2008

Expression and Function of Nicotinic Acetylcholine Receptors in Human Vascular Endothelial Cells

Ryan C. Splittgerber
University Of Alabama At Birmingham

EXPRESSION AND FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS IN HUMAN VASCULAR ENDOTHELIAL CELLS

by

RYAN C. SPLITTGERBER

KENT T. KEYSER, CHAIR
CRAIG ELMETS
PAUL GAMLIN
ALECIA GROSS
LORI MCMAHON-WAKEFIELD

A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2008

EXPRESSION AND FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS IN HUMAN VASCULAR ENDOTHELIAL CELLS

RYAN C. SPLITTGERBER

VISION SCIENCE

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed in neural and non-neural tissues and have been reported to play a role in neovascularization and vascular diseases of the eye, specifically age-related macular degeneration (ARMD). However, the subunit composition of functional nAChRs, their mechanisms of action, and the downstream signaling pathways in non-neuronal tissues are not well understood. Since some nAChR subtypes have high Ca^{2+} permeability, we investigated the possible role of Ca^{2+} in activating signaling cascades that underlie the affects of nAChR activation in Human Dermal Microvascular Endothelial Cells (HDMECs). RT-PCR experiments revealed message for nearly all of the known nicotinic receptor subunits and muscarinic receptors. The effects of nAChR activation were examined using fluorescent Ca^{2+} imaging. The Ca^{2+} indicator dye, Fluo-3, revealed a significant increase in intracellular Ca^{2+} influx after exposure to nicotine. This increase was significantly reduced or eliminated by nAChR antagonists. The effect of nAChR activation on intracellular signaling cascades was assessed by measuring the phosphorylation of signaling proteins. Phosphorylation of ERK1/2 was increased by exposure to nicotine for five minutes but decreased after 60 minutes of exposure. ERK1/2 phosphorylation after nicotine exposure was significantly reduced by both specific and non-specific nAChR antagonists and binding of Ca^{2+} by the Ca^{2+} chelator BAPTA-AM, demonstrating that the phosphorylation was a result of calcium dependent nAChR activation. As a member of

the mitogen-activating protein kinase pathway, it is hypothesized that ERK1/2 activation leads to activation of transcription factors targeting angiogenesis-related genes. Real-time RT-PCR was used to analyze gene expression changes due to acute or chronic exposure to nicotine. At short durations nicotine exhibits anti-angiogenic effects and chronic exposure promotes a pro-angiogenic vascular environment via transcription of inflammatory molecules, adhesion molecules, and growth factors. These results were significantly decreased using nAChR antagonists. Our results suggest nicotinic activation of receptors in non-neuronal cells results in intracellular Ca^{2+} influx and phosphorylation of signaling kinases, leading to modified transcription of angiogenesis-related genes which we hypothesize affects cell proliferation or angiogenesis.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS

First and foremost, I thank God, with whom all things are possible.

I want to thank Brienne, my wonderful and patient wife. Six years ago we moved to Birmingham, nearly 1100 miles from home; my choice, not hers. Well, we're done now- and I do mean "we" because it took hard work from both of us to complete this dissertation. I couldn't have done it without you. Thanks and I love you.

For Carter- thanks for giving me another reason to do my best. Love, Dad.

It was a group effort to get me to this point in my career. With respect to my project, there are three big contributors: Margot Andison, who started me off on the right track and together we laid the foundation for my project. Margot has become a wonderful friend; Chris Strang, who helped me with just about everything since. Chris has been the constant always-there-for-me for-just-about-anything friend; Ginger Wotring, who was crucial to raising my scientific abilities to a higher level.

I would also like to thank Jordan Renna, who came to UAB in the same class and will leave the program at the same time. I was going to thank you for contributing to the delinquency of a co-worker, but I decided to rephrase it as *helping me to become a well-rounded scientist*, especially regarding all things sports related. Also, Dave McDougal, who together with Jordan kept me optimistic throughout these six years because you made be believe that your problems were always worse than mine, even if they weren't. I would also like to thank Marci Smith who is a recent addition to the lab but has become a good friend in such a short amount of time. I wish you all the best of luck.

Finally, I want to thank my mentor, Kent Keyser for bringing me into his lab and entrusting me with his research and ideas. I have learned more from you than you will ever know. I hope my future successes will make you proud and that you view them as a direct result of your guidance. Thank you.

LIST OF TABLES

Table *Page*

LIST OF FIGURES

Figure *Page*

NICOTINIC ACETYLCHOLINE RECEPTOR ACTIVATION CAUSES INCREASES IN [CA^{2+}] AND AFFECTS KINASE PHOSPHORYLATION STATES IN HDMECS

NICOTINIC ACETYLCHOLINE RECPTOR ACTIVATION REGULATES EXPRESSION OF ANGIOGENESIS-RELATED GENES IN HDMECS

INTRODUCTION

Tobacco use is implicated in the initiation and progression of many vascular diseases of the eye. The leading causes of tobacco-related blindness are age-related macular degeneration (ARMD), diabetic retinopathy (DR), and Giant Cell Arteritis (GCA). These vascular diseases are characterized by compromised blood flow to the retina due to neovascularization or alterations to existing blood vessels. In addition, the high concentration of nicotine found in the particulate form of cigarette smoke has been identified as mitogenic for many types of endothelial cells and also has been reported to induce networking of capillaries (Heeschen et al., 2002). Evidence supports a major role for nicotine in the progression of angiogenesis in ARMD, DR, and GCA.

Age-related macular degeneration is classified into two different disease phenotypes: nonexudative and neovascular, that is, dry and wet, repectively. While nonexudative AMD is the most prevalent clinical type, wet AMD results in the most rapid and severe cases of vision loss. The strongest environmental risk factor for all types of AMD is cigarette smoking (Armstrong 2003).

The wet form of ARMD results in vision loss due to abnormal blood vessel growth in the choriocapillaries. This blood vessel growth leads to leakage of vascular contents below the macula, resulting in damage to the photoreceptors and eventual blindness. Case-controlled studies link smoking to increased occurrence of ARMD in males, increased occurrence for individuals that no longer smoke but were heavy smokers no more than 20 years ago, increased occurrence for current smokers, and an even higher risk for heavy smokers (Armstrong 2003; Klein et al., 1993; Christen et al., 1996; Seddon et al., 1996).

Diabetic retinopathy is a vascular disease of the eye found in diabetes patients. In DR, patients show inflammation and ischemia of ocular blood vessels or the abnormal growth of new blood vessels on the surface of the retina, both leading to blindness. Smoking has been identified as a major risk factor for DR (Klein et al., 2006a). Studies have shown that the severity of retinopathy in diabetes patients is associated with increased central retinal venular equilivent (CRVE), a measure of venular caliber. Tobacco users show an increased CRVE over non-smokers and a positive correlation for increased CRVE and packs of cigarettes smoked per day (Klein et al., 2006b). In contrast, the same authors did not show an association between DR and smoking during 10 and 14 year progession studies of diabetic retinopathy (Moss et al., 1996; Klein et al., 1998)

Giant cell arteritis is an inflammatory disease, typically involving arteries of the head, neck, and shoulders, where the inflammation causes the arteries to narrow and impede blood flow or cause ischemia. The vascular blockage can reduce the blood flow through the retinal arteries, resulting in vision loss. Giant cell arteritis is considered an ophthalmic emergency as it carries a high risk of unilateral or bilateral blindness (Hayreh et al., 1998). Machado et al. (1989) in a case-control study of biopsy-proven GCA report a 2:1 odds ratio that smoking is associated with giant cell arteritis. Tobacco smoking has been reported to increase the incidence of GCA in women by 6-fold for casual smokers and 17-fold for heavy smokers (Duhaut et al., 1998).

Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated cation channels that belong to the same superfamily as $GABA_A$ and glycine receptors. cDNAs

encoding nine a subunits (α2- α10) and three β subunits (α2- β4) have been cloned from chicken and rat nervous systems (Nef, et al., 1988; Couturier, et al., 1990; Boulter, et al., 1986, 1990; Goldman, et al., 1987; Wada, et al., 1988; Deneris, et al., 1988, 1989). These subunits can combine to form a potentially large number of nAChR subtypes, each characterized by a unique subunit composition and pharmacological profile (Lindstrom 2000). α7, α8, or α9 subunits can form homomeric nAChRs, or combine with each other (for α7 and α8), or with other as yet unidentified subunits, to form receptors that bind, and are blocked by, α-bungarotoxin (αBgt), a protein from the venom of the banded krait. Neuronal nAChRs that consist of β2- β4 subunits in combination with α2- α6 subunits are *insensitive* to αBgt.

Thus, the known subunits can theoretically yield a vast number of different receptor complexes, each with a unique subunit composition. Studies in expression systems show that the resulting receptors differ in terms of their ligand binding properties and physiological characteristics (see Lindstrom, 1996, 2000). For example: the half-maximal effective concentration (EC_{50}) for acetylcholine for human α4β2 nAChRs in HEK 293 cells is ~3 μM (Buisson, et al., 1996) while the EC_{50} for ACh for rat α7 nAChRs is ~0.13 μM (Gopalakrishnan, et al., 1996). Therefore, cells can be differentially activated by ACh or nicotine depending upon the combination of receptor subtypes expressed and the concentrations of ligand to which they are exposed. In addition, different nAChR subtypes desensitize at different rates, with α7 nAChRs desensitizing very rapidly at moderate to high acetylcholine concentration yielding transient excitation. At similar ligand concentrations, cells that express α3 or β4 nAChRs would experience more tonic activation because of the slower desensitization kinetics.

Desensitization can in turn affect nAChR expression patterns. In expression systems, chronic nicotine exposure at concentrations near 10 nM induces both desensitization and upregulation of α4-containing neuronal nAChR subtypes (Fenster et al., 1997; 1999) and α4β2 and α7 nAChR subtypes are upregulated in the brains of smokers (Buisson & Bertrand, 2001; 2002).

It is important to note that choline, both a requirement for acetylcholine synthesis and break down product of acetylcholine, is a potent agonist for some nAChR subtypes including homomeric α7 nAChRs. The main source of choline is from dietary intake, and plasma choline levels are reportedly stable at about 10 μM (Lockman and Allen, 2002). Choline is taken up into cells by a high-affinity choline transporter expressed by some cells, and can be used as a precursor for ACh synthesis (Apparsundaram et al., 2001;Guo and Chiappinelli, 2002). Since many non-neuronal cells reportedly synthesize and release acetylcholine and there are non-neuronal sources of acetylcholinesterases, choline may be found at higher levels in some tissues than dietary intake might suggest (Wessler et al., 1999). Both endogenous choline and endogenous acetylcholine may activate nAChRs differentially, and α7 nAChRs may be tonically active at endogenous choline levels (Uteshev et al., 2002). This raises the possibility that nicotinic receptors can exert effects through endogenous pathways associated with normal maintenance of vasculature as well as effect changes in vascular endothelial cells associated with vascular diseases. The physiological asymmetries that result from receptor heterogeneity may have important consequences for cholinergic signaling in vascular endothelial cells.

Expression of ACh and nAChRs by non-neuronal cells

There is considerable evidence that ACh is synthesized and stored in non-neuronal tissues. Wessler et al., (1998) found choline acetyltransferase (ChAT), the rate-limiting enzyme in the production of ACh, and nAChRs in human epithelial cells. These authors also report acetylcholine expression in endothelial cells and proposed that ACh of non-neuronal origin is involved in mitosis, trophic functions, cellular activity, and the formation of cell-cell contacts. There are other reports that vascular endothelial cells express nAChRs comprised of neuronal type subunits including $\alpha 7$ and $\alpha 3\beta 4$ nAChRs (Kawashima et al., 1989; 1990; Macklin et al., 1998; Wang, et al., 2001). This has lead to the suggestion that ACh might act as an autocrine factor in blood vessels (Cooke and Bitterman, 2004).

Nicotine is responsible for many of the effects of cigarette smoke and for the addictive properties of tobacco. The high concentration of nicotine found in the particulate form of cigarette smoke has been identified as mitogenic for many types of endothelial cells and also has been reported to induce networking of capillaries (Heeschen et al., 2002). Nicotine and other cholinergic agents have been shown in some studies to contribute to increased angiogenesis (Heeschen et al., 2001; Conklin et al., 2002), and accelerated wound healing (Lu et al., 2001). For example, nicotinic agonists have been shown to enhance the process of angiogenesis in a diabetic wound model (Jacobi et al., 2002). On the other hand, other reports describe cigarette smoking as having a deleterious effect on wound healing (Arredondo, et al., 2001; Hagforsen, et al., 2002). Nicotine has also been implicated in tumor angiogenesis (Kerbel, 2000) and

hypervascularization. Heeschen et al. (2003), reported expression in human umbilical vein endothelial cells (HUVECs) and verified a relationship between α7 nAChRs and vascular endothelial growth factor (VEGF) whereby microvascular tube formation could be significantly decreased with inhibition of either nAChRs or VEGF. Indeed, the angiogenesis effects of nicotine were found to be as potent as those of VEGF or FGF (Cooke and Bitterman, 2004). Still other reports describe different effects based on nicotine concentration and length of exposure (Villablanca 1998; Zia et al., 2000). The diverse effects of nicotinic activation reported in different vascular tissues may be due in part to differential expression of nAChR subtypes on different cell types.

In summary, there is limited information concerning which of the many nAChR subtypes are functional in vascular endothelial cells, and whether this functional expression pattern is stable or changes under different conditions, such as the chronic exposure to nicotine experienced by tobacco, nicotine patch, or nicotine gum users. Together with the lack of a mechanistic explanation of how cation channels on the cell surface effect changes in vascular cell intracellular signaling and gene expression, these gaps in our knowledge comprise major obstacles to a more complete understanding of the pathophysiology of tobacco-related vascular eye diseases.

NICOTINIC ACETYLCHOLINE RECEPTOR ACTIVATION CAUSES INCREASES IN [CA^{2+}] AND AFFECTS KINASE PHOSPHORYLATION STATES IN HDMECS

RYAN C. SPLITTGERBER
MARGOT E. ANDISON
JASON LOWERY
CHRISTIANNE E. STRANG
CRAIG A. ELMETS
KENT T. KEYSER

Submitted to American Journal of Physiology-Cell Physiology

Format adapted for dissertation

ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed in neural and non-neural tissues and have been reported to play a role in neovascularization and vascular disease (24). However, the subunit composition of functional nAChRs, their mechanisms of action, and the downstream signaling pathways in non-neuronal tissues are not well understood. Since some nAChR subtypes have high Ca^{2+} permeability, we investigated the possible role of Ca^{2+} in activating signaling cascades that underlie the affects of nAChR activation in Human Dermal Microvascular Endothelial Cells (HDMECs). RT-PCR experiments revealed message for nearly all of the known nicotinic receptor subunits and muscarinic receptors. The effects of nAChR activation were examined using fluorescent Ca^{2+} imaging. The Ca^{2+} indicator dye, Fluo-3, revealed a significant increase in intracellular Ca^{2+} influx after exposure to nicotine. This increase was significantly reduced or eliminated by nAChR antagonists. The effect of nAChR activation on intracellular signaling cascades was assessed by measuring the phosphorylation of signaling proteins. Phosphorylation of ERK1/2 was increased by exposure to nicotine for five minutes but decreased after 60 minutes of exposure. ERK1/2 phosphorylation after nicotine exposure was significantly reduced by both specific and non-specific nAChR antagonists and binding of Ca^{2+} by the Ca^{2+} chelator BAPTA-AM, demonstrating that the phosphorylation was a result of calcium dependent nAChR activation. Our results suggest nicotinic activation of receptors in non-neuronal cells results in intracellular Ca^{2+} influx and phosphorylation of signaling kinases, which we hypothesize, may affect cell proliferation or angiogenesis.

INTRODUCTION

Smoking has been identified as the single most important cause of many vascular diseases. Previous studies suggest that use of tobacco products is essential to the establishment and development of Thromboangiitis obliterans (Buerger's Disease), a vascular skin disease characterized by decreased blood flow to the extremities accompanied by reorganization of the small and medium sized arteries and veins (41). Case-control studies report smoking to be central to vascular skin diseases such as discoid lupus erythematosus, palmoplantar pustulosis (40), and psoriasis (37, 43). In addition, cigarette smoke has been shown to increase endothelial vascularization in an animal model (45). A confounding factor in these studies is that cigarette smoke contains thousands of toxic compounds (19), making it difficult for any study using smoke inhalation to accurately attribute the results to a single component and correctly label other possible interactions as non-factors. However, the high concentration of nicotine found in the particulate form of cigarette smoke has been identified as mitogenic for many types of endothelial cells and also has been reported to induce networking of capillaries (24).

Nicotine is a potent agonist for neuronal nicotinic acetylcholine receptors (nAChRs). Neuronal nAChRs are pentameric non-specific cation channels composed of α- and β-subunits. cDNA encoding nine a subunits (α2- α10) and three β subunits (β2-β4) have been cloned from chicken and rat nervous systems (6, 10, 11, 15, 18, 22, 38, 50). These subunits can combine to form a potentially large number of nAChR subtypes, each characterized by a unique subunit composition and pharmacological profile (29). α7, α8, or α9 subunits can form homomeric nAChRs or in some cases can combine with

each other to form receptors that bind, and are blocked by α-bungarotoxin (αBGT), a protein from snake venom (23). In contrast, neuronal nAChRs that consist of β2-β4 subunits in combination with a2-a6 subunits are *insensitive* to αBGT. Expression studies suggest that as few as one or two neuronal nAChR genes, each encoding a different subunit, is sufficient to generate nicotinic responses in neurons (2, 6, 10, 11, 15, 28, 50).

There is considerable evidence that components necessary for nicotinic cholinergic signaling are functionally expressed in non-neuronal tissues (52). Studies report that ACh release was non-vesicular and met all criteria to be considered as an autocrine/paracrine hormone in lung epithelial cells (44). Lung epithelial cells express ChAT, the vesicular ACh transporter (VAChT), the high-affinity choline transporter (CHT), and α4, α7, and β2 nAChR subunits. Macklin et al., (1998) demonstrated that vascular endothelial cells express nAChRs with properties similar to the receptors expressed in autonomic ganglionic neurons and epidermal keratinocytes (31). Using *in situ* hybridization, these authors identified α3, α5, β2, and β4 ganglionic-type nAChR subunits in human vascular endothelial cells. Furthermore, studies identified the expression of functional α7 subunits in human bronchial and aortic endothelial cells, and provided indirect evidence for the existence of functional α3β4 nAChRs (51). Activation of nAChRs by cholinergic agents can exert a variety of effects in non-neuronal tissue. For example, nicotine and other cholinergic agents have been shown in some studies to contribute to increased angiogenesis (9, 24), and accelerated wound healing (30). Nicotine has also been implicated in tumor angiogenesis and hypervascularization (27). Zia et al., (2000) demonstrated that long-term nicotine

exposure decreased keratinocyte migration, and nicotine also increased levels of Ca^{2+} influx in keratinocytes (55). Authors reported nAChR expression in human umbilical vein endothelial cells (HUVECs) and verified a relationship between nAChRs and vascular endothelial growth factor (VEGF), whereby microvascular tube formation could be significantly decreased with inhibition of either nAChRs or VEGF (25). The angiogenic effects of nicotine were found to be as potent as those of VEGF or fibroblast growth factor (FGF). Still other reports describe different effects based on nicotine concentration and length of exposure (49, 55). The diverse effects of nAChR activation reported in different vascular tissues may be due in part to differential expression of nAChR subtypes on specific cell types. In particular, the α7 nAChR subtype may play a larger role in some cells because of its high Ca^{2+} permeability and ability to affect second messenger pathways (47).

We show below that nicotine-induced increases in intracellular Ca^{2+} initiate the activation of specific intracellular signaling pathways, including the MAPK pathway in HDMECs through nicotinic activation of non-neuronal acetylcholine receptors.

MATERIALS AND METHODS

Cell Culture

Human dermal microvascular endothelial cells (HDMECs) and human umbilical vein endothelial cells (HUVECs) were isolated from human foreskin and umbilical cord, respectively, obtained from Dr. Robert Swerlick of Emory University and cultured at the UAB Skin Cell Culture Core. Cells were grown in 80 cm^2 flasks incubated with Clonetics' Endothelial Cell Basal Medium (EBM-MV; Cambrex, East Rutherford, NJ).

Flasks that were 80-90% confluent were used for experiments. HDMECs were tested for mycoplasma contamination using Lonza's MycoAlert Assay (Lonza, Rockland, ME).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from cultured HDMECs and HUVECs using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). Briefly, cells were homogenized in lysis buffer containing guanidine thiocyanate and β-mercaptoethanol. The homogenate was prefiltered, captured onto a fiber matrix RNA binding filter and washed in low-salt buffer. Contaminating DNA bound to the fiber matrix was removed by DNase digestion and followed by a series of high and low salt buffer rinses. RT-PCR was performed using a Qiagen One-Step RT-PCR kit (Valencia, CA) and primers for nAChR subunits and muscarinic receptors (26), as listed in Table 1. DNA products were electrophoresed on 2% agarose gels, excised, and purified using Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Sequencing was carried out by the Sequencing Core Facility operated by the University of Alabama at Birmingham (UAB) Comprehensive Cancer Center.

Calcium Imaging

Cells were loaded with Fluo-3 AM [2mM] Ca^{2+} indicator dye (Molecular Probes, Eugene OR) for 30 minutes at 37°C and subsequently washed three times with Hanks Balanced Salt Solution (HBSS). Agonists and antagonists were bath applied by a perfusion pump. Nicotine was used as a non-specific agonist and was used at a working concentration of 10mM. To determine involvement of homomeric α7 nAChRs, αBGT (10 μM) was used

Table 1. Custom nAChR and mAChR primers for RT-PCR

nAChR	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)	Homology (%)	
				HDMEC	HUVEC
α2	GTGGAGGAGGAGGACAGA	CTTCTGCATGTGGGGTGATA	150	90	92
α3	CAAGCAACGAGGGAACG	CCGTCCTGGCAGGGGTAG	121	91	90
α4	GTTCCATGACGGGCGGGTGCAGTGGAC	ATCATCCCGTCCACCTCACTGGTCAT	482	98	98
α5	TTTCTTCACACGCTTCCCAAA	TCACGGACATCATTTTCCTTCA	179	100	100
α6	ACCAATTTGTGGCTGCGTCAC	CCAGAGATGTGGATGGGATGGT	652	--	--
α7	CTCGCAACCACTCACCGTCTACTTC	GATTCCCACTAGGTCCCATTCTCCA	463	100	100
α9	ATTCCCTGTGATCTATTCCAATGTT	CAGTCACCCACCACTACGGTG	213	100	100
α10	GTGCGTGCGGGAAAGAGGG	CCTGGCGGCACAGACATCG	134	98	100
β2	GTGGGAAGATTATCGCCTCA	CAGTTCTGCTGGTCAAATGG	249	98	98
β3	TTGGAAAAAGCTGCTGATTCC	GGTAAAAATCAGAACCGAGCC	168	--	98
β4	AGCAAGTCATGCCGTGACCAAG	GCTGACACCTTCTAATGCCTCC	210	100	100

mAChR	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)	Homology (%)	
				HDMEC	HUVEC
M1*	GAAGAAGAGGAAGAGGACGAA	CAGGAGAGGGGACTATCAGCA	573	100	100
M2*	GGGTCCTCTCTTTCATCCTCT	TCCTGGGTTATTTCATCATCT	469	96	100
M3*	GTCTGGCTTGGGTCATCTCCT	ACTGCTGCTGTGGTCTTGGTC	434	99	99
M4*	TGGGTACTGTCCTTCGTGCT	CACACTCATTGCCTGTCTGCTTCG	592	93	98
M5*	CTCATCAGTGGAATCTTCTCCA	GGTCCTTGGTTCGCTTCTCTGT	451	98	100

*Hellström-Lindahl & Nordberg, 1996

to prevent activation of α7 and α9-containing nAChRs. Methyllycaconitine (MLA) is an antagonist of α6, α7, and α9 containing nAChRs at low nM concentrations and acts as a nonspecific blocker at low μM concentrations. To determine the involvement of other subtypes, MLA was used at non-specific concentrations (10 μM). Ca^{2+} influx via release from intracellular stores was prevented by Ruthenium Red (RR, 5 μM) and Xestospongin C (XeC, 5 μM) which inhibit Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores and inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release, respectively (21, 34, 53). Control cells were incubated with Fluo-3 AM dye and images were obtained after addition of HBSS alone.

Ca^{2+} fluorescence was examined with a Leica TCS-SP confocal laser-scanning microscope using an Argon-Krypton laser at 30 second intervals for up to 300 seconds. Quantification was performed using the Leica confocal software to measure intensity histograms of manually drawn ROIs around individual cells. Two-Way ANOVA was performed with Bonferonni's multiple comparisons post hoc test (GraphPad Prism 4, San Diego, CA) to determine statistically significant differences between average fluorescence intensities for cells at different intervals for the various pharmacological agents.

Protein Phosphorylation

HDMECs (P4) were incubated with nicotine for 5 minutes to emulate acute nicotine exposure or one hour with nicotine or vehicle (sterile ddH_20) to emulate chronic nicotine exposure. Cells were also treated with nAChR and calcium antagonists, as described above. Protein was extracted per Human phospho-MAPK Proteome Profiler Antibody

Array protocol (R&D Systems; Minneapolis, MN). Briefly, cells were solubilized with lysis buffer and microcentrifuged at 4°C for 30 minutes and the lysate was removed. Protein was concentrated from the lysate using a 0.5 mL Millipore Ultrafree Centrifugal Device (Billerica, MA). Individual arrays were washed with washing buffer for one hour prior to addition of proteins on a rocking platform and subsequently incubated overnight at 4°C with 250mL lysate (250mg) per array. After incubation, each array was washed 3 X 10 minutes with wash buffer and then incubated for two hours at room temperature with antibody detection cocktail and the provided array buffer. After washing, Streptavidin-HRP (1:2000) was applied for 30 minutes at room temperature. After washing, a chemiluminescent agent (R&D Systems) was applied to the array for 1 minute and subsequently imaged and quantified using Kodak Image Station 4000MM. ROI's were placed around each blot and compared to the respective blot in the control array. Background was subtracted and raw data were transformed to percent changed from control. One-Way ANOVA using Tukey's multiple comparison post hoc test was used to identify significant differences between conditions.

RESULTS

Acetylcholine receptor expression

NAChR subunit expression by endothelial cells was evaluated by using RT-PCR with human based primers: transcripts for α2, α3, α4, α5, α7, α9, α10, β2, and β4 were identified in HDMECs (Figure 1); and for α2, α3, α4, α5, α7, α9, α10, β2, β3, and β4 in HUVECs, consistent with previous reports (24). Transcripts for nAChR subunit a6 were not detected in either HDMEC or HUVEC lines. Muscarinic receptor subunits M1, M2, M3, M4, and M5 were also identified in both HDMECs and HUVECs. Homology of experimental sequences to GenBank human nAChR and mAChR sequences ranged from 90-100% (Table 1).

Calcium imaging

Because certain nAChR subtypes exhibit high Ca^{2+} permeabilities, intracellular Ca^{2+} was measured in microvascular cells during application of cholinergic agonists and antagonists. In vascular endothelial cells, basal levels of intracellular Ca^{2+} have been reported to range from 70-100nM (Ehringer et al., 2000; David-Dufilho et al., 2003; Dang et al., 2005; Montiel et al., 2006).All nAChR antagonists used were also administered in the absence of nicotine, and did not elicit responses different from the control condition, with the exception of MLA, indicating that the fluorescence increases observed were the direct result of nicotine application and probably did not stem from the action of endogenous agonists. In addition, the morphology of HDMECs did not exhibit any systematic changes after treatment with nicotine or nAChR antagonists. Nicotine

application to passage 6 HDMECs (Figure 2) elicited an increase in Fluo-3 fluorescence within 30 seconds but the increase was not statistically significant until the 60s time point (Figure 3; $p<0.05$). The greatest average intensity was observed 90s following initial administration. When co-applied with MLA (Figure 2b), a non-specific nAChR antagonist when used at micromolar concentrations, the nicotine response was statistically decreased from control cells at the longest time periods (Figure 4). Additionally, application of MLA alone decreased fluorescence levels below control levels, indicating a baseline activation of nAChRs. Pretreatment with α-bungarotoxin, a specific antagonist of homomeric α7 nAChRs, also significantly decreased ($P<0.05$) the response to nicotine as compared to nicotine treatment alone (Figure 2c; Figure 5). The αBGT and MLA data together provide evidence that activation of α7 and non- α7 nAChRs contribute to nicotine-induced calcium increases.

To determine the involvement of intracellular stores in the nicotine-induced Ca^{2+} increase, Xestospongin C (XeC) and Ruthenium Red (RR) were administered to block $Ins(1,4,5)P_3$ and ryanodine receptors, respectively. XeC and RR administered in the presence of nicotine only did not affect the nicotine-induced fluorescence increase (data not shown). XeC and RR were applied in the presence of both nicotine and αBGT. The resulting change in $[Ca^{2+}]_i$ was not significantly different than the αBGT/nicotine treatment (data not shown). Together, these data suggest that Ca^{2+}-induced Ca^{2+} release from intracellular stores activated by $Ins(1,4,5)P_3$ and ryanodine receptors is not a major factor in the Ca^{2+} increases.

Because we identified transcripts for muscarinic acetylcholine receptors (mAChRs), we used the mAChR antagonist atropine to rule out the contribution of

mAChR activation by endogenous ACh. Atropine alone did not result in fluorescence different from the control. Atropine in the presence of nicotine, however, did result in a fluorescence level significantly lower than nicotine alone (Figure 6; $p<0.05$) yet significantly higher than controls ($p<0.05$) and αBGT/nicotine ($p<.05$) from 60 to 300s. Because nicotine has not been shown to activate mAChRs, the effect of atropine cannot be attributed to inhibition of mAChRs. However, previous studies have shown atropine can have an antagonistic effect on α3-containing nAChRs (42), suggesting that the observed effect of atropine is due to the blockade of these receptor subtypes.

The membrane permeable Ca^{2+} chelator, BAPTA-AM, prevented nicotine induced fluorescence increases. After BAPTA-AM application and exposure to nicotine, the resulting fluorescence was similar to control levels and comparable to fluorescence levels observed when BAPTA-AM was administered alone (Figure 7). This condition was used to verify that BAPTA-AM bound all intracellular Ca^{2+} and that the changes in fluorescence detected in other experiments resulted from Ca^{2+}/Fluo-3 interactions.

Phosphorylation of signaling molecules

The downstream effects of nicotine-induced changes in intracellular Ca^{2+} levels were evaluated using proteome arrays to analyze the phosphorylation status of signaling molecules known to be regulated by Ca^{2+}. Previous studies have suggested that increases in cytoplasmic Ca^{2+} can lead to activation of mitogen-activated protein kinases (MAPKs) and other intracellular kinases (1, 16, 20).

Two different treatment durations, five and 60 minutes, were analyzed. Previous studies have shown increased ERK phosphorylation during brief exposure to nicotine

with a decline to baseline after 5 minutes. This has been suggested to be due to desensitization of nAChRs (36). The results of the current study were consistent with previous studies in that the shorter duration resulted in significantly increased phosphorylation of both ERK1 and ERK2 over control conditions (Figure 8; $p<0.05$). Treatment for one hour resulted in a decrease in ERK1/2 phosphorylation that was not statistically different from control. The short duration nicotine-induced increase was blocked by the α7-specific antagonist, α-bungarotoxin, and the antagonist, MLA at non-specific concentrations (Figure 9), indicating that α7 nAChR activation is primarily responsible for ERK1/2 phosphorylation. The application of αBGT with nicotine or MLA with nicotine yielded results that were not different from controls but were significantly lower than nicotine only ($p<0.05$).

BAPTA-AM was used to verify that the nicotine-induced changes in phosphorylation were due to alterations in $[Ca^{2+}]_i$. Phosphorylation of ERK1/2 after BAPTA-AM treatment was significantly lower than both nicotine only and control levels (Figure 9; $p<0.05$) indicting that baseline levels of phosphorylation are calcium dependent. Atropine applied with nicotine reduced nicotine's effect on ERK2 but not ERK1, potentially indicating the contribution of the atropine sensitive nAChR subtypes (42). Atropine alone did not elicit changes that were significantly different than controls.

DISCUSSION

Consistent with Heeschen et al. (2002), we detected α2, α3, α4, α5, α7, α9, α10, β2, β3, and β4 mRNA transcripts in HUVECs and we extended this investigation to HDMECs and detected transcripts for all subunits but β3.

The transcripts appear to be indicative of functional nAChR subtypes because the application of nicotine induced a rapid and sustained increase in intracellular Ca^{2+} in cultured HDMECs and HUVECs. There are several possible mechanisms that might underlie this increase in $[Ca^{2+}]_i$. The nAChRs as a class are mixed cation channels with generally moderate Ca^{2+} permeability; however, the homomeric α7 nAChR subtype has a very high Ca^{2+} permeability (54). Thus, it is possible that intracellular Ca^{2+} could increase via Ca^{2+} influx through the nAChRs themselves. The observed Ca^{2+} increases were eliminated using the nAChR antagonist MLA at receptor subtype non-specific concentrations, resulting in fluorescence levels similar to the control condition in which nicotine was not applied. The application of MLA by itself was an important control since ACh has been reported to function as an autocrine hormone which activates receptors on the same cell from which it was released. MLA, when applied alone, resulted in fluorescence levels similar to the MLA co-application with nicotine at early time points but significantly lower than control levels at later time points. Thus decrease in fluorescence after complete nAChR inhibition provides evidence for the action of endogenous ACh. Additionally, the application of the α7 specific antagonist αBGT resulted in a significant decrease of more than half the total effect of nicotine, indicating that much of the nicotine-induced change in $[Ca^{2+}]_i$ is mediated by α7 nAChRs. The remaining portion of the $[Ca^{2+}]_i$ increase that was MLA-sensitive but αBGT insensitive is likely attributable to other nAChR subtypes. Additional evidence for the involvement of non- α7 nAChRs includes the ability of atropine to partially inhibit the effects of nicotine. Atropine has been reported to block α3-containing nAChRs (42).

Alternatively, an increase in intracellular Ca^{2+} could rely on other mechanisms such as store-operated Ca^{2+} (SOC) channels. Two types of highly Ca^{2+} selective ligand-binding SOC channels have been characterized in intracellular membranes of endothelial cells. One type is activated by ATP and bradykinin (32) or $Ins(1,4,5)P_3$ (39). Results from our studies using antagonists for ryanodine and $Ins(1,4,5)P_3$ receptors, Ruthenium Red (RR) and Xestospongin C (XeC), respectively, to block the release of Ca^{2+} from intracellular stores did not indicate a significant role for intracellular Ca^{2+} store release by $Ins(1,4,5)P_3$ when applied with nicotine. Similar results were obtained when RR and XeC were used in conjunction with αBGT and nicotine and did not prevent an increase in intracellular Ca^{2+}. This suggests a minor role for $Ins(1,4,5)P_3$ or ryanodine activated SOC release following application of nicotine and the influx of extracellular Ca^{2+}.

Another possible mechanism for Ca^{2+} entry into the cells is via transient receptor potential calcium (TRPC) channels. This superfamily has been divided into three subfamilies including Short TRPC (STRPC), Long TRPC (LTRPC), and O(sm-9-like) TRPCs, which rely on Ca^{2+} dependent calmodulin-binding regions or intracellular changes in osmolarity for activation (39). It is unlikely that TRPC channels are responsible for the initial influx of extracellular Ca^{2+} as this has been demonstrated to be due to nAChR activation but TRPC channels could play a role in store-operated Ca^{2+} release in response to the nicotine-induced increase in Ca^{2+}.

Other possible mechanisms for Ca^{2+} increase in vascular endothelial cells include membrane depolarization resulting from activation of nAChRs that in turn activate voltage gated Ca^{2+} channels (VGCC) allowing Ca^{2+} influx in the stimulated cells or even neighboring cells via gap junctions. Endothelial cells are nonexcitable cells and it was

once thought that the likelihood of VGCC expression in endothelial cells was very small. However, there are reports of VGCCs, similar to L- and T-type Ca^{2+} channels, in bovine capillary endothelial cells (4, 5). In addition, a new type of VGCC has been proposed to be important to Ca^{2+} flux in endothelial cells. The R-type Ca^{2+} channel is activated by long duration depolarizations and has been proposed to be responsible for a sustained Ca^{2+} influx (3).

There are several possible mechanisms that might underlie the prolonged duration to peak fluorescence after activation of nAChRs. The density of nAChRs on endothelial cells has yet to be explored, and low density of receptors could result in slower increases in $[Ca^{2+}]_i$. The exact nAChR subunit composition of unusual combinations in non-neuronal vascular endothelial cells has not been determined, but might be responsible for the slower $[Ca^{2+}]_i$ increase. The slow, sustained increase can also be explained by membrane differences between neuronal and non-neuronal cells. Neuronal cells typically have fast Ca^{2+} currents, a component of rapid depolarization, which involves the activation of various other channels. For instance, the activation of nAChRs would cause an influx of cations, subsequently activating voltage sensitive channels and resulting in a rapid increase in $[Ca^{2+}]_i$. In HDMECs, it is possible that there are few, if any, voltage gated ion channels. Thus, nAChRs may be the only channels actively allowing Ca^{2+} into the cell, resulting in slow intracellular Ca^{2+} influx. In addition, neuronal cells use ion pumps to maintain a high driving force for Ca^{2+}, and other positively charged ions, resulting in a fast and strong Ca^{2+} current after activation of nAChRs. Endothelial cells have been reported to be able to maintain a high Ca^{2+} driving force for an extended period

of time after initial Ca^{2+} influx through a combination of K^+, Na^+, and Cl^- channels, in addition to Na^+/Ca^{2+} exchangers (39).

To elucidate the effects of Ca^{2+} influx on intracellular signaling pathways, antibody arrays were used to measure the phosphorylation state of important signaling kinases, specifically the mitogen-activated protein kinase (MAPK) cascade. It is the common paradigm that the MAPK cascade is activated by growth factor ligands binding growth factor receptors that contain protein tyrosine kinase in their intracellular domain (46). This cascade contributes to the amplification of the intracellular signals that eventually activate regulatory proteins in the cytoplasm. Signaling to the nucleus initiates processes such as proliferation and differentiation.

We hypothesized that nicotine would affect the MAPK signaling cascade based on previous studies that revealed an angiogenic role for nicotine. In our study, nicotine alone significantly increased phosphorylation of ERK1/2 after 5 minutes of exposure. In contrast, 60 minutes of nicotine exposure resulted in a decline of ERK1/2 phosphorylation back to baseline levels. These results are consistent with previous literature in which PC12 cells treated with more than 1mM nicotine displayed phosphorylation of ERK at 5 minutes and significantly lower levels at 60 minutes (36). In our study, nicotine-induced phosphorylation was blocked by the addition of MLA. The α7 specific antagonist, α-bungarotoxin, also blocked the nicotine-induced phosphorylation of ERK1/2 suggesting that α7 is the major nAChR subtype involved in the activation of the ERK signaling pathway. Atropine was used to evaluate the role of muscarinic acetylcholine receptors. Although mAChRs do not respond to nicotine, they do respond to ACh which might be endogenously produced, and our Ca^{2+} imaging

experiments indicated the endogenous activation of nAChRs in these cells. Nicotine induced phosphorylation was modified by the addition of atropine in that p-ERK2 was decreased to control levels but p-ERK1 was not significantly different than nicotine treatment alone. Part of this difference may be the result of nAChR antagonism by atropine (42) but it is unclear why the effect on ERK2 was so pronounced.

It was not unexpected that both ERK1/2 phosphorylation states were significantly modified by exposure to nAChR agonist. Members of the MAPK family are activated by a wide spectrum of growth factors and mitogens (8). Upon activation, the ERKs can phosphorylate a wide variety of regulatory proteins including nuclear transcription factors, indicating important roles as regulators of transcriptional activity. Moreover, ERK activity has been shown to control transition from G0 to G1 phases of the cell cycle (7). The ERK mediated increase in mitogenic signaling suggests a mechanism whereby nicotine can effect vascular disease initiation and progression. There may be other mechanisms whereby nicotine can exert its effects on ERK phosphorylation. For example, there are previous reports of nicotine influences on intracellular signaling and cell proliferation that did not investigate $[Ca^{2+}]_i$ changes. Dasgupta et al., (2006) presented evidence that nicotine may influence cell proliferation through $\alpha 7$ mediated metabotropic mechanisms, involving ERK and subsequent induction into the cell cycle S-phase (13).

The magnitude of ERK1/2 phosphorylation following nicotine exposure mirrored the effects observed in the Ca^{2+} imaging experiments. The greatest effect in each experiment was to nicotine alone. The effects of nicotine were partially blocked by atropine and αBGT but fully blocked by MLA, at non-specific doses. This provides

evidence for a direct relationship between the Ca^{2+} influx seen after nAChR activation and activation of the ERK signaling pathway.

CONCLUSIONS

Previous studies suggest that use of tobacco products is essential to the establishment and development of many vascular diseases (30, 33, 36, 37, 39). The evidence presented herein provides insight into the mechanism whereby nicotine, a major constituent of cigarette smoke, may effects changes in cell proliferation and angiogenesis in microvasculature. In summary, nicotine activates nAChRs expressed in HDMECs. Subsequent changes in intracellular Ca^{2+} concentration which is sufficient to activate mitogenic signaling pathways, specifically, the MAPK pathway. The direct effect of MAPK activation on the transcription of angiogenic factors has yet to be determined, therefore the extent to which nicotine-induced activation of nAChRs is involved in cellular proliferation of vascular diseases remains unclear.

ACKNOWLEDGMENTS

We thank Virginia Wotring, PhD, for technical assistance and comments on the manuscript; Laura Timares, PhD, and Sanjay Pradhan, PhD, for assistance with tissue culture.

GRANTS

Funding was made available through NIAMS P30 AR050948 (PI: C. Elmets, MD) and The Rosacea Society.

REFERENCES

1. Atherfold PA, Norris MA, Robinson PJ, Gelfand EW, Franklin RA. Calcium-induced ERK activation in human T lymphocytes. *Molecular Immunology* 36: 543-549, 1999.

2. Ballivet M, Nef P, Couturier S, Rungger D, Bader CR, Bertrand D, Cooper E. Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in Xenopus oocytes after cDNA injection. *Neuron* 1: 847-852, 1988.

3. Bkaily G, d'Orléans-Juste P, Naik R, Pérodin J, Stankova J, Abdulnour E, Rola-Pleszczynski M. PAF activation of a voltage-gated R-type Ca2+ channel in human and canine aortic endothelial cells. *Br J Pharmacol* 110: 519-520, 1993.

4. Bossu JL, Elhamdani A, Feltz A. Voltage-dependent calcium entry in confluent bovine capillary endothelial cells. *FEBS Letters* 299: 239-242, 1992.

5. Bossu JL, Feltz A, Rodeau JL, Tanzi F. Voltage-dependent transient calcium currents in freshly dissociated capillary endothelial cells. *FEBS Letters* 255: 377-380, 1989.

6. Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J. Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor a-subunit. *Nature* 319: 368-374, 1987.

7. Boulton TG, Yancopoulos CD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH. An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249: 64-66, 1990.

8. Campbell JS, Seger R, Graves TD, Graves LM, Jensen AM, Krebs EG. The MAP kinase cascade. *Recent Prog Horm Res* 50: 131-59, 1995.

9. Conklin BS, Zhao W, Zhong DS, Chen C. Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells. *Amer J Path* 160: 413-418, 2002.

10. Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron* 5: 847-856, 1990.

11. Couturier S, Erkman L, Valera S, Rungger D, Bertrand S, Boulter J, Ballivet M, Bertrand D. Alpha 5, alpha 3, and non-alpha 3. Three clustered avian genes encoding neuronal nicotinic acetylcholine receptor-related subunits. *J Biol Chem* 265: 17560-17567, 1990.

12. Dang L, Seale JP, Qu X. High glucose-induced human umbilical vein endothelial cell hyperpermeability is dependent on protein kinase C activation and independent of the Ca2+-nitric oxide signalling pathway. *Clin Exp Pharmacol Physiol* 2005 32:771-6.

13. Dasgupta P, Rastogi S, Pillai S, Ordonez-Ercan D, Morris M, Haura E, Chellappan S. Nicotine induces cell proliferation by b-arrestin-mediated activation of Src and Rb-Raf-1 pathways. *J.Clin.Invest* 116: 2208-2217, 2006.

14. David-Dufilho M, Schussler O, Pernollet MG, Brunet A, Millanvoye-Van Brussel E, Le Quan Sang KH, Rendu F. Therapeutic immunoglobulin reduces Ca^{2+} mobilization and von Willebrand factor secretion, and increases nitric oxide release in human endothelial cells. *Thromb Haemostasis* 2003 90: 1046-1053.

15. Deneris E, Connolly J, Boulter J, Wada K, Swanson L, Patrick J, Heinemann S. Primary structure and expression of beta 2: a novel subunit of neuronal nicotinic acetylcholine receptors. *Neuron* 1: 45-54, 1988.

16. Egea J, Espinet C, Comella JX. Calcium influx activates extracellular-regulated kinase/mitogen-activated protein kinase pathway through a calmodulin-sensitive mechanism in PC12 cells. *J Biol Chem* 274: 75-85, 1999.

17. Ehringer WD, Wang O, Haq A, Miller FN. Bradykinin and α-thrombin increase human umbilical vein endothelial macromolecular permeability by different mechanisms. *Inflammation* 2000 24: 175-193.

18. Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J. Alpha 10: A determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *PNAS* 98: 3501-3506, 2001.

19. Environmental Protection Agency. Respiratory health effects of passive smoking: lung cancer and other disorders. Washington (DC): Environmental Protection Agency, 1992.

20. Fierro AF, Wurth GA, Zweifach A. Cross-talk with Ca^{2+} influx does not underlie the role of extracellular signal-regulated kinases in cytotoxic T lymphocyte lytic granule exocytosis. *J Biol Chem* 279: 25646-25652, 2004.

21. Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN. Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-Trisphosphate Receptor. *Neuron* 19: 723-733, 1997.

22. Goldman D, Deneris E, Luyten W, Kochhar A, Patrick J, Heinemann S. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* 48: 965-973, 1987.

23. Hanley MR, Eterovic VA, Hawkes SP, Hebert AJ, Bennett EL. Neurotoxins of bungarus multicinctus venom purification and partial characterization. *Biochemistry* 16: 5840-5849, 1977.

24. Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest* 110: 527-536, 2002.

25. Heeschen C, Weis M, Cooke JP. Nicotine promotes arteriogenesis. *J Amer Col Cardiology* 41: 489-496, 2003.

26. Hellström-Lindahl E and Nordberg A. Muscarinic receptor subtypes in subpopulations of human blood mononuclear cells as analyzed by RT-PCR technique. *J Neuroimmunology* 68: 139-144, 1996.

27. Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis* 21: 505-515, 2000.

28. Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, Wells G. Structure and function of neuronal nicotinic acetylcholine receptors. [Review]. *Prog Brain Res* 109: 125-137, 1996.

29. Lindstrom JM. The structures of neuronal nicotinic receptors. In Handbook of Experimental Pharmacology: Clementi F, Fornasari D, Gotti C. eds. (Berlin Heidelburg: Springer-Verlag) 144: 101-162, 2000.

30. Lu L, Reinach PS, Kao WW. Corneal epithelial wound healing. [Review] *Exp Biol Med* 226: 653-664, 2001.

31. Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. *Journal of Pharmacology & Experimental Therapeutics* 287: 435-439, 1998.

32. Mendelowitz D, Bacal K, Kunze DL. Bradykinin-activated calcium influx pathway in bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol* 262: 942-948, 1992.

33. Miot HA, Bartoli Miot LD, Haddad GR. Association between Discoid Lupus erythematosus and Cigarette Smoking. *Dermatology* 211: 118-122, 2005.

34. Miyamoto S, Izumi M, Hori M, Kobayashi M, Ozaki H, Karaki H. Xestospongin C, a selective and membrane-permeable inhibitor of IP3 receptor, attenuates the positive inotropic effect of a-adrenergic stimulation in guinea-pig papillary muscle. *Br J Pharmacol* 130: 650-654, 2000.

35. Montiel M, de la Blanca EP, Jiménez E. P2Y receptors activate MAPK/ERK through a pathway involving PI3K/PDK1/PKC-zeta in human vein endothelial cells. *Cell Physiol Biochem* 2006 18:123-34.

36. Nakayama H, Numakawa T, Ikeuchi T, Hatanaka H. Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. *J Neurochem* 79: 489-498, 2001.

37. Naldi L, Peli L, Parazzini F, Psoriasis Study Group of the Italian Group for Epidemiologic Research in Dermatology. Association of early-stage psoriasis with smoking and male alcohol consumption: evidence from an Italian case-control study. *Arch Dermatol* 135: 1479-1484, 1999.

38. Nef P, Oneyser C, Alliod C, Couturier S, Ballivet M. Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. *EMBO J* 7: 595-601, 1988.

39. Nilius B and Droogmans G. Ion channels and their functional role in vascular endothelium. *Physiol Rev* 81: 1415-1459, 2001.

40. O'Doherty CJ and MacIntyre C. Palmoplantar pustulosis and smoking. *Br Med J* 291: 861-864, 1995.

41. Olin JW. Thromboangiitis obliterans (Buerger's Disease). *N Engl J Med* 343: 864-869, 2000.

42. Parker JC, Sarkar D, Quick MW, Lester RAJ. Interactions of atropine with heterologously expressed and native a3 subunit-containing nicotinic acetylcholine receptors. *Br J Pharm* 138: 801-810, 2003.

43. Poikolainen K, Reunala T, Karvonen J. Smoking, alcohol and life events related to psoriasis among women. *Br J Dermatol* 130: 473-477, 1994.

44. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* 145: 2498-2506, 2004.

45. Sarkar R, Gelabert HA, Mohiuddin KR, Thakor DK, Santibanez-Gallerani AS. Effect of cigarette smoke on endothelial regeneration in vivo and nitric oxide levels. *J Surgical Res* 82: 43-47, 1999.

46. Seger R and Krebs EG. The MAPK signaling cascade. *FASEB J* 9: 726-735, 1995.

47. Takada-Takatori Y, Kume T, Sugimoto M, Katsuki H, Sugimoto H, Akaike A. Acetylcholinesterase inhibitors used in treatment of Alzheimer's disease prevent

glutamate neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol 3-kinase cascade. *Neuropharmacology* 51: 474-486, 2006.

48. Vaca L and Kunze DL. IP3-activated Ca2+ channels in the plasma membrane of cultured vascular endothelial cells. *Am J Physiol Cell Physiol* 269: C733-C738, 1995.

49. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Applied Physiol* 84: 2089-2098, 1998.

50. Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* 284: 314-335, 1989.

51. Wang Y, Pereira EFR, Maus ADJ, Ostlie NS, Navaneetham D, Lei S, Albuquerque EX, Conti-Fine BM. Human Bronchial Epithelial and Endothelial Cells Express alpha 7 Nicotinic Acetylcholine Receptors. *Mol Pharmacol* 60: 1201-1209, 2001.

52. Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. [Review] *Pharmacol Therapeut* 77: 59-79, 1998.

53. Xu L, Tripathy A, Pasek DA, Meissner G. Ruthenium Red modifies the cardiac and skeletal muscle Ca2+ release channels (ryanodine receptors) by multiple mechanisms. *J Biol Chem* 274: 32680-32691, 1999.

54. Zhao L, Kuo YP, George AA, Peng JH, Purandare MS, Schroeder KM, Lukas RJ, Wu J. Functional properties of homomeric, human a7-nicotinic acetylcholine receptors heterologously expressed in the SH-EP1 human epithelial cell line. *J Pharmacol Exp Ther* 305: 1132-1141, 2003.

55. Zia S, Ndoye A, Lee TX, Webber RJ, Grando SA. Receptor-mediated inhibition of keratinocyte migration by nicotine involves modulations of Ca^{2+} influx and intracellular concentration. *J Pharm Exper Ther* 293: 973-981, 2000.

Figure 1

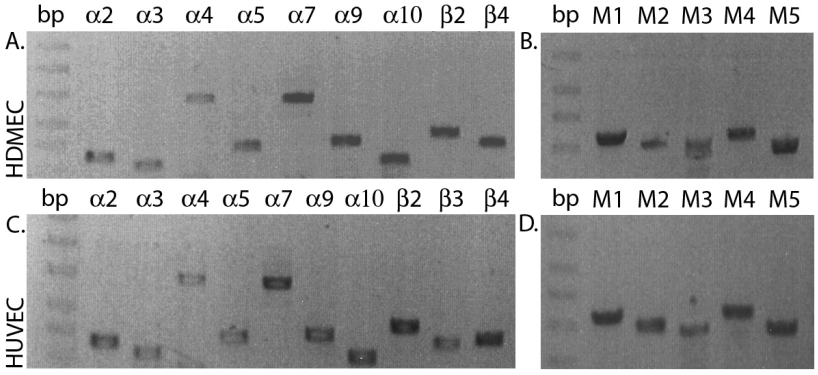

Figure 1. (A) RT-PCR experiments identified transcripts for α2, α3, α4, α5, α7, α9, α10, β2, and β4 nAChR subunits were amplified from HDMECs. (B,D) Muscarinic receptor subunits M1, M2, M3, M4, and M5 were also identified in both HDMECs and HUVECs. (C) Transcripts for α2, α3, α4, α5, α7, α9, α10, β2, β3, and β4 were amplified from HUVECs. Transcripts for nAChR subunit α6 were detected in neither HDMEC nor HUVEC lines. RT-PCR products were sequenced and compared to Genbank entries for their respective human nAChR entries; homology of sequences ranged from 90-100%.

Figure 2

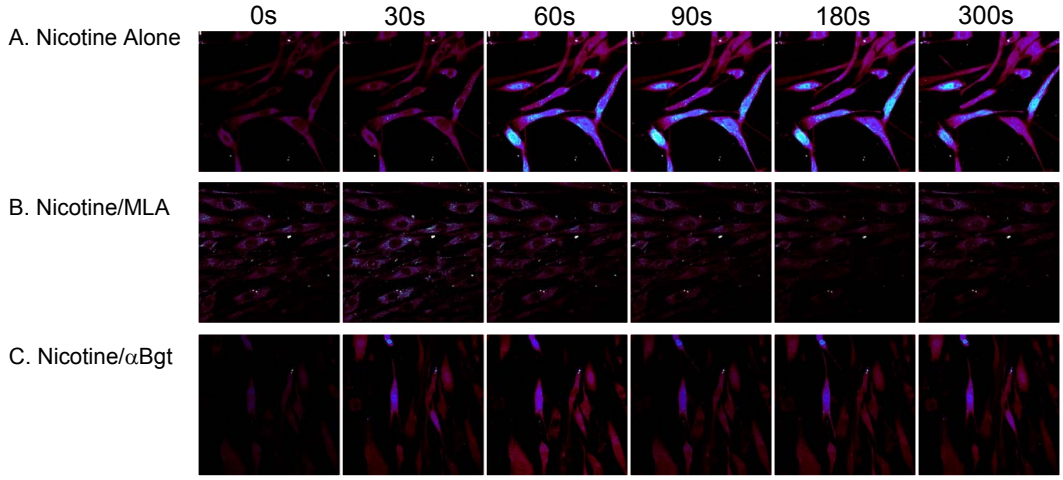

Figure 2. The effect of nicotine and cholinergic antagonists using fluorescent calcium imaging on nAChRs in passage 6 (P6) HDMECs. (A) Treatment with nicotine [10 μM], top row, resulted in increased fluorescence between 0s and 180s after administration. $[Ca^{2+}]$ increases are pseudocolored blue to green. Average fluorescence between activated cells continued to increase until 180s post administration, when fluorescence plateaued. (B) Co-application of MLA [10 μM] and nicotine blocked the nicotine-induced increase in fluorescence as measured over time. (C) Treatment with a-bungarotoxin significantly decreased nicotine-induced fluorescence but did not eliminate the response to nicotine.

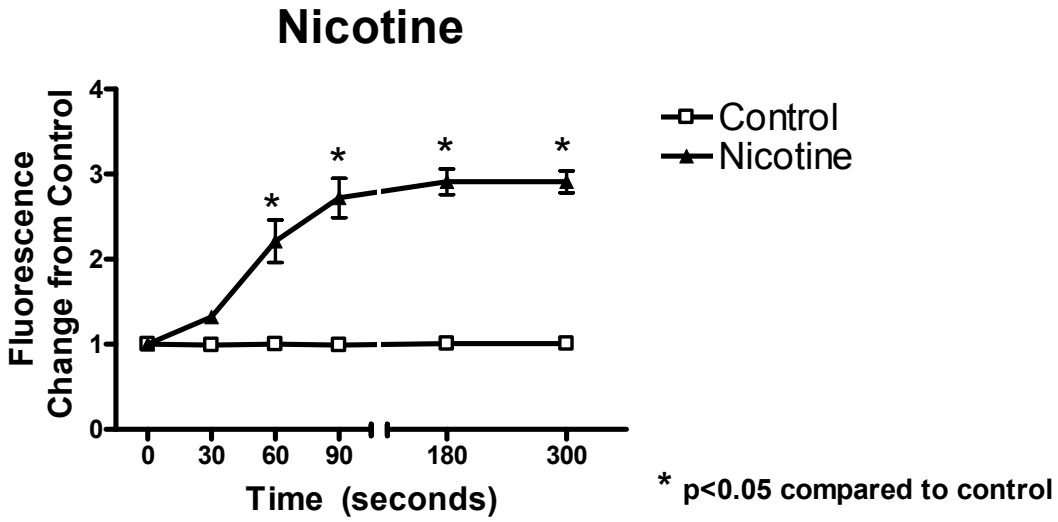

Figure 3. Nicotine increased Ca^{2+} influx in HDMECs. Bath application of nicotine (10 μM) increased intracellular $[Ca^{2+}]_i$, measured by fold change in Fluo-3 reactivity with Ca^{2+}. Fluorescence was measured every 30 seconds for 90 seconds, and then at 180s and 300s. Fluorescence levels were significantly higher than control at 30s and thereafter ($p<0.05$; $n=30$).

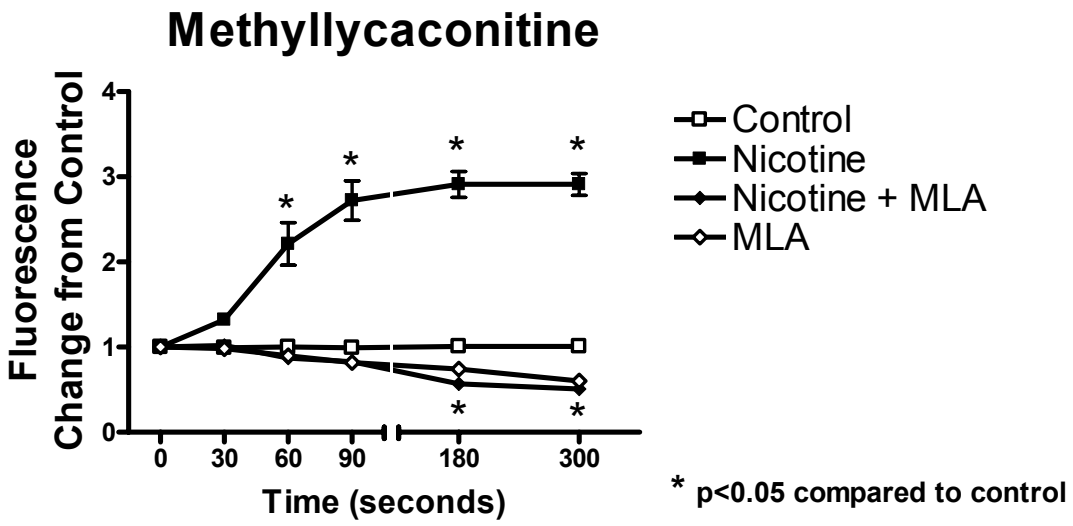

Figure 4. MLA inhibited nicotine-induced Ca^{2+} influx in HDMECs. Co-application of MLA and nicotine resulted in fluorescence levels significantly decreased as compared to nicotine alone, measured by fold change (n=22; $p<0.05$). At 180s and 300s fluorescence in both MLA conditions was significantly lower than controls (n=21; $p<0.05$), suggesting blockade of nAChRs activated by endogenous agonist.

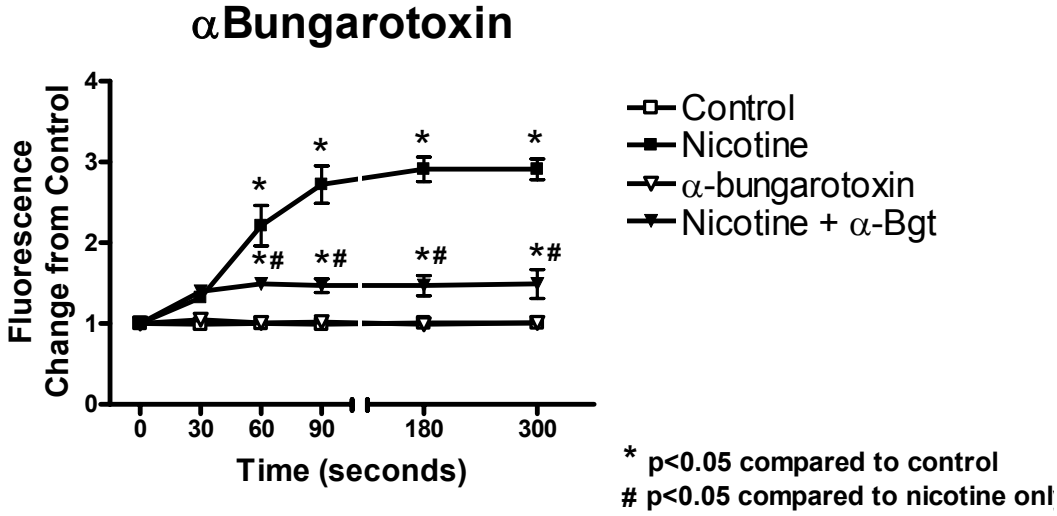

Figure 5. α-Bungarotoxin inhibited nicotine-induced Ca^{2+} influx in HDMECs. Co-application of αBGT and nicotine partially prevented the increase of $[Ca^{2+}]_i$, measured by fold change in fluo-3 reactivity with Ca^{2+}. Fluorescence levels were significantly elevated as compared to control (n=22; $p<0.05$), indicating that the activation of both αBGT sensitive and insensitive nAChRs contributed to nicotine-induced Ca^{2+} increases.

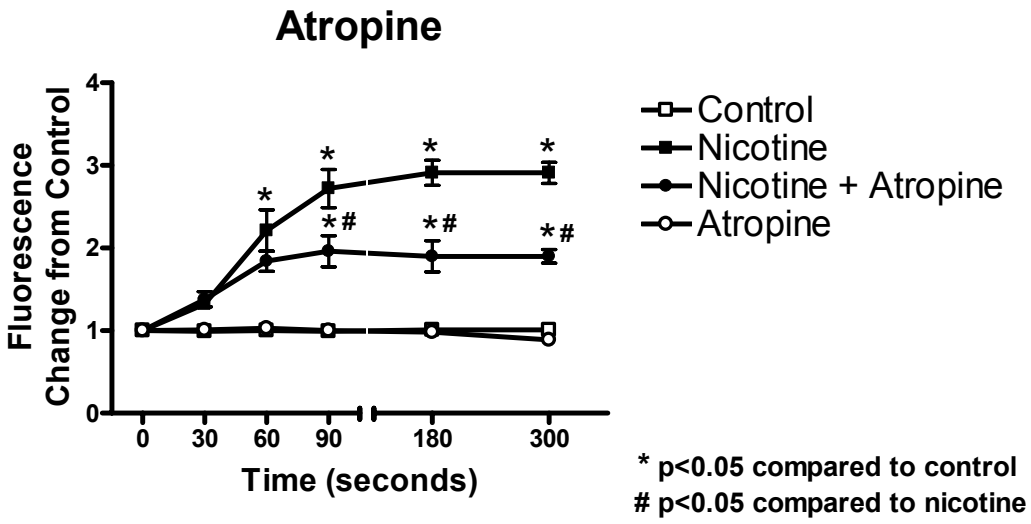

Figure 6. Atropine effects nicotine-induced Ca^{2+} influx in HDMECs. Co-application of atropine and nicotine partially inhibited nicotine-induced Ca^{2+} increase. Fold change in fluorescence levels were significantly higher than controls (n=22; $p<0.05$) and significantly lower than nicotine alone ($p<0.05$).

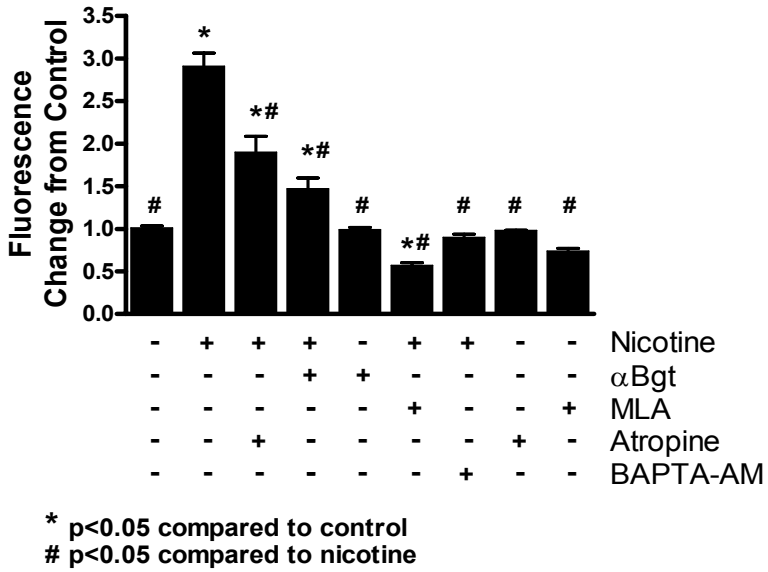

Figure 7. Summary of nicotine treatments with antagonists at peak fluorescence, 180 seconds. Nicotine alone elicited the highest degree of fluorescence, followed by nicotine co-application with atropine or α-bungarotoxin. All other treatments failed to gain fluorescence levels greater than baseline levels. In addition to nAChR and mAChR antagonists, BAPTA-AM was used to bind free Ca^{2+} and effectively blocked increased fluorescence ($n=28$; $p<0.05$). Treatment conditions labeled with (+) indicate the agonist/antagonist was applied; (-) indicates the agonist/antagonist was omitted.

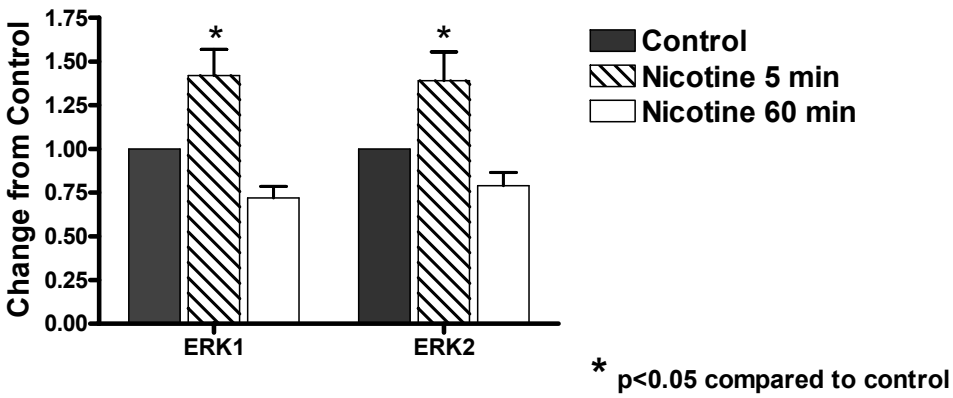

Figure 8 . Effect of nicotine treatment on phosphorylation of ERK1/2 for 5 minutes and 60 minutes. Cell cultures were treated with nicotine [10 μM] and compared to controls. Differences in ERK1/2 phosphorylation were reported as fold change. Levels of phosphorylated ERK1/2 increased after 5 minutes of exposure to nicotine (n=8; $p<0.05$). After 60 minutes, phosphorylation levels of ERK1/2 were not different than control (n=8) suggesting a transient effect of nicotine in protein phosphorylation.

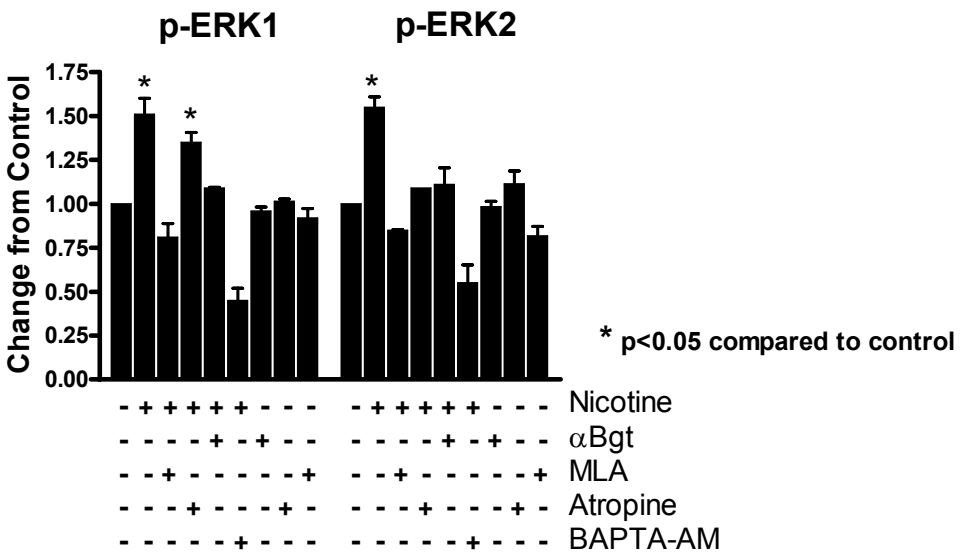

Figure 9. Effect of 5 minute nicotine exposure on phosphorylation of ERK1/2 using nAChR antagonists. Levels of phosphorylated ERK increased in response to nicotine and reported as fold change. The effects were blocked by co-application of nicotine and αBGT or MLA. Nicotine treatment with atropine remained significantly higher than controls for ERK1, however, ERK2 returned to control levels. BAPTA-AM blocked phosphorylation of ERK1/2.

NICOTINIC ACETYLCHOLINE RECEPTOR ACTIVATION REGULATES EXPRESSION OF ANGIOGENESIS-RELATED GENES IN HDMECS

RYAN C. SPLITTGERBER
VIRGINIA E. WOTRING
CRAIG A. ELMETS
KENT T. KEYSER

In preparation for Investigative Ophthalmology and Visual Science

Format adapted for dissertation

INTRODUCTION

Tobacco use is implicated in the initiation and progression of many vascular diseases of the eye. Age-related Macular Degeneration (AMD) is classified into two different disease phenotypes: nonexudative and neovascular, that is, dry and wet, respectively. While nonexudative AMD is the most prevalent clinical type, wet AMD results in the most rapid and severe cases of vision loss.

The wet form of ARMD results in vision loss due to abnormal blood vessel growth in the choriocapillaries. This blood vessel growth leads to leakage of vascular contents below the macula, resulting in damage to the photoreceptors and eventual blindness. The strongest environmental risk factor for all types of AMD is cigarette smoking. Case-controlled studies link smoking to increased occurrence of ARMD in males, increased occurrence for individuals that no longer smoke but were heavy smokers no more than 20 years ago, increased occurrence for current smokers, and an even higher risk for heavy smokers (Armstrong 2003; Klein et al., 1993; Christen et al., 1996; Seddon et al., 1996).

Previous studies have shown that vascular endothelial cells synthesize and store acetylcholine (ACh; Kawashima et al., 1989; 1990) which exerts its effects through neuronal type nicotinic acetylcholine receptors (nAChRs) also expressed by vascular endothelial cells (Macklin et al., 1998). This has lead to the suggestion that ACh might act as an autocrine factor in blood vessels (Proskocil et al., 2004). Reported effects of cholinergic agents on normal vasculature include initiation of a cholinergic anti-inflammatory response which has been shown to reduce inflammatory cytokines, including tumor necrosis factor (TNF), and interleukins as typical cytokine targets for

down regulation (Tracey, 2002; Gallowitsch & Tracey, 2005; Pavlov et al., 2005). On the other hand, nicotine, a potent ligand for nAChRs, has the ability to cause neovascularization and to remodel existing blood vessels, and can cause proliferation of human umbilical vein endothelial cells (Heeschen et al., 2001). In addition, long term nicotine exposure is reported to stimulate the growth of atherosclerotic lesions (Heeschen et al., 2001). Nicotine has also been shown to stimulate choroidal neovascularization (CNV) after laser-induced rupture of Bruch's membrane (Kiuchi et al., 2008). These authors also suppressed nicotine and basal levels of CNV with the non-specific nAChR antagonist, mecamylamine. Unfortunately, the mechanisms that underlie nicotine's effects on human vascular endothelial cells have not been explained. We hypothesize that nicotine-medicated activation of nAChRs, at concentrations similar to the blood serum nicotine levels found in cigarette smokers, induces nAChR-dependent increase in angiogenesis-related gene expression.

MATERIAL AND METHODS

Cell Culture

Cultured human dermal microvascular endothelial cells (HDMECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Dr. Robert Swerlick of Emory University and cultured at the UAB Skin Cell Culture Core. Cells were grown in 80 cm^2 flasks incubated with Clonetics' Endothelial Cell Basal Medium (EBM; Cambrex, East Rutherford, NJ). HDMECs were supplemented with microvascular growth factor (Cambrex). All cells were simultaneously plated at 50-60% confluence and treated with

agonist/antagonist for 72 hours or 1 hour. All cultures were simultaneously harvested after 72 hours; cultures were 80-90% confluent.

Pharmacological Treatment

Cell cultures were treated with cholinergic agonist and antagonists. Nicotine was used as a non-specific agonist and was used at a working concentration of 100 nM. To determine involvement of homomeric α7 nAChRs, αBGT (10 μM) was used to prevent activation of α7 and α9-containing nAChRs. Methyllycaconitine (MLA) is an antagonist of α6, α7, and α9 containing nAChRs at low nM concentrations and acts as a nonspecific blocker at low μM concentrations. To determine block all nAChR, MLA was used at a non-specific concentration (10 μM). Control cultures were treated with sterile water.

RNA Extraction

RNA was isolated from treated cell cultures using TRI REAGENT (Molecular Research, Inc.; Cincinnati, OH). Cells were homogenized with 1 ml/10cm^2 of culture plate and phases were separated with chloroform; RNA was precipitated with isopropanol, washed with 75% ethanol and solubilized with nuclease-free water. RNA solution was treated with DNAse I (Ambion) following the manufacturer's protocol.

RT^2 Profiler PCR Array

Single strand DNA for SuperArray experiments was created using RT^2 First Strand Kit (SuperArray Bioscience Corporation; Frederick, MD). Briefly, RNA (100 ng) was mixed with a genomic DNA Elimination Buffer and incubated for 5 minutes at 42°C. RNA was

then added to a reverse transcription buffer, primer controls, reverse transcriptase, RNase-free water. Mixture was incubated for 15 minutes at 42°C and 5 minutes at 95°C to inactivate the reverse transcriptase. All Real-Time RT-PCR experiments were performed using the iQ5 Real-Time Detection System (Bio-Rad Laboratories; Hercules, CA).

RT^2 nAChR PCR Assay

For real-time RT-PCR using nAChR primers, cDNA was created using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Briefly, RNA (100 ng) was mixed with iSript Reaction Mix, reverse transcriptase and nuclease-free water. Reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. All Real-Time RT-PCR experiments were performed using the iQ5 Real-Time Detection System (Bio-Rad Laboratories; Hercules, CA).

RT^2 PCR Analysis

For each gene: **Average C_t = C_{t1}, C_{t2}, C_{t3} …** To standardize cycle threshold values, subtract average C_t for Gene of Interest from average C_t for the selected housekeeping genes.

$\Delta C_{t\ (treat)}$ = Average $C_{t\ (GOI\ treat)}$ − Average $C_{t\ (HK\ treat)}$

$\Delta C_{t\ (con)}$ = Average $C_{t\ (GOI\ cont)}$ − Average $C_{t\ (HK\ con)}$

$\Delta\Delta C_t = \Delta C_{t\ (treat)} - \Delta C_{t\ (con)}$

Fold change = $2^{(-\Delta\Delta Ct)}$

95% confidence intervals are calculated using:

Lower: $2^{[-(\Delta\Delta Ct + (1.96* SD\Delta\Delta Ct)/SQRT(n)]}$

Upper: $2^{[-(\Delta\Delta Ct - (1.96* SD\Delta\Delta Ct)/SQRT(n)]}$

For SuperArray: Average cycle threshold for three housekeeping genes was subtracted from the average cycle threshold for the gene of interest. For nAChR primers: Average cycle threshold for two housekeeping genes, β-actin and GAPDH, was subtracted from the average cycle threshold for the nAChR gene of interest. Statistical significance was determined using One-Way Analysis of Variance (ANOVA). Significance values less than 0.05 was considered statistically significant.

RESULTS

Angiogenesis-related Gene Expression

Cells were treated with nicotine and nicotinic antagonists for 1 (table 1) or 72 hours (table 2) and angiogenesis-related gene expression was analyzed. After 1 hour nicotine exposure (100 nM; figure 1) there was a greater than a three fold reduction in mRNA for genes encoding: inhibitor of DNA binding 3 (ID3; -87 fold), integrin b3 (ITGB3; -27 fold), chemokine CXCL6 (-7 fold), angiopoietin-like 4 (ANGPTL4; -5.1 fold), hepatocyte growth factor (HGF; -4.4 fold), ephrin α3 (EFNA3; -3.5 fold), chemokine CCL2 (-3.5 fold), thrombospondin 2 (THBS2; -6.5 fold), sphingosine kinase 1 (SPHK1; -2.28 fold). One hour nicotine exposure increased mRNA for transforming growth factor-beta receptor 1 (TGFBR1; 7 fold) and prostaglandin-endoperoxide synthase1 (PTGS1; 3 fold). The application of the non-specific nAChR antagonist MLA (figure 2) did not completely block gene expression changes but resulted in a new pattern of changes, specifically, reduced expression of EFNA3 (-3.1 fold), jagged 1 (JAG1; -5.3), and ITGB3

Angiogenesis-Related Gene Expression- 1 hour

	Control	Nicotine		MLA		MLA NIC		BGT		BGT NIC	
	Ct	Ct	Fold	Ct	Fold	Ct	Fold	Ct	Fold	Ct	Fold
ANGPT1	34.3	33.9	1.3	33.6	1.6	33.3	1.8	33.7	1.2	33.8	**3.2**
ANGPTL3	32.8	34.5	-3.5	34.3	-2.9	33.2	-1.5	33.5	-2.2	34.1	-1.0
ANGPTL4	32.2	34.5	**-5.1**	32.6	-1.3	32.1	-1.0	33.0	-2.2	34.0	-1.6
CCL11	35.0	34.5	1.3	34.9	1.0	34.8	1.0	34.4	1.2	35.0	2.0
CCL2	29.7	31.4	**-3.5**	28.0	**3.2**	27.2	**4.8**	27.1	**4.6**	27.8	**7.7**
CXCL3	34.4	35.0	-1.6	35.0	-1.6	35.0	-1.7	35.0	-2.0	35.0	1.2
CXCL6	31.5	34.4	**-7.9**	32.2	-1.7	31.7	-1.3	32.0	-1.9	32.5	1.1
EFNA3	31.6	32.7	-2.3	33.2	**-3.1**	31.7	-1.2	32.2	-2.1	33.2	-1.4
EFNB2	29.6	31.5	**-3.7**	30.5	-1.9	28.8	1.6	29.0	1.2	30.2	1.5
ENG	27.8	27.3	1.3	28.1	-1.3	27.5	1.0	27.8	-1.4	28.9	-1.1
EPHB4	28.0	29.2	-2.5	27.9	1.0	27.7	1.1	27.9	-1.3	29.8	-1.7
EREG	30.5	31.5	-2.0	29.9	1.5	29.6	1.7	29.6	1.5	30.4	2.3
FLT1	30.4	30.5	-1.1	30.9	-1.5	30.3	-1.1	30.5	-1.4	31.7	-1.3
HGF	28.1	30.2	**-4.4**	30.0	-3.8	28.3	-1.3	28.3	-1.6	29.1	1.1
ID3	27.3	33.7	**-87.0**	26.8	1.4	26.8	1.2	26.7	1.1	27.3	1.9
IL1B	32.5	33.7	-2.4	32.3	1.1	31.8	1.4	31.8	1.2	32.6	1.4
IL6	33.0	33.1	-1.1	33.7	-1.7	32.7	1.0	33.1	-1.4	33.9	1.1
ITGB3	28.4	33.1	**-27.4**	30.8	**-5.6**	29.2	-2.0	29.6	**-3.0**	30.8	-2.6
JAG1	29.4	31.3	-3.8	31.7	**-5.3**	31.2	-3.9	30.8	**-3.7**	34.3	**-13.8**
LAMA5	30.0	30.5	-1.5	29.9	1.0	29.6	1.2	30.1	-1.5	31.3	-1.7
MMP9	34.7	34.3	1.3	32.1	**6.0**	31.9	**5.9**	31.9	**5.1**	32.8	**7.0**
PECAM1	34.2	35.0	-1.9	35.0	-1.9	35.0	-2.1	35.0	-2.4	35.0	1.0
PF4	34.1	33.2	1.8	34.3	-1.2	34.4	-1.4	34.2	-1.5	34.3	1.8
PGF	31.0	30.0	1.9	30.7	1.2	30.6	1.2	30.8	-1.2	31.2	-1.2
SERPINF1	24.2	24.9	-1.7	25.5	-2.5	25.0	-2.0	24.9	-2.2	25.7	-1.4
SPHK1	28.9	31.1	**-4.9**	30.2	-2.6	29.7	-2.1	30.5	**-4.1**	31.7	**-3.6**
STAB1	33.0	34.4	-2.8	34.2	-2.5	34.5	**-3.4**	34.2	**-3.2**	34.9	-1.9
TEK	34.8	35.0	-1.2	33.8	2.0	33.0	**3.2**	33.3	2.1	34.3	2.9
TGFBR1	32.4	29.5	7.0	31.7	1.5	31.6	1.5	31.5	1.4	32.5	1.5
THBS2	26.3	29.0	**-6.5**	27.5	-2.3	26.9	-1.7	26.9	-2.0	28.4	-2.2
TIMP1	22.3	22.5	-1.2	21.7	1.5	21.6	1.4	21.2	1.6	22.1	2.3

Table 1. Expression of angiogenesis-related gene after 1 hour treatment. Cycle threshold (Ct) represent the average cycle where fluorescence detection was significantly greater than baseline and began linear increases. Fold change was determined by subtracting standardized experimental Ct values from Control Ct values (see Methods). Figures in bold are statistically significant ($P<0.05$) and greater than 3-fold magnitude compared to controls.

Angiogenesis-Related Gene Expression- 72 hours

	Control	Nicotine		MLA		MLA NIC		BGT		BGT NIC	
	Ct	Ct	Fold	Ct	Fold	Ct	Fold	Ct	Fold	Ct	Fold
ANGPT1	33.3	33.0	2.1	34.4	1.4	34.4	1.4	33.6	1.5	34.2	1.1
ANGPTL3	33.6	33.7	1.7	33.1	1.2	34.0	-1.6	33.9	-2.2	34.5	**-3.3**
ANGPTL4	35.1	34.9	2.6	32.0	1.7	32.2	1.5	31.9	1.2	32.8	-1.5
CCL11	35.4	32.8	**7.8**	35.0	1.5	34.5	2.0	35.0	-1.0	34.7	1.2
CCL2	31.1	26.4	**49.0**	30.0	1.2	30.5	-1.2	29.8	-1.1	30.7	-2.0
CXCL3	37.3	33.3	**6.1**	34.7	1.2	35.0	-1.1	35.0	-1.6	35.0	-1.5
CXCL6	31.2	31.6	1.4	31.7	1.3	31.9	1.1	31.6	-1.2	32.3	-1.8
EFNA3	33.5	32.6	3.5	31.6	1.5	32.5	-1.3	32.0	-1.3	32.9	-2.5
EFNB2	29.6	29.0	2.9	29.8	1.3	29.8	1.3	29.6	-1.0	30.0	-1.3
ENG	27.8	26.6	**4.3**	28.4	-1.1	28.2	1.1	27.3	1.4	27.6	1.1
EPHB4	29.7	28.4	**4.4**	28.6	-1.0	28.4	1.1	27.9	1.1	28.2	-1.1
EREG	28.1	26.8	**4.4**	30.8	1.2	31.4	-1.3	31.3	-1.8	29.9	1.5
FLT1	29.2	27.7	**5.2**	30.6	1.3	30.6	1.2	30.6	-1.2	31.4	-2.0
HGF	27.7	29.3	-1.7	28.5	1.1	29.2	-1.5	28.4	-1.3	28.2	-1.1
ID3	36.5	36.3	1.8	27.8	1.0	28.2	-1.3	27.9	-1.6	28.6	-2.5
IL1B	32.6	30.0	**11.2**	32.2	1.8	32.8	1.2	32.0	1.3	32.1	1.3
IL6	32.4	30.9	**5.2**	33.2	1.2	33.4	1.1	32.6	1.3	32.9	1.1
ITGB3	31.0	33.3	-1.6	28.4	1.4	29.3	-1.3	29.1	-1.6	29.9	-2.8
JAG1	31.5	31.1	2.4	29.4	1.5	29.5	1.3	28.9	1.3	29.1	1.2
LAMA5	30.7	29.4	**4.6**	29.6	2.0	29.3	2.4	28.9	2.1	30.0	1.0
MMP9	33.6	30.3	1.6	34.6	1.6	35.0	1.2	34.9	-1.2	33.8	1.8
PECAM1	35.4	32.6	**9.0**	35.0	-1.2	35.0	-1.3	35.0	-1.9	35.0	-1.8
PF4	31.7	36.1	**-5.4**	34.7	-1.1	34.8	-1.2	34.4	-1.3	34.3	-1.2
PGF	31.8	29.0	**13.6**	31.3	1.2	31.3	1.2	30.2	1.7	29.8	2.3
SERPINF1	23.4	29.3	**-31.6**	25.4	-1.6	25.6	-1.9	24.5	-1.3	26.9	-6.4
SPHK1	30.3	29.5	3.4	29.4	1.0	30.0	-1.5	29.2	-1.3	29.1	-1.2
STAB1	34.6	34.7	2.0	33.6	-1.1	34.3	-1.7	33.5	-1.6	33.7	-1.7
TEK	35.4	37.4	1.6	35.0	1.3	35.0	1.3	35.0	-1.2	34.7	1.1
TGFBR1	28.1	26.9	**4.2**	32.6	1.2	32.5	1.3	31.7	1.6	31.4	2.0
THBS2	29.1	28.4	2.9	26.3	1.5	26.6	1.2	26.2	1.0	26.7	-1.3
TIMP1	21.3	23.8	**-3.3**	23.0	-1.1	23.1	-1.2	22.5	-1.2	22.6	-1.3

Table 2. Expression of angiogenesis-related gene after 72 treatment. Cycle threshold (Ct) represent the average cycle where fluorescence detection was significantly greater than baseline and began linear increases. Fold change was determined by subtracting standardized experimental Ct values from Control Ct values (see Methods). Figures in bold are statistically significant ($P<0.05$) and greater than 3-fold magnitude compared to controls.

(-5.61 fold) but increased mRNA expression of matrix-metalloproteinase 9 (MMP-9; 6 fold) and chemokine CCL2 (3.2 fold). This pattern was also evident with MLA alone and MLA with nicotine (figure 3). Specific antagonism of α7 containing subtypes using αBGT (figure 4) yielded a similar response with the additional reduction of stabilin 1 (STAB1; -3.2 fold). Alpha-bungarotoxin in the presence of nicotine (figure 5) reduced the mRNA expression of JAG1 (-13 fold) and SPHK1 (-3.6 fold) and increased expression of MMP-9 (7 fold), CCL2 (7.7 fold), and ANGPT1 (3.2 fold).

Alternatively, after 72 hours of nicotine exposure (100 nM; figure 6), only platelet factor 4 (PF4; -5.4 fold), serine protease 1 (SERPINF1; -31 fold), and tissue inhibitor of metalloproteinase 1 (TIMP1; -3.29) were significantly decreased over three-fold magnitude; mRNA expression of chemokine CCL2 (49 fold), placental growth factor (PGF; 13 fold), interleukin 1-beta (IL1B; 11.2 fold), platelet-endothelial cell adhesion molecule 1 (PECAM1; 9 fold), chemokine CCL11 (7.8 fold), chemokine CXCL3 (6.1 fold), laminin alpha-5 (LAMA5; 4.6 fold), endoglin (ENG; 4.32), interleukin-6 (IL6; 5 fold), epiregulin (EREG; 4.4 fold), FMS-related tyrosine kinase 1 (FLT1; 5.2 fold), and Ephrin β4 (EPHB4; 4.4 fold) were significantly increased above three-fold magnitude. MLA alone (figure 7) and MLA with nicotine (figure8) blocked this expression pattern and resulted in no significant changes in gene expression beyond three-fold magnitude. Similar results were obtained using αBGT (figure 9) or αBGT and nicotine together (figure 10).

nAChR Gene Expression

Cell cultures were treated with nicotine and nicotinic antagonists for 1 or 72 hours and expressions of nAChRs was analyzed. After 1 hour nicotine exposure (figure 11) there was a significant increase in α7 and α9 subunits and decreases in α10 and β2 subunits, the remaining subunits were not significantly different than controls. These results were blocked using the nAChR non-specific antagonist MLA or MLA and nicotine together (figures 12a, b). Again, these results were blocked using α7 specific nAChR antagonist αBGT or αBGT and nicotine together (figures 12c, d).

Similarly, after 72 hours of nicotine treatment (100 nM; figure 13), there was increased expression of α7, α9, and β4 subunits and a decrease in α10 and β2 subunits. This expression change was blocked using MLA, MLA/nicotine, αBGT and αBGT/nicotine (figure 14).

DISCUSSION

Angiogenesis has been shown to involve a complex biochemical response to the interaction of different cell types, inflammatory cytokines, adhesion molecules, and growth factors. Stadelman et al. (1998) describes the wound healing response through the relationships of three phases- inflammation, cellular proliferation, and angiogenesis.

The body maintains inflammatory homeostasis through a balance of pro-inflammatory and anti-inflammatory cytokines; typically low levels of cytokines are required to maintain homeostasis. Overproduction results in disease and tissue damage (Tracey, 2007). The body regulates inflammation through humeral methods such as

concentration gradients. Cytokines accumulate at the site of infection or damage. When a cytokines levels reach threshold, pro-inflammatory cytokines are inhibited and damage is prevented. An alternative method of inflammation control is the cholinergic anti-inflammatory pathway. Studies demonstrate that vagal innervation of blood vessels provide signals that suppress pro-inflammatory cytokine release and inhibit inflammation. Activation of the nerve releases ACh, which activates α7 nAChRs on endothelial cells and immune cells (such as macrophages) blocking activation NF- κB and thereby inhibiting release of cytokines (Borovikova et al. 2000a, b). This results in attenuated systemic anti-inflammation.

After the initial inflammatory response, the target zone begins a proliferation phase followed by an angiogenesis phase, both of which involve fibroblast production of glycosaminoglycans, collagen, and fibroblast proliferation (Stadelman et al., 1998). These phases are mediated by adhesion molecules, growth factors, components of the extracellular matrix. For example, a major component of this phase includes the use of collagen as building material, however, it but must also be degraded to provide room for blood vessels, which then requires the activation of plasminogen activators and collagenases, such as MMP-9.

Short-Term Anti-Angiogenic Expression

Short-term treatment with nicotine resulted in statistically significant decreases of many promoters of angiogenesis. Inhibitor of DNA binding 3 (ID3) was reduced more than 50-fold. ID3 interferes with DNA binding of transcription factors and have been shown to maintain vasculature outgrowth (Lyden et al. 1999). In addition, ID3 deficient

mice have no obvious abnormalities but have compromised immunity (Pan et al, 1999). Because immune function plays a major role in neovascularization, ID3 is considered a pro-angiogenic molecule.

Another increase was seen for integrin β3 (ITGB3). This is a gene that encodes glycoprotein III, a receptor subunit in the class of integrin adhesion molecules. Integrins are known to mediate interactions with extracellular matrix and initiate intracellular signals that regulate cell cycle. Integrins promote the growth of both normal and tumorigenic cells and they induce proliferation by specifically causing the transcription of cyclins and cyclin dependent kinases which are required for transition past the G1 cell cycle start point (Kozlava et al. 2000; Coppolino et al., 2000; Assoian et al., 2001).

CXCL6 and CCL2 are members of chemokine the families, CXC and CC, respectively. These chemokines are responsible for trafficking various types of immune cells in the vasculature and playing basic roles in immune function and endothelial angiogenesis (Anisowicz et al, 1987).

Other significant decreases include:

- Hepatocyte growth factor (HGF) which stimulates the growth of hepatocytes and has been shown to limit tumor progression when over expressed (Schmidt et al., 1995; Uehara et al., 1995).
- Ephrin β3 (EFNB3) is a ligand for the EPH family of receptor tyrosine kinases. Ephrin knockouts display disrupted angiogenesis, especially regarding vascular remodeling (Wang et al., 1998).
- Thrombospondin 2 (THBS2) has been shown to inhibit tumor growth and angiogenesis in THBS2-expressing tumors (Streit et al., 1999).

- Sphingosine kinase 1 (SPHK1) catalyzes the conversion of sphingosine to sphingosine-1-phosphate, a lipid messenger that has been shown to regulate cell proliferation (Kohama et al., 1998).

Short-Term Pro-Angiogenic Expression

While a majority of the short-term nicotine exposure affects are anti-angiogenic there were several pro-angiogenic changes. Short-term nicotine treatment resulted in the increase of two pro-angiogenic mediators and the reduction of one tumor-reducing growth factor.

Transforming growth factor-beta receptor 1 (TGFBR1) was increased by seven-fold over control. TGFBR1 has been shown to activate the phosphorylation of SMAD2 and SMAD3 which leads to downstream transcription (Inman et al, 2002). Larsson et al., 2001, described TGF- β1 enhancement of cell proliferation and irregular migration in vitro and vascular defects in vivo.

Another increase was seen for prostaglandin-endoperoxide synthase-1 (PTGS1) which has been studied in cell culture systems of endothelial cells and colon carcinoma. When treated with aspirin, a nonsteriodal anti-inflammatory drug which blocks the enzymatic activity of PTGS1, tube formation was suppressed (Tsujii et al., 1998).

Transcripts for angiopoietin-like 4 (ANGPTL4), a negative promoter of angiogenesis, were reduced after 1 hour exposure to nicotine. The angiopoietin family is a group of growth factors exclusively targeting vascular endothelium (Camenisch et al., 2002). ANGPTL4 has been shown to prevent spreading of mouse lung carcinoma in vivo

(Galaup et al., 2006). Melanoma cells in vitro showed reduced migration and invasion when compared to controls (Galaup et al., 2006).

Short-term nicotine exposure reduced transcription of inflammatory chemokines, pro-angiogenic adhesion molecules, and growth factors. This anti-inflammatory and overall anti-angiogenic endothelial cell response to cholinergic stimulation is not unexpected when considered in the context of the cholinergic anti-inflammatory pathway. The application of nAChR antagonists MLA and αBGT did not return the gene expression changes to control levels. The consistent down-regulation of stabilin 1 (STAB1), a protein used for immune cell function; jagged 1 (JAG1), a receptor for the Notch ligand; ITGB3; and the up-regulation of CCL2 and matrix metalloproteinase-9 (MMP-9), a collagenase involved in the breakdown and remodeling of collagen involved in angiogenesis, suggest a role for basal signaling through endogenous ACh. There is evidence that activation through endogenous ACh regulates cell proliferation and angiogenesis in the vasculature. Kiuchi et al (2008) observed suppressed basal and nicotine-stimulated neovascularization in a mouse model of choroidal neovascularization (CNV) using mecamylamine, a non-specific nAChR antagonist. We suggest that gene expression changes following use of nAChR antagonists are remnants of endogenous ACh signaling after 1 hour inhibition. Moreover, these changes are absent after 72 hours of inhibition.

Long-Term Anti-Angiogenic Expression

After chronic 72 hour nicotine exposure, we find results opposite to the acute, 1 hour response. Specifically, we observed a significant increase of inflammatory

chemokines, growth factors, and pro-angiogenic adhesion molecules. However, there were a few anti-angiogenic gene expression changes.

First, long-term exposure to nicotine resulted in decreased mRNA expression for platelet factor 4 (PF4), which has been suggested to play a role in inflammation and vascular repair due to its neutrophil chemoattractant capabilities (Eisman et al., 1990). In addition, we observed a decrease in a serine protease inhibitor family member (SERPINF1), which is known to attenuate inflammatory responses.

Seventy-two hour nicotine exposure was associated with increased expression of the anti-angiogenic gene, FMS-related tyrosine kinase 1 (FLT1). FLT1 binds with high affinity to vascular endothelial growth factor (VEGF) and regulates VEGF activity by working as a trap or antagonist (Wulff et al., 2001; He et al., 1999).

Long-Term Pro-Angiogenic Expression

HDMEC exposure to nicotine resulted in a modest anti-inflammatory response but a large increase in pro-angiogenic gene expression pattern. First, TIMP1, an inhibitor of the pro-angiogenic protein MMP-9 was decreased below control. The expressions of the inflammatory chemokines CCL2, CCL11, CXCL3, which are responsible for immune cell recruitment and are essential for new blood vessel growth, were increased.

Placental growth factor (PGF), a 50% homolog to VEGF (Maglione et al., 1991) was increased by 13-fold. PGF stimulates angiogenesis in heart and limbs with comparable efficiency to VEGF (Luttun et al. 2002).

Two members of the interleukin cytokine family, IL1-B and IL-6, were also increased. IL-1B has been shown to increase expression of vascular cell adhesion

molecules resulting in adherence of cancer cells (Vidal-Vanaclocha et al., 2000) and is required for in vivo angiogenesis in of different varieties of tumors (Voronov et al., 2003).

Platelet-endothelial cell adhesion molecule 1 (PECAM1) is a surface receptor found in high concentrations on circulating immune cells and endothelial cells. Previous work has shown that PECAM1 knockout mice do not exhibit NF- κB activation and thus activation of downstream inflammatory genes was prevented (Tzima et al., 2005).

Other significant angiogenic increases:

- Laminin alpha-5 (LAMA5), a protein found in the extracellular matrix.
- Endoglin (ENG) is a protein involved in vascular remodeling and organization both of which are diminished in ENG-/- mice (Li et al., 1999; Bourdeau et al, 1999). In a mouse model of age-related macular (ARMD), endoglin was found to be over expressed in advanced stage neovascular tissue (Grisanti et al., 2004)
- Epiregulin (EREG) is a member of the epidermal growth factor family and plays a role in progression of vascular remodeling through upregulation by dedifferention factors in an ERK-dependent manner (Takahashi et al. 2003).
- Endothelial tyrosine kinase (TEK) binds angiopoietin ligands and is critical for endothelial cell-smooth muscle cell communications where overexpression results in venous malformations (Vikkula et al., 1996).

Nicotinic Acetylcholine Receptor Expression

Gene expression of angiogenesis-related genes differed between acute and chronic exposure to nicotine. In expression systems, chronic nicotine exposure at concentrations near 10 nM induces both desensitization and upregulation of α4-containing neuronal nAChR subtypes (Fenster et al., 1997; 1999); in addition, α7 nAChR subtypes are upregulated in the brains of smokers (Buisson & Bertrand, 2001; 2002). Because different nAChR subtypes have different affinities to cholinergic agonist and also have different activation kinetics, we hypothesize that changes in nAChR expression will result in changes in intracellular signaling and downstream gene transcription. Real-time RT-PCR analyses show upregulation of both α7 and α9 nAChRs for both short- and long-term durations. In addition, both nicotine exposure durations resulted in decreases of nAChR subunits α10 and β2. While nicotine exposure leads to up regulation of nAChRs as expected, the similar patterns in acute and chronic conditions suggest the differential expression patterns seen for angiogenesis-related gens is not based on up or down regulation of nAChRs.

CONCLUSION

Angiogenesis is a normal process in growth and development, as well as in wound healing. However, this is also a fundamental step in the initiation and progression of tobacco-related diseases. Nicotine, a major component of cigarette smoke, modifies expression of angiogenesis–related genes in HDMECS after 1 hour or 72 hours exposure. Acute nicotine treatment results in an anti-angiogenic gene expression pattern while

chronic exposure leads to a pro-angiogenic gene expression pattern. The difference in expression patterns is not due to a change in nAChR gene expression. These data provide clinical implications for the use of cholinergic agents for treatment of vascular eye diseases. At short durations nicotine activates the cholinergic anti-inflammatory pathway and exerts anti-angiogenic effects. Chronic durations of nicotine exposure implicate nicotine as an angiogenic factor that modifies the vascular environment to promote vascular growth via transcription of inflammatory molecules, adhesion molecules, and growth factors.

REFERENCES

1. Armstrong RA. Is smoking a risk factor for AMD? *Ophthalmology Times* 42-45, 2003.
2. Klein R, Klein BEK, Moss SE, Cruickshanks KJ. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in Type 1 diabetes. *Ophthalmology* 105: 1801–15, 1998.
3. Christen WG, Glynn RJ, Manson JE, Ajani UA, Buring JE. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 276: 1147-51, 1996.
4. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA* 276: 1141-6, 1996.
5. Kawashima K, Oohata H, Fujimoto K, Suzuki T. Extraneuronal localization of acetylcholine and its release upon nicotinic stimulation in rabbits. Neurosci Lett. 1989 Oct 9;104(3):336-9.
6. Kawashima K, Watanabe N, Oohata H, Fujimoto K, Suzuki T, Ishizaki Y, Morita I, Murota S. Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett 1990 Nov 13;119(2):156-8.
7. Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. *Journal of Pharmacology & Experimental Therapeutics* 287: 435-439, 1998.

8. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine Is an Autocrine or Paracrine Hormone Synthesized and Secreted by Airway Bronchial Epithelial Cells. *Endocrinology* 145:2498-2506, 2004.
9. Tracey KJ. The inflammatory reflex. *Nature* 420:853-859, 2002.
10. Gallowitch-Puerta, MARG, Tracey KJ. Immunologic Role of the Cholinergic Anti-Inflammatory Pathway and the Nicotinic Acetylcholine a7 Receptor. *Ann NY Acad Sci* 1062: 209-219, 2005.
11. Pavlov VA, Tracey KJ. The cholinergic anti-inflammatory pathway. *Brain, Behavior, and Immunity* 19: 493-499, 2005.
12. Heeschen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, Tsao PS, Johnson FL, Cooke JP. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nature Medicine* 7: 833-839, 2001.
13. Kiuchi K, Matsuoka M, Wu JC, Lima e Silva R, Kengatharan M, Verghese M, Ueno S, Yokoi K, Khu NH, Cooke JP, Campochiaro PA. Mecamylamine suppresses Basal and nicotine-stimulated choroidal neovascularization. Invest Ophthalmol Vis Sci. 2008 Apr; 49(4): 1705-11.
14. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg. 1998 Aug;176(2A Suppl):26S-38S.
15. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest. 2007 Feb;117(2): 289-96.
16. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000 May 25;405 (6785):458-62.

17. Borovikova LV, Ivanova S, Nardi D, Zhang M, Yang H, Ombrellino M, Tracey KJ. Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. Auton Neurosci. 2000 Dec 20;85(1-3):141-7.

18. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benazra R. (1999) Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenograts. Nature. 401; 670-677.

19. Pan L, Sato , Fredrick JP, Sun XH, Zhuang Y. (1999) Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. Molec Cell Biol. 19; 5969-5980.

20. Kozlova NI. Integrins: structure and functions. *Membr Cell Biol.* 2000;13(2):207–244.

21. Coppolino M G, Dedhar S. Bi-directional signal transduction by integrin receptors. *Int J Biochem Cell Biol.* 2000;32(2):171–188.

22. Assoian R K, Schwartz M A. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Curr Opin Genet Dev.* 2001;11(1):48–53.

23. Anisowicz A, Bardwell L, Sager R. 1987. Constituative overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc Nat Acad Sci. 84; 7188-7192.

24. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. Nature. 373: 699-702.

25. Uehara Y, Minowa O, Mori C, Shiota L, Kuno J, Noda T, Kitamura N. 1995. Placental defect and embryonic lethality in mice lackin ghepatocyte growth factor/scatter factor. Nature. 373: 702-705.

26. Wang HU, Chen ZF, Anderson DJ. Molecualr distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-b2 and its receptor Eph-B4. 1998. Cell. 93:741-753.

27. Streit M, Riccardi L, Velasco P, Brown LF, Hawighorst T, Bornstein P, Detmar M. 1999. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. Proc Nat Acad Sci. 96: 14888-14893.

28. Kohama T, Olivera A, Edsall L, Nagiec MM, Dickson R, Spiegel S. 1998. Molecular cloning and functional charaterization of murine sphingosine kinase. J Biol Chem. 273: 23722-23728.

29. Inman GJ, Nicolas FJ, Hill CS. 2002. Nucleocytoplasmic shuttling of SMADS 2, 3, and 4 permits sensing of TGF-beta receptor activity. Molec Cell. 10: 283-294.

30. Larsson J, Goumans MJ, Sostrand LJ, van Rooijen MA, Ward D, Levenn P, Xu X, Dijke P, Mummery CL, Karlsson S. 2001. Abnormal angiogenesis but intact hemotopoietic potential in TGF-beta type 1 receptor-deficient mice. EMBO J. 20: 1663-1673.

31. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell. 93: 705-716.

32. Camenisch G, Pisabarro MT, Sherman D, Kowalski J, Nagel M, Hass P, Xie MH, Gurney A, Bodary S, Liang XH, Clark K, Beresini M, Ferrara N, Gerber HP. 2002. ANGPTL3 stimulates endothelial cell adhesion and migration via integrin-v-beta-3

and induces blood vessel formation in vivo. J Biol Chem. 277: 17281-17290.

33. Galaup A, Cazes A, Le Jan S, Philippe J, Connault E, Le Coz E, Mekid H, Mir LM, Opolon P, Corvol P, Monnot C, Germain S. 2006. Angiopoitin-like 4 prevenets metasis through inhibition of vascular permeability and tumor cell motility and invasiveness. Proc Nat Acad Sci. 103: 18721-18726.
34. Eisman R, Surrey S, Ramachandran B, Schwartz E, Poncz M. 1987. Structural and functional comparison of the genes for human platelet factor-4 and PF4-alt. Blood. 76: 336-344.
35. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. 2001. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trapA40. *J. Clin. Endocr. Metab.* 86: 3377-3386, 2001
36. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. 1999. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Molec. Endocr.* 13: 537-545.
37. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. 1991. Proc Nat Acad Sci. 88: 9267-9271.
38. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, et al. Revascularization of ischemic tissues by PIGF treatment, and inhibition of tumor angiogenesis, arthritis, and atherosclerosis. 2002. Nature Med. 8: 831-840.
39. Vidal-Vanaclocha F, Fantuzzi G, Mendoza L, Fuentes AM, Anasagasti M.J, Martin J, Carrascal T, Walsh P, Reznikov LL, Kim SH, Novick D, Rubinstein M, Dinarello

CA. 2000. IL-18 regulates IL-1-beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. *Proc. Nat. Acad. Sci.* 97: 734-739.

40. Voronov E.; Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. 2003. IL-1 is required for tumor invasiveness and angiogenesis. *Proc. Nat. Acad. Sci.* 100: 2645-2650.
41. Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, Cao G, DeLisser H, Schwartz MA. 2005. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature. 437:426-431.
42. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP. 1999. Defective angiogenesis in mice lacking endoglin. *Science* 284: 1534-1537.
43. Bourdeau A, Dumont DJ, Letarte M. 1999. A murine model of hereditary hemorrhagic telangiectasia. *J. Clin. Invest.* 104: 1343-1351.
44. Grisanti S, Canbek S, Kaiserling E, Adam A, Lafaut B, Gelisken F, Szurman P, Henke-Fahle S, Oficjalska-Mlynczak J, Bartz-Schmidt KU. 2004. Expression of endoglin in choroidal neovascularization. *Exp. Eye Res.* 78: 207-213.
45. Takahashi M, Hayashi K, Yoshida K, Ohkawa Y, Komurasaki T, Kitabatake A, Ogawa A, Nishida W, Yano M, Monden M, Sobue K. 2003. Epiregulin as a major autocrine/paracrine factor released from ERK- and p38MAPK-activated vascular smooth muscle cells. *Circulation* 108: 2524-2529.
46. Vikkula M, Boon LM, Carraway KL III, Calvert JT, Diamonti AJ, Goumnerov B, Pasyk KA, Marchuk DA, Warman ML, Cantley LC, Mulliken JB, Olsen BR. 1996. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine

kinase TIE2. *Cell* 87: 1181-1190.

47. Fenster CP, Rains MF, Noerager B, Quick MW, Lester RA. Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. *Journal of Neuroscience* 17: 5747-5759, 1997.
48. Fenster CP, Whitworth TL, Sheffield EB, Quick MW, Lester RA. Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. *Journal of Neuroscience* 4804-4814, 1999.
49. Buisson B, Bertrand D. Nicotine addiction: the possible role of functional upregulation. *Trends in Pharmacological Sciences* 23: 130-136, 2002.
50. Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, Bertrand D. Human alpha4beta2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J. Neuroscience* 16: 7880-7891, 1996.

Figure 1

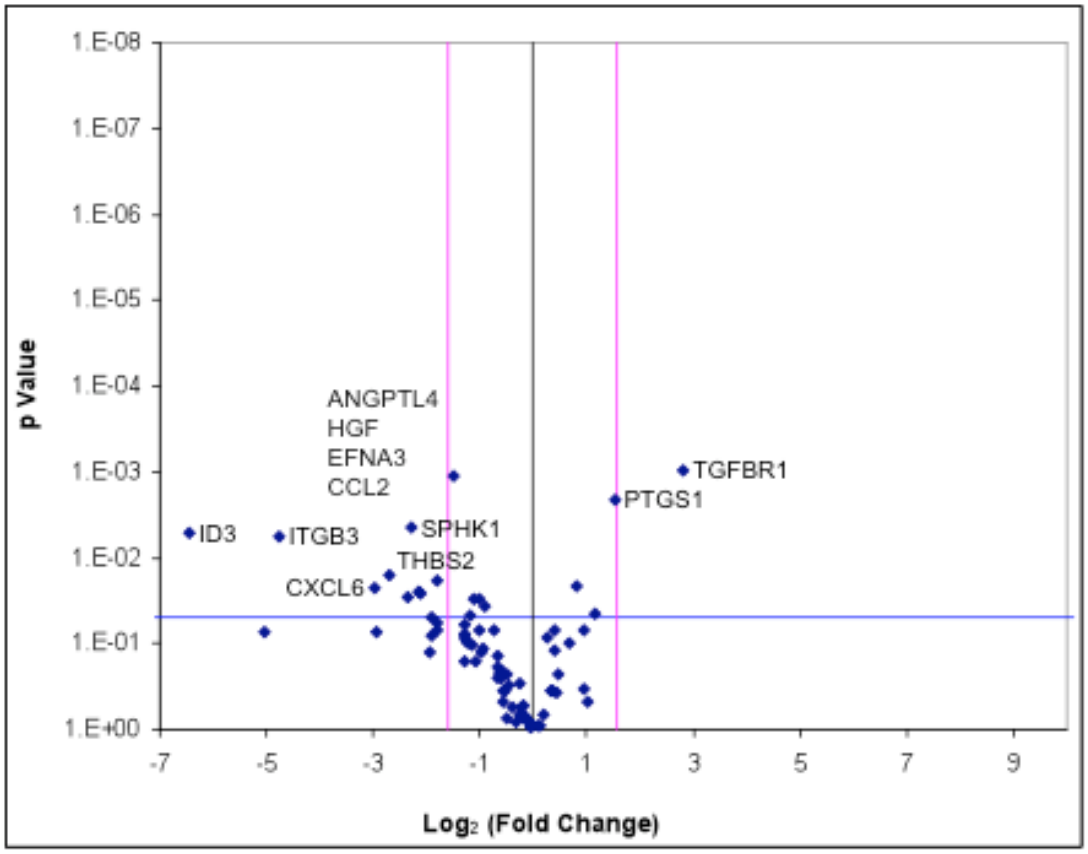

Figure 1. Angiogenesis-related gene expression after 1 hour exposure to 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 2

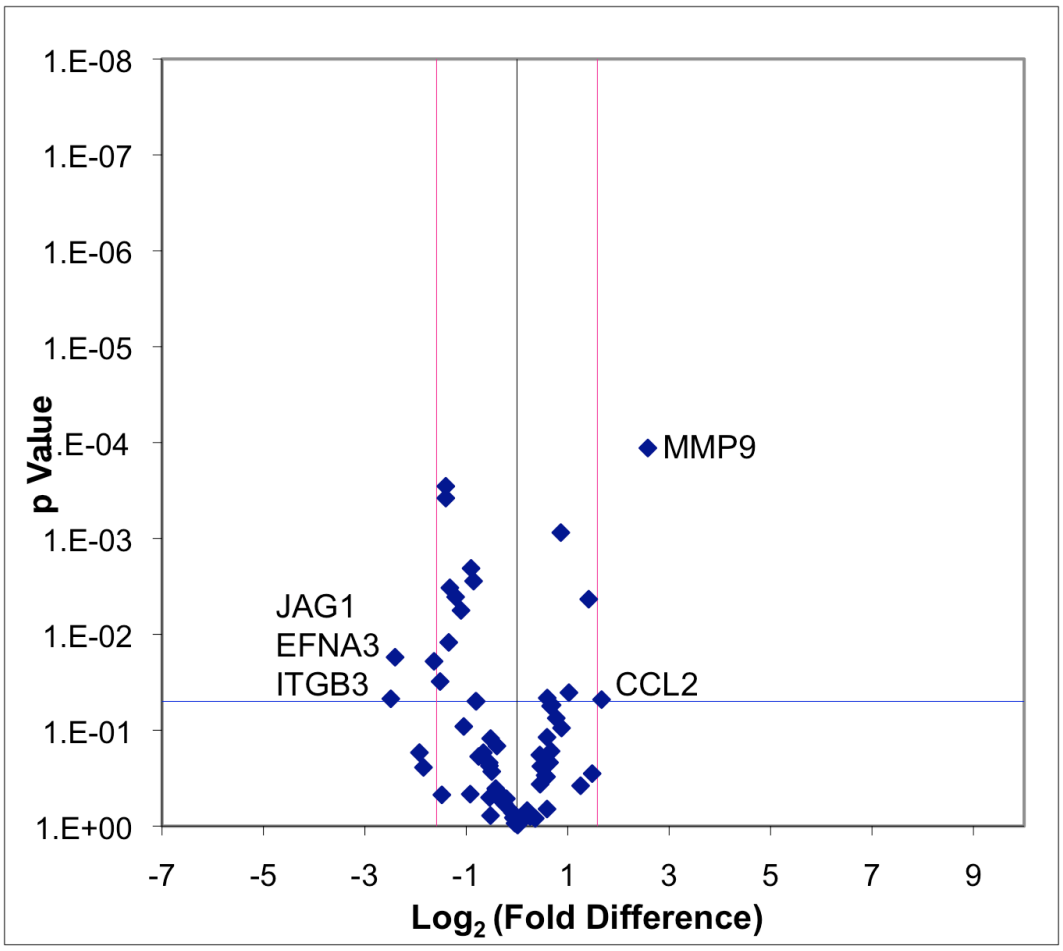

Figure 2. Angiogenesis-related gene expression after 1 hour exposure to 10 μM MLA. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 3

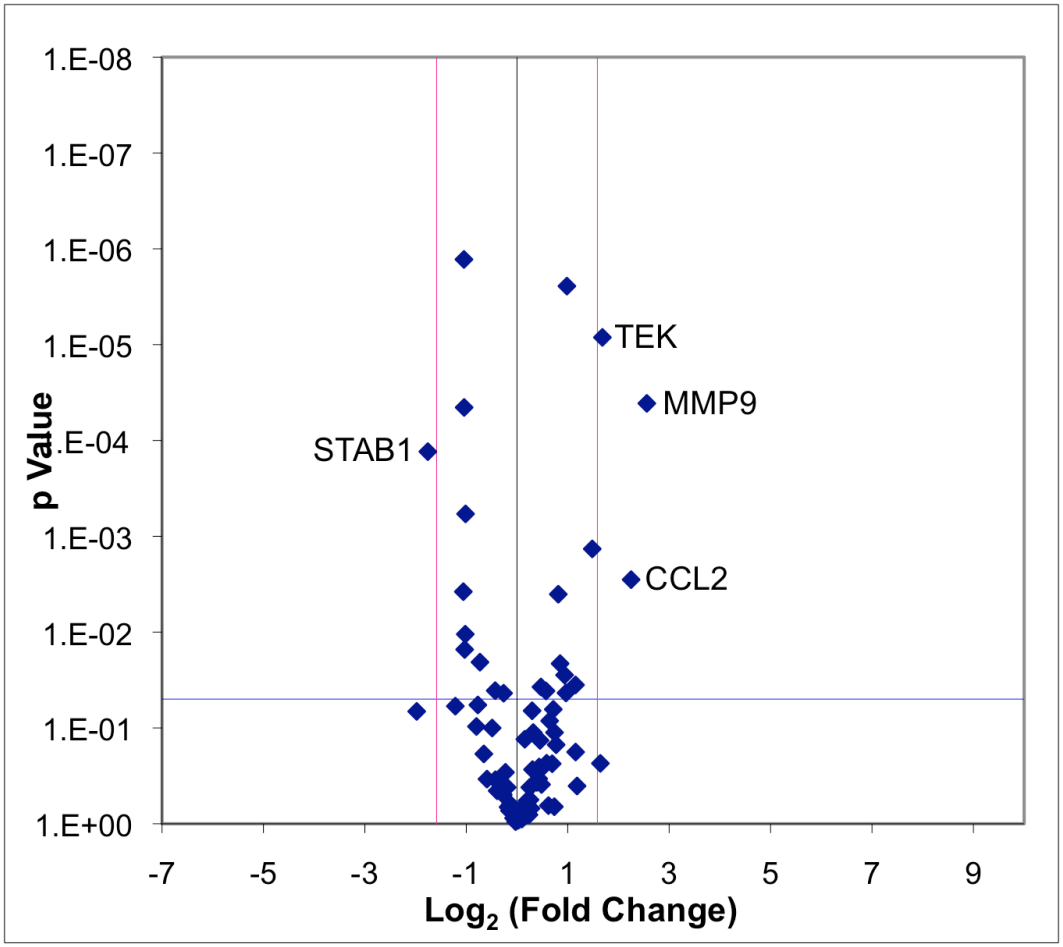

Figure 3. Angiogenesis-related gene expression after 1 hour exposure to 10 μM MLA and 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 4

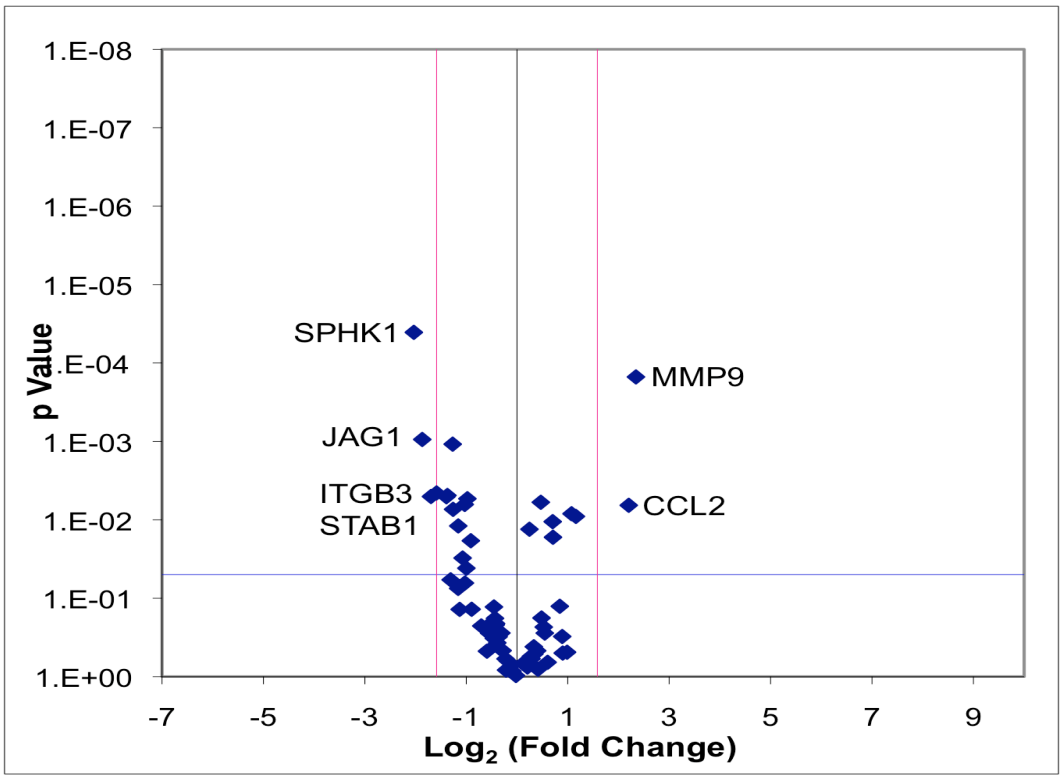

Figure 4. Angiogenesis-related gene expression after 1 hour exposure to 10 μM αBGT. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 5

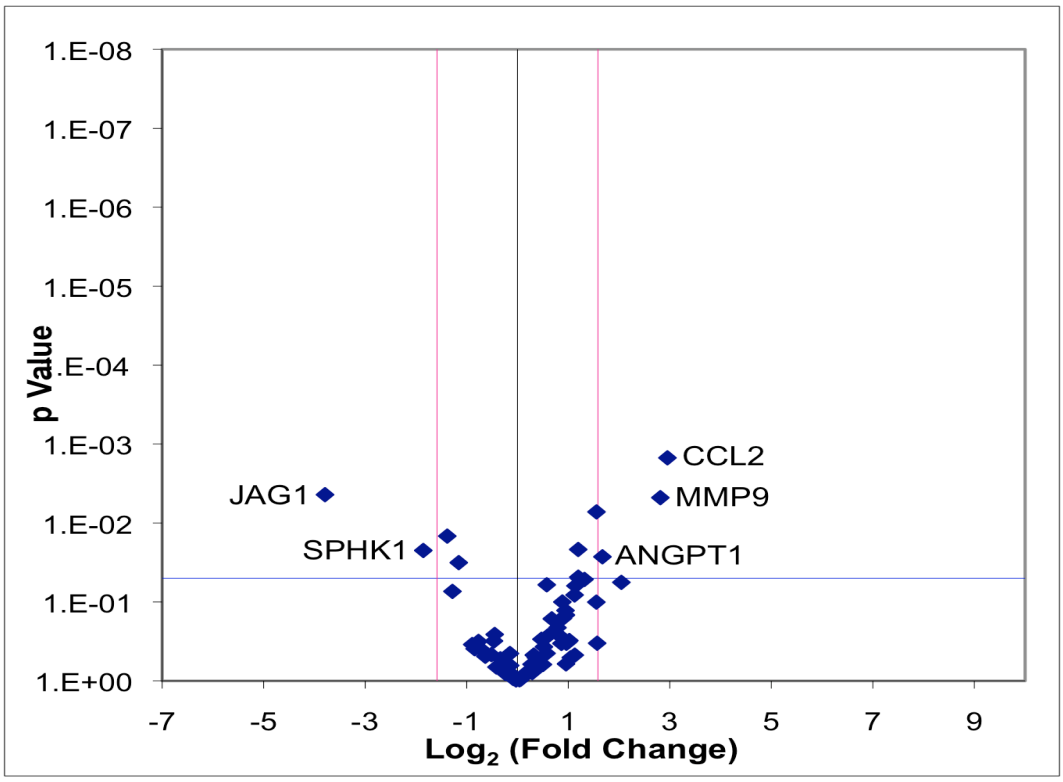

Figure 5. Angiogenesis-related gene expression after 1 hour exposure to 10 μM αBGT and 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 6

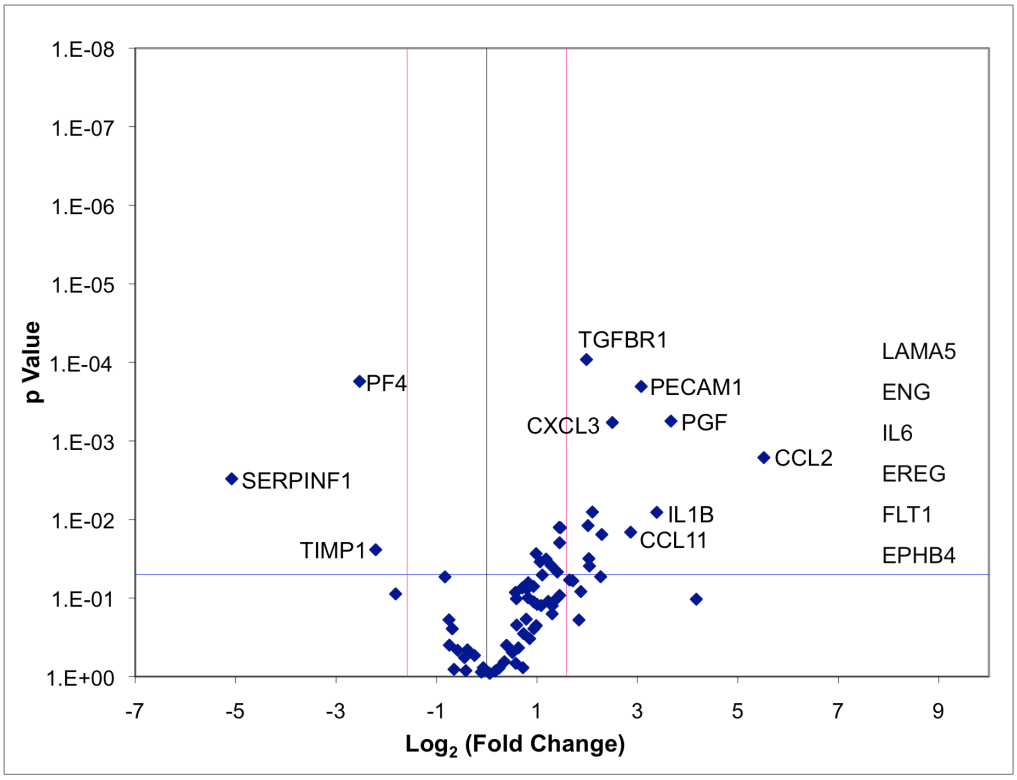

Figure 6. Angiogenesis-related gene expression after 72 hour exposure to 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 7

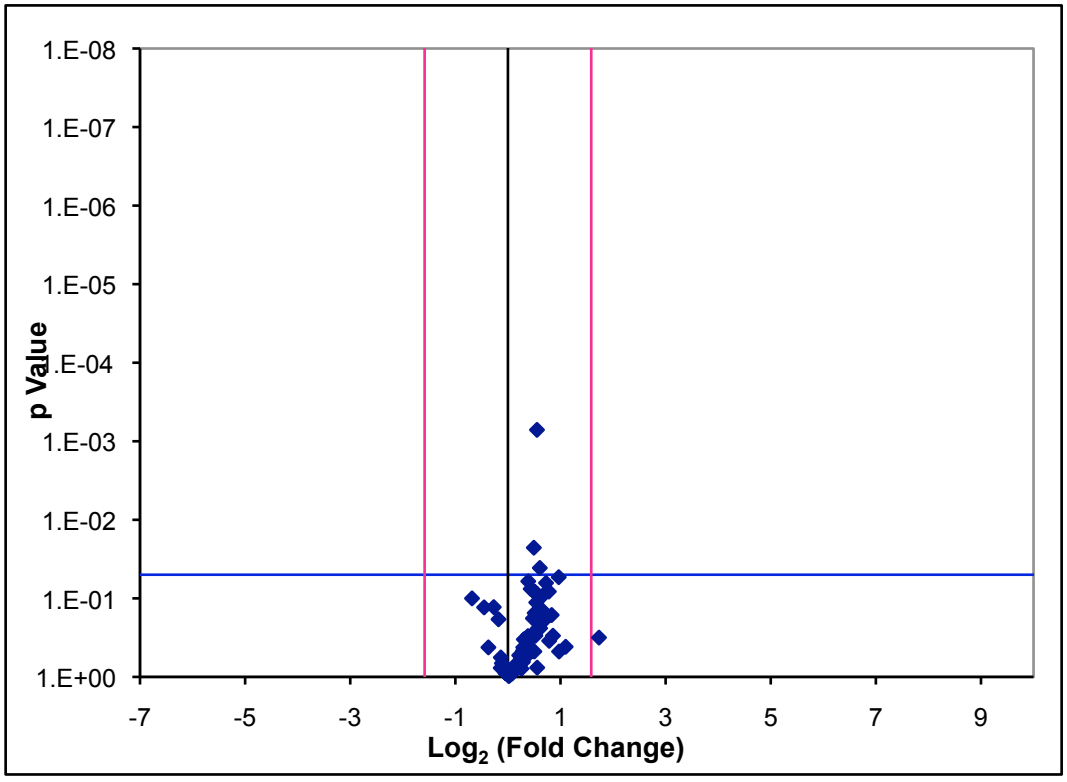

Figure 7. Angiogenesis-related gene expression after 72 hour exposure to 10 μM MLA. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 8

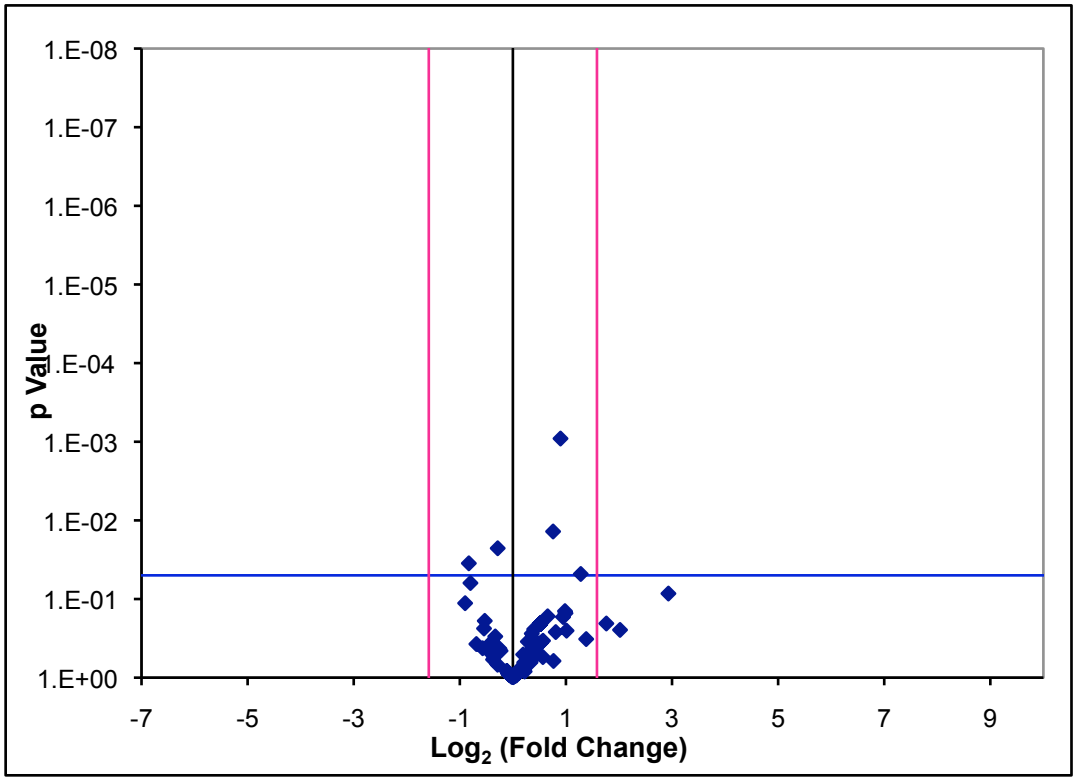

Figure 8. Angiogenesis-related gene expression after 72 hour exposure to 10 μM MLA and 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 9

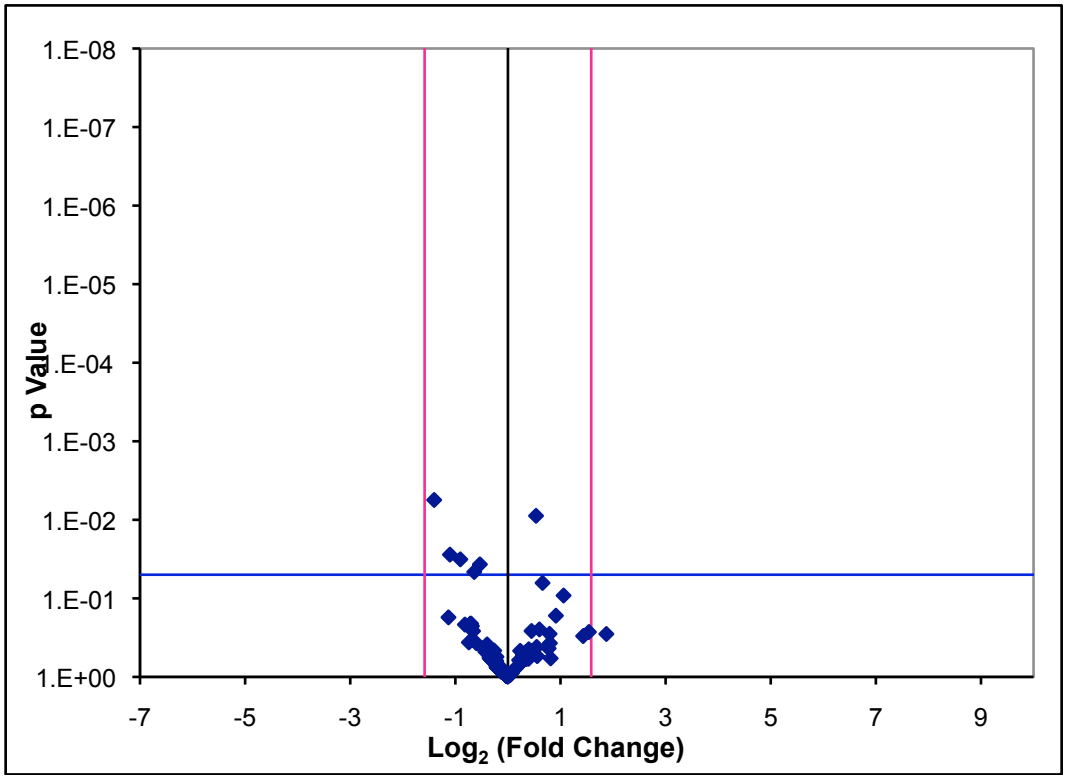

Figure 9. Angiogenesis-related gene expression after 72 hour exposure to 10 μM αBGT. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 10

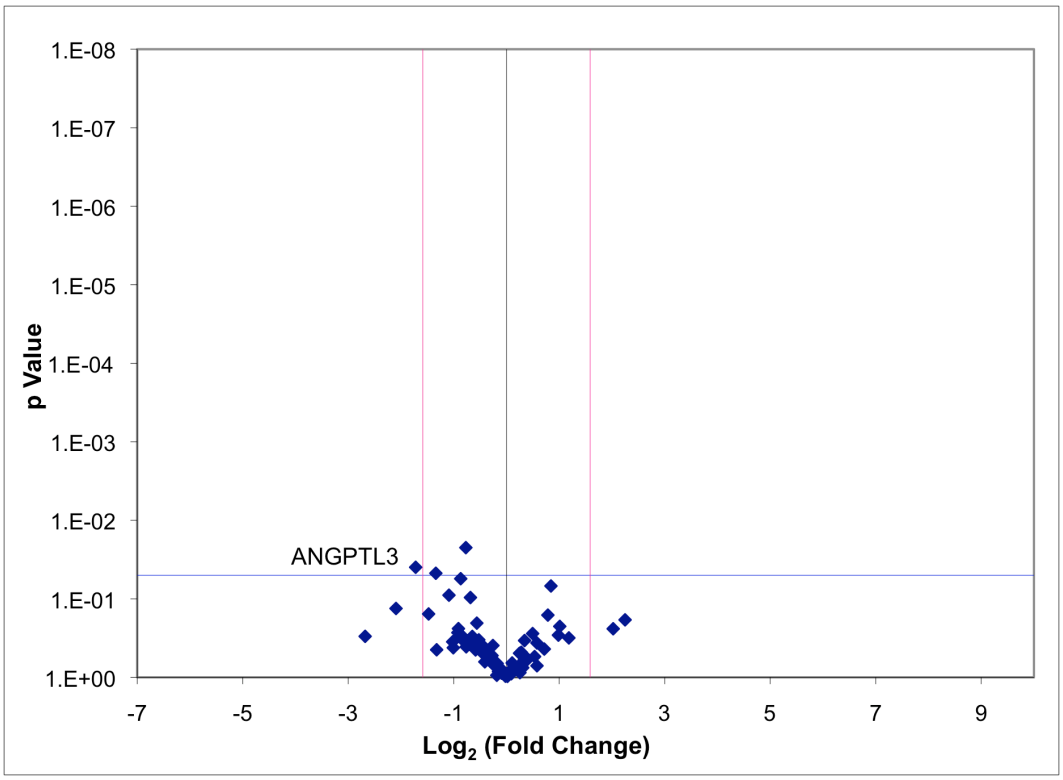

Figure 10. Angiogenesis-related gene expression after 72 hour exposure to 10 μM αBGT and 100 nM nicotine. Blue horizontal line represents a significance level of .05, genes above blue line are statistical different than control. Pink vertical lines represent a 3-fold increase or decrease in gene expression.

Figure 11

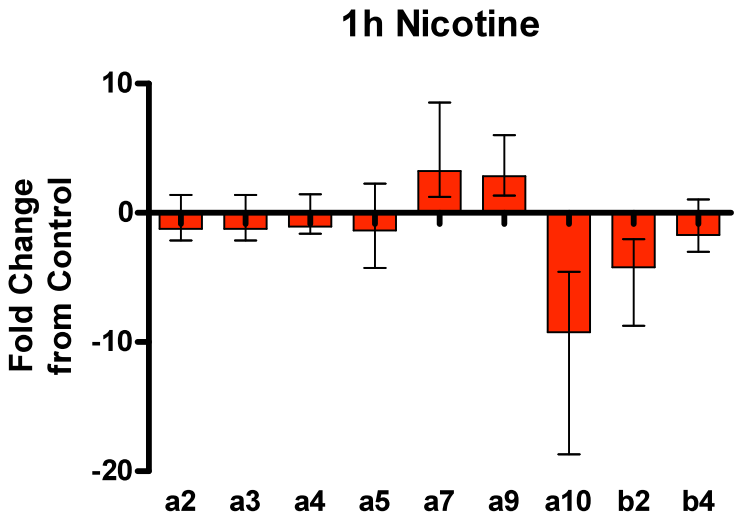

Figure 11. Real-Time RT-PCR expression of nAChRs after 1 hour exposure to 100 nM nicotine. Error bars represent 95% confidence interval.

Figure 12

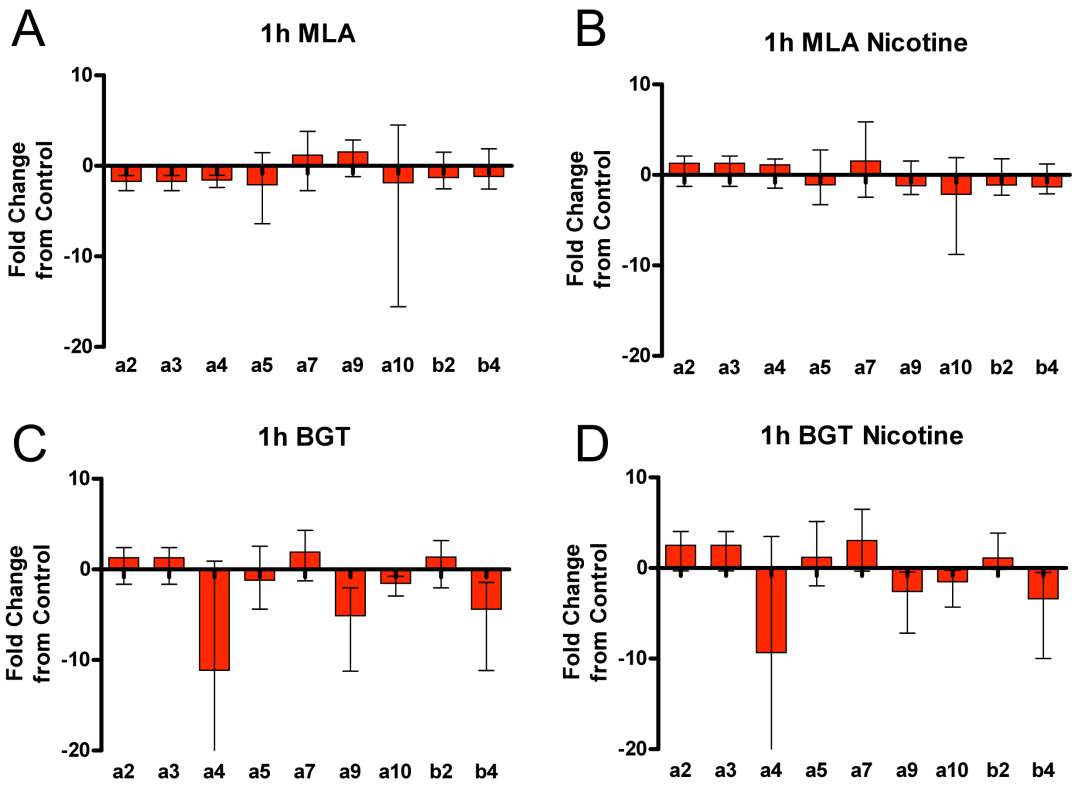

Figure 12. Real-Time RT-PCR expression of nAChRs after one hour exposure to 100 nM nicotine and/or nAChR antagonist. Error bars represent 95% confidence interval.

Figure 13

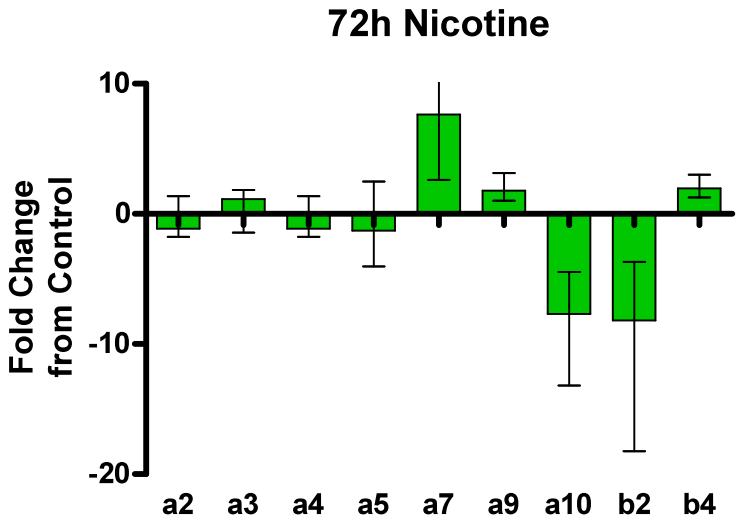

Figure 13. Real-Time RT-PCR expression of nAChRs after 72 hour exposure to 100 nM nicotine. Error bars represent 95% confidence interval.

Figure 14

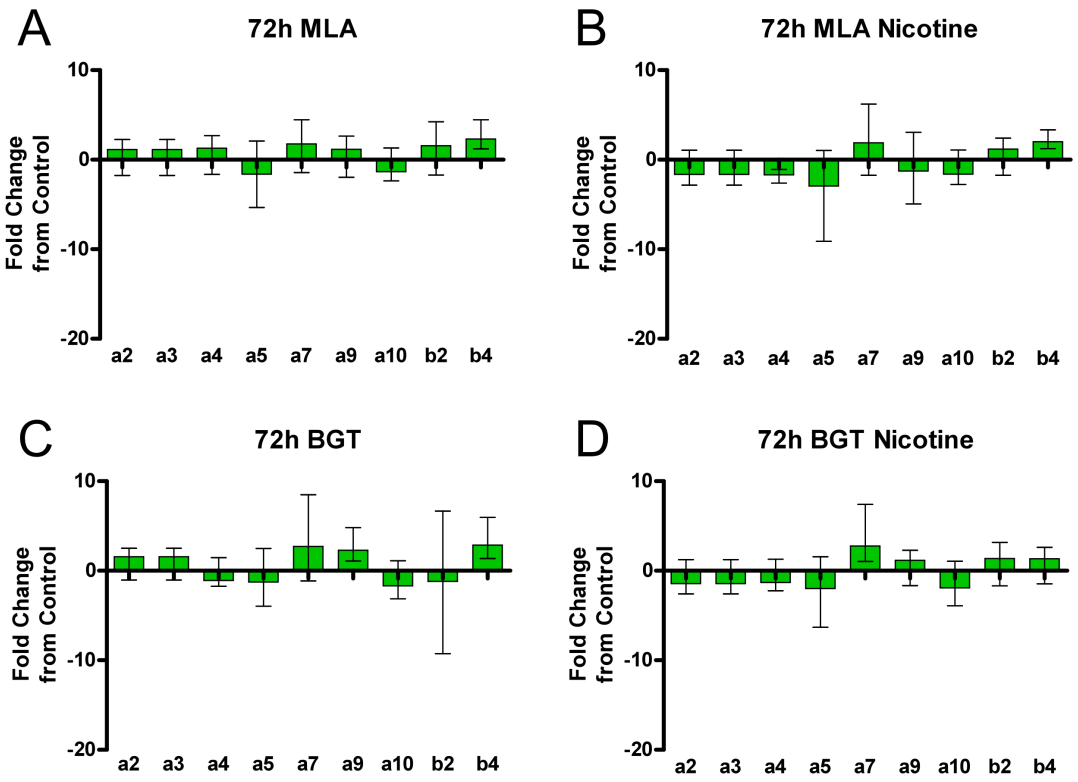

Figure 14. Real-Time RT-PCR expression of nAChRs after one hour exposure to 100 nM nicotine and/or nAChR antagonist. Error bars represent 95% confidence interval.

SUMMARY

The aims of this study were to determine 1) if functional nAChRs are expressed in human microvascular endothelial cells, 2) the effect of nAChR activation on downstream signaling cascades, and 3) the effect of nAChR activation on angiogenesis-related gene transcription. We hypothesize that nAChRs are expressed in HDMECs and upon nicotinic activation engage intracellular signaling pathways that lead to increased transcription of genes involved in angiogenesis.

We investigated the expression of nAChR transcript in HDMECs with RT-PCR, resulting in detection of α2, α3, α4, α5, α7, α9, α10, β2, and β4 subunits. The transcripts appear to be indicative of functional nicotinic receptors because the application of nicotine induced a sustained increase in intracellular Ca^{2+} in cultured HDMECs, as measured by increased fluorescence of the calcium indicator dye, Fluo-3. There are several possible mechanisms that might underlie this increase in $[Ca^{2+}]_i$. It is possible that intracellular Ca^{2+} could increase via Ca^{2+} influx through the nAChRs themselves because nAChRs are mixed cation channels with moderate Ca^{2+} permeability (54). The nicotine-induced Ca^{2+} increases were eliminated using the nAChR antagonist MLA at receptor subtype non-specific concentrations- indicating that the Ca^{2+} increases were mediated by nicotinics. MLA, when applied alone, resulted in fluorescence that was not different from control levels at early time points but significantly lower than control levels at later time points. The application of MLA by itself was an important control since ACh has been reported to function as an autocrine hormone, which activates

receptors on the same cell from which it was released (Proskocil et al, 2004). The decrease in fluorescence after complete nAChR inhibition provides evidence for the actions of endogenous ACh. Additionally, the application of the α7 specific antagonist αBGT also resulted in a significant fluorescence decrease as compared to nicotine alone, indicating that much of the nicotine-induced change in $[Ca^{2+}]_i$ is mediated by α7 nAChRs. The remaining portion of the $[Ca^{2+}]_i$ increase that was MLA-sensitive but αBGT-insensitive is likely attributable to other nAChR subtypes. Additional evidence for the involvement of non- α7 nAChRs includes the ability of atropine to partially inhibit the effects of nicotine because atropine has been reported to block α3-containing nAChRs (42).

Alternatively, an increase in intracellular Ca^{2+} could rely on other mechanisms such as store-operated Ca^{2+} (SOC) channels. Two types of highly Ca^{2+} selective ligand-binding SOC channels have been characterized in intracellular membranes of endothelial cells. One type is activated by ATP and bradykinin (32) or $Ins(1,4,5)P_3$ (39). Results from our studies using antagonists for ryanodine and $Ins(1,4,5)P_3$ receptors, Ruthenium Red (RR) and Xestospongin C (XeC), respectively, to block the release of Ca^{2+} from intracellular stores did not indicate a significant role for intracellular Ca^{2+} store release by $Ins(1,4,5)P_3$, because when RR and XeC were applied with nicotine, the fluorescence was not different than nicotine alone.

Another possible mechanism for Ca^{2+} influx into the cells is via transient receptor potential calcium (TRPC) channels, which rely on Ca^{2+} dependent calmodulin-binding or intracellular changes in osmolarity for activation (39). It is unlikely that TRPC channels are responsible for the initial influx of extracellular Ca^{2+} as the initiation of Ca^{2+} influx

has been demonstrated to be due to nAChR activation, but TRPC channels could play a role in store-operated Ca^{2+} release in response to the nicotine-induced increase in $[Ca^{2+}]$.

Other possible mechanisms for Ca^{2+} increase in vascular endothelial cells include membrane depolarization resulting from activation of nAChRs that in turn activate voltage gated Ca^{2+} channels (VGCC) allowing Ca^{2+} influx. There are reports of VGCCs, similar to L- and T-type Ca^{2+} channels, in bovine capillary endothelial cells (4, 5). In addition, a new type of VGCC has been proposed to be important to Ca^{2+} flux in endothelial cells. The R-type Ca^{2+} channel is activated by long duration depolarizations and has been proposed to be responsible for a sustained Ca^{2+} influx (Bkaily et al., 1993).

Ca^{2+} is a potent second messenger and has been shown to activate intracellular signaling pathways. The MAPK family has been shown to activate regulatory proteins in the cytoplasm and thereby signaling the nucleus to initiate processes such as proliferation and differentiation (Seger & Krebs, 1995). To test whether the MAPK pathway was activated by nicotine-induced changes in intracellular $[Ca^{2+}]$, measured MAPK family member phosphorylation levels. Nicotine alone significantly increased phosphorylation of ERK1/2 after 5 minutes of exposure. In contrast, 60 minutes of nicotine exposure resulted in a decline of ERK1/2 phosphorylation similar to baseline levels. Nicotine-induced phosphorylation was blocked by the addition of MLA. The α7 specific antagonist, α-bungarotoxin, also blocked the nicotine-induced phosphorylation of ERK1/2 suggesting that α7 is the major nAChR subtype involved in the activation of the ERK signaling pathway.

Upon activation, the ERKs can phosphorylate a wide variety of regulatory proteins including nuclear transcription factors. Gene expression changes resulting from

ERK activation of transcription factors were determined using real-time RT-PCR. Because angiogenesis has been shown to involve a complex biochemical response to the interaction of different elements, inflammatory cytokines, adhesion molecules, and growth factors were measured.

Short-term treatment with nicotine resulted in significant decreases of many angiogenesis promoters including chemokines such as CXCL6 and CCL2, growth factor (HGF), and pro-angiogenic signaling molecules (EFNA3, THBS2, SPHK1). While the majority of short-term nicotine exposure effects were anti-angiogenic, there were several pro-angiogenic changes including up-regulation of TGFBR1 and PTSG1 and down-regulation of ANGPTL4.

The application of nAChR antagonists MLA and αBGT did not inhibit gene expression changes with 60 minute incubation. There was consistent down-regulation of STAB1, JAG1, ITGB3, and up-regulation of CCL2 and MMP-9. We suggest that these gene expression changes following use of nAChR antagonists are remnants of endogenous ACh signaling after 1 hour inhibition; the changes are absent after 72 hours of inhibition.

After chronic, 72 hour, nicotine exposure, we observed a significant increase of inflammatory chemokines, growth factors, and pro-angiogenic adhesion molecules: CCL2, CCL11, CXCL3, IL1-B, IL-6, PGF, PECAM1, ENG, and EREG. A pro-angiogenic decrease of TIMP1 was also observed. A modest anti-angiogenic response was observed and included decreased PF4, SERPINF1 and increased FLT1.

We observed a different expression pattern of genes associated with angiogenesis after short and long term nicotine exposure. Chronic nicotine exposure has been shown to

up-regulate α4 and α7-containing nAChR subtypes in neurons (Fenster et al., 1997; 1999; Buisson & Bertrand, 2001; 2002). Because different nAChR subtypes have different affinities to cholinergic agonist and also have different activation kinetics, we hypothesized that changes in nAChR expression would result in changes in intracellular signaling and downstream gene transcription. Real-time RT-PCR analyses of nAChR subunits show upregulation of both α7 and α9 nAChRs for both short- and long-term durations to nicotine exposure. In addition, both nicotine exposure durations resulted in decreases of nAChR subunits α10 and β2. Thus, nicotine exposure leads to up regulation of nAChRs as predicted, the similar patterns in acute and chronic conditions suggest regulation of nAChRs by nicotine exposure is not correlated with nicotine-induced changes in expression levels of pro- and anti-angiogenic genes.

These results suggest nAChRs expressed on endothelial cells in human vasculature are activated by nicotine and influence expression of genes important to vascular growth. Nicotine-induced changes in gene expression can be implicated as an important component in the initiation and progression of many tobacco-related ocular diseases such as ARMD, DR, and GCA. The role of nAChRs in the progressions of these diseases provides the possibility of anti-nicotinic agents to prevent vascular overgrowth. We have shown that extended durations of nicotine exposure increases pro-angiogenic gene expression and extended exposure to nicotinic antagonists reverses the pro-angiogenic shift. While anti-muscarinic and anti-nicotinic, agents are currently administered for ailments ranging from urinary incontinence to Parkinson's disease and cerebral palsy, to our knowledge, the effect on vascular diseases of the eye has not been tested. While systemic administration might not be favored due to the high risk of side

effects, it would be interesting to measure the effect of intraocular or subconjunctival administration of an anti-nicotinic agent for reducing the severity of choroidal overgrowth found in vascular eye diseases.

GENERAL LIST OF REFERENCES

1. Armstrong RA. Is smoking a risk factor for AMD? *Ophthalmology Times* 42-45, 2003.
2. Arredondo J, Nguyen VT, Chernyavsky AI, Jolkovsky DL, Pinkerton KE, Grando SA. A receptor-mediated mechanism of nicotine toxicity in oral keratinocytes. *Laboratory Investigation* 81: 1653-1668, 2001.
3. Bkaily G, d'Orléans-Juste P, Naik R, Pérodin J, Stankova J, Abdulnour E, Rola-Pleszczynski M. PAF activation of a voltage-gated R-type Ca2+ channel in human and canine aortic endothelial cells. *Br J Pharmacol* 110: 519-520, 1993.
4. Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J. Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor a-subunit. *Nature* 319: 368-374, 1987.
5. Buisson B, Bertrand D. Nicotine addiction: the possible role of functional upregulation. *Trends in Pharmacological Sciences* 23: 130-136, 2002.
6. Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, Bertrand D. Human alpha4beta2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J. Neuroscience* 16: 7880-7891, 1996.
7. Christen WG, Glynn RJ, Manson JE, Ajani UA, Buring JE. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 276: 1147-51, 1996.

8. Conklin BS, Zhao W, Zhong DS, Chen C. Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells. *Amer J Path* 160: 413-418, 2002.
9. Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron* 5: 847-856, 1990.
10. Couturier S, Erkman L, Valera S, Rungger D, Bertrand S, Boulter J, Ballivet M, Bertrand D. Alpha 5, alpha 3, and non-alpha 3. Three clustered avian genes encoding neuronal nicotinic acetylcholine receptor-related subunits. *J Biol Chem* 265: 17560-17567, 1990.
11. Deneris E, Connolly J, Boulter J, Wada K, Swanson L, Patrick J, Heinemann S. Primary structure and expression of beta 2: a novel subunit of neuronal nicotinic acetylcholine receptors. *Neuron* 1: 45-54, 1988.
12. Duhaut P, Pinede L, Demolombe-Rague S, Loire R, Seydoux D, Ninet J, Pasquier J, The Groupe de Recherche sur L'artérite à Cellules GéAntes. Giant cell arteritis and cardiovascular risk factors: A multicenter, prospective case-control study. Arthritis and Rheumatism 41: 1960-1965, 1998.
13. Fenster CP, Rains MF, Noerager B, Quick MW, Lester RA. Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. *Journal of Neuroscience* 17: 5747-5759, 1997.

14. Fenster CP, Whitworth TL, Sheffield EB, Quick MW, Lester RA. Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. *Journal of Neuroscience* 4804-4814, 1999.
15. Goldman D, Deneris E, Luyten W, Kochhar A, Patrick J, Heinemann S. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* 48: 965-973, 1987.
16. Hagforsen E, Edvinsson M, Nordlind K, Michaelsson G. Expression of nicotinic receptors in the skin of patients with palmoplantar pustulosis. *British Journal of Dermatology* 146: 383-391, 2002.
17. Hayreh SS, Podhajsky PA, Zimmerman B. Occult giant cell arteritis: ocular manifestations. Am J Ophthalmol 125: 521-6, 1998.
18. Heeschen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, Tsao PS, Johnson FL, Cooke JP. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nature Medicine* 7: 833-839, 2001.
19. Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest* 110: 527-536, 2002.
20. Heeschen C, Weis M, Cooke JP. Nicotine promotes arteriogenesis. *J Amer Col Cardiology* 41: 489-496, 2003.
21. Jacobi J, Jang JJ, Sundram U, Dayoub H, Fajardo LF, Cooke JP. Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. *American Journal of Pathology* 161: 97-104, 2002.

22. Kawashima K, Watanabe N, Oohata H, Fujimoto K, Suzuki T, Ishizaki Y, Morita I, Murota S. Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett 1990 Nov 13;119(2):156-8.

23. Kawashima K, Oohata H, Fujimoto K, Suzuki T. Extraneuronal localization of acetylcholine and its release upon nicotinic stimulation in rabbits. Neurosci Lett. 1989 Oct 9;104(3):336-9.

24. Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis* 21: 505-515, 2000.

25. Klein R, Klein BEK, Moss SE, Cruickshanks KJ. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in Type 1 diabetes. *Ophthalmology* 105: 1801–15, 1998.

26. Klein R, Klein BEK, Knudtson MD, Wong TY, Tsai MY. Are Inflammatory Factors Related to Retinal Vessel Caliber?: The Beaver Dam Eye Study. *Arch Ophthalmol* 124: 87-94, 2006.

27. Klein R, Klein BEK, Moss SE, Wong TY, Sharrett AR. Retinal Vascular Caliber in Persons with Type 2 Diabetes: The Wisconsin Epidemiological Study of Diabetic Retinopathy: XX. *Ophthalmology* 113: 1488-1498, 2006.

28. Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, Wells G. Structure and function of neuronal nicotinic acetylcholine receptors. [Review]. *Prog Brain Res* 109: 125-137, 1996.

29. Lindstrom J. The structures of neuronal nicotinic receptors. In Handbook of Experimental Pharmacology: Clementi F, Fornasari D, Gotti C. eds. (Berlin Heidelburg: Springer-Verlag) 144: 101-162, 2000.

30. Lu L, Reinach PS, Kao WW. Corneal epithelial wound healing. [Review] *Experimental Biology & Medicine* 226: 653-664, 2001.

31. Machado EBV, Gabriel SE, Beard CM, Michet CJ, O'Fallon WM, Ballard DJ. A Population-Based Case-Control Study of Temporal Arteritis: Evidence for an Association between Temporal Arteritis and Degenerative Vascular Disease? *Int.J.Epidemiol.* 18: 836-841, 1989.

32. Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. *Journal of Pharmacology & Experimental Therapeutics* 287: 435-439, 1998.

33. Moss SE, Klein R, Klein BE. Cigarette smoking and ten-year progression of diabetic retinopathy. *Ophthalmology* 103: 1438-42, 1996.

34. Nef P, Oneyser C, Alliod C, Couturier S, Ballivet M. Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. *EMBO J* 7: 595-601, 1988.

35. Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER. Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. *Endocrinology* 145: 2498-2506, 2004.

36. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA* 276: 1141-6, 1996.

37. Seger R and Krebs EG. The MAPK signaling cascade. *FASEB J* 9: 726-735, 1995.

38. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Applied Physiol* 84: 2089-2098, 1998.

39. Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* 284: 314-335, 1989.

40. Wang Y, Pereira EFR, Maus ADJ, Ostlie NS, Navaneetham D, Lei S, Albuquerque EX, Conti-Fine BM. Human Bronchial Epithelial and Endothelial Cells Express alpha 7 Nicotinic Acetylcholine Receptors. *Mol Pharmacol* 60: 1201-1209, 2001.

41. Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. [Review] *Pharmacology & Therapeutics* 77: 59-79, 1998.

42. Zia S, Ndoye A, Lee TX, Webber RJ, Grando SA. Receptor-mediated inhibition of keratinocyte migration by nicotine involves modulations of Ca^{2+} influx and intracellular concentration. *J Pharm Exper Ther* 293: 973-981, 2000.