Effect of hyperglycemia and neuropeptides on interleukin-8 expression and angiogenesis in dermal microvascular endothelial cells

Monica Jain, BS, Frank W. LoGerfo, MD, Patrick Guthrie, BS, and **Leena Pradhan, PhD,** *Boston, Mass*

Background: **Impaired wound healing is a major complication associated with diabetes, involving a dysregulation and impairments in the inflammatory and angiogenic phases of wound healing. Here, we examine the effects of the neuropeptides substance P (SP) and neuropeptide Y (NPY) on dermal microvascular endothelial cell (DMVEC) angiogenesis and interleukin-8 (IL-8) expression, a known effector of the neuropeptide pathways in normal and hyperglycemic conditions in vitro.**
Methods: **DMVECs are treated with one of four glucose concentrations: 1) 5 mM glucose; 2) 10 mM glucose; 3) 30 mM glucose; or 4) 30 mM mannitol and cotreated with 100 nM NPY, 100 nM SP, or 10 ng/mL IL-8. Angiogenesis is assessed with proliferation and tube formation assays. IL-8 mRNA and protein expression are evaluated at days 1 and 7.**
Results: **As compared with noromoglycemia (5 mM glucose), hyperglycemia (30 mM glucose) decreases DMVEC proliferation and tube formation by 39% and 42%, respectively. SP cotreatment restores DMVEC proliferation (211%) and tube formation (152%), and decreases IL-8 expression (34%) in DMVECs exposed to hyperglycemic conditions. These effects are not observed with NPY. However, IL-8 treatment by itself does not affect proliferation or tube formation, suggesting that the effect of SP on DMVEC angiogenesis is unlikely through changes in IL-8 expression.**
Conclusion: **Hyperglycemic conditions impair DMVEC proliferation and tube formation. SP mitigates the effect of hyperglycemia on DMVECs by increasing DMVEC proliferation and tube formation. These findings are not likely to be related to a dysregulation of IL-8 due to the lack of effects of hyperglycemia on IL-8 expression and the lack of effect of IL-8 on DMVEC proliferation and tube formation. The effect of SP on DMVECs makes SP a promising potential target for therapy in impaired wound healing in diabetes, but the exact mechanism remains unknown. (J Vasc Surg 2011;53:1654-60.)**

Clinical Relevance: **Chronic diabetic foot ulceration, as a result of impaired wound healing in diabetes, is the source of a vast quantity of hospitalizations and lower extremity amputations across the nation. Normal wound healing involves the bidirectional signaling and interaction between a multitude of inflammatory cytokines and neuropeptides, and previous research has demonstrated a dysregulation in the expression and activity of these molecules in diabetes. This work evaluates the effect of hyperglycemic conditions and neuropeptides on DMVECs, a cell type fundamental to the wound healing process, but in which research is currently limited. The discovery of a molecule, which promotes angiogenesis in hyperglycemic conditions would be a promising therapeutic target for impaired wound healing in diabetes. In addition, the determination of the effects of these neuropeptides and cytokines on DMVECs could elucidate other potential therapies, which could decrease wound-healing time and improve overall wound healing in other realms of medicine.**

Impaired wound healing, and subsequently chronic diabetic foot ulceration (DFU), is a major complication associated with both Type I and Type II diabetes that significantly impairs the quality of life of diabetic patients. In particular, DFU is the leading complaint in all diabetic hospitalizations, resulting in a rate increase of over 10-fold of nontraumatic lower-extremity amputations in diabetic versus nondiabetic patients.[1]

Normal wound healing is a complex and precise process that consists of four phases: hemostasis, inflammation, proliferation, and remodeling. The integration and well-timed, linear progression through these phases, as well as the appropriate interaction between the cells and the inflammatory cytokines, is necessary for proper wound healing.[2] Most frequently implicated as alterations in both Type I and Type II diabetes and observed as the sources of impaired wound healing in diabetes are peripheral vascular disease and peripheral sensory neuropathy.[3] In diabetes, there is a chronic upregulation of inflammatory cytokines.

From the Division of Vascular and Endovascular Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School.
This work in part is funded by the William J. von Liebig Foundation award to Dr LoGerfo.
Competition of interest: none.
Reprint requests: Leena Pradhan, PhD, Instructor in Surgery, Division of Vascular and Endovascular Surgery, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave., Dana 805, Boston, MA 02215 (e-mail: lpradhan@bidmc.harvard.edu).
The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.
0741-5214/$36.00

doi:10.1016/j.jvs.2011.02.019

In addition, current work in our laboratory demonstrates that the initial acute inflammatory phase that leads to the secretion of cytokines, chemokines, and growth factors from immune cells is disrupted due to hyperglycemia, leading to abnormal expression and activity of these essential inflammatory mediators in wound healing.[4-6] Recent studies have shown that in normal wound healing, the neuromodulators interact with a variety of cell types involved in wound repair and regulate cytokine expression and activity, eliciting the downstream signaling of the inflammation and proliferation phases of wound healing. Moreover, in diabetics, these neuropeptides are decreased.[7] Therefore, a general impairment of the natural wound healing process, due to the nonsynchronous and incomplete progression through the various phases of wound healing, can be attributed to alterations in the interaction between cytokines and neuropeptides.[7-9]

The neuropeptides and inflammatory cytokines commonly associated with the success of normal wound healing and found to have abnormal expression in diabetic patients include the neuropeptides, neuropeptide Y (NPY) and substance P (SP), and the cytokines, interleukin (IL)-6, IL-8, tumor necrosis factor-α, platelet-derived growth factor, fibroblast growth factor, vascular endothelial growth factor, transforming growth factor (TGF)-α, and TGF-β.[7,10,11] It is unknown whether these neuropeptides influence the expression of cytokines in endothelial cells and/or whether these neuropeptides act through those cytokines to regulate angiogenesis and thus wound healing in hyperglycemic conditions.

IL-8 is a potent chemoattractant for keratinocytes, neutrophils, and other leukocytes, is a promoter of neutrophil adhesion, and is a known and potent autocrine proangiogenic molecule.[7] IL-8 is also one of the key inflammatory cytokines in wound healing that is chronically upregulated in diabetes and whose expression in the acute inflammatory phase following injury in diabetic patients is disrupted.[4,7] Moreover, SP is known to increase the secretion of IL-8 from different cell types, including colonic epithelial cells, airway epithelial cells, and mesenteric adipocytes.[12-14]

The goal of this study is to investigate the role of IL-8 in the effects of NPY and SP on the dermal microvascular endothelial cells (DMVECs) angiogenesis in normal and hyperglycemic conditions.

METHODS

Cell culture. Primary DMVECs were obtained from Lonza (Walkersville, Md) and grown in Lonza microvascular endothelial cell media (EGM-2MV). Cells were cultured to passages between 4 and 8. For the proliferation, mRNA expression, and protein expression assays, cells were plated at a density of 10,000 cells/cm^2; for the tube formation assay, cells were plated according to the assay protocol. Cells were subjected to one of four different glucose concentrations: 1) 5 mM glucose (standard EGM-2MV media); 2) 10 mM glucose 10 mM (EGM-2MV + 5 mM D-glucose); 3) 30 mM glucose (EGM-2MV + 25 mM D-glucose); or 4) 30 mM mannitol (osmolarity control; EGM-2MV + 25 mM mannitol). In addition, DMVECs were cotreated with 100 nM NPY (Sigma-Aldrich, St. Louis, Mo) or 100 nM SP (Sigma-Aldrich).

Proliferation assay. DMVECs were plated in replicates of six in 96-well plates. Twenty-four hours after plating, DMVECs were treated as listed above. On days 1, 2, 3, 5, and 7 after treatment, treatment media was aspirated off, and cells were subjected to 200 μL of 10% Alamar Blue (Trek Diagnostic Systems) in EGM media (Lonza) for 4 hours. Data were read at OD_{570} and OD_{600} and presented as ΔOD relative to day 1, representing fold change, as previously described.[15]

Tube formation assay. DMVECs were treated, as listed above, and plated at a density of 20,000 cells/well, onto the BD BioCoat Angiogenesis System – Endothelial Cell Tube Formation assay plate (BD Biosciences, Bedford, Mass). After 18 hours, the plates were washed twice with Hank's Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, Calif). Cells were then stained for 30 minutes with 8 mg/mL Calcein AM (Invitrogen) in HBSS and were again washed twice with HBSS. Finally, plates were imaged using a Nikon Eclipse Ti inverted fluorescent microscope (Melville, NY). Images were analyzed for total DMVEC tube fluorescence as a percentage of the entire well area using National Institutes of Health ImageJ software (Bethesda, Md).

Quantitate reverse transcription polymerase chain reaction (QRT-PCR). DMVECs were plated in triplicates on 24-well plates. Twenty-four hours after plating, DMVECs were treated as listed above. Total RNA was extracted from the DMVECs using a Qiagen RNeasy Mini Kit (Valencia, CA) at 24 hours and 7 days after treatment. cDNA was prepared from 10 ng of RNA using the Bio-Rad iScript cDNA synthesis kit (Hercules, Calif). IL-8 mRNA levels were then quantified, using a Stratagene MX3000P real-time PCR machine (La Jolla, Calif) and the following thermal cycling protocol: Stage 1: 10 minutes at 95°C – 1 cycle; Stage 2: 15 s at 95°C and 1 minute at 65°C, and 30 s at 72°C – 40 cycles. Primers and probes from Integrated DNA Technologies (Coralville, Iowa), at concentrations of 300 nM, and Stratagene Brilliant QPCR Master Mix reagents were used. Target gene levels were normalized to β(2) -microglobulin (β2M), as previously described.[15] All control treatments were compared to the 5-mM glucose control treatment within day 1 or day 7, and NPY 100 nM and SP 100-nM treatments were compared with their respective glucose control treatment within day 1 or day 7. Data are presented as relative IL-8 mRNA level.

Enzyme-linked immunosorbent assay (ELISA). Assay was performed on cell culture supernatants from the mRNA expression assay plates at 1 and 7 days after treatment. A BCA Protein Assay (Thermo Scientific, Waltham, Mass) was performed to quantify total protein content. An IL-8 ELISA kit from R&D Systems (Minneapolis, Minn) was used to quantify IL-8 protein levels. Plates were read with a Bio-Tek U-Quant Microplate Reader (Winooski, Vt). IL-8 levels were standardized to total protein content, and data are presented as standardized IL-8 protein level.

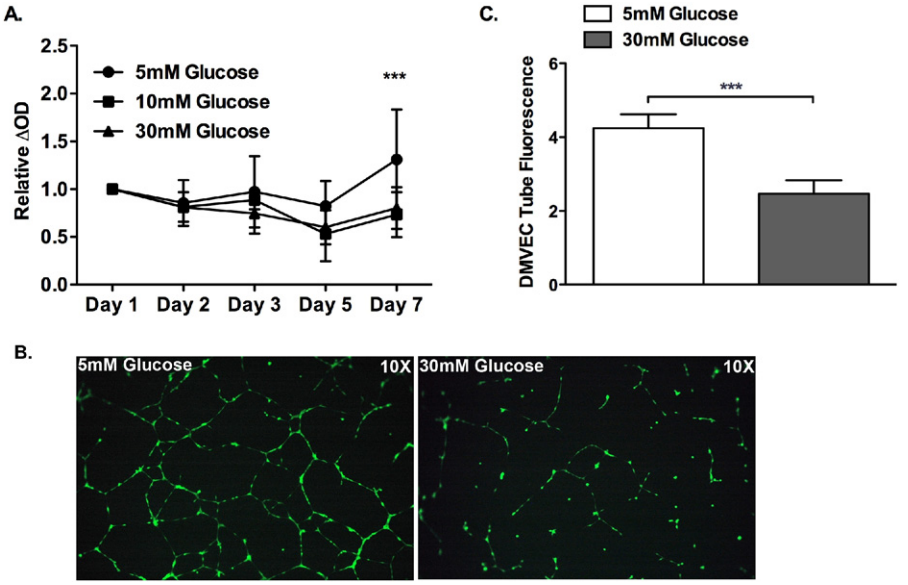


Fig 1. Effect of glucose on dermal microvascular endothelial cell (*DMVEC*) proliferation and tube formation. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose on DMVEC proliferation at 1, 2, 3, 5, and 7 days. Data are represented as relative ΔOD compared to day 1 for each treatment, respectively (n = 3, mean ± SD; ***$P < .001$). **B,** Representative pictures of DMVEC tube formation upon treatment with 5 mM and 30 mM glucose. **C,** Quantification of DMVEC tube formation upon treatment with 5 mM and 30 mM glucose. Data are represented as area of intact fluorescent tubes normalized to total well area (n = 3, mean ± SD; ***$P < .001$).

Statistical analysis. All experiments were repeated at least three times, and each treatment condition was performed in at least triplicate. All data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software (La Jolla, Calif). Significance of association was assessed using two-way analysis of variance (ANOVA) with Bonferroni post-hoc test or one-way ANOVA with Bonferroni post-hoc test. $P < .05$ was considered to be statistically significant.

RESULTS

Hyperglycemia decreases DMVEC proliferation and tube formation. As compared with the 5-mM glucose control treatment, DMVECs treated with 10 mM and 30 mM glucose demonstrate statistically significant decreases in proliferation by day 7, (5 mM glucose [1.309 ± 0.523] vs 10 mM glucose [0.734 ± 0.235] and 30 mM glucose [0.802 ± 0.219], n = 3; $P < .001$). No significant differences in proliferation are observed between DMVECs treated with 10 mM and 30 mM glucose (Fig 1, *A*).

Representative pictures of DMVECs treated with 5 mM and 30 mM glucose demonstrate decreases in tube formation in DMVECs treated with 30 mM glucose (Fig 1, *B*).

As compared with the 5-mM glucose control treatment, DMVECs treated with 30 mM glucose demonstrate statistically significant decreases in tube formation (5 mM glucose [4.245% ± 0.377%] vs 30 mM glucose [2.460% ± 0.365%], n = 3; $P < .001$).

No significant differences in proliferation or tube formation are observed in DMVECs treated with 30 mM mannitol as compared with control glucose treatment (data not shown; Fig 1, *C*).

SP treatment restores DMVEC proliferation and tube formation in cells exposed to hyperglycemia. As compared with DMVECs only treated with 10 mM or 30 mM glucose, DMVECs cotreated with 100 nM SP demonstrate statistically significant increases in proliferation (10 mM glucose [0.734 ± 0.235] vs 10 mM glucose + 100 nM SP [1.388 ± 0.258], n = 3; $P < .01$; 30 mM glucose [0.802 ± 0.219] vs 30 mM glucose + 100 nM SP [1.708 ± 0.546], n = 3; $P < .001$; Fig 2, *A*).

As compared with DMVECs only treated with 30 mM glucose, DMVECs cotreated with 100 nM SP demonstrate statistically significant increases in tube formation (30 mM glucose [2.460% ± 0.365%] vs 30 mM glucose + 100 nM SP [3.764% ± 1.354%], n = 3; $P < .05$; Fig 2, *B* and Supplemental Fig 1, online only).

No significant differences in proliferation or tube formation are observed between DMVECs cultured in 5 mM glucose and cotreated with 100 nM SP versus the 5-mM glucose control treatment (Fig 2, *A* and *B*).

NPY cotreatment does not affect DMVEC proliferation and tube formation. DMVECs cotreated with 100 nM NPY did not demonstrate any statistically significant changes in proliferation (Fig 3, *A*) or tube formation (Fig 3, *B* and Supplemental Fig 1, online only) at any glucose concentrations. However, a trend toward increased tube formation with NPY cotreatment in both the 5-mM glucose and 30-mM glucose concentrations is observed (Fig 3, *B*).

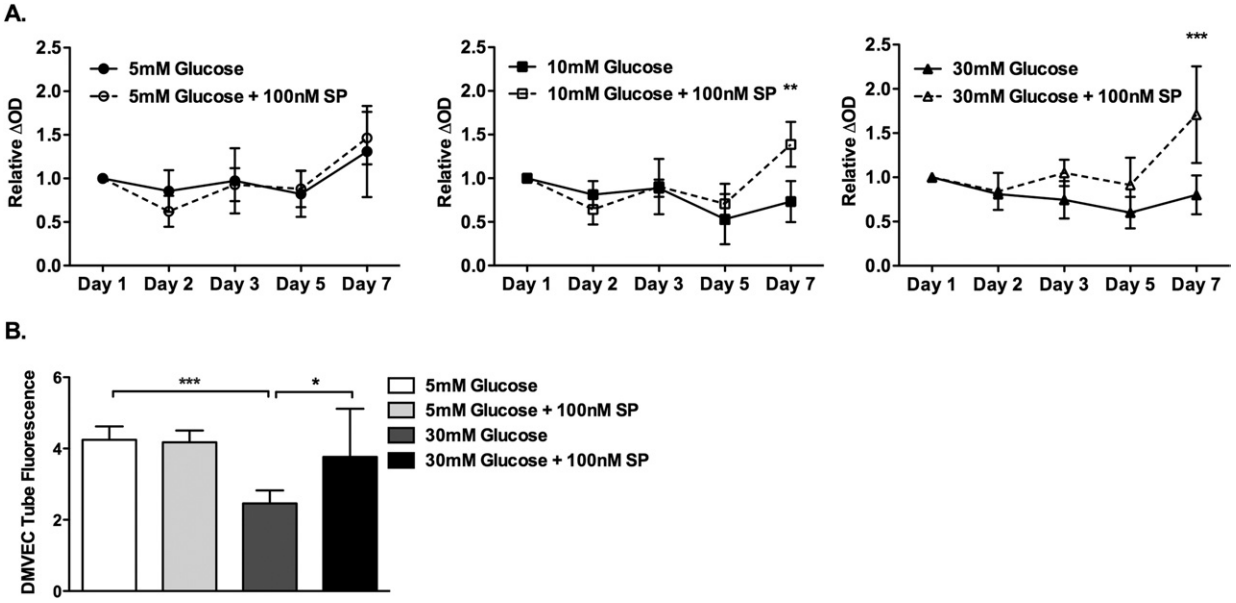


Fig 2. Effect of substance P (*SP*) cotreatment on dermal microvascular endothelial cell (*DMVEC*) proliferation and tube formation. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose and cotreatment with 100 nM SP on DMVEC proliferation at 1, 2, 3, 5, and 7 days. Data represented as relative ΔOD compared to day 1 for each treatment, respectively (n = 3, mean ± SD; **P < .01, ***$P < .001$). **B,** Quantification of DMVEC tube formation upon treatment with 5 mM or 30 mM glucose and cotreatment with 100 nM SP. Data are represented as area of intact fluorescent tubes normalized to total well area (n = 3, mean ± SD; *$P < .05$, ***$P < .001$).

SP treatment decreases while NPY treatment does not affect DMVEC IL-8 gene and protein expression in cells exposed to hyperglycemia. As compared with the 5-mM glucose control treatment on day 1, DMVECs cotreated with 100 nM SP demonstrate statistically significant decreases in IL-8 gene expression (5 mM glucose [1.000 ± 0.000] vs 5 mM glucose + 100 nM SP [0.654 ± 0.051], n = 3; $P < .01$). As compared with the 30 mM glucose treatment on day 7, DMVECs cotreated with 100 nM SP demonstrate statistically significant decreases in IL-8 gene expression (30 mM glucose [1.000 ± 0.000] vs 30 mM glucose + 100 nM SP [0.391 ± 0.167], n = 3, $P < .001$; Fig 4, *A*).

Furthermore, compared with 5-mM, 10-mM, or 30-mM glucose treatments on day 7, DMVECs cotreated with 100 nM SP demonstrate statistically significant decreases in IL-8 protein expression (5 mM glucose [0.102 ± 0.013] vs 5 mM glucose + 100 nM SP [0.069 ± 0.015], n = 3; $P < .001$; 10 mM glucose [0.084 ± 0.005] vs 10 mM glucose + 100 nM SP [0.054 ± 0.009], n = 3; $P < .001$; 30 mM glucose [0.108 ± 0.014] vs 30 mM glucose + 100 nM SP [0.037 ± 0.010], n = 3; $P < .001$; Fig 4, *B*).

DMVECs treated with NPY 100 nM demonstrate no significant differences in IL-8 gene or protein expression at days 1 or 7 (Fig 4, *A* and *B*).

IL-8 cotreatment does not affect DMVEC proliferation or tube formation in cells exposed to hyperglycemia. DMVECs cotreated with 10 ng/mL IL-8 do not demonstrate any statistically significant changes in proliferation (Fig 5, *A*) or tube formation (Fig 5, *B* and Supplemental Fig 1, online only) at any glucose concentrations. However, similar to NPY, a trend toward increased tube formation with IL-8 cotreatment in both the 5-mM glucose and 30-mM glucose concentrations is observed (Fig 5, *B*).

DISCUSSION

This study delineates the effects of neuropeptides on DMVEC proliferation, tube formation, and cytokine expression associated with normal and hyperglycemic conditions. Diabetes is associated with a high incidence of neuropathy and altered neuropeptide function. Accordingly, research has brought attention to the dysregulation of neuropeptides and cytokines in wound healing in diabetes. DMVECs are fundamental to the wound healing process, but research on the effects of hyperglycemic conditions and neuropeptides on these cells is limited. Thus, the identification of the effects of neuropeptides and cytokines on DMVECs, is essential to the development of viable treatment options for impaired wound healing in diabetes.

Previous research has demonstrated that diabetes causes a chronic inflammatory state in which the expression of cytokines is significantly increased and that this chronic inflammatory state disrupts the wound healing process.[4,7] In other disease models, SP is known to increase the expression of IL-8, a known autocrine, proangiogenic marker, and hence, it was logical to investigate the effects of SP on IL-8 expression in DMVECs. NPY, on the other hand, is known to be proangiogenic, but the exact mechanism by which it exerts its effect is not known.

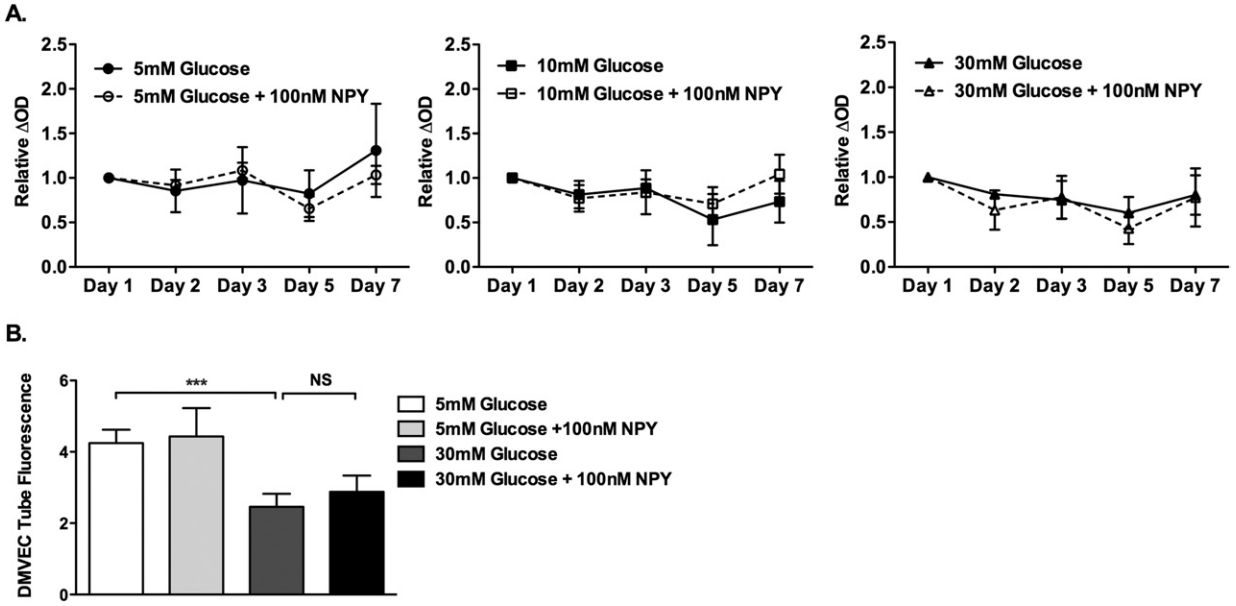


Fig 3. Effect of neuropeptide Y (*NPY*) cotreatment on dermal microvascular endothelial cell (*DMVEC*) proliferation and tube formation. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose and cotreatment with 100 nM NPY on DMVEC proliferation at 1, 2, 3, 5, and 7 days. Data represented as relative ΔOD compared to day 1 for each treatment, respectively (n = 3, mean ± SD). **B,** Quantification of DMVEC tube formation upon treatment with 5 mM or 30 mM glucose and cotreatment with 100 nM NPY. Data represented as area of intact fluorescent tubes normalized to total well area (n = 3, mean ± SD; $***P < .001$, NS = not significant).

In the present study, although hyperglycemic conditions caused significant decreases in DMVEC proliferation and tube formation, they did not change IL-8 expression (Supplemental Fig 2, online only). This suggests that the impairment of DMVEC proliferation and tube formation in hyperglycemic conditions is not related to a dysregulation of cytokine expression by DMVECs but an impairment of other processes in endothelial cells. Furthermore, it appears that DMVECs do not contribute to the chronic inflammatory state in diabetes through the upregulation of cytokine expression. As only one cytokine out of the numerous cytokines involved in inflammation is studied, this effect could be exclusive to IL-8 expression by DMVECs, and further studies would need to be performed to observe the effects of a hyperglycemic environment on the expression of other inflammatory cytokines by DMVECs. Still, given that IL-8 is a key inflammatory cytokine, it is unlikely that other cytokines would be affected while IL-8 expression is not. These findings have implications for further studies of cellular processes in endothelial cells that are affected in diabetes.

Here, we found that SP significantly increased DMVEC proliferation and tube formation in hyperglycemic conditions. This correlated with a decrease in IL-8 gene and protein expression in the hyperglycemic conditions. It is important to note that in normoglycemic conditions, there is an initial decrease in IL-8 gene expression with SP cotreatment at day 1, which is not sustained at the day 7 time point. On the other hand, the protein expression in the same treatment group is unchanged at day 1 time point but decreased at day 7 time point. One reason for this could be that SP acutely affects IL-8 gene expression, but these effects are not immediately translated into protein expression. However, with sustained SP cotreatment, an effect on protein expression is eventually observed. Thus, as with other important proteins, there is potentially a lag period between changes in gene expression and subsequent protein translation. Also, there could be a rebound effect on gene expression after the initial decrease observed, which could explain the normalization of gene expression at the day-7 time point. Future studies will include earlier time points (2, 6, and 18 hours) and also intermediary points (36, 48, and 72 hours) to obtain a complete picture of SP's regulation of IL-8 in DMVECs.

Furthermore, distinct dose-response trends following SP treatment, in which increased proliferative response and decreased IL-8 protein expression with increased glucose concentrations were identified. These findings demonstrate that SP modulates IL-8 expression in DMVECs. However, since IL-8 cotreatment did not influence proliferation or tube formation of DMVECs, it is evident that although SP affects IL-8 expression, SP does not affect DMVEC angiogenic function through IL-8. Moreover, the protein expression of the SP receptor, NK1R, was not altered by hyperglycemia (data not shown). Thus, the mechanism of SP's effects on DMVECs is unclear, and further studies into the signaling processes related to these effects are warranted.

As opposed to SP, NPY did not promote DMVEC proliferation or tube formation in normal or hyperglycemic conditions, with no dose-response trend related to glucose

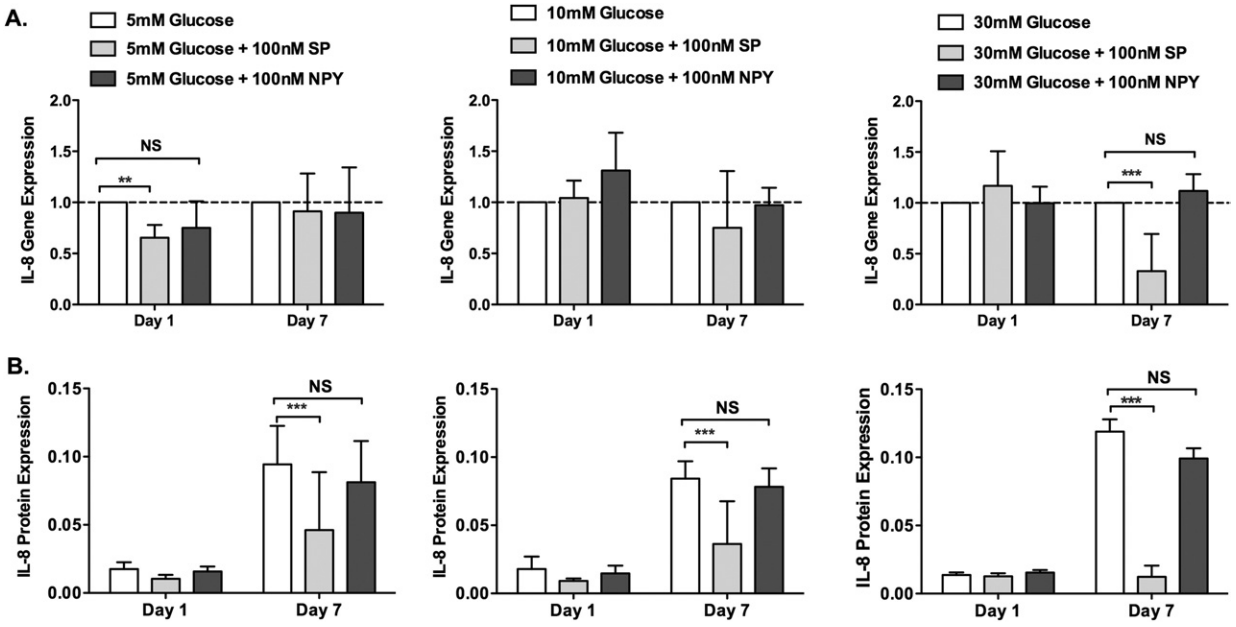


Fig 4. Effect of substance P (*SP*) or neuropeptide Y (*NPY*) cotreatment on interleukin-8 (*IL-8*) gene and protein expression. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose and cotreatment with 100 nM SP or 100 nM NPY on IL-8 gene expression at 1 and 7 days. Data expressed as fold change in gene expression compared to 5-mM, 10-mM, or 30-mM glucose control treatments, respectively (n = 3, mean ± SD; $^{**}P < .01$, $^{***}P < .001$, NS = not significant). **B,** Effect of 5 mM, 10 mM, and 30 mM glucose and cotreatment with 100 nM SP or 100 nM NPY on IL-8 protein expression at 1 and 7 days. Data normalized to total protein (n = 3, mean ± SD; $^{***}P < .001$).

concentration identified. Given that other studies have found that NPY induces human umbilical vein endothelial cell migration, proliferation, and tube formation, our results indicate that DMVECs are unlike other types of endothelial cells and that NPY could affect other cellular processes.[16] Additional research demonstrates that the protein expression of one of the NPY receptors, NPY5R, which is related to the angiogenic function of NPY, was not altered by hyperglycemia (data not shown), which lends support to the conclusion that changes in glucose concentration did not influence treatment outcomes in this study, and that NPY could affect other processes in these cells. The fact that NPY did not affect DMVEC IL-8 expression further attests to the fact that IL-8 is not involved in the pathways by which DMVEC proliferation and tube formation are promoted.

We observed that DMVEC proliferation continued over the 7-day period studied, with proliferative activity increasing over time and significant differences in proliferation identified by day 7. Moreover, with most treatment conditions, significant differences in IL-8 gene or protein expression, which were not present on day 1 were established by day 7. In normal wound healing, the proliferative phase, ultimately resulting in angiogenesis and re-epithelialization, begins about 3 days following injury, peaks about 7 days following injury, and is marked by the migration and proliferation of endothelial cells, keratinocytes, fibroblasts, and other cell types.[2,9] Thus, the increasing proliferative activity observed in the DMVECs over time, as well as the results observed on day 7 following treatment, imply a possible mechanism related to wound healing in which endothelial cells require longer exposure to the wound microenvironment before significant effects are detected.

The aim of this study was to characterize the effects of neuropeptides and cytokines on DMVECs in normal and hyperglycemic conditions. It is understood that there are limitations with research on wound biology with in vitro conditions. The complete wound microenvironment cannot be accurately simulated, and hence, a thorough evaluation of the complex molecular signaling and interaction involved in wound healing is restricted. Nevertheless, identifying the mechanisms underlying the effects of these neuropeptides and cytokines provides a basis from which to guide the development of potential therapeutics for impaired wound healing in diabetes.

Further studies are warranted to investigate the effects of SP on other known effectors of angiogenesis, such angiopoietins and other cytokines, in DMVECs using multiplex assays. Finally, understanding the effects of NPY and SP on inflammatory cell cytokine expression will be important in characterizing other contributors to the wound microenvironment and possible causes of impaired wound healing in diabetes.

In conclusion, this study demonstrates impaired DMVEC proliferation and tube formation in hyperglycemic environments, which is not associated with a change in

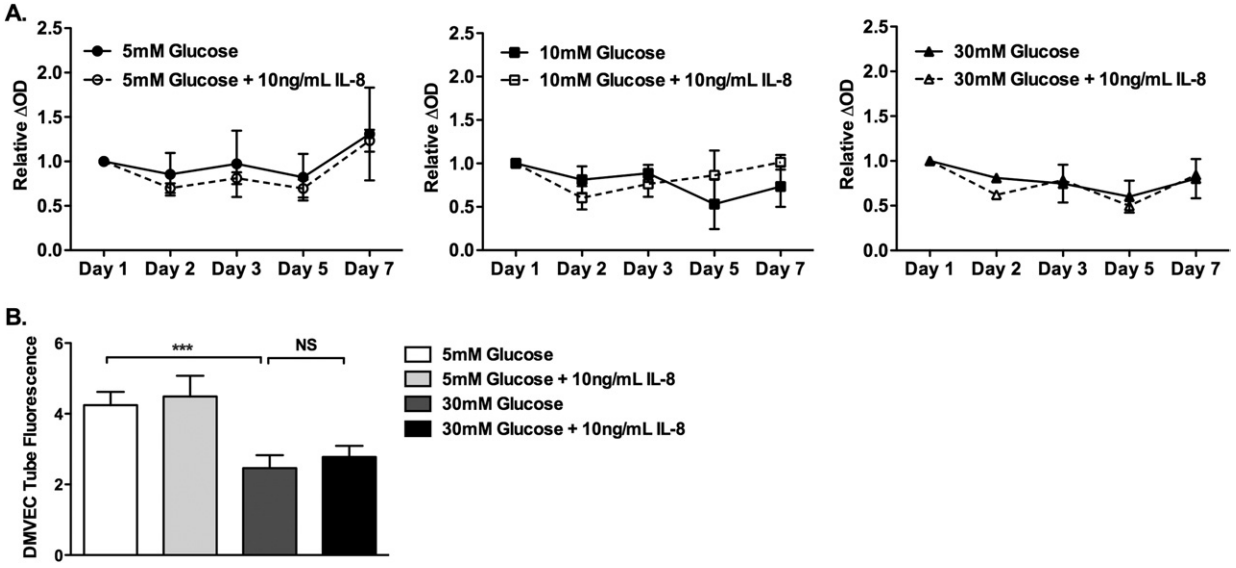


Fig 5. Effect of interleukin-8 (*IL-8*) cotreatment on dermal microvascular endothelial cell (*DMVEC*) proliferation and tube formation. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose and cotreatment with 10 ng/mL IL-8 on DMVEC proliferation at 1, 2, 3, 5, and 7 days. Data represented as relative ΔOD compared to day 1 for each treatment, respectively (n = 3, mean ± SD). **B,** Quantification of DMVEC tube formation upon treatment with 5 mM or 30 mM glucose and cotreatment with 10 ng/mL IL-8. Data represented as area of intact fluorescent tubes normalized to total well area (n = 3, mean ± SD; ***$P < .001$, NS = not significant).

DMVEC IL-8 expression. Furthermore, we demonstrate increased DMVEC proliferation and tube formation with SP treatment, which does not appear to be associated with decreased DMVEC IL-8 expression. Overall, we identify SP as a potential therapeutic target for the promotion of the angiogenesis phase of wound healing, and future studies are warranted to further define the interplay between neuropeptides and inflammatory cytokines in diabetes.

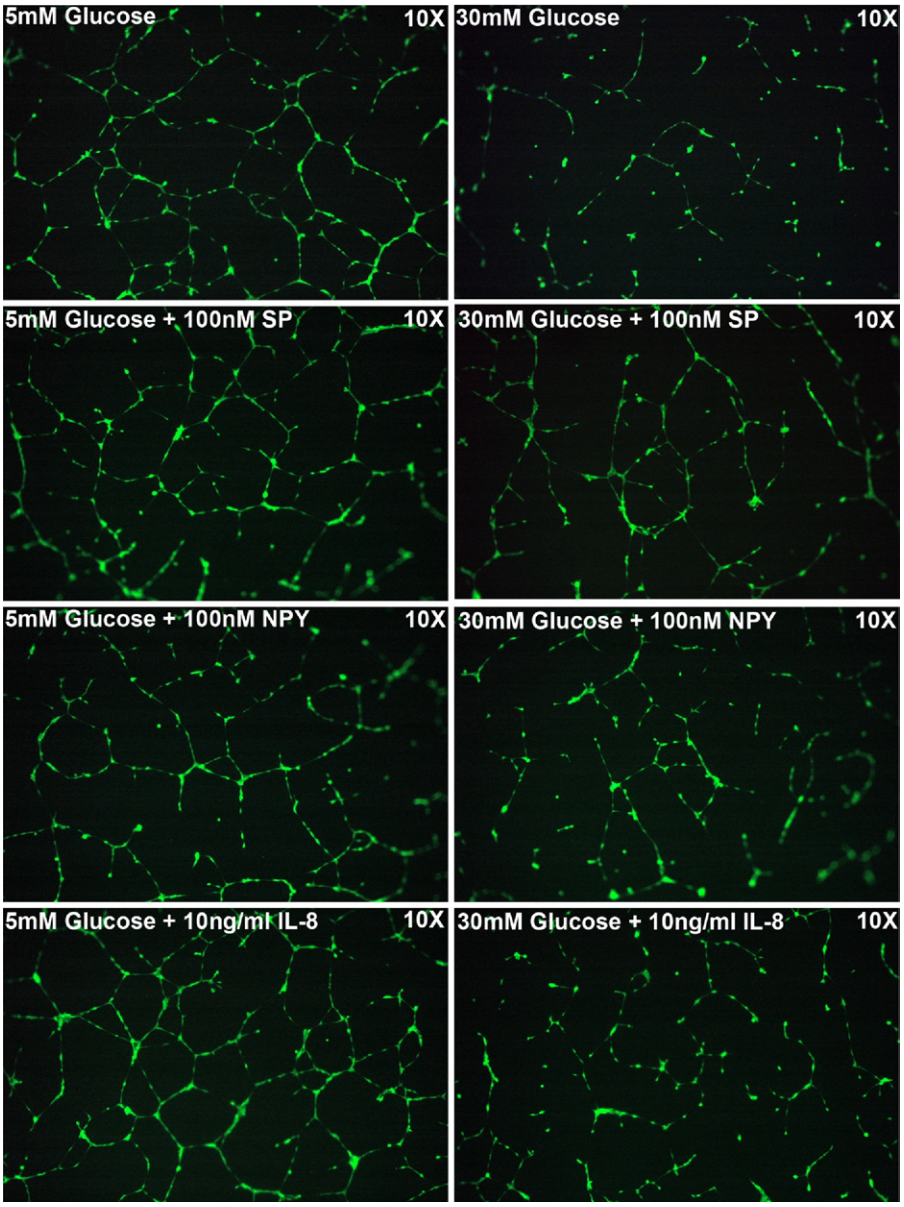
AUTHOR CONTRIBUTIONS

Conception and design: LP, FL
Analysis and interpretation: LP, MJ
Data collection: MJ, PG
Writing the article: MJ, LP
Critical revision of the article: MJ, LP
Final approval of the article: LP
Statistical analysis: MJ, LP
Obtained funding: FL
Overall responsibility: LP

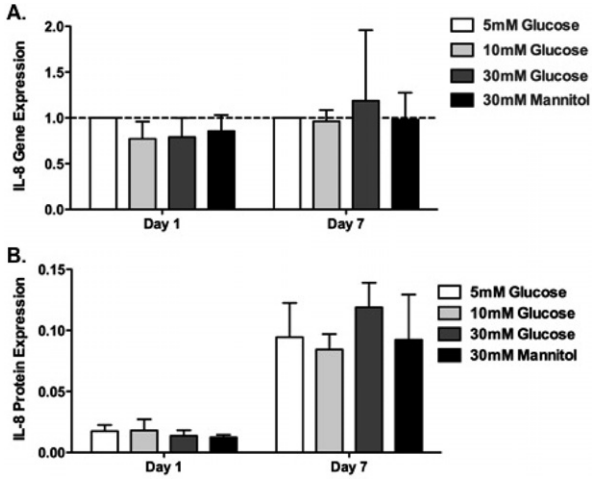
REFERENCES

1. Gordois A, Scuffham P, Shearer A, Oglesby A, Tobian JA. The health care costs of diabetic peripheral neuropathy in the US. Diabetes Care 2003;26:1790-5.
2. Baum CL, Arpey CJ. Normal cutaneous wound healing: clinical correlation with cellular and molecular events. Dermatol Surg 2005;31:674-86; Discussion:86.
3. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010;89:219-29.
4. Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, et al. Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 2011;167:336-42.
5. Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF. Impaired wound healing. Clin Dermatol 2007;25:19-25.
6. Armstrong DG, Lavery LA. Diabetic foot ulcers: prevention, diagnosis and classification. Am Fam Physician 1998;57:1325-32:37-8.
7. Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A. Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med 2009;11:e2.
8. Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M. Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. Physiol Rev 2006;86:1309-79.
9. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet 2005;366:1736-43.
10. Ochoa O, Torres FM, Shireman PK. Chemokines and diabetic wound healing. Vascular 2007;15:350-5.
11. Schaffer M, Beiter T, Becker HD, Hunt TK. Neuropeptides: mediators of inflammation and tissue repair? Arch Surg 1998;133:1107-16.
12. Koon HW, Zhao D, Zhan Y, Simeonidis S, Moyer MP, Pothoulakis C. Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves protein kinase Cdelta activation. J Pharmacol Exp Ther 2005;314:1393-400.
13. Zhao D, Kuhnt-Moore S, Zeng H, Pan A, Wu JS, Simeonidis S, et al. Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves Rho family small GTPases. Biochem J 2002;368:665-72.
14. Veronesi B, Carter JD, Devlin RB, Simon SA, Oortgiesen M. Neuropeptides and capsaicin stimulate the release of inflammatory cytokines in a human bronchial epithelial cell line. Neuropeptides 1999;33:447-56.
15. Monahan TS, Andersen ND, Martin MC, Malek JY, Shrikhande GV, Pradhan L, et al. MARCKS silencing differentially affects human vascular smooth muscle and endothelial cell phenotypes to inhibit neointimal hyperplasia in saphenous vein. FASEB J 2009;23:557-64.
16. Movafagh S, Hobson JP, Spiegel S, Kleinman HK, Zukowska Z. Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors. FASEB J 2006;20:1924-6.

Submitted Jan 9, 2011; accepted Feb 11, 2011.



Supplemental Fig 1 (online only). Dermal microvascular endothelial cell (DMVEC) tube formation. Representative pictures of DMVEC tube formation upon treatment with 5 mM or 30 mM glucose and cotreatment with either 100 nM substance P (*SP*), 100 nM neuropeptide Y (*NPY*), or 10 ng/mL interleukin-8 (*IL-8*).



Supplemental Fig 2 (online only). Effect of glucose treatment on IL-8 gene and protein expression. **A,** Effect of 5 mM, 10 mM, and 30 mM glucose on interleukin-8 (*IL-8*) gene expression at 1 and 7 days. Data are expressed as fold change in gene expression compared with 5-mM glucose treatment (n = 3, mean ± SD). **B,** Effect of 5 mM, 10 mM, and 30 mM glucose on IL-8 protein expression at 1 and 7 days. Data normalized to total protein (n = 3, mean ± SD).