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Nicotine and Pathological Angiogenesis

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Abstract

Aims—This paper describes the role of endothelial nicotinic acetylcholine receptors (nAChR) in diseases where pathological angiogenesis plays a role.

Main methods—An extensive review of the literature was performed, focusing on studies that investigated the effect of nicotine upon angiogenesis.

Key findings—Nicotine induces pathological angiogenesis at clinically relevant concentrations (i.e. at tissue and plasma concentrations similar to those of a light to moderate smoker). Nicotine promotes endothelial cell migration, proliferation, survival, tube formation and nitric oxide (NO) production *in vitro*, mimicking the effect of other angiogenic growth factors. These *in vitro* findings indicate that there may be an angiogenic component to the pathophysiology of major tobacco related diseases such as carcinoma, atherosclerosis, and age-related macular degeneration. Indeed, nicotine stimulates pathological angiogenesis in pre-clinical models of these disorders. Subsequently, it has been demonstrated that nicotine stimulates nAChRs on the endothelium to induce angiogenic processes; that these nAChRs are largely of the α7 homomeric type; and that there are synergistic interactions between the nAChRs and angiogenic growth factor receptors at the phosphoproteomic and genomic levels.

Significance—These findings are of potential clinical relevance, and provide mechanistic insights into tobacco-related disease. Furthermore, these findings may lead to novel therapies for diseases characterized by insufficient or inappropriate angiogenesis.

Keywords

Nicotine; atherosclerosis; angiogenesis; nAChR (nicotinic acetylcholine receptor); choroidal neovascularization

Introduction

Tobacco smoke contains over 7000 different chemicals, some of which are known to be toxic or mutagenic (Fowles and Dybing, 2003). These include the polycyclic aromatic hydrocarbons (PAH), N-nitrosamines, 1,2 butadiene, cyanide, arsenic, cresols, oxidizing agents and particulate matter (Fowles and Dybing, 2003, US Department of Health and human services. Public health Service, 2010). Nicotine is the habituating agent in tobacco. Recently, it has been discovered that nicotine has angiogenic properties (Heeschen et al., 2001), at clinically relevant concentrations (i.e. at tissue and plasma concentrations similar to those of a light to moderate smoker). Nicotine promotes endothelial cell migration,

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proliferation, survival, tube formation and nitric oxide (NO) production *in vitro*, mimicking the effect of other angiogenic growth factors. These *in vitro* findings suggest that there may be an angiogenic component to the pathophysiology of major tobacco related diseases such as carcinoma, atherosclerosis, and age-related macular degeneration. Indeed, nicotine stimulates pathological angiogenesis in pre-clinical models of these disorders (*see following sections*). These findings are of potential clinical relevance, and justify further characterization of the mechanisms underlying the angiogenic effect of nicotine.

Role of nicotine in atherosclerosis

Atherosclerosis is the major cause of heart attack, stroke and peripheral arterial disease. Cigarette smoke promotes pathophysiological processes that contribute to atherosclerosis, including thrombosis, insulin resistance and dyslipidemia, vascular inflammation, and endothelial dysregulation (Benowitz, 2003). In addition, the nicotine in tobacco smoke stimulates the release of catecholamines, and thereby increases heart rate and systemic resistance, raising the blood pressure. Furthermore, nicotine-induced catecholamine release increases platelet aggregability. Platelets contribute to the growth of plaque through the accretion of thrombus, as well as through the release of growth factors (such as platelet derived growth factor; PDGF) that induce vascular smooth muscle cell proliferation.

In addition, nicotine acts directly on cellular elements participating in plaque formation. Nicotine induces the proliferation of vascular smooth muscle cells, in part by stimulating their release of fibroblast growth factor (Carty et al., 1996). This is significant because proliferating vascular smooth muscle cells contribute to the growth of the lesion. Under the influence of PDGF they migrate into the intima and undergo phenotypic modulation into myofibroblasts and osteoblast-like cells. There they elaborate extracellular matrix (collagen and osteopontin), and even take up lipid to resemble macrophage-derived foam cells. Zhang and colleagues (Zhang et al., 2007) recently found that a “muscle-type” nAChR may play a direct role in regulating vascular smooth muscle cell proliferation and migration in the apo E deficient mouse model of atherosclerosis. In this study, silencing of the α1 subunit was associated with an 80% reduction in myofibroblasts in the lesion, a reduction in the accumulation of extracellular matrix, an attenuation of calcification, and a reduction in immune cells in the aortic wall.

Notably, nicotine enhances endothelial cell proliferation, migration and tube formation in concentrations that are clinically relevant (Heeschen, 2001). This effect of nicotine may also play a role in the pathophysiology of atherosclerosis.

Indeed, previous workers had proposed a role of neovascularization in the progression of atherosclerotic plaque. In 1984, Barger and colleagues published their observation that the vasa vasorum was expanded in the immediate vicinity of atherosclerotic plaque in human coronary arteries (Barger et al., 1984). Their findings were supported by microscopic computerized tomographic images of the coronary arteries from hypercholesterolemic swine, which confirmed the association of neovascularization with atherosclerotic plaque (Kwon et al., 1998). Subsequently, Judah Folkman’s group showed that endostatin and other anti-angiogenic agents could block the progression of plaque growth in apolipoprotein (ApoE)-deficient hypercholesterolemic mice (Moulton et al., 1999). Furthermore, inhibition of plaque angiogenesis reduced macrophage accumulation in the atheroma. Conversely, administration of the angiogenic cytokine vascular endothelial growth factor (VEGF) promoted plaque neovascularization and growth in the cholesterol-fed rabbit model (Celletti et al., 2001). Accordingly, we assessed the effect of nicotine on plaque neovascularization and growth in the ApoE deficient mouse model of atherosclerosis. After 20 weeks of a high cholesterol diet, animals were given nicotine or vehicle in their drinking water, in a

concentration documented to increase cotinine levels in the plasma similar to those of moderate smokers. We found that nicotine increased plaque progression and neovascularization (Heeschen, 2001). This effect of nicotine was independent of plasma lipid values and was blocked by the anti-angiogenic agent rofecoxib.

Thus, nicotine itself can promote atherosclerotic cardiovascular disease, in part by promoting plaque neovascularization. A role for nicotine in the pathophysiology of atherosclerosis is supported by recent genomic evidence, which indicates that a sequence variant in the cluster of genes on chromosome 15 that encode nicotinic acetylcholine receptors is associated with increased risk of peripheral arterial disease (Thorgeirsson et al., 2008). It is possible that this genetic variation affects the vascular response to nicotine, and/or nicotine dependency.

Role of nAChR in tumor angiogenesis

Smoking is a major preventable risk factor for malignancy. Nicotine has been observed to accelerate the pathological angiogenesis necessary for tumor growth and metastasis (Heeschen, 2001). *In vitro*, nicotine does not stimulate proliferation of Lewis lung cancer cells, which appear to lack functional nAChRs. By contrast, when these cells are injected into mice, we observed that systemic administration of nicotine accelerated tumor growth (Heeschen, 2001). The effect of nicotine to enhance tumor growth was associated with a 5-fold increase in capillary density in the tumor nodules. These findings suggested an effect of nicotine to promote tumor angiogenesis, rather than a direct effect on tumor cell proliferation, in the Lewis lung cancer model. In other tumor types, such as non-small cell lung cancer, activation of α7-nAChRs on the tumor cells may also directly enhance their survival and proliferation (Grozio et al., 2008).

The effect of nicotine to increase tumor growth and vascularity is mimicked by second hand smoke (SHS). In the Lewis lung cancer model, exposure to SHS increased tumor angiogenesis and tumor growth, an effect that was associated with elevated plasma VEGF (Zhu et al., 2003). Notably, these effects of SHS were abrogated by systemic administration of mecamylamine. Thus, the effect of SHS to promote tumor growth may be due in part to an angiogenic effect of nAChR activation. In this regard, we also found that exposure to SHS increased the numbers of circulating endothelial progenitor cells. This observation is consistent with later work showing that nicotine increases the recruitment of endothelial progenitor cells to ischemic sites in mice (Heeschen et al., 2006). In a separate study of human endothelial progenitor cells, incubation with 10 nM nicotine enhanced the viability, migratory and adhesive behavior, as well as the in vitro vasculogenesis capacity, of these cells (Yu et al., 2011). The effect of nicotine on human endothelial progenitor cells was attenuated by mecamylamine or α-bungarotoxin (Yu, 2011). To summarize, the mutagenic and cytotoxic properties of tobacco smoke, combined with the angiogenic and vasculogenic effects of nicotine, provide for a lethally effective synergy in the initiation and progression of malignancy.

Role of nAChRs in choroidal neovascularization (CNV)

Less widely known is the fact that tobacco is the primary preventible risk factor for age-related macular degeneration (AMD), the major cause of blindness in the developed world. In its most severe form, AMD is associated with a choroidal neovascularization (CNV) that can lead to retinal edema, hemorhage and fibrosis, with loss of central vision. Because CNV is responsible for the blinding effects of AMD, we determined if nicotine would promote CNV. We tested the hypothesis in a murine model of AMD (Kiuchi et al., 2008). In brief, mice were given nicotine (100 μg/mL) or vehicle (2% saccharine) for 2 weeks orally (in the drinking water ad libitum). This amount of nicotine administration achieves serum cotinine

levels similar to those observed in moderate smokers (Heeschen et al, 2001). Some animals received mecamylamine or vehicle by an osmotic Alzet minipump. Mice underwent laser-induced rupture of Bruch's membrane (which initiates CNV), and 14 days later mice were perfused with fluorescein-labeled dextran to image and quantitate the lesion area. We found that CNV lesions were larger in mice that received nicotine, an effect which was blocked by co-administration of mecamylamine. In parallel studies, human choroidal and retinal arterial endothelial cells were shown to express nAChRs, and that nicotine stimulated proliferation, migration and tube formation in these cells.

These studies support the notion that endothelial nAChRs may promote choroidal neovascularization. Retinal edema also contributes to the pathobiology of AMD. In preclinical studies, nicotine increases cerebrovascular permeability, an effect that can be blocked by the nAChR antagonist hexamethonium (Suner et al., 2004). Thus, the association of tobacco smoke with AMD may be due to the effect of nicotine to promote abnormal permeability as well as growth of choroidal vessels.

The suppression of CNV by mecamylamine suggests that it could potentially be used as a therapeutic agent in AMD and other ocular vasoproliferative diseases. To avoid the known systemic side effects of mecamylamine, we developed a topical formulation. In mice, rabbits and primates, we found that topical mecamylamine penetrated into the cornea and sclera at high levels, and migrated through the sclera to the posterior eye. Subsequently we demonstrated that daily topical application of mecamylamine (0.1 and 1%) for two weeks could suppress CNV in the murine model.

Notably, the effects of mecamylamine to inhibit CNV also occurred in the absence of nicotine. In this regard, we have observed that mecamylamine can suppress other forms of neovascularization in the absence of nicotine, as in tumor angiogenesis, or with the fibrovascular growth in response to a foreign body (Heeschen et al., 2002). Endogenous activation of the endothelial nAChR invokes the existence of an autocrine or paracrine factor that interacts with endothelial nAChRs to alter their activity. The most likely candidate for this endogenous factor is acetylcholine, released by endothelial cells, as discussed immediately below.

Mechanisms by which nicotine induces pathological angiogenesis

The endothelial cholinergic pathway

Notably, much of the cholinergic machinery for synthesis and metabolism of acetylcholine (Ach) exists in endothelial cells (Figure 1). Endothelial choline acetyltransferase (ChAT) synthesizes ACh from acetyl coenzyme A and choline (Nachmansohn and Machado, 1943). Re-uptake of choline is essential for cellular ACh synthesis (Haga and Noda, 1973) and a high affinity choline transporter has been demonstrated in endothelial and vascular smooth muscle cells (Lips et al., 2003). Vascular cells also express acetylcholinesterase (AChE) and butyrylcholinesterases (BChE). This hydrolyzing activity spatially restricts ACh to local paracrine or autocrine effects. Of note, the homomeric α7-nAChRs are also activated by choline. Thus, even after ACh has been cleaved by the esterase, some signaling may persist (Papke et al., 1996).

Nevertheless, one might expect that antagonists of the cholinesterases might enhance cholinergic signaling, and promote nAChR-mediated angiogenic processes. Indeed, neostigmine increases endothelial cell migration (Cooke, 2007). Furthermore, the acetylcholinesterase inhibitor Donepezil, promotes angiogenesis in the murine hindlimb ischemia model (Kakinuma et al., 2010).

In addition to the machinery for synthesis and disposition of ACh, the endothelium contains receptors to respond to this endogenous transmitter. There are two major types of cholinergic receptors, the muscarinic and the nicotinic. Whereas ACh stimulates both receptor types, nicotine preferentially stimulates the nicotinic receptor. The muscarinic receptors are 7-transmembrane spanning G protein–coupled receptors. By contrast, the nicotinic ACh receptors (nAChRs) are each composed of 5 subunits, forming a barrel-like channel in the cell membrane. There are 16 isoforms of the subunits (α1-α10, β1- β4, δ, γ and ε), which may assemble into homomers or heteromers. Each subunit contains four transmembrane domains (M1–M4). The M2 transmembrane domain of each subunit contributes to the channel of the receptor. Although nAChRs were first described in excitable cells, they have now been identified in many cell types (Arredondo et al., 2007a), including endothelial cells (Heeschen, 2001, Heeschen, 2002, Villablanca, 1998), vascular smooth muscle cells (Macklin et al., 1998); keratinocytes (Grando et al., 1995) and immune cells (Richman and Arnason, 1979). Activation of the nAChRs by endogenous ACh, or exogenous nicotine, increases permeability of these ligand-gated channels to cations. To summarize, endothelial cells express each of the key elements for nicotinic cholinergic signaling, including the receptors and the enzymatic machinery for synthesis and metabolism of the endogenous ligand ACh. This endogenous cholinergic pathway is active in pathological angiogenesis, and can be further stimulated by nicotine.

An endothelial nAChR mediates the angiogenic effects of nicotine

Atropine is a typical antagonist of muscarinic receptors, whereas mecamylamine is an established antagonist of the nAChR. We found that mecamylamine, but not atropine, blocked the angiogenic effects of nicotine in vitro (Heeschen, 2002), findings that were later validated *in vivo* as discussed above. The anti-angiogenic effect of mecamylamine was mimicked by other nAChR antagonists, including hexamethonium and α-bungarotoxin. These studies indicated that endothelial nAChRs mediated the angiogenic effects of nicotine. [That being said, it is very possible that *endogenous acetylcholine* may act on both *nicotinic* and *muscarinic* receptors to induce angiogenesis. M 1 - and M 3 - mAChR are found in most vessels, and muscarinic stimulation of the endothelium is known to release the angiogenic factor, nitric oxide; Kurzen et al, 2007]

In the endothelial cell, the dominant isoform is the α7 homomer, and it is this homomeric nAChR that is believed to predominate as the mediator of cholinergic angiogenesis (Heeschen, 2002), although other nAChR types may modulate cholinergic angiogenesis as discussed below. Notably, the α7 homomer is upregulated in endothelial cells exposed to hypoxia *in vitro*. Similarly, the expression of the α7 homomer is increased in the ischemic hindlimb of the mouse (Heeschen, 2002). The α7-nAChR antagonist α-bungarotoxin suppresses the effect of nicotine to increase EC migration, proliferation and tube formation *in vitro*. Consistent with the notion that cholinergic angiogenesis is primarily mediated by the α7-nAChR, in mice deficient in this subtype the angiogenic effect of nicotine is blunted (Heeschen, 2002).

The dominant role of the α7-nAChR in angiogenesis has been supported by recent pharmacological and molecular observations. The α7-nAChR antagonist MG624 attenuates the effect of nicotine to promote angiogenic processes in vitro (Brown et al., 2011). In addition, MG624 inhibits the effect of nicotine to promote angiogenesis in the chorioallantoic membrane, and in human small cell lung carcinoma implanted in nude mice. The effect of MG624 is mediated in part by its effect to suppress nicotine-induced release of FGF2, possibly by blocking the effect of nicotine to increase activity of the early growth response gene 1 (Brown, 2011). In a separate investigation, Dom and colleagues found that nicotine promotes tubulogenesis of human retinal endothelial cells, an effect that is

associated with increased expression of metalloproteinase. Both effects are inhibited by siRNA directed against the α7-nAChR (Dom et al., 2011).

Other nAChRs involved in angiogenesis

Although the studies described above clearly indicated a major role for the α7-nAChR in angiogenesis, these studies also indicated a residual angiogenic response to nicotine that was insensitive to pharmacological suppression or genetic knockdown of the α7-nAChR. Accordingly, we attempted to identify all of the nAChR subunits present in four human endothelial cell types (pulmonary artery, dermal microvascular, umbilical vein, and retinal endothelial cells). Using subunit-specific RT-PCR we detected endothelial mRNA for each of the known mammalian nAChR subunits with the exception of α_2, α_4, γ and δ in the four different types of endothelial cells (Wu et al., 2009). There were some differences in the levels of expression of various nAChR subunits between the cell types. To assess the significance of the expressed nAChR subunits, we performed RNAi screening.

These studies indicated that RNAi against α_7 markedly reduced basal and nicotine-induced EC proliferation. By contrast, RNAi against the other subunits either had no effect, or even enhanced basal or nicotine-induced EC proliferation. Notably, RNAi directed against α_9, (the other subunit known to form calcium-selective homomeric channels), appeared to enhance nicotine induced EC proliferation. In subsequent studies, comparing the effect of knocking down either of these receptors, α7-nAChR had a dominant role in nicotine-induced cell signaling (assessed by intracellular calcium and NO imaging, and studies of protein expression and phosphorylation), as well as nicotine-activated EC functions (proliferation, survival, migration, and tube formation). The endothelial α9-nAChR appears to oppose this signaling pathway, as well as nicotine-induced cell proliferation and survival (Wu, 2009).

The α7-nAChR differs from the heteromeric nAChRs in that it is preferentially permeable to calcium, rather than sodium. Stimulation of endothelial cells by nicotine causes a large but delayed (20s) increase in intracellular calcium (Wu, 2009). This nicotine-induced increase in intracellular calcium is nearly abolished by RNAi against the α7-nAChR. Little is known about the downstream signaling of EC α7-nAChRs and the importance of calcium permeability in nAChR-induced angiogenesis. However, calcium fluxes seem to play a role in the action of other angiogenic factors such as FGF (Kohn et al., 1995). Our calcium imaging studies show that nicotine causes a sustained increase in intracellular calcium, with a time course of about 60 seconds. Our data suggests that this response is initiated by activation of the α7-nAChR. Whereas the initial calcium entry may be due to opening of the α7-nAChR, the sustained increase in cytosolic calcium must be from the subsequent opening of a secondary calcium channel, and/or release from intracellular stores. This conclusion is based on electrophysiological studies of the α7-nAChR in neurons, which show that the receptor is quickly desensitized after its activation, with an open state of only 0.23 ms at single channel conductance (Shao and Yakel, 2000). Accordingly, we hypothesize that the short burst of calcium entry through the α7-nAChR triggers the opening of a receptor-and/or store operated calcium channel, which sustains the increase in intracellular calcium (Figure 2). We propose that the most likely candidate for this secondary calcium channel is one in the class of canonical Transient Receptor Potential Channels (TRPC), which are known to be expressed in endothelial cells. These channels participate in a diverse range of vascular functions, including control of vascular tone, regulation of vascular permeability, secretion, EC proliferation, and EC apoptosis and death, and response to growth factors (Firth et al., 2007, Nilius et al., 2003).

Interaction of the nAChR with other receptors mediating angiogenesis

Activation of the endothelial nAChR reinforces other angiogenic pathways. For example, endothelial cells cultured in matrigel form tubes. This effect is mediated by the multiple growth factors present in matrigel including VEGF and FGF. Notably, in the absence of exogenous nicotine, endothelial tube formation is partially dependent upon nAChR activity. Specifically, nAChR antagonists such as mecamylamine, hexamethonium or bungarotoxin attenuate endothelial tube formation in Matrigel (Heeschen, 2002).

Other angiogenic processes stimulated by VEGF or FGF are also suppressed by antagonists of the endothelial nAChR. For example, the effect of VEGF or FGF to induce endothelial cell proliferation and migration *in vitro* is inhibited by nAChR antagonists. These effects are not reproduced by muscarinic antagonists. These findings suggest that the growth factors exert their effects in part by activating a cholinergic pathway mediated by endothelial nAChRs. This concept of interdependence between the angiogenic signaling pathways mediated by the nAChR and other angiogenic cytokines is supported by microarray studies, which demonstrate that nicotine, VEGF and FGF each induce transcriptional changes that are highly concordant (Ng et al., 2007). Within the transcriptional profiles induced by nicotine, VEGF or bFGF, we identified 6 clusters with concordant gene expression (3 clusters of commonly activated and 3 commonly co-repressed genes). These results suggest positive interactions between angiogenic growth factors and cholinergic signaling.

Mechanisms underlying interaction between pathways activated by nAChR or growth factors

The interactions between the nAChR- and growth factor-mediated pathways occur at the levels of signaling and transcription. When porcine coronary arteries are perfused with a solution containing nanomolar concentrations of nicotine, endothelial expression of VEGF is increased (Macklin, 1998). Furthermore, the activation state of the endothelial VEGF receptor KDR is increased by nicotine (Conklin et al., 2002). Second hand smoke increases plasma levels of VEGF, an effect which is reversed by the nAChR antagonist mecamylamine. Nicotine also stimulates endothelial cells to release FGF (Carty, 1996), as well as endothelin (Lee and Wright, 1999); under certain conditions; endothelin is a pro-angiogenic factor (Salani et al., 2000). In addition, nanomolar concentrations of nicotine release prostacyclin and NO from the endothelium (Boutherin-Falson and Blaes, 1990, Heeschen, 2001), which are known to be small molecule mediators of VEGF- and FGF-induced angiogenesis. It is also possible that growth factor induced release of acetylcholine might play a role in the interaction of the growth factors and nAChR activation with respect to angiogenesis, but this remains undetermined.

The interaction of the signaling pathways mediated by endogenous activation of cholinergic and growth factor receptors have been validated in vivo. For example, in the absence of exogenous nicotine, mecamylamine (but not atropine) reduces fibrovascular growth into polyvinyl discs implanted subcutaneously in mice. Furthermore, in the α7-nAChR knockout mouse, this fibrovascular growth is reduced. In the absence of nicotine, mecamylamine inhibits tumor vascularity and growth in animals implanted with Lewis lung cancer cells (Heeschen, 2002). Furthermore, mecamylamine reduces plasma VEGF levels in tumor-bearing animals (Zhu, 2003). These data are clinically relevant, as antagonists of VEGF action have been used to treat cancer and pathological neovascularization of the eye (de Gramont A and Van Cutsem, 2005, Gragoudas et al., 2004). Therefore, agents that target the endothelial nAChRs could represent a novel class of drugs to treat diseases characterized by pathological angiogenesis.

Subtleties of nAChR-mediated angiogenesis

Although the focus of this review is on pathological angiogenesis mediated by nicotine, as one might predict, nicotine also acutely enhances physiological angiogenesis, as observed in wound healing or limb ischemia (Heeschen, 2006, Heeschen, 2001, Kiuchi, 2008). In the murine model of wound healing, topical application of the nAChR activator epibatidine promoted wound angiogenesis, and accelerated wound healing. The effect of nicotine or epibatidine on wound angiogenesis and healing was similar to that of topical application of FGF (Jacobi et al., 2002). These observations seem counter-intuitive to those of us working in regenerative medicine, where smoking is known to be a risk factor for delayed wound healing. Of course, there are substantial differences between tobacco smoke and nicotine, as pathological stimuli. As previously discussed, tobacco smoke is estimated to contain over 7000 different chemicals and compounds, some of which are cytotoxic or mutagenic. However, it is also possible that the chronicity of exposure, and/or route of delivery, is an important factor in the angiogenic response to nicotine. Recently, it has been shown that chronic oral administration of nicotine impairs nAChR-mediated angiogenesis (Konishi et al., 2010). When mice were exposed to nicotine for 16 weeks, the effect of nicotine to augment the angiogenic response to limb ischemia was abolished. Impairment of cholinergic angiogenesis was associated with a reduction in vascular nAChR expression, plasma VEGF levels, and aortic ring sprouting in vitro. Similarly, when human umbilical vein endothelial cells are exposed to nicotine for prolonged periods of time (2 wks) impaired proangiogenic functions (decreased cell migration and tubular structure formation) are observed, although the antiapoptotic effect of nicotine is preserved (Park et al., 2011).

Unanswered questions and the future directions

Although evidence suggests that α7 homomeric nAChRs primarily regulate cholinergic angiogenesis, other nAChR subunits are expressed by endothelial cells. It is likely that these other EC subunits aggregate into functional receptors, but in what configurations is unknown (perhaps even with α7 subunits in a heteromeric configuration?). Furthermore, the role of other nAChR subtypes in reinforcing, modulating or opposing the action of the α7-nAChR needs further analysis. Of course, we desperately need better tools for these studies, such as more specific antibodies for each of the nAChR subunits to improve our immunohistochemistry, FACS and immunoprecipitation assays.

We know that acetylcholine is synthesized in endothelial cells (Arredondo et al., 2007b, Wessler et al., 1999), and the evidence that acetylcholine is an autocrine angiogenic factor in the vascular system has been presented above. However, there are other potential endogenous nAChR agonists include choline, and the peptides SLURP-1 and -2 (lymphocyte antigen 6/urokinase-type plasminogen activator receptor related protein-1 and -2) (Moriwaki et al., 2007). The SLURP peptides are allosteric modulators of the nAChRs. Whether these peptides are expressed in the vessel wall, and contribute to angiogenesis, is unknown.

Much more needs to be understood regarding the function and signaling of nAChRs on other cell types that participate in angiogenesis. For example, we know that nicotine can recruit endothelial progenitor cells from the bone marrow to incorporate into the vasculature of ischemic tissue (Heeschen, 2006). Is it possible that other hematopoietic cells that release angiogenic cytokines are recruited by nicotine (or endogenous acetylcholine)? Indeed, it is known that the α7 subunit is expressed on hematopoietic stem cells (HSCs), and that nicotine can increase the proliferation of HSCs and the generation of leukocytes (Chang et al., 2010).

Is there a role of the cholinergic nervous system in angiogenesis? It seems possible that nerve-released ACh might play a role in the development and/or maintenance of the microvasculature. For example, in diabetic patients, a profound neuropathy precedes skin ulcerations of the foot. Could the loss of nerve-released acetylcholine contribute to the impaired angiogenesis and wound healing in these patients? Other questions remain about the nature and function of nAChRs throughout the circulation. The nAChRs of venous, lymphatic, arterial or microvascular endothelium are possibly of different subtypes or proportions of subtypes, and may have similar or divergent functions. It is not known if these endothelial nAChRs are up- or down-regulated in various stages of development or disease. How important is cholinergic regulation in the pathological angiogenesis observed in different tumors, or in other forms of aberrant neovascularization (such as the retinopathy of prematurity or the inflammatory angiogenesis of rheumatoid arthritis)?

Finally, if nicotine might augment pathological angiogenesis, what ramifications does this have for nicotine replacement therapies (NRTs) for smokers? In October 2010, the FDA held a public workshop to discuss the risks and benefits of approving long-term use of NRT (i.e. beyond the current approved maximum period of 12 weeks). This workshop revealed that, whereas short-term use of NRTs has been shown to be safe and effective in tobacco cessation, there are little clinical data on the safety and efficacy of long-term use of NRTs. Until clinical data are available on long-term use of nicotine, the cautious physician may wish to substitute other approaches toward tobacco cessation if short-term use of medicinal nicotine fails.

To conclude, there is strong pre-clinical evidence supporting the existence of cholinergic angiogenesis, mediated at least in part by an endothelial nAChR. The significance of the nAChR in various forms of pathological neovascularization in human disease remains to be determined. This knowledge will be critical for the development of new anti-angiogenic therapies for malignancy, atherosclerosis, and retinal neovascularization, as well as other diseases where pathological angiogenesis plays a role.

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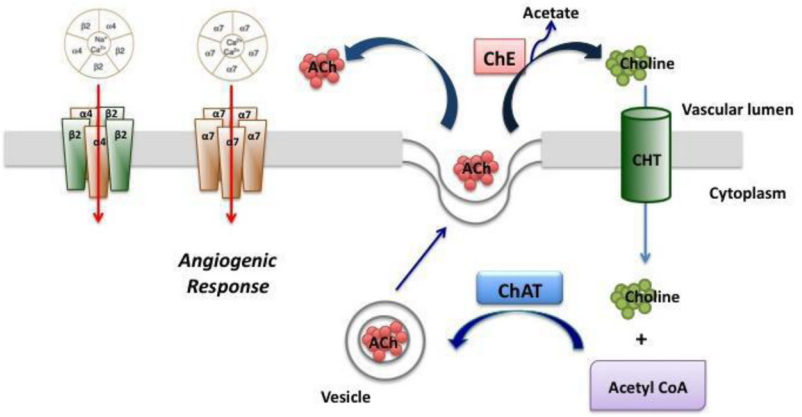

Figure 1. Endothelial cells contain components for nicotinic signaling

The nicotinic acetylcholine receptor (nAChR) consists of five subunits. The homomeric α7 nAChR consists of five α7 subunits. Representative of the heteromeric receptors is the α4β2 nAChR, composed of two α4 subunits and three β2 subunits. The endothelial cell (EC) contains most of the components for nicotinic signaling. EC α7 nAChRs are activated by the endogenous agonist acetylcholine (Ach) or by other nicotinic agonists. Within the EC, ACh is synthesized from acetyl coenzyme A (Acetyl-CoA) and choline by choline acetyltransferase (ChAT). An ACh molecule activates the nAChR in an autocrine or paracrine manner to induce an angiogenic response. Acetylcholinesterase (ChE) degrades ACh into acetate and choline, to spatially and temporally constrains cholinergic signaling. Choline is transported by the high-affinity transporter (CHT) to the intracellular compartment for a new cycle of ACh synthesis. It is not known if acetylcholine is stored in endothelial cells, as it is in neurons.

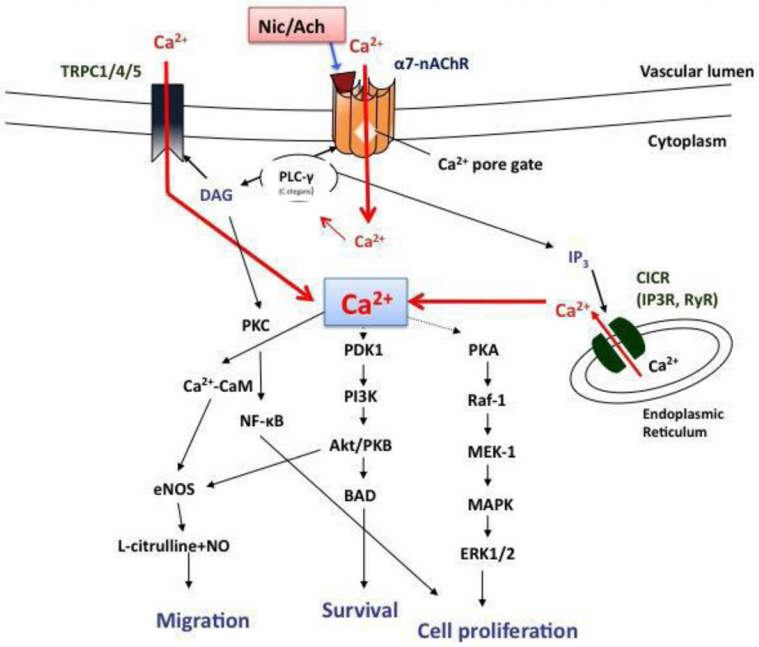

Figure 2. The EC angiogenic nAChR signaling pathway

Figure shows a schematic for mechanisms by which calcium entry may induce cell signaling based on the literature and our hypothesis. We hypothesize that when nicotine or ACh binds to the nAChR, it causes conformational changes. This conformational change induces calcium permeability and recruits phospholipase C (PLC-γ), which acts on its substrate to form inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C mediated signaling, whereas IP3 acts on its receptor on the endoplasmic reticulum (ER) to release Ca^{2+} from intracellular stores. This transient release of calcium may activate some kinase cascades, which induce cellular migration, survival and proliferation.