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Endothelial Nicotinic Acetylcholine Receptors and Angiogenesis

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Abstract

Nicotinic acetylcholine receptors (nAChRs) were first described in non-excitabile cells just over a decade ago. The nAChRs on endothelial cells (ECs) modulate key angiogenic processes, including EC survival, proliferation, and migration. The receptors may be stimulated by endogenous agonists such as acetylcholine, or exogenous chemicals such as nicotine, to activate physiological angiogenesis (such as in wound healing) or pathological angiogenesis (such as retinal neovascularization or tumor angiogenesis). The endothelial nAChRs may represent a target for therapeutic modulation of disorders characterized by insufficient or pathological angiogenesis.

The nicotinic acetylcholine receptor (nAChR)

Several years ago we were surprised to discover that nicotine is a potent angiogenic agent (Heeschen et al. 2001), at clinically relevant concentrations (i.e. at tissue and plasma concentrations similar to those generated by light to moderate smoking). We observed that nicotine enhanced tumor angiogenesis and tumor growth, which indicated a mechanistic link to tobacco-related disease. The effect of nicotine mimicked that of other angiogenic cytokines; in an *in vivo* model of fibrovascular growth the effect of nicotine was similar in magnitude to that of fibroblast growth factor. Furthermore, like other angiogenic cytokines, nicotine promoted endothelial cell (EC) migration, proliferation, survival, tube formation and nitric oxide (NO) production *in vitro*. These unexpected findings were of potential clinical relevance, and led us to characterize the mechanisms underlying the angiogenic effect of nicotine.

Cholinergic receptors are of two types, the nicotinic and the muscarinic, each of which is expressed by ECs. The muscarinic cholinergic receptors are seven transmembrane spanning G protein gated receptors. By contrast, the nicotinic acetylcholine receptors (nAChR)s are pentameric ligand-gated cationic channels. The classic antagonist of muscarinic receptors is atropine, whereas mecamylamine is an established antagonist of the nAChR. We found that mecamylamine, but not atropine, blocked the effects of nicotine on angiogenic processes *in vitro*, findings that were later confirmed *in vivo* (*vide infra*). The inhibitory effects of mecamylamine were mimicked by other nAChR antagonists, including hexamethonium, as well as α -bungarotoxin. These studies confirmed that EC nAChRs mediated the angiogenic effects of nicotine.

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The nAChRs are composed of five subunits, arranged in a barrel-like configuration in the cell membrane (Figure 1). The subunits exist as 16 isoforms ($\alpha 1$ – $\alpha 10$, $\beta 1$ – $\beta 4$, δ , γ and ϵ), which may be arranged in homomeric or heteromeric configurations. Each subunit contains four domains (M1–M4) in the cell membrane, with the M2 transmembrane domain of each subunit contributing to the channel of the receptor. First described in excitable cells, nAChRs have recently been identified in many cell types (Kumar et al. 2005, Arredondo et al. 2007), including endothelial cells (ECs) (Villablanca 1998, Heeschen et al. 2001, 2002), vascular smooth muscle cells (Macklin et al. 1998); keratinocytes (Grando et al. 1995) and immune cells (Richman and Arnason, 1979).

In neurons, the subunits that make up the nAChRs may consist of a combination of α subunits ($\alpha 1$ – $\alpha 10$) and β subunits ($\beta 1$ – $\beta 4$), whereas in the neuromuscular junction the nAChR is composed of $\alpha 1_{(2)}$, $\beta 1$, δ , and ϵ subunits (in the fetus, a γ subunit replaces the ϵ subunit) (Lindstrom et al. 1997). The combinatorial association of various α - and β - subunits result in functionally diverse nAChR subtypes that vary in ion permeability, open time, ligand affinity and other functions (Conti-Tronconi et al. 1994, Changeux et al. 1995). There is cellular diversity in expression, dominance and subunit composition of the nAChRs (Heeschen et al. 2001, Elgoyhen et al. 1994). For example, mature keratinocytes predominantly express the $\alpha 7$ subunit (Grando et al. 1995), whereas $\alpha 9$ is the main subunit in cochlear hair cells (Elgoyhen et al. 1994). For a discussion of other non-neuronal nAChRs and their functions, we refer the readers to several other reviews of this subject (Sharma and Vijayaraghavan, 2002, Gahring and Rogers 2006, Skok 2007). In the EC, the $\alpha 7$ homomeric nAChR is believed to predominate as the mediator of cholinergic angiogenesis, although other nAChR types also contribute.

An endothelial nAChR mediates the angiogenic effects of nicotine

We have demonstrated the angiogenic effect of nicotine in murine models of pathological angiogenesis such as cancer, atherosclerosis and age-related macular degeneration (AMD), discussed in more detail below. However, as befitting a potent angiogenic agent, nicotine also acutely enhances physiological angiogenesis, as observed in wound healing or limb ischemia (Heeschen et al. 2001, 2006, Kiuchi et al. 2008). In the murine model of wound healing, topical application of epibatidine (like nicotine, a nAChR agonist) 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 fibroblast growth factor (FGF) (Jacobi et al. 2002).

The predominant endothelial nAChR involved in angiogenesis appears to be the $\alpha 7$ homomer (Heeschen et al. 2001, 2002). The effect of nicotine to increase EC migration, proliferation and tube formation *in vitro* are largely antagonized by α -bungarotoxin, an antagonist of the $\alpha 7$ nAChR (Figure 2). Notably, the $\alpha 7$ homomer is upregulated in EC exposed to hypoxia *in vitro*. Similarly, in the ischemic hindlimb of the mouse, the expression of the $\alpha 7$ homomer is increased (Heeschen et al. 2002). In keeping with a dominant role of the $\alpha 7$ nAChR, in mice deficient in this subtype the angiogenic effect of nicotine in hindlimb ischemia is blunted (Heeschen et al. 2002).

Role of nAChR in tumor angiogenesis

Smoking is a major preventable risk factor for malignancy. Tobacco smoke contains about 4000 different chemicals, some of which are known to be directly mutagenic (Prefontaine et al. 2006). Our work suggests that nicotine may contribute to the risk of smoking by accelerating the pathological angiogenesis necessary for tumor growth and metastasis. We have used the murine lung cancer model to study the effects of nicotine. In this model, Lewis lung cancer cells are injected subcutaneously, and tumor nodules harvested 2–4 weeks later. *In vitro*, nicotine does not stimulate proliferation of the Lewis lung cancer cells, which appear to lack

functional nAChRs. By contrast, we observed that systemic administration of nicotine to mice injected with tumor cells accelerated tumor angiogenesis and tumor growth (Heeschen et al. 2001). These findings suggested an angiogenic effect of nicotine that enhanced tumor growth, rather than a direct effect on tumor proliferation. (In other tumor types, such as nonsmall cell lung cancer, $\alpha 7$ -nAChRs may be found on the tumor cells, and directly enhance tumor cell survival and proliferation) (Song et al. 2003, Grozio et al. 2008). Intriguingly, we observed much the same effect with second hand smoke (SHS). In the Lewis lung cancer model, clinically relevant levels of SHS increased tumor angiogenesis and tumor growth (Zhu et al. 2003). This effect was associated with elevated plasma vascular endothelial growth factor (VEGF) and increased numbers of circulating endothelial progenitor cells. Notably, these effects of SHS were abrogated by systemic administration of mecamylamine. These studies indicated that the pro-angiogenic effects of SHS are in large part due to nicotine, acting on EC nAChRs. Thus, in addition to the known mutagenic properties of tobacco smoke, conferred by such compounds as nitrosamines, tobacco smoke has angiogenic properties secondary to nicotine. The mutagenic and angiogenic properties of tobacco smoke are a deadly combination.

Role of nAChRs in choroidal neovascularization (CNV)

Tobacco is the major preventable risk factor for age-related macular degeneration (AMD). This disease is 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, hemorrhage and fibrosis, with loss of central vision. Recently, we found that nicotine promotes CNV in a murine model of AMD. This effect of nicotine was blocked by systemic administration of the nAChR antagonist mecamylamine (Kiuchi et al. 2008). In brief, mice were given nicotine in their drinking water in doses that we have previously documented to provide for clinically relevant concentrations of nicotine. Some animals received mecamylamine or vehicle by an osmotic Alzet minipump. After 2 days, these mice and a control group of mice that did not receive nicotine, underwent laser-induced rupture of Bruch's membrane at 3 locations in each eye. This procedure is an established method to induce CNV. Fourteen days after laser treatment, the mice were perfused with fluorescein-labeled dextran and the area of CNV measured by image analysis. By comparison to the CNV lesions in untreated mice, the lesions were larger in mice that received nicotine. This effect of nicotine was blocked in mice receiving mecamylamine. These studies support the notion that EC nAChRs may promote choroidal neovascularization.

Retinal edema also contributes to the pathobiology of AMD. Notably, evidence indicates that activation of EC nAChRs may promote retinal edema. In preclinical studies, nicotine has been shown to increase cerebrovascular permeability, an effect that can be blocked by the nAChR antagonist hexamethonium (Sunner et al. 2004). Thus, the association of tobacco smoke with AMD may be due to the effect of nicotine to promote abnormal permeability and 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 such as proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP); however, systemic administration is not ideal. Mecamylamine has significant systemic side effects (orthostatic hypotension, impaired cognition and mood, gastrointestinal effects) related to its inhibition of nAChRs in the autonomic nervous system. We therefore sought to determine if CNV could be inhibited by topical administration of mecamylamine to the eye. In preliminary studies, it was found that topical delivery of 0.1 or 1% mecamylamine resulted in significant levels in retina/choroid in mice, rabbits and primates. Mecamylamine penetrates into the cornea and sclera at high levels, and migrates through the sclera to the posterior eye (Kiuchi et al. 2008). Subsequent

studies in the murine model of AMD revealed that that topical mecamlamine (0.1 and 1%) suppressed CNV.

Notably, the effects of mecamlamine to inhibit CNV also occurred in the absence of exogenous administration of nicotine. This observation suggested that endogenous activation of endothelial nAChRs may occur under pathological conditions. Similarly, we have observed that mecamlamine can suppress other forms of neovascularization in the absence of nicotine, as in tumor angiogenesis, as well as 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.

Endogenous ligands for nAChRs

The prototypical endogenous nAChR agonist is acetylcholine (ACh). Acetylcholine is believed to be one of the most ancient of biological mediators (Kawashima et al. 1989, Wessler et al. 1999). Acetylcholine and its receptors have been evolutionary conserved among prokaryotes and eukaryotes (Bocquet et al. 2007). Intriguingly, the entire cholinergic machinery for synthesis, storage, and metabolism of ACh is present in ECs. Choline acetyltransferase (ChAT), the enzyme that synthesizes ACh from acetyl coenzyme A and choline, is expressed by ECs, which can synthesize and release ACh (Aiello et al. 1994). High affinity re-uptake of choline is essential for a new cycle of cellular ACh synthesis, and in cholinergic neurons this re-uptake is the rate limiting step in cholinergic signaling (Kuhar and Murrin, 1978). Intriguingly, a high affinity choline receptor has been demonstrated in rat and human endothelial and vascular smooth muscle cells (Mosinger and Olney 1989, Feller 2002). ACh is metabolized by acetylcholinesterase (AChE) and butyrylcholinesterases (BChE) in neuronal and extraneuronal tissues, including vascular cells (Alles and Hawes 1940, Norel et al. 1993, Kirkpatrick et al. 2003). This widespread hydrolyzing activity spatially restricts ACh to local paracrine or autocrine effects. However, the homomeric $\alpha 7$ nAChRs are activated by choline (released by AChE after its metabolism of acetylcholine). Thus, even after ACh has been cleaved by AChE there is an opportunity for signaling to persist (Alkondon et al. 1997). 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.

Interaction of the nAChR with other receptors mediating angiogenesis

Recent work from our laboratory suggests that the angiogenic pathway mediated by EC nAChRs reinforces other angiogenic pathways. For example, ECs cultured in matrigel form endothelial tubes. This effect is mediated by the multiple growth factors present in matrigel including VEGF and FGF. Intriguingly, in the absence of exogenous nicotinic stimulation, endothelial tube formation in this model appears to be partially dependent upon nAChR activity. Specifically, we observe that the nAChR antagonist mecamlamine blocks endothelial tube formation in matrigel (Heeschen et al. 2002). The effect of mecamlamine was mimicked by the $\alpha 7$ -nAChR antagonist α -bungarotoxin, indicating that the $\alpha 7$ -nAChR is involved in this response. The effect of mecamlamine was not reproduced by the muscarinic antagonists tubocurarine or atropine.

We have observed that the activity of nAChRs seems to be important for other angiogenic processes stimulated by VEGF or FGF. For example, the effect of VEGF or FGF to induce EC proliferation and migration in vitro was inhibited by the nAChR antagonist hexamethonium. The effects of hexamethonium were mimicked by mecamlamine, as well as α -bungarotoxin. These findings suggest that the growth factors exert their effects in part by activating a cholinergic pathway mediated by EC nAChRs. Microarray studies demonstrate that nicotine,

VEGF and FGF each induce transcriptional changes that are highly concordant, indicating some interdependence of the three angiogens (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 additive interactions between VEGF 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. Perfusate containing nicotine increases EC expression of VEGF in porcine coronary arteries (Macklin et al. 1998). Nicotine induces phosphorylation of the endothelial VEGF receptor KDR, thereby increasing its activity (Conklin et al. 2002). Nicotine stimulates FGF release from EC and increases metalloproteinase expression (Carty et al. 1996). Second hand smoke increases plasma levels of VEGF, an effect which is reversed by the nAChR antagonist mecamylamine. Nicotine releases endothelin from endothelial cells (Lee et al. 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 EC (Boutherin-Falson et al. 1990, Heeschen et al. 2001), which are known to be small molecule mediators of VEGF- and FGF- induced angiogenesis. We validated these findings in vivo using our disc assay for angiogenesis. Subcutaneous implantation of a polyvinyl alcohol disc causes fibrovascular ingrowth. After 4 weeks, the mouse is injected with fluorescent microspheres to quantify the intravascular space. The discs are removed for measurement of fibrovascular area and disc fluorescence, which are highly correlated to vascularity (Jang et al. 2000). Mecamylamine reduced fibrovascular growth but atropine had no effect. Furthermore, in the $\alpha 7$ -nAChR knockout mouse, fibrovascular growth was reduced, and the angiogenic effect of nicotine was impaired. In a subsequent study, mecamylamine inhibited tumor vascularity and growth in animals implanted with Lewis lung cancer cells (Heeschen et al. 2002). These studies confirmed the existence of an endogenous cholinergic pathway for angiogenesis. The cholinergic pathway is mediated by EC nicotinic receptors, primarily of the $\alpha 7$ subtype. Furthermore, the nAChR antagonist mecamylamine can reduce plasma VEGF levels in tumor-bearing animals (Zhu et al. 2003). These data regarding interaction of the pathways is clinically relevant, as antagonists of VEGF action have been used to treat cancer, PDR, AMD as well as retinopathy of prematurity (Gragoudas et al. 2004, de Gramont and Van Cutsem 2005, Lalwani et al. 2008). Therefore, agents that target the endothelial nAChRs could represent a novel class of drugs to treat diseases characterized by abnormal or insufficient angiogenesis (Pieramici and Rabena 2008), as demonstrated in pre-clinical animal models (Table 1).

Directions of Future nAChR and Angiogenesis Research

Although evidence suggests that the $\alpha 7$ nAChR plays an important role in the cholinergic modulation of angiogenesis, other nAChR subunits are expressed by ECs. It is likely that these other EC subunits aggregate into functional receptors, but in what configurations is unknown. Furthermore, the role of other nAChR subtypes in reinforcing, modulating or opposing the action of the $\alpha 7$ nAChR is not known. The endogenous sources of nAChR agonists (and possibly antagonists) need further exploration. Is it possible that nerve-released acetylcholine (or other longer-lived nAChR agonists) play a role in the development and/or maintenance of the microvasculature? For example, in diabetic patients, a profound neuropathy precedes the impaired healing ability of the foot. Could the loss of nerve-released trophic factors (such as acetylcholine) cause a regression of the microvasculature, and/or contribute to the impaired angiogenesis in these patients? Other questions remain about the nature and function of nAChRs throughout the circulation. The nAChRs of venous, lymphatic, arterial or microvascular ECs 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. Additional unknowns surround the pathobiological role of EC nAChRs. How important is cholinergic modulation in the pathological angiogenesis observed in different tumors, or in other forms of aberrant neovascularization (such as the inflammatory angiogenesis of rheumatoid arthritis, or the expansion of the vasa vasorum observed in atherosclerosis)? The significance of the nAChR in various forms of pathological neovascularization in man is unknown, and this knowledge could be critical for the development of new anti-angiogenic therapies. Further research may reveal that the vascular nAChRs are as interesting and functionally relevant as their neuronal counterparts.

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Figure 1.

The endothelial cell contains all the components for nicotinic signalling. Endothelial cell $\alpha 7$ -nAChRs are activated by the endogenous agonist acetylcholine (ACh) or by other nicotinic agonists. Within the endothelial cell, 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 acetylcholine into acetate and choline, to spatially and temporally constrain cholinergic signaling. Choline is transported by the high affinity transporter (CHT1) to the intracellular compartment for a new cycle of ACh synthesis.

A Control **B Mecamylamine**

Figure 2. Mecamylamine inhibits VEGF-induced endothelial tube formation in matrigel (B) compared to the vehicle treated control cells (A). Figure reproduced with permission from Kiuchi et al, IOVS (2008); 49:1705–1711. The Association for Research in Vision and Ophthalmology is the copyright holder of the source of the figure.

Table 1

Pre-clinical evidence for role of nAChRs in angiogenic disorders

Pathological Angiogenesis	Physiological Angiogenesis
Age-related Maculopathy ¹	Capillary density in myocardial Infarction ¹⁰
Lung Cancer ^{2,3}	Limb Ischemia ^{11,12}
Atherosclerosis ⁴	Wound Healing ^{13,14}
Colon Cancer ^{5,6,7}	Enhancement of EPC survival ¹⁵
Restenosis ⁸	Osteogenesis ¹⁶
Breast cancer ⁹	

¹ Kiuchi et al. 2008² Zhang et al. 2007³ Zhu et al. 2003⁴ Heeschen et al. 2001⁵ Natori et al. 2003^{6,7} Wong et al. 2007⁸ Ruixing et al. 2007⁹ Mousa et al. 2006¹⁰ Li et al. 2006^{11,12} Heeschen et al. 2002, 2003¹³ Jacobi et al. 2002¹⁴ Morimoto et al. 2008¹⁵ Sugimoto et al. 2007¹⁶ Zheng et al. 2008

EPC = Endothelial Progenitor Cells