Pola, Marta Bosch-Marce, Rudolf Kirchmair, Young-sup Yoon, Cynthia Curry, Marcy Silver, Marianne Kearney, Takayuki Asahara, Douglas W. Losordo

Objective—The embryonic morphogen sonic hedgehog (SHh) has been shown to induce neovascularization of ischemic tissue but has not been shown to play a role in regulating vascular nerve supply. Accordingly, we investigated the hypothesis that systemic injection of SHh protein could improve nerve blood flow and function in diabetic neuropathy (DN).

Methods and Results—Twelve weeks after induction of diabetes with streptozotocin, motor and sensory nerve conduction velocities (MCV and SCV) of the sciatic nerves were significantly reduced in diabetic rats. SHh-treated diabetic rats demonstrated marked improvement of both MCV and SCV (P<0.05). Laser Doppler perfusion imaging showed that nerve blood flow was significantly reduced in the diabetic rats but was restored in SHh-treated diabetic rats (P<0.05 versus diabetic saline-treated rats) to levels similar to those achieved with vascular endothelial growth factor-2 (VEGF-2) gene therapy. In vivo perfusion of Bandeuraea simplicifolia (BS)-1 lectin showed marked reduction in the nerve blood flow supply can mitigate neuropathy despite persistent diabetes, and that SHh can exert angiogenic effects that could mitigate DN. Accordingly, we performed a series of investigations to test the hypothesis that SHh could replenish vasa

In the United States alone, >18 million people have diabetes.1 Diabetic neuropathy (DN) is a frequent complication of diabetes, affecting 1 to 7 million people, including 7% within 1 year of diagnosis and 50% of patients after 25 years. It has also been reported that up to 90% of patients have subclinical levels of neuropathy.2 Although several factors have been reported to contribute to diabetic polyneuropathy,3–5 the pathogenic basis has remained uncertain.6 An association between changes in the vasa nervorum and DN has been noted in multiple previous reports;11–17 however, the pathophysiologic importance of these observations remains uncertain. The possibility that attenuation of the vasa nervorum might be a major factor in the development of DN is suggested by several recent studies. Impaired ischemia-induced angiogenesis was noted in animal models of diabetes,18 and more recently we have reported that ischemia-induced angiogenesis was noted in animal models of diabetes.19,20 are associated with attenuation of the vasa nervorum and that local delivery of naked DNA encoding for vascular endothelial growth factor (VEGF-1 and VEGF-2) restores the vascular supply and has a favorable effect on the nerve conduction velocities. These observations, documenting the loss of vasa nervorum in diabetic animals, and restoration of neural vascularity by VEGF, associated with a return of nerve function, suggested that the microangiopathic abnormality is one of the critical factors that cause DN.

Sonic hedgehog (SHh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development of limb, lung, gut, hair follicles, and bone.21–23 The hedgehog (Hh) pathway also plays an essential inductive and morphogenetic role in the developing central and peripheral nervous system.24 Recently, we have also reported that SHh protein has an indirect but powerful angiogenic effect in a mouse hind-limb ischemia model.25 Together, these previous studies suggested to us the possibility that diabetic polyneuropathy results, at least in part, from attenuation of vasa nervorum, that restoration of nerve blood flow supply can mitigate neuropathy despite persistent diabetes, and that SHh can exert angiogenic effects that could mitigate DN. Accordingly, we performed a series of investigations to test the hypothesis that SHh could replenish vasa...
nervorum in diabetes, thereby restoring nerve blood flow and nerve function in DN.

Methods

Animal Models
All protocols were approved by St. Elizabeth’s Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to identify of the treatment administered.

Rats
Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 200 to 225 grams were used. Rats were fed standard laboratory rodent chow and water ad libitum and housed individually.

Induction of Diabetes
Rats were made diabetic by a single intraperitoneal injection of streptozotocin (75 mg/kg in 0.9% sterile saline) into anesthetized rats (5 mg/100 g pentobarbital).

Systemic Treatment With SHh Protein
Human SHh proteins were used to construct SHh rat IgG fusion proteins to increase the half-life, as described.20 Systemic injection of SHh–rat IgG fusion protein was started 12 weeks after the induction of diabetes. After completion of baseline nerve conduction measurements, animals received subcutaneous injection of SHh proteins (1.0 mg/kg) or saline using 27-gauge needle 3 times per week for 4 weeks.

phVEGF-2 Plasmid and Gene Transfer
As a positive control, we used naked plasmid DNA encoding human VEGF-2 (phVEGF-2), as described previously.20

Electrophysiological Measurements
Nerve motor and sensory conduction velocity was measured as described previously20,30 in all rats at baseline (before treatment) and then at 2 and 4 weeks after treatment. All procedures and analyses were performed by an experienced researcher who was blinded to treatment.

In Vivo Assessment of Perfusion and Vascularity: Laser Doppler Imaging of Vasa Nervorum
Blood Flow
Blood perfusion of the sciatic vasa nervorum was measured unilaterally in the hind limb of the rats with a laser Doppler perfusion imager (LDPI) system (Moor Instruments, Wilmington, Del) as described previously,20 programmed to measure perfusion of surrounding tissue as zero, or background. All perfusion measurements, as well as neurophysiological examinations, were performed with the animal placed on a heating blanket underneath a warming lamp controlled by a thermistor probe applied to the proximal nerve to maintain temperature at 37°C. All procedures and analyses were performed by an experienced researcher who was blinded to treatment assignment.

Hemodynamic Assessment
To insure that blood pressure was not affected by treatment, subgroups of animals from all treatment groups underwent analysis of blood pressure and heart rate. At the time of euthanization, a 2.0-French high-fidelity Millar pressure catheter (Millar Instruments) was inserted from the left ventricular apex to the ascending aorta, and systolic aortic pressure and heart rate were recorded. Calibration of the Millar catheter was verified before and after each measurement.

Sciatic Nerve Histology: Fluorescent Imaging of Vasa Nervorum
Vascularity of sciatic nerves from both normal and diabetic rats were assessed by in situ fluorescent staining using the endothelial cell-specific marker Bandeiraea simplicifolia (BS)-1 lectin conjugated to fluorescein isothiocyanate (Vector Laboratories, Burlingame, Calif) as described previously.20

Immunohistochemistry
Sciatic nerves were fixed in 100% methanol and paraffin-embedded sections of 5-μm thickness were stained for murine-specific endothelial marker isoelectin B4 (Vector Laboratories), factor VIII (Signet Laboratories, Dedham, Mass), or alpha-smooth muscle actin (Sigma Chemical Co. St. Louis, Mo) and counterstained with eosin to detect capillary endothelial cells or smooth muscle cells in the vasa nervora.

Reverse-Transcription Polymerase Chain Reaction
Total RNA was extracted from sciatic nerves or L4,5 dorsal root ganglia 1 week after treatment using the Ambion Isolation kit (RNAqueousTM) according to the manufacturer’s instructions. DNAase digestion was performed after RNA extraction. Reverse-transcription polymerase chain reaction was performed according to the manufacturer’s instructions (Clontech, Palo Alto, Calif). All procedures and analyses were performed by an experienced researcher who was blinded to treatment assignment.

Cultured Nerve Fibroblasts
Primary cultured nerve fibroblasts were obtained from 250- to 350-gram male Sprague Dawley rats according to the method of Bolin.31 Cells were harvested after 48 hours and reverse-transcription polymerase chain reaction was performed.

Statistics
All results were expressed mean±SD. Statistical comparisons between groups were performed by ANOVA with Bonferroni correction. P<0.05 was considered statistically significant.

Results

DN Model: Treatment With SHh Versus VEGF Versus Saline
As shown in Table I (available at http://atvb.ahajournals.org), the serum glucose and blood urea nitrogen were elevated in the diabetic versus nondiabetic rats, as expected. Weight was also reduced in all diabetic animals. There were no significant differences between any of the diabetic treatment groups in these parameters or in blood pressure or heart rate (Table I).

Depletion of Vasa Nervorum Accompanies DN: SHh Replenishes Nerve Vascular Supply: In Vivo Staining of Vasa Nervorum by BS-1 Lectin Perfusion
Whole-mount staining reveals restoration of vasa nervorum by SHh (Figure 1A). Four weeks after treatment, an endothelial-specific marker, fluorescein isothiocyanate-conjugated BS-1 lectin was injected to permit documentation of vasa nervora. The nondiabetic rat in both saline and SHh showed a regular pattern of vascularity including a superficial longitudinal network and penetrating branches responsible for providing blood flow to the endoneurial vascular network. However, in nerves of diabetic rats treated with saline, the total number of vasa nervora was markedly decreased and the vascular network was substantially destroyed, resulting in an irregular distribution pattern and areas of nonvascularized nerve tissue. In SHh-treated diabetic rats, the vascular net-
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nerve capillaries, we counted the number of vessels using recovery of vasa nervora in transfer, which was included as a positive control.20 rats showed replenished vascularity. The total number of SHh-treated (and phVEGF-2 gene therapy as a positive control) (Figure 1A). Endoneurial vessels were also counted using factor VIII staining. As shown in Figure 1C, factor VIII-positive vessels were also reduced in saline-treated diabetic rats (34.2±4.5 in nondiabetic plus saline, n=5, and 37.4±7.9 in nondiabetic plus SHh, n=6, and 14.2±3.5 per section in diabetic, n=5; P<0.01). SHh treatment resulted in recovery of endoneurial capillaries (epineurial/perineurial: 35.8±4.8, n=5) similar to the results of VEGF-2 gene therapy (25.6±6.4 per section, n=5).

**LDPI of Sciatic Nerve Blood Flow**

LDPI was performed to evaluate blood flow in the sciatic nerves of rats in all treatment groups (Figure 2). This blinded analysis revealed markedly reduced nerve blood flow in saline-treated diabetic rats (401.0±106.3 LDPI units versus 1185.2±370.1 LDPI units in nondiabetic controls; P<0.01) as described previously.20 SHh treatment in diabetic rats resulted in substantial restoration of sciatic nerve perfusion (791.0±351.4 LDPI units, P<0.05, versus saline-treated diabetic rats; Figure 2B). VEGF-2 gene transfer also restored perfusion of sciatic nerves to a level similar to that seen in SHh-treated diabetic rats (816.8±310.1 LDPI units, P<0.05, versus saline-treated diabetic rats). To further validate the usefulness of LDPI measurements as an indicator of vascular recovery, the capillary counts and LDPI measurements were correlated in randomly selected subgroups from all treatment groups. As shown in Figure 2C, there was a significant (P<0.01) correlation between total (epineurial/perineurial and endoneurial) capillary density in the nerve and LDPI measurements in each animal.

SHh-induced neovascularization is morphologically distinct (Figure 3). During our initial analysis of capillary density, we noted that the vasculature of the epineurium/perineurium appeared larger in size than the vessels in the other treatment groups (Figure 3A top and middle) We measured vessel diameter and found that the epineurial/perineurial vessels in the SHh group were significantly larger than those in the phVEGF-2–treated rats and were similar in size to those in the nondiabetic control rats (mean vessel diameter 15.3 μm in phVEGF-2 group versus 26.4 μm in SHh-treated group, P<0.05) (Figure 3B). Moreover, staining for α-smooth muscle actin revealed that the SHh-treated nerves contained a greater number of α-actin-positive cells colocalized in the epineurial/perineurial vessels than in nerves work was restored, with both superficial and penetrating branches. Similar findings were disclosed with VEGF-2 gene transfer, which was included as a positive control.20 Quantification of epineurial/perineurial and endoneurial capillaries in sciatic nerves documents recovery of vasa nervora in SHh-treated rats (Figure 1B and 1C). To analyze the sciatic nerve capillaries, we counted the number of vessels using cross-section slides. Figure 1B clearly showed much more epineurial/perineurial capillaries in the nondiabetic nerves compared with saline-treated diabetic nerves (epineurial/perineurial vasa/cross-section: 138.0±8.0 in nondiabetic plus saline, n=7; 142.0±12.0 in nondiabetic plus SHh, n=6; and 62.2±11.0 per section in diabetic plus saline, n=5; P<0.01). There was no significant difference between saline-treated and SHh-treated nondiabetic nerves. Endoneurial capillaries were also significantly reduced in saline-treated diabetic rats (endoneurial vasa/ cross-section: 37.8±3.3 in nondiabetic plus saline, n=7; 38.2±4.5 in nondiabetic plus SHh, n=6; and 21.0±2.4 per section in diabetic plus saline, n=5; P<0.01). SHh treatment resulted in recovery of both epineurial/perineurial and endoneurial capillaries (epineurial/perineurial: 105.7±14.0; endoneurial: 36.3±2.4 in SHh; n=6 per section). Similar findings were noted in VEGF-2 gene therapy-treated animals as shown previously (epineurial/perineurial: 108.3±22.3; endoneurial: 35.8±7.1 per section in phVEGF-2; n=5) (Figure 1B). Endoneurial capillaries were also counted using factor VIII staining. As shown in Figure 1C, factor VIII-positive vessels were also reduced in saline-treated diabetic rats (34.2±4.5 in nondiabetic plus saline, n=5, and 37.4±7.9 in nondiabetic plus SHh, n=6, and 14.2±3.5 per section in diabetic, n=5; P<0.01). SHh treatment resulted in recovery of endoneurial capillaries (25.8±4.8, n=5) similar to the results of VEGF-2 gene therapy (25.6±6.4 per section, n=5).
We then measured the total area of \( \alpha \)-actin–positive vasculature in all treatment groups and found that the \( \alpha \)-actin–positive vasculature in SHh-treated nerves was significantly closer to the nondiabetic nerves than after VEGF gene therapy. These data indicated that treatment with SHh resulted in a vessel morphology that was distinct from that induced by gene transfer of a single angiogenic cytokine.

SHh restores nerve function in DN (Figure 4). Within 12 weeks of the onset of diabetes induced by streptozotocin, a severe peripheral neuropathy developed in rats, as described previously.\(^{20}\) Electrophysiological recordings revealed that significant slowing of motor nerve conduction velocity (MCV) and sensory nerve conduction velocity (SCV) was observed in diabetic rats (MCV = 35.0 ± 2.9 m/s versus 46.2 ± 3.1 m/s [nondiabetic], SCV = 34.2 ± 2.5 m/s [diabetic], and 48.1 ± 3.7 m/s [nondiabetic]; \( P < 0.01 \) for both). Saline-treated diabetic rats showed no change in nerve conduction velocities during the 4 weeks of treatment (MCV = 35.2 ± 2.5 m/s and SCV = 35.6 ± 3.0 m/s). In contrast, 4 weeks after treatment with systemic injection of SHh protein, all nerve conduction velocities demonstrated a marked improvement (Figure 4). Specifically, MCV in diabetic rats treated with SHh protein increased to 44.9 ± 4.2 m/s and SCV increased to 47.5 ± 7.0 m/s (both \( P < 0.01 \) versus saline- from phVEGF-2–treated rats. (Figure 3A bottom). We then measured the total area of \( \alpha \)-actin–positive vasculature in all treatment groups and found that the \( \alpha \)-actin–positive vasculature in SHh-treated nerves was significantly closer to the nondiabetic nerves than after VEGF gene therapy. These data indicated that treatment with SHh resulted in a vessel morphology that was distinct from that induced by gene transfer of a single angiogenic cytokine.

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SHh upregulates expression of multiple angiogenic and neurotrophic cytokines (Figures I and II, available online at http://atvb.ahajournals.org). To identify potential mechanisms responsible for the therapeutic effect of SHh, we evaluated the expression of the Hh-related transcriptional factor Gli-1 and certain neurotrophic factors (BDNF and IGF-1) and angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) in treated and control rats. As shown in Figure I, endogenous Gli expression was downregulated in saline-treated diabetic rats, suggesting that the Hh pathway was inactivated in the nerves of diabetic rats. The expression of angiogenic factors and neurotrophic cytokines were also downregulated (Figure I). However, SHh treatment resulted in a significant increase in the expression of mRNA of both endogenous angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) and neurotrophic factors (BDNF, IGF-1), as well as upregulation of Gli-1 mRNA expression to nondiabetic levels. In contrast, phVEGF-2 did not upregulate the expression angiogenic cytokines or neurotrophic factors, except BDNF.

To verify these findings and to establish a direct effect of SHh on gene expression, we repeated reverse-transcription polymerase chain reaction on primary cultured rat nerve fibroblasts. Expression of Gli-1 was not detected in the cultured fibroblasts (Figure II). However, the expression of mRNA for angiogenic cytokines (VEGF-1, angiopoietin-1, and angiopoietin-2) and neurotrophic factors (BDNF, IGF-1), as well as Gli-1, were upregulated by SHh protein (Figure II) in a dose-dependent manner (1, 5, 10 μg/mL), suggesting that SHh stimulation of neural fibroblasts can modulate expression of multiple factors with the potential to promote nerve recovery.

**Discussion**

The peripheral neuropathy that complicates diabetes results in major morbidity, contributing to the leading cause of hospitalization among diabetic subjects and loss of tissue integrity in the lower extremities. The magnitude of this public health problem has led to aggressive efforts to define the cause and develop preventative measures or treatment strategies for this disabling condition. Despite the identification of multiple potential mechanisms, no therapy attempting to address individual causative factors has proven successful.

Our results demonstrate that SHh induces functional recovery in DN by simultaneously normalizing a repertoire of vascular and neural growth and survival factors and cytokines and replenishing a more mature-appearing vasa nervorum in both endoneurial and epineurial/perineurial capillaries. Notably, and in contrast to a recent report,29 our data reveal that DN is associated with vascular pathology. Specifically, disruption and loss of vasa nervorum accompany the onset of neuropathy in multiple animal models of DN (and ischemic neuropathy).20,32 and restitution of vascular architecture and nerve perfusion have now been repeatedly shown to be a consistent component of neurological recovery. These findings are consistent with development models that have verified the requirement for coordination between vascular and neurological elements.33 The role of vascular recovery in the restoration of neurophysiologic function induced by SHh in diabetes is underscored by the observed decrease in angiogenic factor expression in the affected nerves in diabetic animals and the recovery of expression after SHh treatment. Consistent with the central role of vascular recovery, direct replenishment of an angiogenic cytokine by VEGF-2 gene therapy also results in significant neurophysiologic recovery. Although the neovasculature induced by SHh and VEGF was different in appearance, the recovery of perfusion is similar, as are the degree and rate of physiological recovery. These data support a vasculogenic cause of DN.

The ability of the morphogen SHh to normalize expression of numerous factors downregulated in diabetic subjects resulted in the restoration of vasa nervora that appeared morphologically distinct and more similar in appearance to normal vessels than did the VEGF induced vessels. This is consistent with previous studies in which multiple cytokines were shown to induce formation of multilayered vessels.34 This observation regarding the vasculature induced by SHh may provide clues to the cause of diabetes-induced attrition of the vasa nervorum and to a better understanding of the mechanisms of neovascularization in vivo.

**Downregulation of Angiogenic Cytokines, Neurotrophic Factors, and Hh Pathway in the Diabetic Sciatic Nerve**

Multiple mechanisms have been implicated in the pathogenesis of DN, including modification and inactivation of proteins critical to neural function by nonenzymatic glycosylation,8 altered neural polyol metabolism,6,7 reductions in neurotrophin or the availability of neurotrophic factors, and microvascular disease including reduced vasa nervora in the diabetic nerve.20,35 However, debate still oscillates between propositions based on neurochemical versus vascular events. Our data demonstrate that not only neurotrophic factors but also various angiogenic cytokines were significantly reduced in the diabetic sciatic nerves. These data reveal that downregulation of both neurochemical and of vascular factors is related to the development of DN. After injection of SHh, expression of the Gli-1 transcription factor was upregulated and the expression of multiple endogenous angiogenic cytokines (angiopoietin-1, angiopoietin-2, and VEGF-1) and neurotrophic factors (BDNF and IGF-1) was restored to nondiabetic levels. These observations were also confirmed in vitro. However, phVEGF-2 treatment did not induce upregulation of endogenous cytokines or neurotrophic factors but did restore the vasa nervora with an equal impact on nerve physiology. These data suggest that the vascular pathology plays a key role in the advent of DN.

Anatomically, in situ fluorescent imaging of whole-mounted nerves (Figures 1 and 3) revealed that diabetes resulted in attrition of the vasa vasorum (both epineurium/perineurium and endoneurium) and disruption of the nerve architecture that is also characteristic of ischemic neuropathy, as has been documented previously in this model,20 resulting in decreased nerve perfusion. All of these phenomena were reversed by SHh. Interestingly, the morphological features of the vasa in SHh-treated rats seemed to more closely resemble the native vasculature, with a range of vessels sizes, in comparison to the restored vasculature in phVEGF-2–treated
rats. Because we show here that SHh upregulates multiple endogenous angiogenic cytokines, including VEGF and angiopoietin-1, the observed differences in morphology appear consistent with the effect of SHh on multiple downstream targets. Similar observations were reported in a model of acute hind-limb ischemia in mice.28

In conclusion, these data suggest that SHh targets multiple signaling pathways that can influence the recovery of nerve perfusion in DN. These findings also highlight the potential for SHh to promote the development of a neovascularature that exhibits morphological features of the mature native vasculature and may therefore provide clues to the signaling mechanisms that distinguish arteriogenesis from angiogenesis.

Acknowledgments
This work supported by National Institutes of Health grants HL 53354, 63695, 66957, and 57516. The authors gratefully acknowledge the assistance of Mickey Neely and Deirdre Costello in the preparation of this manuscript.

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Arterioscler Thromb Vasc Biol. 2004;24:2102-2107; originally published online September 9, 2004;
doi: 10.1161/01.ATV.0000144813.44650.75
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure Legends

(Figure I) SHh induces in vivo expression of multiple angiogenic and neurotrophic cytokines: RT-PCR.
Tissue samples were harvested 1 week after treatment and mRNA expression was examined in dorsal root ganglia [Gli-1, brain-derived neurotrophic factor (BDNF), and insulin-like growth factor (IGF)-1: upper] and sciatic nerves (VEGF-1, angiopoietin-1 and 2: lower). Expression of all factors was significantly reduced in the saline-treated diabetic rats. Only SHh-treated sciatic nerves showed significant restoration of mRNA for Gli-1, as expected. Importantly, SHh also induced expression of multiple angiogenic cytokines and neurotrophic factors, whereas VEGF-2 gene therapy had more limited effects. All experiments were repeated at least five times and results of 3 representative experiments are shown.

(Figure II) SHh induces expression of angiogenic and neurotrophic factors in primary cultured nerve fibroblasts.
SHh effect on gene expression in primary cultured nerve fibroblasts. Gli-1 mRNA were not detected in nerve fibroblasts before SHh supplementation. However, rapid and marked upregulation of mRNA expression for endogenous neurotrophic cytokines (BDNF and IGF-1) and angiogenic cytokines (VEGF-1, angiopoietin-1 and 2) as well as Gli-1 was observed following SHh protein supplementation of the culture medium (1, 5, 10 µg/ML). All experiments were repeated at least 3 times.
Figure I

Gli-1
BDNF
IGF-1
GAPDH

Non-diabetic + saline  Diabetic + saline  Diabetic + SHH  Diabetic + VEGF-2  positive control

Gli-1/GAPDH  BDNF/GAPDH  IGF-1/GAPDH

Non-diabetic + saline  Diabetic + saline  Diabetic + SHH  Diabetic + VEGF-2  positive control

VEGF-1
angiopeptin-1
angiopeptin-2
GAPDH

Non-diabetic + saline  Diabetic + saline  Diabetic + SHH  Diabetic + VEGF-2  positive control

VEGF-1/GAPDH  angiopeptin-1/GAPDH  angiopeptin-2/GAPDH

*P<0.05, **P<0.01 vs. Non-diabetic-saline
#P<0.05, ##P<0.01 vs. Diabetic-saline
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Values are expressed as mean ±SD. *<0.05, **P<0.01 versus saline treated non-diabetic (ANOVA).
*P<0.05 vs. Non-diabetic
**P<0.01 vs. Non-diabetic