A Central Role for Nicotinic Cholinergic Regulation of Growth Factor–Induced Endothelial Cell Migration

Martin K.C. Ng, Jenny Wu, Edwin Chang, Bing-yin Wang, Regina Katzenberg-Clark, Akiko Ishii-Watabe, John P. Cooke

Objective—An endothelial nicotinic acetylcholine receptor (nAChR) participates in atherogenesis and tumorigenesis by promoting neovascularization. To date, the mechanisms of nAChR-mediated angiogenesis and their relationship to angiogenic factors, eg, VEGF and bFGF, are unknown.

Methods and Results—Nicotine induced dose-dependent human microvascular endothelial cell (HMVEC) migration, a key angiogenesis event, to an extent which was equivalent in magnitude to bFGF (10 ng/mL) but less than for VEGF (10 ng/mL). Unexpectedly, nAChR antagonism not only abolished nicotine-induced HMVEC migration but also abolished migration induced by bFGF and attenuated migration induced by VEGF. Transcriptional profiling identified gene expression programs which were concordantly regulated by all 3 angiogens (nicotine, VEGF, and bFGF), a notable feature of which includes corepression of thioredoxin-interacting protein (TXNIP), endogenous inhibitor of the redox regulator thioredoxin. Furthermore, TXNIP repression by all 3 angiogens induced thioredoxin activity. Silencing thioredoxin by small interference RNA abrogated all angiogen-induced migration while silencing TXNIP strongly induced HMVEC migration. Interestingly, nAChR antagonism abrogates growth factor (VEGF and bFGF)–mediated induction of thioredoxin activity.

Conclusions—Nicotine promotes angiogenesis via stimulation of nAChR-dependent endothelial cell migration. Furthermore, growth factor–induced HMVEC migration, a key angiogenesis event, requires nAChR activation—an effect mediated in part by nAChR-dependent regulation of thioredoxin activity. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: nicotine ■ angiogenesis ■ endothelium ■ vascular endothelial growth factor ■ fibroblast growth factor

The nicotinic acetylcholine receptor (nAChR) is a pentameric ligand-gated cationic channel. The nAChR was first described in neurons, but has recently been identified in many cell types including endothelial cells (ECs) and vascular smooth muscle cells. Intriguingly, ECs also synthesize and store acetylcholine. Recently, we serendipitously discovered that nAChR activation causes ECs to form capillary tubes in vitro, and promotes angiogenesis in vivo.4,5 Pathological as well as physiological forms of angiogenesis are mediated by EC nAChRs. For example, by activating the EC nAChR, nicotine accelerates tumor angiogenesis and tumor growth in a murine Lewis lung cancer model.4 The acceleration of tumor growth by environmental tobacco smoke is also mediated by nAChR-induced angiogenesis.4 Furthermore, nAChR activation by nicotine stimulates the neovascularization and progression of atherosclerotic plaque.4 On the other hand, activation of the nAChR in a murine model of diabetic ulceration enhances wound angiogenesis and healing.7

To date, the mechanisms of nAChR-mediated angiogenesis and their relationship to established angiogenic growth factors, such as VEGF and bFGF, are unknown. We therefore sought to study and compare the effects of nAChR activation on EC migration, a key event in angiogenesis, alongside those induced by VEGF or bFGF. In this article, we report an unexpected observation: pharmacological antagonism of the nAChR fully blocks bFGF-induced EC migration, and substantially suppresses the endothelial response to VEGF. Furthermore, by microarray analysis, we identify gene expression programs which are concordantly regulated by nicotine, VEGF, or bFGF, and confirm the role of one of these genes in the cholinergic component of growth factor–induced endothelial cell migration.

Methods

For Methods, please refer to the Data Supplement, available online at http://atvb.ahajournals.org.

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mol/L, producing cell migration that was 216±9% that of vehicle-treated ECs (P<0.001 versus control; Figure 1B). Stimulation with VEGF or bFGF also induced EC migration with maximal effects at 10 ng/mL (data not shown). Nicotine-induced EC migration at 10⁻⁸ mol/L was equivalent in magnitude to that observed for bFGF (10 ng/mL) but less than for VEGF (10 ng/mL; P=NS for nicotine versus bFGF; P<0.01 for nicotine versus VEGF; Figure 1C). To further investigate the effects of nAChR-dependent pathways on EC migration, we studied the effect of the nAChR antagonist, hexamethonium (HEX, 10⁻⁴ mol/L), on EC migration induced by nicotine, VEGF, and bFGF. Hexamethonium abrogated nicotine-induced EC migration (Figure 1C). Unexpectedly, in addition to abolishing NIC-induced EC migration, HEX abolished migration induced by bFGF (P=NS versus control) and reduced migration induced by VEGF (P<0.01 for VEGF+HEX versus VEGF alone; Figure 1C). Similar results were observed with the nAChR antagonist, mecamylamine (10⁻⁶ mol/L; supplemental Figure I). The nAChR-related effect was dose-dependent: in the case of VEGF, cell migration was attenuated by 26±12%, 43±9%, and 52±12% by HEX concentrations of 10⁻⁶ mol/L, 10⁻⁷ mol/L, and 10⁻⁸ mol/L, respectively (P<0.05 for trend). Nicotinic receptor activation is known to stimulate EC proliferation. Interestingly, nAChR antagonism also significantly attenuated VEGF- and bFGF-mediated EC proliferation as assessed by bromodeoxyuridine incorporation (supplemental Figure II). The nAChR antagonist-related effects were not the result of cellular toxicity as addition of hexamethonium or mecamylamine alone did not induce cell death as examined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays, nor did mecamylamine or hexamethonium induce apoptosis in VEGF- or bFGF-treated cells as assessed by annexin V staining (data not shown). Previous in vitro and in vivo data have implicated a central role for the α7-nAChR isoform in mediating nAChR-induced neovascularization. Consistent with this, selective inhibition of the α7-nAChR isoform by α-bungarotoxin (10⁻⁹ mol/L) abrogated nicotine effects (P=NS versus control) and significantly attenuated bFGF and VEGF-induced EC migration by 50±11% and 38±13%, respectively (P<0.05 for stimulus versus stimulus + α-bungarotoxin; Figure 2A). We also studied HMVEC α7-nAChR mRNA expression in response to nicotine, VEGF, and bFGF over a 24-hour time course. At 1 hour, nicotine, VEGF, and bFGF all induced >10-fold downregulation of α7-nAChR expression compared with vehicle-treated conditions (P<0.05 versus control for all angiogens; Figure 2B). However, by 6 hours, there was significant upregulation of α7-nAChR expression by nicotine (12±0.8-fold), VEGF (11±1.3-fold), and bFGF (4±0.2-fold; P<0.001 versus control; Figure 2B). By 24 hours, HMVEC α7-nAChR expression in all conditions had returned to levels similar to control (P=NS versus control). These data suggest that nicotine, VEGF, and bFGF induce acute stimulation of α7-nAChR with subsequent early downregulation of α7-nAChR expression by negative feedback followed by upregulation at 6 hours. These findings are consistent with a role for nAChR in growth factor signaling pathways.

Results

A Cholinergic Component of Growth Factor–Mediated Endothelial Cell Migration

The effects of nicotine on human microvascular endothelial cell (HMVEC) migration were studied using standard wounding migration assays. Figure 1A depicts typical microphotographs of in vitro HMVEC migration in the presence of vehicle or nicotine. Nicotine stimulated EC migration in a dose-dependent manner with maximal stimulation at 10⁻⁸ M nicotine (10⁻⁸ mol/L), producing cell migration that was 216±9% that of vehicle-treated ECs (P<0.001 versus control; Figure 1B).
Identification of Shared Transcriptional Responses to Nicotine, bFGF, and VEGF by Microarray Analysis

To further evaluate a cholinergic contribution to growth factor–induced EC migration, and to identify commonly regulated genes that may be required for HMVEC migration, we performed microarray analysis of HMVECs after exposure to nicotine, VEGF, or bFGF. At 24 hours after treatment, each of these stimuli induced profound transcriptional changes in HMVECs (Figure 3), resulting in differential expression of a total of 3072 genes uniquely identified by UniGene, as well as 312 expressed-sequence tags, all of which were represented by 4070 nonredundant cDNA clones.

To study relationships between gene expression programs induced by nicotine, VEGF, or bFGF, data for all differentially expressed genes at 24 hours were hierarchically clustered by gene and by array, thereby organizing genes and experimental samples on the basis of similarity of expression patterns (Figure 3A through 3F). The cluster dendrogram shows that all 3 stimuli induced distinct transcriptional signatures which cluster within 3 distinct groups, but there is a closer relationship between the VEGF- and bFGF-induced expression profiles, which cluster together on the same dendrogram branch (Figure 3A).

Within the distinct transcriptional profiles induced by nicotine, VEGF, or bFGF, we identified 6 clusters with concordant gene expression (3 clusters of commonly activated and 3 commonly corepressed genes; Figure 3B through 3F). The characteristics of these clusters provide insights into shared cellular processes that may be requisite for angiogen-induced cell migration. The first activation cluster (Figure 3B), the “migration cluster,” was enriched for genes associated with cytoskeletal processes including migration-associated G protein signaling (Rho GTPase regulatory proteins and RIN2), integrin binding (ERBB2IP and ADAM9), cell cycle regulation and proliferation (RRM2, MDM2, AHR, MLLT4, and MUTYH), NF-κB activation (BCL10 and ADAM9), cell cycle regulation and proliferation (RRM2, MDM2, AHR, MLLT4, and MUTYH), and migration-associated oxidoreductase activity (LOX and ASPH). Significantly, three Rho GTPase activating proteins (GAPs) including ARHGAP5, ARHGAP21, and ARHGAP24 and one Rho guanine nucleotide exchange factor (GEF), ARHGEF7, were concordantly upregulated in this cluster. Rho GEFs and GAPs, by respectively controlling the activation and inactivation of small Rho GTPases (Cdc42 and Rac), regulate the orchestration of cytoskeletal and adhesive changes during cytokinesis. A smaller second coactivation cluster (Figure 3C) includes the p21-activated kinase PAK1, an effector for the Rho
GTases Rac and Cdc42, that facilitates cell migration by coordinating formation of new adhesions at the leading edge of the cell with detachment at the trailing edge.\(^\text{10}\) Other genes in this activation cluster comprise zinc finger proteins and genes involved in nucleic acid metabolism. Interestingly, all three angiogens induced activation of the VEGF receptor, FLT1, an effect that was stronger for bFGF-treated cells than for nicotine or VEGF (Figure 3D, which contains 3 nonredundant cDNA clones for FLT1). In addition to the coinduction of FLT1 by all three stimuli, we found that several isoforms of nAChR subunits are upregulated by VEGF at 24 hours (supplemental Table I), suggesting other potential synergistic interactions between VEGF and cholinergic signaling pathways.

The first repression cluster (Figure 3E) contains genes that are strongly downregulated by bFGF, many of which are also concordantly repressed by nicotine and VEGF. A dominant theme among concordantly repressed genes is the downregulation of chemokine genes (principally of CC class) involved in leukocyte chemotaxis (CCL2, CCL7, CCL8, CCL20, and CX3CL1). Another prominent feature is the robust repression of thioredoxin interacting protein (TXNIP) (Figure 3E), a protein that binds and inhibits thioredoxin, a major intracellular antioxidant. Other corepressed genes in this cluster have been implicated in apoptosis (TNFRSF1B, EP300), signal transduction (CD53, SQSTM1), and cell adhesion (ICAM1). The second repression cluster (Figure 3F) included two Ephrin receptors: EPHB4, a marker of venous differentiation and EPHA2, an inhibitor of cell migration which suppresses intracellular antioxidant activity significantly above vehicle-treated cells.**\(^{\text{P}<0.01}\).

In summary, the transcriptional signatures of nicotine, VEGF, and FGF, while distinct, demonstrate many overlapping features. By hierarchical cluster analysis, we have identified a series of shared angiogen-dependent EC transcriptional programs, with implications for understanding shared mechanisms in EC migration/angiogenesis.

**Role of Thioredoxin Interacting Protein in the Cholinergic Contribution to Growth Factor–Induced EC Migration**

As nAChR antagonism modulates VEGF- and bFGF-dependent EC migration, we hypothesized that some of the transcriptional effects shared by nicotine, VEGF, and bFGF may be nAChR-dependent. Thioredoxin interacting protein (TXNIP), a gene not previously associated with EC migration, was downregulated by all three angiogens. TXNIP is the endogenous inhibitor of thioredoxin. Thioredoxin is a major redox regulator of protein function increasingly implicated in tumorigenesis.\(^{\text{12,13}}\) In microarray data from 3 nonredundant cDNA clones for TXNIP, nicotine, VEGF, and bFGF consistently decreased TXNIP expression—a finding confirmed by RT-PCR, which demonstrated decreased expression by 42±4%, 33±6%, and 26±7% relative to control, respectively (\(^{\text{P}<0.001}\) for all stimuli; Figure 4A). As in vivo reduction of TXNIP expression in the order of 30% to 40% has been associated with >3-fold increases in thioredoxin activity,\(^{\text{14}}\) we hypothesized that TXNIP downregulation may influence thioredoxin activity and play a role in angiogen-mediated EC migration.

Using a standard assay for thioredoxin activity,\(^{\text{15}}\) we found that addition of nicotine or VEGF induced thioredoxin activity significantly above vehicle-treated cells (\(^{\text{P}<0.001}\) versus control for all stimuli; Figure 4B). The addition of bFGF induced a less robust (\(^{\text{P}<0.05}\) versus control) but significant increase in thioredoxin activity (Figure 4B). Notably, coadministration of hexamethonium inhibited nicotine-, VEGF-, or bFGF-induced thioredoxin activity (Figure 4B; \(^{\text{P}<0.001}\) for each stimulus versus stimulus + hexamethonium). Hexamethonium alone had no significant effect on thioredoxin activity. Consistent with these results, nAChR antagonism abrogated nicotine, VEGF, and bFGF-mediated repression of TXNIP mRNA expression (\(^{\text{P}=\text{NS}}\) versus control for each stimulus + hexamethonium; Figure 4A). Transfection of small interference RNA (siRNA) against
thioredoxin abrogated nicotine-, VEGF-, or bFGF-induced thioredoxin activity and abolished cell migration induced by nicotine, VEGF, or bFGF (P=NS versus control for each stimulus + siRNA; Figure 5A and 5B). Furthermore, in the absence of angiogenic stimuli, siRNA against TXNIP significantly stimulated thioredoxin activity (P<0.0001 versus control; Figure 6A) and strongly stimulated HMVEC migration (Figure 6B, P<0.0001 versus control). These studies indicate that inhibition of TXNIP by cholinergic or growth factor activation promotes endothelial cell migration, via derepression of thioredoxin activity. Furthermore, VEGF- or bFGF-mediated regulation of TXNIP expression is dependent on activation of nAChR (Figure 4A).

**Discussion**

We report a cholinergic contribution to growth factor-induced endothelial cell migration. The salient observations are that: (1) activation of nAChR induces EC migration similar in magnitude to that observed for bFGF or VEGF; (2) antagonism of nAChR markedly attenuates the migragenic effects of bFGF or VEGF on ECs; (3) the nAChR-dependent effects of bFGF and VEGF on EC migration are due, in large part, to activation of the α7-nAChR isoform; (4) nAChR activation induces a transcriptional profile that has many overlapping features to those induced by bFGF or VEGF, particularly for genes involved in EC migration; (5) downregulation of TXNIP with subsequent induction of thioredoxin activity is shown to be important to the migragenic effects exerted by each of the stimuli; (6) antagonism of the nAChR abrogates VEGF- or bFGF-mediated regulation of TXNIP expression. In toto, our findings identify a novel role for the nicotinic cholinergic pathway in growth factor-mediated EC migration, a critical event in angiogenesis.

Previous studies have demonstrated that ECs synthesize, store, and release acetylcholine and express functional nAChRs. Increasing evidence suggests that such nonneuronal nAChRs are involved in the regulation of vital cell functions, such as mitosis, differentiation, organization of the cytoskeleton, cell–cell contact, locomotion, and migration. Thus, acetylcholine, originally identified as a neurotransmitter, may function as an autocrine factor that modulates migration of endothelial cells. We and others have previously shown that exogenous nicotine, at pathophysiologically relevant concentrations, promotes angiogenesis in a number of in vivo settings, including inflammation, wound healing, ischemia, tumor, and atherosclerosis. Furthermore, inhibition of nAChR, in the absence of exogenous nicotine, reduces the angiogenic response in vitro and in vivo, indicating that there exists an endogenous cholinergic pathway for angiogenesis. In contrast, recent work has shown that VEGF and FGF, originally identified as angiogenic growth factors, exert neurotrophic effects and promote neurogenesis. These and other data suggest that there may be interdependence between “vascular” and “neuronal” factors and processes.

In this study, we found that nicotine induced dose-dependent nAChR-mediated EC migration which was maximal at concentrations consistent with those found in moderate smokers (10⁻⁸ mol/L). Surprisingly, coadministration of nAChR antagonists, hexamethonium, or mecamylamine, significantly attenuated the migragenic response of ECs to both VEGF and bFGF. Although several nAChR isoforms exist, we have previously identified a principal role for the α7-nAChR isoform in nAChR-mediated angiogenesis in vitro and in vivo. Consistent with these findings, we now find that VEGF and bFGF both induced changes in α7-nAChR ex-
pression consistent with activation of \( \alpha7 \)-nAChR. Moreover, the \( \alpha7 \)-nAChR selective antagonist, \( \alpha \)-bungarotoxin, attenuated VEGF and bFGF-induced EC migration to a similar extent as for the nonselective antagonists, hexamethonium, and mecamylamine. These latter findings suggest that nAChR-dependent pathways, particularly via \( \alpha7 \)-nAChR activation, are involved in the modulation of growth factor–induced EC migration.

To study the relationship between nicotine and angiogenic growth factors at a genomic level, microarray analysis was performed after HMVEC exposure to nicotine, VEGF, or bFGF. By hierarchical clustering, we found that each stimulus induced distinct but overlapping transcriptional responses, with concordant gene expression being concentrated within six largely functionally coherent gene clusters. A major functional theme among concordantly expressed genes was the coregulation of cell motility-related processes by all three angiogens. In particular, a “migration” cluster of concordantly activated genes was strongly enriched for genes involved in cytokinetic-related processes such as the Rho GTPase cell motility pathways, integrin binding, cell cycle regulation, and NF-\( \kappa \)B activation. Our findings with regard to activation of Rho GTPase pathways by VEGF and bFGF are consistent with previous studies and reinforce the central role of Rho-related regulation of actomyosin cytoskeletal organization EC migration during angiogenesis. We previously demonstrated that nAChR-dependent endothelial tube formation in vitro is dependent on NF-\( \kappa \)B activation. The migration cluster included two genes associated with NF-\( \kappa \)B activation: BCL10, an important activator of NF-\( \kappa \)B downstream of protein kinase C, and CASP8AP2 (aka FLASH), which coordinates downstream NF-\( \kappa \)B activity via a TRAF2-dependent pathway. In addition, many genes within the migration cluster have been implicated in oncogenesis (MDM2, ADAM9, BCL10, etc), a finding pathogenetically consistent with the role of angiogenesis in cancer.

The majority of concordantly regulated genes revealed by our microarray analyses have not been previously associated with angiogenesis. These include the p53 inhibitor MDM2 (activated by all three stimuli) and TXNIP, C-C chemokines, and metallothioneins (repressed by all three stimuli). These concordant transcriptional profiles provide further evidence for a cholinergic component of the angiogenic pathways. For example, our findings of coinduction of FLT1 by all three stimuli, and of nAChR subunit induction by VEGF are consistent with interaction between the signaling pathways.

Of the coregulated genes identified by hierarchical clustering, we focused our attention on TXNIP. Originally identified in HL-60 leukemia cells treated with 1,25 dihydroxyvitamin \( \text{D}_3 \) (and previously known as Vitamin \( \text{D}_3 \) upregulated protein 1), TXNIP is an endogenous inhibitor of the ubiquitous redox protein thioredoxin. Thioredoxin, a major redox regulator of protein function and signaling via thiol redox control, has been implicated in the regulation of cellular responses to oxidative stress and apoptosis. Thioredoxin selectively regulates the activity of DNA-binding proteins; for example, two transcription factors concordantly regulated by the three stimuli, NF-\( \kappa \)B, and p53, require thioredoxin reduction for stimulation of DNA binding. Increasing evidence implicates TXNIP and thioredoxin in tumorigenesis. Thioredoxin expression is increased in several human primary cancers, whereas TXNIP is strongly downregulated in human tumor tissues. Inhibition of thioredoxin signaling with experimental antitumor agents such as PX-12 (1-methylpropyl 2-imidazolyl disulfide) and pleurotin reduces tumor cell production of HIF-1\( \alpha \) and VEGF in vitro and inhibits tumor angiogenesis in vivo. We hypothesized that repression of TXNIP may play a role in mediating growth factor–mediated EC migration.

In the present study, we found that nicotine, VEGF, and bFGF stimulated thioredoxin activity—a finding consistent with their common suppression of TXNIP. Gene knockdown of thioredoxin by siRNA reversed the effect of growth factor stimulation and abrogated the effect of nicotine, VEGF, or bFGF on EC migration. These findings are consistent with a critical role for thioredoxin in mediating growth factor–induced EC migration. Interestingly, the increase in thioredoxin activity induced by each of the three stimuli could be blocked by nAChR antagonism. Finally, gene knockdown of TXNIP alone, without addition of growth factors, induced EC migration. Our findings indicate that TXNIP, by regulating of thioredoxin activity, may play an important role in angiogenesis mediated by growth factor receptors or nAChRs. The mechanism whereby thioredoxin mediates EC migration is poorly understood but may involve stimulation of hypoxia-inducible factor-1\( \alpha \) (HIF-1\( \alpha \)), a transcription factor that plays a central role in mediating the angiogenic response to hypoxia. Overexpression of thioredoxin in a variety of malignant cells has been shown to induce HIF-1\( \alpha \) expression and VEGF production. Induction of HIF-1\( \alpha \) in human endothelial cells upregulates the expression of multiple angiogenic factors including the angiopoietins which are potent stimulators of cell migration via Tie-2 signal transduction pathways. Moreover, as cellular redox state is an important determinant of Rho GTPase activity, it is possible that thioredoxin may play a role in Rho-mediated cytoskeletal remodelling during cell migration.

In summary, our data show that activation of nicotinic acetylcholine receptors (nAChRs) induces endothelial cell migration. Furthermore, growth factor (VEGF and bFGF)–induced endothelial cell migration involves nAChR activation. By transcriptional profiling we have identified convergent genomic responses of ECs to nicotine, VEGF, and bFGF. Identification of concordantly regulated genes may provide novel insights into molecular processes mediating EC migration and angiogenesis. Indeed, using this approach we found that TXNIP, by regulating thioredoxin activity, is centrally involved in nAChR-meditated EC migration. Our studies provide evidence for a cholinergic contribution to growth factor–induced EC migration. The nAChRs may play an important role in growth factor–induced angiogenesis, and thus may be a target for therapeutic modulation in disorders of pathological or insufficient angiogenesis.

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Disclosures
Dr Cooke holds equity in Athenagen Inc, which has licensed Stanford University patents for the use of nAChR agonists and antagonists for disorders of angiogenesis. Dr Cooke is an inventor on these patents, and receives royalties from the licenses. A patent is being filed based upon the intellectual property described in this manuscript that may benefit J.P.C., M.K.C.N., E.C., and J.W.

References
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Methods

Materials and Reagents

All cells and growth media were purchased from Cambrex-BioSciences (Walkersville, MD). Nicotine (free base), hexamethonium bromide, mecamylamine and α-bungarotoxin were purchased from Sigma-Aldrich (St. Louis, MO). Human VEGF165 and human bFGF were purchased from R&D Systems Inc (Minneapolis, MN). cDNA microarray slides were obtained from the Stanford Functional Genomics Facility (SFGF, Stanford, CA), and all microarray reagents were from Invitrogen (Carlsbad, CA), Stratagene (La Jolla, CA) or Amersham Biosciences (Piscataway, NJ).

Cell Culture

Adult dermal microvascular cells (HMVEC) were purchased from Cambrex-BioSciences (Walkersville, MD) as frozen aliquots at passage 3. The cells were expanded and used for both cell culture and microarray investigations at no later than passage seven. Cells were grown under standard conditions of 37°C in a 95% O₂/5% CO₂ atmosphere in EBM-MV2 media using 5% Fetal Bovine Serum (FBS) with supplements and antibiotics as per manufacturer’s protocol.
Cell Migration Assay

We employed a monolayer-wounding cell migration assay on early passage (P<7) HMVECs. HMVECs were pre-incubated with 1%FBS/EBM for 24 hours during which angiogenic factors (or vehicle) were added in the following concentrations: nicotine (10^{-10}M to 10^{-4}M), VEGF (1-10ng/ml), or bFGF (1-10ng/ml). In some cases, antagonists of the nAChR were co-administered including hexamethonium bromide (10^{-8}M to10^{-4}M), mecamylamine (10^{-6}M), or α-bungarotoxin (10^{-9}M). After 24h pre-incubation, cell layers are scraped with a sterile single-edged razor blade as previously described and reincubated their respective treatments for 24 h The extent of migration was quantified by counting the number of cells mobilized into the denuded area for a total of twelve microscopic fields of view (125X) per 60 mm plate (Leitz Labovert FS visible light microscope; Leitz, Wetzlar, Germany). All migration assays were performed by individuals blinded to treatment conditions and repeated using at least two independent vials of primary cells, with each experiment reproduced at least in triplicate.

HMVEC Proliferation by Bromodeoxyuridine (BrdU) Incorporation Assay

HMVEC cell proliferation was assessed by BrdU incorporation assay using a commercially available kit (ABSOLUTE-S SBIP Cell Proliferation assay, Molecular Probes, Eugene, OR) in accordance to manufacturer's instructions. In brief, HMVECs, grown to 50-70% confluence, were treated for 24 hours with
either: vehicle, nicotine (10^{-8} M), VEGF (10ng/ml), or bFGF (10ng/ml) with or without co-administration of hexamethonium bromide (10^{-4} M). After 24 hours, cells were incubated with BrdU for 30 minutes and then photolysed and labelled. BrdU was detected using an anti-BrdU antibody by standard immunohistochemical techniques. The fraction of BrdU positive cells was expressed as a percentage relative to control.

Microarray Analyses

HMVECs were grown to 50-70% confluency. Subconfluent rather than confluent HMVEC cultures were studied because they more closely resemble conditions associated with cell migration in vivo, and because this condition is associated with increased EC expression of α7-nAChRs (6). The concentration of the FBS in the media was then reduced to 1% so as to render the endothelial cells quiescent. After 24 hours, we added nicotine (10^{-8} M), VEGF (10ng/ml), bFGF (10ng/ml) or vehicle. Total RNA was then harvested at 24 hours. Five separate experiments per condition were processed for cDNA microarray analysis. We fluorescently hybridized labeled cDNAs (angiogen-treated HMVECs versus vehicle-treated HMVECs) on cDNA microarrays containing 39711 nonredundant cDNA clones, representing 26,260 unique UniGene clusters (SFGF, Stanford, CA) ². Scanning of processed microarray slides was performed using a GenePix4000A scanner and images analysed with GENEPIX PRO software (Axon Instruments, Foster City, CA). Microarray data were stored in the Stanford Microarray Database (Stanford, CA). We removed spots with signal <2.5-fold
above background in both channels and/or a regression correlation of $<0.6$. Genes with $<80\%$ evaluable data were excluded and the remaining data was then normalized by total intensity normalization. The significance of microarrays (SAM) algorithm was used with a one-class design to identify genes differentially expressed by treatment with each stimulus. Delta values giving approximately 5\% false discovery were chosen. To analyse convergent gene expression profiles, all differentially expressed genes at 24 hours were hierarchically clustered by gene and by array (Cluster software, version 2.11), and the results analysed with Treeview software (version 1.6).

**Real-time RT-PCR analysis of Thioredoxin Interacting Protein (TXNIP) and $\alpha 7$ Nicotinic Acetylcholine Receptor ($\alpha 7$-nAChR)**

Primer and fluorogenic probe sets for TXNIP and GAPDH were designed using Primer Express V2.0 software (Applied Biosystems, Foster City, CA). The TXNIP forward and reverse primers and fluorescent-labeled probes were 5'-AGATCAGGTCTAAAGCAGCAGAAC-3', 5'-TCAGATCTACCCAACTCATCTCAGA-3', and 5'-CCAGCATGGCCAGCCGAACC-3', respectively. The $\alpha 7$-nAChR forward and reverse primers and fluorescent-labeled probes were 5'-ATGCTGCTCGTGCTGAGAT-3', 5'-TGCGAAGTACTGGGCTATCA-3', and 5'-CCGCAACATCCGATTCCGATCC-3', respectively. The 5' fluorogenic reporter probe was 6-carboxy-fluorescein (FAM), and 3' fluorogenic quencher was 6-carboxytetramethyl-rhodamine (TAMRA). Primers and probes were synthesized by the Peptide and Nucleic Acid (PAN) Facility at Stanford University. cDNA was
synthesized and amplified from 10ng of total RNA using TaqMan One-Step RT-PCR master Mix Reagents Kit (Applied Biosystems). Amplification was performed on ABI Prism 7900HT Sequence Detection System. All samples were in triplicate. The reaction conditions were at 48°C for 30 minutes and at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Reactions without template and/or enzyme were used as negative controls. GAPDH was used as an internal control. RNA quantity was expressed relative to the corresponding GAPDH control. Fold induction over control is determined by normalizing treated samples to the control.

**Thioredoxin Bioassay**

Thioredoxin was assayed based upon previously published colorimetric protocols. The level of thioredoxin activity is assessed at 412 nm via the oxidation of a colorimetric substrate DTNB (5,5′-dithiobis(2-nitrobenzoic acid)). Protein extracts were obtained by subjecting cells to a lysis buffer (20mM HEPES (pH 7.9), 300mM NaCl, 100mM KCl, 10mM EDTA, 0.1% Nonidet P-40, PMSF protease inhibitors.).

**Gene Silencing by small interfering RNAs (siRNAs) for Thioredoxin (Trx) and Thioredoxin Interacting Protein (TXNIP)**

Double-stranded siRNA (PAN, Stanford University) for selective silencing of thioredoxin (GCAGAUCGAGGCAAGACUdtt) and TXNIP (ACAGACUUCGGAGUACCUGdtt) were transfected into cells
(Lipofectamine2000 reagent, Invitrogen). Twenty four hours after transfection, HMVEC migration experiments were performed, in the presence of vehicle or stimuli. Cell migration and thioredoxin activity were assayed as described above. Scrambled (randomly arranged) RNAi (GUUGGCCAUUCUACUUCGCTTdtt) was used as control.

**Statistical Analysis**

Results are expressed as mean±SEM. Differences between groups were analyzed by t-test (two-sided) or by ANOVA with assessment of intergroup differences by Bonferroni multiple comparisons test as appropriate. Probability values of < 0.05 were considered statistically significant.

**References**


**Figure Legends for Supplemental Figures**

**Supplemental Figure I.** Effects of the nicotinic acetylcholine receptor (nAChR) antagonist, mecamylamine (MEC), on growth factor-induced human microvascular endothelial cell (HMVEC) migration. Nicotine (10^-8M)-induced migration is abrogated by MEC (10^-6M) (P=NS for nicotine+MEC vs control). HMVEC migration induced by bFGF or VEGF (10ng/ml) is also significantly attenuated by MEC (P<0.001 for all angiogens versus angiogen + MEC). **P<0.001.

**Supplemental Figure II.** Role of nAChR in the regulation of endothelial cell proliferation as assessed by BrdU incorporation. Nicotine (10^-8M), VEGF (10ng/mL) and bFGF (10ng/mL) all stimulated human microvascular endothelial cell (HMVEC) proliferation (P<0.001 vs. control for all stimuli). Co-administration of hexamethonium (HEX, 10^-4M) abrogated nicotine-induced HMVEC proliferation.
(P=NS vs. control for nicotine + HEX). HEX also significantly attenuated the cell proliferative responses to bFGF and VEGF (P<0.05 for growth factor vs. growth factor + HEX for both VEGF and bFGF). *P<0.05;**P<0.001.
**Supplemental Table 1.**

VEGF upregulates Acetylcholine Receptor Subunits (24 hr)

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Supplemental Figure I

Migration Activity (% of Control)

NICOTINE
bFGF
VEGF
MEC