The Nicotinic Cholinergic Pathway Contributes to Retinal Neovascularization in a Mouse Model of Retinopathy of Prematurity

Sean F. Hackett,1 Christopher Seidel,1 Sheena Abraham,2 Rishi Chadha,1 Seth D. Fortmann,1 Peter A. Campochiaro,1 and John P. Cooke3

1Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
2Division of Cardiovascular Medicine, Stanford University, Stanford, California, United States
3Department of Cardiovascular Sciences, Methodist Hospital System, Houston, Texas, United States

Correspondence: John P. Cooke, Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, Houston Methodist Research Institute, 6670 Bertner Avenue, Mail Stop: R10-South, Houston, TX 77030, USA; jpcooke@houstonmethodist.org.

Submitted: September 2, 2016
Accepted: December 16, 2016
Citation: Hackett SE, Seidel C, Abraham S, et al. The nicotinic cholinergic pathway contributes to retinal neovascularization in a mouse model of retinopathy of prematurity. Invest Ophtalmol Vis Sci. 2017;58:1296–1303. DOI:10.1167/iovs.16-20670

PURPOSE. To investigate the role of nicotinic acetylcholine receptors (nAChRs) in retinal vascular development and ischemia-induced retinal neovascularization (NV).

METHODS. The expression of nAChR subtypes and VEGF signaling pathway components was assessed in mice with and without oxygen-induced ischemic retinopathy by comparing expression levels at postnatal day (P) 14 and P17 in mice exposed to 75% oxygen from P7 to P12 and returned to room air versus mice pups that were exposed to ambient oxygen levels during the same period. The effect of topical or intraocular injection of mecamylamine, a nonspecific nAChR antagonist, or targeted deletion of α7- or α9-nAChRs on ischemia-induced retinal NV was determined by comparing the amount of retinal NV at P17 in these mice versus appropriate controls.

RESULTS. The expression of nAChR subunits and components of the VEGF signaling pathways was increased in ischemic retina. Topical application or intraocular injection of mecamylamine decreased retinal NV in this model. Mecamylamine had no effect on normal retinal vascular development or on revascularization of the central retinal area of nonperfusion in mice with ischemic retinopathy. Targeted deletion of α9, but not α7, nAChR receptor subunits reduced retinal NV in mice with ischemic retinopathy.

CONCLUSION. These data suggest that nAChR signaling, primarily through the α9 nAChR subunit, contributes to ischemia-induced retinal NV, but not retinal vascular development. Mecamylamine or a specific α9 nAChR antagonist could be considered for treatment of retinopathy of prematurity and other ischemic retinopathies.

Keywords: retinopathy of prematurity, cholinergic pathway, nicotinic acetylcholine receptors, VEGF, retinal neovascularization

There are two major types of ocular neovascularization (NV) that affect the retina: ischemia-induced retinal NV and subretinal NV. Ischemia-induced retinal NV occurs in humans in retinopathy of prematurity (ROP) and diabetic retinopathy and affects the vessels of the inner retina. Subretinal NV occurs in the subretinal space and occurs in diseases of the RPE and Bruch’s membrane such as AMD with vessels arising from the choroid. These two types of ocular NV share some characteristics with each other and with NV elsewhere in the body, but they also have unique features. It cannot be assumed that molecular signals implicated in one play a role in the other.

Nicotine stimulates endothelial cell proliferation, migration, and survival, due to the activation of nicotinic acetylcholine receptors (nAChRs).1,2 Activation of these receptors stimulates angiogenesis in adult animals, in part by increasing VEGF expression as well as phosphorylation of VEGF receptor 2 (VEGFR2).3–5 However, the function of these receptors in normal vascular development in unclear. Mice deficient in expression of several nAChR subunits are viable, without reported vascular anomalies, suggesting that these subunits are not indispensable for vascular development.6–8 In adult animals, both physiological angiogenesis as well as pathologic NV are modulated by nAChRs. For example, activation of nAChRs in the bed of an experimental wound accelerates wound healing.9,10 and enhances endothelial cell survival.11 In addition, nAChR may also mediate pathologic angiogenesis including NV of atherosclerotic plaque, tumor angiogenesis, and choroidal NV.12–15

Endogenous acetylcholine activates these receptors to induce endothelial cell proliferation, migration, and tube formation in vitro, and angiogenesis in vivo. In the eye specifically, activation of nAChRs by exogenous nicotine contributes to choroidal NV while inhibition of the pathway by mecamylamine suppresses it.5 Mecamylamine hydrochloride is a secondary amine and a well characterized post-ganglionic sympathetic system inhibitor that has been shown to block nicotine-induced stimulation of nAChRs and was extensively used as an antihypertensive.16–19 Studies in mammalian brain and amphibian neuromuscular tissue demonstrated that mecamylamine is a noncompetitive inhibitor that binds to the nAChR ion channel region and decreases the longevity of channel opening rather than by blocking nicotine.
Nicotinic Pathway Contributes to Retinal NV

Effect of Mecamylamine on Normal Vascular Development

On the first day after birth (P1), mouse pups were given a periorbital injection of 0.03% mecamylamine in PBS or PBS alone using a Harvard microinjection apparatus with a pulled glass needle inserted just below the closed eyelid so as not to puncture the eye or enter the venous plexus. The mice were killed at P7, P10, or P18. The retinas were fixed, dissected out, stained, and flat mounted as above. For P7 retinas, the length of vascular development of the superficial retinal vessels was measured from the optic nerve head (ONH) to the edge of the peripheral vascular bed in all four quadrants per retina and the results were averaged to yield one measurement per retina. At P10 and P18, measurements were performed in the same manner focusing on the deeper capillaries that grow into the retina from the superficial vessels in a pattern starting from the central vessels to the periphery.

METHODS

Mice

All experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee or the Stanford University Administrative Panel on Laboratory Animal Care. Knock-out mice for the nAChR7 (B6.129S-Chrna7tm1-Bay/J) and nAChR9 (129S-Chrna9tm1Bedv/J) genes were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). C57Bl/6 mice were purchased from Charles River, Harlan Laboratories (both in Frederick, MD, USA), or Jackson Laboratories (Sacramento, CA, USA).

Oxygen-Induced Ischemic Retinopathy in Mice

Retinal neovascularization (rNV) was induced in neonatal mice as previously described. Briefly, at postnatal day 7 (P7), mothers and pups were placed in a hyperoxia chamber (75% O2) to interrupt normal postnatal development of the retinal vasculature in the pup eyes, and returned to room air at P12. For some experiments, immediately upon removal from the hyperoxic chamber, mice were given a 1-μL intravitreal injection of PBS or mecamylamine (Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in PBS at concentrations ranging from 0.001% to 0.1% in the right eye and PBS alone in the left eye using a Harvard microinjection apparatus (Harvard Apparatus, Holliston, MA, USA) with a pulled glass needle and a dissecting microscope. In other animals, mecamylamine was administered topically by dropping 2 μL to each eye daily from P12 to P16 at concentrations of 0.01%, 0.03%, 0.1%, and 0.3% w/vol reconstituted in PBS, while control animals received vehicle alone on both eyes. At P17 the mice pups were killed, the eyes were removed and fixed in 10% formalin, and the retinas were dissected out and stained using fluorescein-GSA Isolectin B4 (Invitrogen, Carlsbad, CA, USA) to identify the neovascular tufts. Digital photographs were obtained with a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) of the flat mounted retinas. ImagePro Plus software (Media Cybernetics, Rockville, MD, USA) was used to highlight and measure the area of retinal NV per retina by an investigator blinded with respect to treatment group.

In other experiments, mouse pups were killed at P12 and P17 following 5-day exposure to hyperoxia and the retinas were fixed, isolated, and stained as above. Flat-mounted retinas were measured as above to delineate the central area of nonperfusion (ANP) that results from vessel regression during the hyperoxia phase.

Quantitative PCR

Real-time quantitative PCR was performed using a Rotor Gene Q instrument (Qiagen, Hilden, Germany) and Rotor-Gene SYBR Green PCR Kit (Qiagen) to investigate the expression levels of angiogenesis-related genes and nicotinic acid receptor alpha subunits in the retinas of normal and hypoxic mice. Cyclophilin A expression was used to standardize expression levels of the test genes. Fold changes in gene expression were calculated using ΔΔCt values. Primers used in these experiments are listed in the Table.

In other experiments, mouse pups from nAChR 7/+ or nAChR 7−/− mice were exposed to hyperoxia as above, returned to room air, and killed at P17. This mating paradigm produces experimental mice as well as controls in the same litters. Retinas were isolated and cDNA was prepared from total retinal RNA using the AllScript RNA PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions. Real-time quantitative PCR was performed using a Rotor Gene Q instrument (Qiagen, Hilden, Germany) and Rotor-Gene SYBR Green PCR Kit (Qiagen) to investigate the expression levels of angiogenesis-related genes and nicotinic acid receptor alpha subunits in the retinas of normal and hypoxic mice. Cyclophilin A expression was used to standardize expression levels of the test genes. Fold changes in gene expression were calculated using ΔΔCt values. Primers used in these experiments are listed in the Table.

In other experiments, mouse pups from nAChR 7/+ or nAChR 7−/− mice were exposed to hyperoxia as above, returned to room air, and killed at P17. This mating paradigm produces experimental mice as well as controls in the same litters. Retinas were isolated and cDNA was prepared from total retinal RNA using the AllScript RNA PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions. Real-time quantitative PCR was performed using a Rotor Gene Q instrument (Qiagen, Hilden, Germany) and Rotor-Gene SYBR Green PCR Kit (Qiagen) to investigate the expression levels of angiogenesis-related genes and nicotinic acid receptor alpha subunits in the retinas of normal and hypoxic mice. Cyclophilin A expression was used to standardize expression levels of the test genes. Fold changes in gene expression were calculated using ΔΔCt values. Primers used in these experiments are listed in the Table.

In other experiments, mouse pups from nAChR 7/+ or nAChR 7−/− mice were exposed to hyperoxia as above, returned to room air, and killed at P17. This mating paradigm produces experimental mice as well as controls in the same litters. Retinas were isolated and cDNA was prepared from total retinal RNA using the AllScript RNA PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the instructions. Real-time quantitative PCR was performed using a Rotor Gene Q instrument (Qiagen, Hilden, Germany) and Rotor-Gene SYBR Green PCR Kit (Qiagen) to investigate the expression levels of angiogenesis-related genes and nicotinic acid receptor alpha subunits in the retinas of normal and hypoxic mice. Cyclophilin A expression was used to standardize expression levels of the test genes. Fold changes in gene expression were calculated using ΔΔCt values. Primers used in these experiments are listed in the Table.
Nicotinic Pathway Contributes to Retinal NV

RESULTS

Mecamylamine Suppresses Ischemia-Induced Retinal NV

Oxygen-induced ischemic retinopathy (OIR) is a model of rNV. Mouse pups with their mothers were placed in a hyperoxic chamber with oxygen maintained at a concentration of 75% from P7 to P12 when they were returned to room air. Immediately after removing them from the chamber, the pups received a 1-μl intraocular injection of mecamylamine in PBS at a concentration ranging from 0.001% to 0.1% in the right eye and PBS alone in the left eye. In a dose-dependent manner, mecamylamine reduced ischemia-induced NV significantly compared with PBS alone (Fig. 1a). Similar results were seen in mice that received daily topical administration of mecamylamine (Supplementary Fig. S1).

Expression of Components of the VEGF Pathway and nAChR Alpha Subtypes in Retinal Endothelial Cells

Because nAChRs are expressed on both neuronal cells as well as vascular cells, and because ischemic retinopathy can alter VEGFA and VEGFR2 levels in both cell populations, it was desirable to isolate these populations and investigate any changes that may occur, especially with regard to nAChR subtypes. To investigate the expression of VEGFA, VEGFR2, and the nAChR alpha subunits in retinal neuronal and endothelial cell populations, retinal endothelial cells were isolated from dissociated total retinal cell suspensions from mice with OIR at P14 and P17 using anti-PECAM-1–coated magnetic beads. The average enrichment factor of PECAM (þ) cell fraction was 27.4 ± 4.7 compared with the PECAM (þ) cell fraction as well as in the PECAM (þ) cell fraction (Fig. 2a). The change in VEGFA and VEGFR2 expression levels was investigated in PECAM (þ) and (–) cell populations from the retinas of OIR mice. Two days after the onset of ischemia at P12, VEGFA expression levels were elevated in both PECAM (þ) neuronal and PECAM (þ) endothelial cell populations and the elevation was sustained at P17 (Fig. 2b). Vascular endothelial growth factor receptor 2 is expressed on several cell types in the retina, including ganglion cells, rod photoreceptors, and endothelial cells, but its expression was only significantly increased at P14 or P17 in the PECAM (þ) fraction of retinal cells, indicating that it is only upregulated by OIR in endothelial cells.

We next investigated the expression levels of the nAChR subtypes in control and OIR retinas. A previous study indicated that eight subtypes are expressed in the retina. Our study demonstrates that the eight subtypes are expressed in both the PECAM (þ) endothelial cell fraction as well as in the PECAM (–) neuronal fraction of retinal cells and that they are modulated by hypoxia (Figs. 2c, 2d). The nAChR subtypes α2, 9, and 10 were transiently upregulated at P14 in the PECAM (–) fraction but returned to control levels at P17 in retinas from OIR mice compared with controls. The nAChRα5 subtype was increased at P17, while the α5 subtype was increased at both P14 and P17 in the PECAM (–) fraction. In the PECAM (þ) cell fraction, the α9 and α10 subtypes were significantly increased at P14 but not at P17 compared with controls while the α2 and α5 subtypes were significantly increased at P17. Interestingly, the α2, α4, and α6 subtypes were all significantly suppressed at
P17 in the OIR mice. And surprisingly the α7 subtype, which has been shown repeatedly to play a role in proliferation of endothelial cells from other circulations, was unmodulated at either time-point for either cell fraction.

Effect of Targeted Deletion of nAChRα7 or nAChRα9 on Ischemia-Induced Retinal NV and Gene Expression

Previous studies in vitro using siRNA to knockdown expression of specific nAChR subtypes demonstrated that suppression of α7 decreased endothelial cell proliferation and increased apoptosis while suppression of α9 increased proliferation without affecting apoptosis. Therefore, in order to discern whether these two subtypes played similar roles in vivo, we investigated the effect of systemic ablation of α7 or α9 in mice on the development of rNV in the OIR model using commercially available knockout mice. Mice hemizygous for either deletion were mated to produce litters that contained (+/−), (+/−)/C0, and (−/−)/C0 littermate controls to correct for any interlitter variations. Compared with wild-type mice, there was no difference in the amount of rNV in OIR retinas from either hemizygous or homozygous nAChRα7 knockout mice (Fig. 3a). In the nAChRα9 knockout mice, hemizygous deletion resulted in a 14% decrease on the amount of rNV compared with controls while homozygous ablation resulted in a 34% decrease in the amount of rNV that developed (Figs. 3a, 3b). Because the amount of rNV that develops in the OIR model depends upon the amount of vessel ablation that occurs in the hyperoxic phase, we measured the central ANP in nAChRα9 (+/−) and (−/−) mice at P12 immediately after removal from hyperoxia. Both populations of mice showed identically sized ANP. Additionally, when the central ANP remaining at P17 was measured in a separate cohort of mice, there was no difference compared with controls. Thus the regrowth of normal vasculature to fill in the ablated zone occurred at the same rate in nAChRα9 (−/−) mice compared with (+/−) mice suggesting that nAChRα9 is not necessary for normal vessel formation.

Previous work has demonstrated that activation of nAChr receptors can alter the ratio of the proangiogenic VEGF and the antiangiogenic pigment epithelial derived factor (PEDF) in favor of VEGF. To investigate whether a change in the VEGF:PEDF ratio could account for the decreased rNV in nAChRα9 (−/−) mice compared with controls, mRNA levels were compared for VEGF, PEDF, and VEGFR2 in P17 OIR knockout mice versus OIR wild-type controls (Fig. 4). Gene expression was not significantly different for any of these. However, when expression levels for the different nAChRα subtypes were measured, we found significant increases in the expression levels for α2, α4, and α5 in the retinas of knockout mice compared with controls. This suggests that there may be some compensatory upregulation of these subtypes in the absence of nAChRα9.

Figure 1. Mecamylamine reduces rNV in OIR but does not affect normal vascular development. (a) Mouse pup litters (n = 4–9 pups per litter) were treated by intraocular injection of mecamylamine or PBS at the onset of hypoxia (P12). *P < 0.05, **P < 0.001 for control versus treated rNV. (b) Distance from the optic nerve head (asterisk) to the peripheral edge of vascularization (arrow) was measured at P7 in the superficial vascular bed and at P10 and P18 for the deep capillary vascular bed. The distance was measured in each of four quadrants in the flat-mounted retinas and averaged to generate one value per eye. n = 4 to 10 per group. (c) Images of representative retinal quadrants for superficial vessels at P7 of mecamylamine- and PBS-treated eyes.
DISCUSSION
Overview

The salient findings of this study are that in the murine OIR model of rNV, the expression of nAChR subunits and components of the VEGF signaling pathways are increased in the ischemic retina. Furthermore, topical or intraocular administration of the nAChR antagonist mecamylamine decreased retinal NV in this model. Additionally, targeted deletion of \( \alpha_9 \), but not \( \alpha_7 \), nAChR receptor subunits reduced retinal NV in OIR. These data suggest that nAChR signaling, primarily through the \( \alpha_9 \) nAChR, contributes to ischemia-induced...
Nicotinic Pathway Contributes to Retinal NV

ROP Versus OIR

Retinopathy of prematurity is a vasoproliferative retinal disease affecting premature infants (for reviews see Refs. 27–31). Normally, retinal vascular development occurs prenatally and is complete around gestational age 37 weeks. In infants born prematurely, normal vascular development is interrupted due to the loss of maternally derived factors such as insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and ω-3 polyunsaturated fatty acids (PUFAs), as well as the downregulation of proangiogenic factors such as VEGF due to increased oxygen tension relative to that in utero. This interruption of normal vascular development is Phase 1 of ROP. Phase 2 occurs as the neural retina develops and oxygen requirements increase leading to hypoxia of the retina, and upregulation of VEGF and other angiocytokines. This leads to dysregulated vascular cell proliferation into the epiretinal vitreous space forming membranes that can cause retinal detachment and/or neuronal defects that can result in poor visual acuity for life. Interventions to decrease the severity and progression of the disease include careful regulation of oxygen to prevent ROP, and the use of cryotherapy or laser ablation. More recently, anti-VEGF therapies have been assessed in ROP, but concerns remain about possible systemic effects of this approach.32

To model the human disease, we used the well-characterized mouse OIR model.22,33 As in ROP, the mouse OIR model has a biphasic pathology whose initial phase includes loss of proangiogenic stimuli due to increased oxygen tension resulting in a cessation of retinal vascular development, which phase is followed by a hypoxia-induced upregulation of proangiogenic factors leading to aberrant NV. However, there are some key differences.30,34,35 First, retinal vascular development in humans occurs by a combination of vasculogenesis.
and angiogenesis and is normally complete by gestational age of 37 weeks (i.e., prenatal) while that in mice is entirely postnatal and is now believed to involve only angiogenesis. As a result of this difference, while human ROP is exacerbated by the loss of maternally derived factors such as IGF1 and o-s PUFAs, murine OIR does not involve alterations in maternal trophic support. Additionally, human ROP involves a disruption of vessel formation in the peripheral retina during phase 1, while mouse OIR in addition includes ablation of central retina vessel. Lastly, murine OIR does not cause retinal detachment, resolves without intervention, and is not reported to cause neuronal cell death. Thus, the OIR model has been helpful to increase our understanding of the mechanisms of ROP and to identify potential therapeutic interventions, but may not be entirely predictive of the benefit of such new therapeutic approaches.

The Angiogenic Effect of Endothelial nAChRs

We previously discovered that endothelial cell (EC) nicotinic acetylcholine receptors (nAChRs) induce EC survival, proliferation, and function.2 Notably, endothelial cells have the capacity to synthesize and degrade acetylcholine, the endogenous ligand for nAChRs.15,36-40 In vivo, activation of the nAChRs may enhance physiological angiogenesis or contribute to pathologic NV.2-5,9,10

We have shown that there are positive, reinforcing interactions between the cholinergic and VEGF pathways. Stimulation of EC nAChRs increases VEGF expression and metalloproteinase expression41,42; and induces phosphorylation of the VEGFR.43 Vascular endothelial growth factor-induced EC migration is inhibited by nAChR antagonists. Reciprocally, VEGF stimulation increases the expression of EC nAChR.47,44 These findings indicate that the cholinergic and VEGF system are synergistic proangiogenic pathways.

Antagonism of nAChR Blocks Pathologic NV

Kiuchi and colleagues5 showed that pretreating mice with topical administration of mecamylamine, a nonselective nAChR antagonist, effectively reduced choroidal NV in the mouse model of ruptured Bruch’s membrane. Our present results demonstrate that mecamylamine can also mitigate the prolific vascularization seen in OIR. Notably, mecamylamine did not inhibit the revascularization of the central vaso-obliterrated region, indicative of the restoration of normal physiological vascularization in this animal model. Likewise, administration of mecamylamine during normal development did not produce any changes in normal vascular development, as assessed by analyzing the rate of radial outgrowth from the ONH. These studies indicate that normal vascular development cannot proceed in the presence of a nAChR antagonist, whereas pathologic NV is inhibited. Previous observations of human retinal endothelial cells in vitro using nAChR-specific siRNAs suggested that nAChR7 activation induces a proangiogenic response while 9 activation decreases endothelial cell proliferation.4 Therefore, it was predicted that nAChR7 (–/–) mice would have less rNV than wild-type (+/+), mice, whereas nAChR9 (–/–) mice would have greater NV. However, the reverse is true in the mouse eye. In the pathologic NV associated with murine OIR, it appears that 9 nAChRs play a prominent role in the development of rNV. Indeed, mice that lack the 9 nAChR subtype developed less rNV than control mice. We found no difference in the extent of neovascularization between wild-type and 97 (–/–) mice, which suggests that this subtype plays a less important role in the development of rNV than the in vitro experiments would lead one to believe. The roles played by the different subtypes thus appear to be context dependent. Our data also suggest that multiple subtypes play a role in the neovascular response because inhibition of all of the nAChR subtypes by mecamylamine reduced rNV by greater than 95% at the highest concentration while nAChR9 ablation resulted in only a 34% reduction. Other nAChR subtypes may compensate for the loss of the 9 nAChR subtype. Indeed, there was a 2-fold increase in the mRNA expression of nAChRn2 in the n9(–/–) mice, and a 2.5-fold increase for nAChRn4 and z5 mRNA. Further studies will need to be done to determine how much each subtype contributes to the neovascular response.

CONCLUSIONS

In summary, we have shown the existence of nAChRs in the murine neural retina and the retinal vasculature, and their upregulation in the murine OIR model of retinal NV. Furthermore, pharmacologic or genetic suppression of nicotinic acetylcholinergic receptors inhibited the development of retinal NV in this model. Our work justifies a more comprehensive analysis of the role of different nAChR subunits in rNV by genetic manipulations or through use of specific subtype inhibitors to further define the specific receptors that may be antagonistic and agonistic to the pathologic NV process in the retina. Finally, nAChR antagonists such as mecamylamine may represent a novel therapeutic approach to alleviate ischemic retinopathies that develop in diseases such as ROP and diabetic macular edema. Partial evidence for this was suggested by a small phase I/II trial investigating the efficacy of topical 1% mecamylamine drops twice per day in alleviating macular edema and improving visual acuity (VA).45 The mixed results demonstrated that a subset of patients showed improvements while others showed no improvement or even worsening of VA. Much like our results, the authors suggest that the heterogeneity of effect may be a reflection of the differing and possibly opposing roles the nAChR subtypes are reported to play and a better understanding of these differing roles is necessary to develop therapies that can effectively target this angiomodulatory system.

Acknowledgments

Supported by grants from National Heart Lung and Blood Institute U01 HL100397 and the National Eye Institute R01 HL0260901 (JPC).

Disclosure: S.F. Hackett, None; C. Seidel, None; S. Abraham, None; R. Chadda, None; S.D. Fortmann, None; P.A. Campo-chiaro, None; J.P. Cooke, None

References

1. Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J Appl Physiol. 1998;84:2089–2098.

2. Heeschen C, Jang JJ, Weis M, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. Nat Med. 2001;7:833–839.

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

4. Wu JC, Chruscienski A, De Jesus Perez VA, et al. Cholinergic modulation of angiogenesis: role of the 7 nicotinic acetylcholine receptor. J Cell Biochem. 2009;108:433–446.

5. Kiuchi K, Matsuoka M, Wu JC, et al. Mecamylamine suppresses basal and nicotine-stimulated choroidal neovascularization. Invest Ophtalmol Vis Sci. 2008;49:1705–1711.

6. Caffery PM, Krishnaswamy A, Sanders T, et al. Engineering neuronal nicotinic acetylcholine receptors with functional
sensitivity to alpha-bungarotoxin: a novel alpha3-knock-in mouse. Eur J Neurosci. 2009;30:2064–2076.

7. Orr-Urtreger A, Gøldner FM, Saeki M, et al. Mice deficient in the α7 neuronal nicotinic acetylcholine receptor lack α-bungarotoxin binding sites and hippocampal fast nicotinic current. J Neurosci. 1997;17:9165–9171.

8. Vetter DE, Liberaman MC, Mann J, et al. Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. Neuron. 1999;23:93–105.

9. Jacobi J, Jang J, Sundram U, Dayoub H, Fajardo LF, Cooke JP. Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. Am J Pathol. 2002;161:97–104.

10. Morimoto N, Takemoto S, Kawazoe T, Suzuki S. Nicotine at a low concentration promotes wound healing. J Surg Res. 2008;145:199–204.

11. Smedlund K, Tano JY, Margiotta J, Vazquez, G. Evidence for nicotinic Pathway Contributes to Retinal NV.

12. Tang J, Li Z, Lu L, Cho CH. Nicotinic Pathway Contributes to Retinal NV.

13. Lee J, Cooke JP. Nicotine and pathological angiogenesis. Life Sci. 2006;78:2129–2140.

14. Fieber LA, Adams DJ. Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. J Physiol. 1991;434:215–237.

15. Nooney JM, Peters JA, Lambert JJ. A patch clamp study of the nicotinic acetylcholine receptor of bovine adrenomedullary chromaffin cells in culture. J Physiol. 1992;455:503–527.

16. Gyermek L. Methods for the examination of ganglion-blocking activity. In: Kharkевич DA, ed. Pharmacology of Ganglionic Transmission. New York, New York: Springer; 1980:63–121.

17. Varanda WA, Aracava Y, Cho CH. β-Adrenergic system, a backstage player regulating tumour progression and drug target in cancer therapy. Semin Cancer Biol. 2013;23:533–542.

18. Lee J, Cooke JP. Nicotine and pathological angiogenesis. Life Sci. 2012;91:1058–1064.

19. Schaal C, Chellappan SP. Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. Mol Cancer Ther. 2011;12:14–23.

20. Tang J, Li Z, Lu L, Cho CH. β-Adrenergic system, a backstage player regulating tumour progression and drug target in cancer therapy. Semin Cancer Biol. 2013;23:533–542.

21. Fieber LA, Adams DJ. Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. J Physiol. 1991;434:215–237.

22. Nooney JM, Peters JA, Lambert JJ. A patch clamp study of the nicotinic acetylcholine receptor of bovine adrenomedullary chromaffin cells in culture. J Physiol. 1992;455:503–527.

23. Gyermek L. Methods for the examination of ganglion-blocking activity. In: Kharkевич DA, ed. Pharmacology of Ganglionic Transmission. New York, New York: Springer; 1980:63–121.

24. Varanda WA, Aracava Y, Sherby SM, VanMeter WG, Eldefrawi ME, Albuquerque EX. The acetylcholine receptor of the adrenal medulla: a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther. 1998;77:59–79.

25. Carty CS, Soloway PD, Kayastha S, et al. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. J Vasc Surg. 1996;24:927–935.

26. Dom AM, Buckley AW, Brown KC, et al. The α7-nicotinic acetylcholine receptor and MMP-2/9 pathway mediate the proangiogenic effect of nicotine in human retinal endothelial cells. Invest Ophthalmol Vis Sci. 2011;52:4428–4438.

27. Luyt GA, Chan-Ling T, Phelps DL, et al. Proceedings of the Third International Symposium on Retinopathy of Prematurity: an update on ROP from the lab to the nursery. (November 2003, Anaheim, California). Mol Vis. 2006;12:532–580.

28. Heidary G, Vanderveen D, Smith LE. Retinopathy of prematurity: current concepts in molecular pathogenesis. Semin Ophthalmol. 2009;24:77–81.

29. Rivera JC, Sapieha P, Joyal JS, et al. Understanding retinopathy of prematurity: update on pathogenesis. Neonatology. 2011;100:343–353.

30. Hartnett ME, Penn JS. Mechanisms and management of retinopathy of prematurity. N Engl J Med. 2012;367:2515–2526.

31. Hellstrom A, Smith LE, Dammann O. Retinopathy of prematurity. Lancet. 2013;382:1445–1457.

32. Klufas MA, Chan RV. Intravitreal anti-VEGF therapy as a treatment for retinopathy of prematurity: what we know after 7 years. J Pediatr Ophthalmol Strabismus. 2015;52:77–84.

33. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. Nat Protoc. 2009;4:1565–1573.

34. Kim CB, D’Amore PA, Connor KM. Revisiting the mouse model of oxygen-induced retinopathy. Eye Brain. 2016;8:67–79.

35. Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. Prog Retin Eye Res. 2010;29:500–519.

36. Kirkpatrick CJ, Bittinger F, Unger RE, Kriegsmann J, Kilbinger H, Wessler I. The non-neuronal cholinergic system in the endothelium: evidence and possible pathological significance. Jpn J Pharmacol. 2001;85:24–28.

37. Lips KS, Pfeil U, Reiners K, et al. Expression of the high-affinity choline transporter CHT1 in rat and human arteries. J Histochem Cytochem. 2003;51:1645–1654.

38. Mosinger JL, Olney JW. Photothrombosis-induced ischemic neuronal degeneration in the rat retina. Exp Neurol. 1989;105:110–113.

39. Parnevales JG, Kelly W, Burnstock G. Ultrasrtuctural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature. 1985;316:724–725.

40. Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther. 1998;77:59–79.

41. Carty CS, Soloway PD, Kayastha S, et al. Nicotine and cotinine stimulate secretion of basic fibroblast growth factor and affect expression of matrix metalloproteinases in cultured human smooth muscle cells. J Vasc Surg. 1996;24:927–935.

42. Dom AM, Buckley AW, Brown KC, et al. The α7-nicotinic acetylcholine receptor and MMP-2/9 pathway mediate the proangiogenic effect of nicotine in human retinal endothelial cells. Invest Ophthalmol Vis Sci. 2011;52:4428–4438.