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Sent: Tuesday, May 14, 2013 11:56 AM
To: SCHNICHELS Dominik (SANCO)
Subject: British Medical Association (BMA) meeting outcome

Dear Dominik,

I trust you are well. I am writing to express my gratitude for meeting with me last week, it is much appreciated and very useful. Please accept once again my apologies for the other two delegates who were unable to attend the meeting due to the health emergency that occurred on their way. Following our conversation on the TPD, please find attached the BMA's briefing on e-cigarettes that speaks about our views and concerns about NRT and NCP.

In addition, I have also attached various papers that were brought to our attention by the BMA's Occupational Medicine Committee regarding the role of nicotine in causing vascular endothelial dysfunction, producing atherogenic lipid profiles and enhancing promotion and progress in human cancers.

Keep in touch and please do not hesitate to contact me should you require further information.

Best,

Robert

- -
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BMA calls for stronger regulation of e-cigarettes

March 2012 (updated January 2013)

A briefing from the Board of Science and the Occupational Medicine Committee

Summary

- There is emerging evidence that e-cigarettes are being used by some smokers to help cut down or quit; yet, they are subject to limited regulation, are not licensed as a medicine in the UK, and there is no peer-reviewed evidence that they are safe or effective for this purpose.
- While e-cigarettes have the potential to reduce tobacco-related harm (by helping smokers to cut down and quit), a strong regulatory framework is required for the sale and use of e-cigarettes to:
  - ensure they are safe, quality assured and effective at helping smokers to cut down or quit
  - restrict their marketing, sale and promotion so that it is only targeted at smokers as a way of cutting down and quitting, and does not appeal to non-smokers, in particular children and young people
  - prohibit their use in workplaces and public places to limit secondhand exposure to the vapour exhaled by the user, and to ensure their use does not undermine smoking prevention and cessation by reinforcing the normalcy of cigarette use.
- Health professionals should encourage their patients to use a regulated and licensed nicotine replacement therapy to help quit smoking. Where a patient is unable or unwilling to use or continue to use an approved and tested nicotine replacement therapy, health professionals may advise patients that while e-cigarettes are unregulated and their safety cannot be assured, they are likely to be a lower risk option than continuing to smoke.

Background

Electronic cigarettes (e-cigarettes), or electronic nicotine delivery systems (ENDS), have become increasingly popular since the mid-2000s with their own advocacy groups, marketing and increasing online interest.[1,2,3] The legal status of e-cigarettes varies around the world. In some countries (eg Denmark, Canada, Israel, Singapore, Australia and Uruguay) the sale, import, or marketing of e-cigarettes is either banned, regulated in various ways, or the subject of health advisories by government health organisations.[4,5] In others (eg New Zealand), e-cigarettes are regulated as medicines and can only be purchased in pharmacies.[4] The UK has few restrictions on the sale and use of e-cigarettes.

What is an e-cigarette?

E-cigarettes are battery-powered products designed to replicate smoking behaviour without the use of tobacco – some look like conventional cigarettes, while others appear more like an electronic device (see **Figure 1**).[6,7] They consist of a cartridge containing liquid nicotine, an atomiser (heating element), a rechargeable battery, and electronics.[7] They turn nicotine, flavour and other chemicals into a vapour that

is inhaled by the user.[4,6,7] The exhaled vapour can be seen, and some products have a light emitting diode (LED) at the tip that lights up when the user inhales.[8]

Figure 1

Who uses e-cigarettes?

While e-cigarettes have become increasingly popular since the mid-2000s, there are limited data on usage levels in the UK. Emerging evidence suggests that they are mainly used in attempts to quit smoking.[8,9,10,11] A 2012 Action on Smoking and Health (ASH) online survey of 10,000 adults (aged 18+) in England found that:

- one fifth of smokers had tried e-cigarettes, but only a third of those who had tried them were still using them
- one out of five users of e-cigarettes have quit smoking altogether
- four out of five e-cigarette users continue smoking, and use e-cigarettes primarily as a substitute where smoking is not allowed, and to help them quit and to cut down
- less than 1 per cent of never smokers had tried them.[9]

According to the findings of the Smoking Study Toolkit, in 2012, e-cigarettes were the most popular single type of nicotine product, with seven per cent of cigarette smokers in England also using e-cigarettes (as of October 2012).[10,11] The study has also found that:

- e-cigarette use has substituted for use of licensed nicotine products rather than growing the market

- compared to users of licensed nicotine product users, e-cigarette users are slightly less motivated to stop smoking and less likely to have tried to stop; more likely to be male, older and to be 'white collar'; and less cigarette dependent.[10,11]

E-cigarettes and harm reduction

A 2011 review of the evidence regarding the safety of e-cigarettes concluded that they are a safer alternative to tobacco cigarettes.[12] Their use has therefore been suggested as a way of reducing the harm[a] associated with smoking tobacco.[12.13] Despite this, there has been little research into the efficacy of e-cigarettes as aids to stop smoking or cutting down,[4,13] and they are subject to limited regulation in the UK (see **Box 1**).

Box 1 – e-cigarette regulation in the UK

In the UK, e-cigarettes are subject to regulation under the General Product Safety Regulations 2005, the Chemicals (Hazard Information & Packaging for Supply) Regulations 2009, and by trading standards.[4,14] There are no regulations on the sale of e-cigarettes as age restricted products, including their sale to children.[14] The UK Medicines and Healthcare products Regulatory Agency (MHRA) – which is tasked with ensuring that medicines and medical devices work and are safe – is currently considering how e-cigarettes and other nicotine containing products should be regulated. The MHRA have stated that a final decision will be made in Spring 2013, and in the interim, have committed to work with the e-cigarette industry to develop a self-regulatory code.[15]

Safety and efficacy

A 2008 review by the World Health Organization (WHO) does not exclude the possibility that the e-cigarette could be useful as a smoking cessation aid, but concluded that no rigorous, peer-reviewed studies have been conducted showing that the e-cigarette is a safe and effective nicotine replacement therapy.[16] There is evidence that e-cigarette products are highly variable in the efficacy of their vaporisation of nicotine,[b,17] and that the labelling of nicotine levels may be inconsistent and misleading.[18] An analysis of the total level of nicotine generated by e-cigarettes which vaporise nicotine effectively found that the amount inhaled from 15 puffs was lower compared with smoking a conventional cigarette.[b] In 2009, the United States Food and Drug Administration (FDA) released results of an analysis of some e-cigarette products.[18] The analysis found that the e-cigarette cartridges contained carcinogens and toxic chemicals. Analysis of two leading brands revealed:

- diethylene glycol (a toxic chemical) in one cartridge at approximately 1 per cent

[a] The National Institute for Health and Clinical Excellence (NICE) is currently developing guidance on 'Tobacco: harm reduction approaches for smoking'. The BMA supports the development of a tobacco-free harm reduction approach as a part of a structured programme leading to permanent smoking cessation, focusing on the use of licensed and regulated pure nicotine products.

[b] This study analysed sixteen e-cigarette brands (based on their popularity in the Polish, UK and US markets) – the total level of nicotine in vapor generated by 20 series of 15 puffs varied from 0.5 to 15.4 mg. Most of the analysed e-cigarettes effectively delivered nicotine during the first 150-180 puffs. On an average, 50- 60 per cent of nicotine from a cartridge was vaporised.

- tobacco-specific nitrosamines (which are human carcinogens) in half of the samples
- tobacco-specific impurities suspected of being harmful to humans (anabasine, myosmine, and ß-nicotyrine) in a majority of the samples.[18]

The tests also suggested that quality control was inconsistent or non-existent:

- cartridges with the same label emitted a markedly different amount of nicotine with each puff
- one high-nicotine cartridge delivered twice the amount of nicotine compared to a nicotine inhalation product approved by the FDA.[18]

The Trading Standards Institute and others have stated that safety concerns have come to light around some brands of e-cigarettes, including electrical safety, the need for proper labelling, and the provision of child resistant packaging.[14,19]

Promotion and sales

With the exception of statements about the product needing to be substantiated, the promotion of e-cigarettes – which includes point-of-sale displays, and advertising via television, radio, in print media and online – is not specifically controlled.[14] Their promotion ranges from being advertised as 'cigarette substitutes' and 'a healthier alternative to smoking traditional tobacco products', to evocative advertising with phrases such as 'an exceptional alternative smoking experience', 'vape with style', and 'add flavour to your lifestyle'. The advertising also frequently makes positive associations with recreational activities and can incorporate celebrity endorsements. It is worth noting that the provisions of the 2002 Tobacco Advertising and Promotion Act (TAPA) prohibit any brandsharing or connections with tobacco products.

E-cigarettes are sold online and can be bought from a variety of high street outlets, ranging from pubs, chemists and newsagents to specialist shops. The cost of using e-cigarettes is comparatively lower than using tobacco cigarettes – while the initial cost of the e-cigarette starter kits can be four or five times higher than a pack of 20 tobacco cigarettes, the ongoing costs (of cartridge refills and other components) is lower than that of purchasing tobacco cigarettes. This lower cost is commonly highlighted as a benefit to using e-cigarettes compared to smoking.

Strengthening the regulatory framework

It is clear that the existing regulatory framework is inadequate in ensuring that e-cigarettes are safe and effective as a nicotine replacement therapy. This may in turn undermine cessation attempts. To be used as part of a harm reduction approach, there is a need to strengthen the regulation of e-cigarettes to ensure they are safe, quality assured and effective at helping smokers to cut down or quit. This includes the requirement for clear unambiguous labelling and packaging that details the contents of the cartridges and the conditions for its safe use. There is also a need to restrict the marketing, sale and promotion of e-cigarettes so that it is only targeted at smokers as a way of cutting down and quitting, and does not appeal to non-smokers, in particular children and young people. Until this regulatory framework is in

place, e-cigarettes should not be considered as a smoking cessation aid or a lower risk option than continuing to smoke.

E-cigarettes in workplaces and enclosed public places

Restrictions on where e-cigarettes can be used are limited and variable in the UK – ranging from being prohibited in some restaurants and workplaces, to restrictions in controlled environments.

Stronger controls are needed on where e-cigarettes can be used in order to:

- protect others from being exposed to e-cigarette vapours. While the concentrations of the constituents of these vapours (propylene glycol, glycerine, flavouring substances, and nicotine) are lower than with smoked cigarettes, 'passive vaping' has been found to occur with the use of e-cigarettes.[20,21,22]
- ensure their use does not undermine existing restrictions on smokefree public places and workplaces, by leading people to believe it is acceptable to smoke. Of particular concern to BMA members is their use by patients, visitors and staff in hospitals and other healthcare settings. Exposure to nicotine from e-cigarettes (either directly through their use by an individual or indirectly from the vapours they produce) may adversely impact on patients, such as those with heart or circulatory conditions, and their use may also become a source of conflict between staff and patients. Similar concerns exist in other settings, such as the use of e-cigarettes on airplanes.
- ensure their use does not undermine the success of conventional tobacco control measures by reinforcing the normalcy of smoking behaviour in a way that other nicotine containing products do not.[23] This specifically relates to the way these devices commonly resemble tobacco cigarettes, in terms of appearance, nomenclature and the way they are used, as well as features such as flavouring and styling that are potentially highly attractive to children, and may include cigarette brand reinforcement.

In light of these concerns, the BMA believes the existing smokefree legislation in place in the UK should be extended to include vapour from e-cigarettes. As an interim measure, we also encourage employers to implement organisation-wide policies prohibiting the use of e-cigarettes in their workplaces.

Advice for health professionals

In light of the lack of scientific evidence about the efficacy and safety of e-cigarettes, coupled with the absence of a robust regulatory framework in the UK, health professionals should encourage their patients to use a regulated and licensed nicotine replacement therapy to help quit smoking. Where a patient is unable or unwilling to use or continue to use an approved and tested nicotine replacement therapy, health professionals may advise patients that while e-cigarettes are unregulated and their safety cannot be assured, they are likely to be a lower risk option than continuing to smoke.

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Nicotine aggravates the brain postischemic inflammatory response

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Nicotine aggravates the brain postischemic inflammatory response

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Bradford ST, Stamatovic SM, Dondeti RS, Keep RF, Andjelkovic AV. Nicotine aggravates the brain postischemic inflammatory response. *Am J Physiol Heart Circ Physiol* 300: H1518–H1529, 2011. First published January 14, 2011; doi:10.1152/ajpheart.00928.2010.—A substantial body of evidence suggests that nicotine adversely affects cerebral blood flow and the blood-brain barrier and is a risk factor for stroke. The present study investigated the effect of nicotine on cerebrovascular endothelium under basal and ischemia/reperfusion injury under in vivo condition. Nicotine (2 mg/kg sc) was administered to mice over 14 days, which resulted in plasma nicotine levels of ~100 ng/ml, reflecting plasma concentrations in average to heavy smokers. An analysis of the phenotype of isolated brain microvessels after nicotine exposure indicated higher expression of inflammatory mediators, cytokines (IL-1β, TNF-α, and IL-18), chemokines (CCL2 and CX_3CL1), and adhesion molecules (ICAM-1, VCAM-1, and P-selectins), and this was accompanied by enhanced leukocyte infiltration into brain during ischemia/reperfusion ($P < 0.01$). Nicotine had a profound effect on ischemia/reperfusion injury; i.e., increased brain infarct size ($P < 0.01$), worse neurological deficits, and a higher mortality rate. These experiments illuminate, for the first time, how nicotine regulates brain endothelial cell phenotype and postischemic inflammatory response at the brain-vascular interface.

inflammation

CIGARETTE SMOKING IS WIDELY recognized as a major modifiable risk factor for stroke (5, 12). There is a dose-response relationship between cigarette consumption and stroke risk, whereas smoking cessation leads to a prompt stroke risk reduction (33). Chronic exposure to tobacco or nicotine, a major active component of cigarettes, can cause cerebral vasoconstriction, decrease cerebral blood flow (CBF) and enhance ischemic brain injury following transient middle cerebral artery occlusion (MCAO) in rats (13, 38). In addition, some recent findings have indicated that cigarette smoking, and particularly nicotine, has a profound proinflammatory effect, causing a chronic inflammatory state with increased levels of circulating leukocytes, C-reactive protein, and fibrinogen, as well as enhanced leukocyte rolling and adhesion in the cerebral microcirculation and chemoattractant activity for neutrophil migration (7, 17, 47).

Proinflammatory effects of nicotine have been described at several target sites, including the vascular interface, leukocytes, and respiratory and intestinal epithelia. At these targets, nicotine alters expression of proinflammatory mediators, directly or indirectly aggravating the outcome of inflammation (2, 27, 34, 35). There is also evidence that nicotine can induce ICAM-1 and VCAM-1 expression on human umbilical vein endothelial cells (HUVEC) (2). At the level of the central nervous system and the blood-brain barrier (BBB), the effects of nicotine are still unclear. Some studies have indicated that nicotine regulates leukocyte rolling and adhesion mediated by P-selectin and CD18 (47). Nicotine may also alter nitric oxide levels, a molecule critical in regulating cerebrovascular tone and endothelial cell-leukocyte interactions (14).

Postischemic inflammation is considered a significant contributor to secondary brain injury after ischemic stroke (9, 30). The central event in postischemic inflammation is the recruitment of neutrophils that arrive first at the site of inflammation, followed by monocyte/macrophages. This is a multifactorial process involving chemotactic signals that promote the directional migration of leukocytes, adhesion, receptor/ligand interaction at the microvascular endothelial surface, and matrix metalloproteinase production needed for extracellular matrix breakdown and leukocyte extravasation (9, 30). Leukocyte infiltration into the ischemic territory is also associated with activation of microglia and astrocytes that have the potential to contribute further to the inflammatory cascade (9). Despite the fact that smoking and nicotine are indicated as risk factors for stroke and that nicotine is involved in the upregulation of some essential proinflammatory mediators (e.g., IL-8, IL-1β, TNF-α, and ICAM-1) during ischemia/reperfusion (I/R) injury in kidney, liver, coronary artery endothelial cells, and HUVEC, the effect of nicotine on cerebral postischemic inflammation is still unclear (3, 44).

The present study investigates the impact of nicotine on the postischemic inflammatory response at the BBB. Our results show that exposing the BBB to nicotine levels reflecting those in plasma of average to heavy smokers upregulates a broad range of cytokines, chemokines, and adhesion molecules at the vascular interface and significantly alters the inflammatory response during basal and I/R conditions.

MATERIALS AND METHODS

All procedures were performed in strict accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of University of Michigan.

Nicotine treatment. C56BL/6 mice (22–28 g) were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg ip) and miniosmotic pumps (infusion rate 0.5 μl/h; model 2002 Azlet osmotic pump; DURECT, Cupertino, CA) were implanted in a subcutaneous pocket created by making a small incision in the skin between the scapulae. The pumps were filled with normal saline (0.9% NaCl, vehicle) or nicotine (Sigma Aldrich, St Louis, MO) dissolved in saline at a concentration sufficient to deliver 0.5, 2.0, and 5.0 mg/kg of nicotine over 14 days. Nicotine levels were evaluated in plasma samples collected by cardiac puncture (4–6 mice per group). Nicotine was measured using a liquid chromatography/tandem mass spectrometry (23) by the Biomedical Mass Spectrometry facility at the University of Michigan using a Finnigan TSQ Quantum Ultra AM. The duration

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of exposure, 14 days, was chosen on the basis of our evaluation of physiological parameters of nicotine-treated mice and their survival rate after transient MCAO. Fourteen days of exposure provided a stable level of nicotine in plasma and did not affect animal physiological parameters.

MCAO. Experiments were performed on male C57BL/6 (22–25 g) mice (Jackson Laboratory, Bar Harbor, MA). Mice were anesthetized with ketamine and xylazine (100 and 10 mg/kg ip). Body temperature was maintained at 37 ± 0.5°C by means of a heating blanket and a heating lamp during the entire experimental procedure. Focal cerebral ischemia was induced by left MCAO using an intraluminal filament technique (10). Briefly, the common carotid artery was exposed through a midline incision in the neck. A 6–0 silicon suture was next introduced into the external carotid artery and advanced into the internal carotid artery a distance of 10–11 mm from the common carotid artery bifurcation according to animal weight. MCAO was confirmed by a Laser Doppler Flow probe (Model BPM System; Vasomedics, St. Paul, MN) positioned at 3 mm posterior and 5 mm lateral to bregma. After 30 min of MCAO, the mice were reperfused by suture withdrawal and then allowed to awake from anesthesia. Sham-operated animals underwent all procedures except the actual MCAO. Physiological parameters (P_{O_2}, P_{CO_2}, pH, blood glucose, and regional CBF) were monitored before, during, and after MCAO. A reperfusion period was 3 days. During reperfusion, neurological deficits were evaluated with the following scoring scheme: 0, no deficits; 1, flexion of the torso and contralateral forelimb when lifted by the tail; 2, contralateral forelimb weakness upon application of pressure to the side of the body; 3, circling to the affected side; 4, no spontaneous locomotor activity.

Brain water content and electrolytes. Brain water content was measured by the wet/dry weight method. Samples were taken from ischemic and nonischemic hemispheres. After decapitation under deep isoflurane anesthesia, brains were weighed wet and then oven dried at 100°C for 48 h and reweighed. Brain water content (%) was calculated as [(wet weight − dry weight)/wet weight] × 100%. (10).

Morphometric measurement of infarct volume. Animals were euthanized from 1 to 5 days after transient MCAO, and the brain was removed and sliced. Slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich) solution for 1 h at 37°C. The area of infarction in each slice was determined by a computerized image-analysis system, and the volume of infarction was calculated by multiplying the distance between sections. In addition, to account for cerebral edema or resolution of the infarct, an indirect measurement of infarction was performed. Infarct volume was calculated as [contralateral hemisphere volume − (ipsilateral hemisphere volume − measured injury volume)] (10). Cresyl violet staining of 200-μm-thick serial sections was also used to examine infarct size after 3 days of reperfusion (10).

RT^2 profiler PCR array and real-time PCR. Isolated microvessels and whole brain with or without nicotine treatment were collected at the end of experiments. For analysis of I/R injury, isolated brain microvessels and brain area (penumbra) around the ischemic lesion (1 mm thick) were collected using "pinch-out" method (10). The corresponding contralateral region was also collected. For isolated microvessels brain tissue was mechanically dissociated and homogenized in the Dounce type of homogenizer. After washing with Hanks balanced solution, the myelin and erythrocytes were cleaned by 18% Dextran solution and Percol gradient retrospectively (9). Total RNA was prepared using TRIZOL (Invitrogen, Carlsbad, CA). Single-strand cDNA from 2 μg total RNA was synthesized using RT^2 first strand kit, and real-time PCR was performed according to the RT^2 Profiler PCR Array System (SABioscience, Frederick, MD) using SYBR Green PCR Master Mix in an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY). The PCR arrays Mouse Inflammatory Cytokines and Receptors (SABioscience) were repeated three times and the data analyzed using PCR Array analysis software (SABioscience). In addition, real-time PCR analysis was performed to compare RNA expression of select genes (IL-1β, TNF-α, IL-18, CX_3CL1, CCL2, CXCL5, CD40, CCL4, and IL-6ra) between nicotine-treated and non-treated experimental groups with or without I/R injury. All primer sets were from SABioscience.

Cytokine antibody array. The Mouse Cytokine Antibody Array 3 (RayBiotech, Norcross GA) was used to simultaneously detect and semiquantify 62 cytokines in samples collected from all experimental groups. For tissue, samples were homogenized in 1.8 ml Tris buffer solution (pH 8.5) supplemented with 1% Triton X-100. Protein level was evaluated using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL) and for each sample was adjusted to 2 mg/ml. The cytokine antibody array was performed according to the manufacturer's instructions. Membranes were developed with the Pierce ECL substrate kit (Thermo Fisher Scientific) and underwent densitometric analysis using ImageJ analysis software (NIH, Bethesda, MD). The relative level of inflammatory cytokines was evaluated using software provided by the manufacturer. In addition, IL-1β, IL-1α, IL-6, IL-12, IFN-γ, TNF-α, CCL5, CCL2, CCL3, CXCL12, and CCL11 protein levels were quantified by ELISA assay kit (SABioscience).

Quantitative immunohistochemistry. Brain samples were fixed in 4% paraformaldehyde for 18 h and then cryoprotected with sequential immersions in 10% and 20% sucrose solutions and then cut into 50-μm-thick coronal sections with a freezing microtome. After that samples were preincubated in blocking solution (5% bovine serum albumin, 5% normal goat serum, 0.05% Tween and PBS), and then incubated overnight with primary antibody rat anti-mouse Ly6G antibody (BD Bioscience, San Jose, CA) and anti-myeloperoxidase (MPO) antibody (HyCult Biotechnology, Uden, The Netherlands) at 4°C. Reaction was visualized by Texas red-conjugated anti-rat (Sigma-Aldrich) or anti-rabbit antibody (Vector Laboratory, Burlingame, CA). All samples were viewed on a confocal microscope (LSM 510, Zeiss, Jena, Germany). For quantification, 50 coronal brain slices (25 slices in front and 25 behind the "middle line" of the visible lesion). Microscope data were acquired with a ×10 objective numerical aperture with constant laser power (45% of laser power), pinhole, zoom, focus, gain, and duration of image capturing. A total of 20 images was randomly selected and captured per slide. The immunolabeled cells were counted in areas surrounding ischemic lesion. Five mouse brains per group were analyzed. In the sham-operated group, brain areas were analyzed corresponding to those analyzed in ischemic mice. Slides were coded so that the counter was blind to the identity of the slides being counted.

Statistical analysis. All values are expressed as means ± SD. One-way ANOVA followed by Bonferroni post hoc analysis, Chi-squared tests (neurological scores), and Kaplan-Meier survival curves were used (Prism analysis software). A P value <0.05 was regarded as statistically significant.

RESULTS

Plasma nicotine and cotinine concentrations. Initial studies were performed where mice received either one of three doses of nicotine (0.5, 2.0 or 5.0 mg/kg sc) or vehicle (0.9% NaCl) for 14 days. In the nicotine-treated groups, at *days 3*, *7*, and *13* the plasma nicotine levels were 0.52 ± 0.03, 2.7 ± 0.1, and 4.5 ± 0.9 ng/ml in the 0.5 mg/kg group, 67 ± 12, 98 ± 10, and 99 ± 3 ng/ml in the 2.0 mg/kg group, and 87 ± 10, 155 ± 14, and 184 ± 21 ng/ml in the 5.0 mg/kg group. The plasma concentrations of the nicotine metabolite cotinine at the same time points were 3.2 ± 1, 6.3 ± 1.1, and 10.2 ± 2.1 ng/ml in the 0.5 mg/kg group, 96 ± 5, 139 ± 7, and 169 ± 19 ng/ml in the 2.0 mg/kg group, and 201 ± 23, 302 ± 34, and 523 ± 36 ng/ml in the 5.0 mg/kg group (Supplemental Fig. S1, *A* and *B*; supplemental material for this article is available online at the *American Journal of Physiology Heart and Circulatory Phys-*

iology website). These nicotine and cotinine levels are similar to those found in moderate, average, and heavy smokers (>5 mg/ml, 10–100 ng/ml, and <200 ng/ml) on the basis of epidemiological and biochemical studies (20). All nicotine-treated mice did not show changes in pH, P_{O_2}, P_{CO_2}, and glucose, but they did have significantly reduced body weight from 10 days of nicotine treatment (e.g., 89% and 75% of initial body weight at 10 and 14 days, respectively in the 2 mg/kg group; Supplemental Fig. S1*C*). In addition, the mice did not display symptoms of nicotine intoxication like excitability, shivering, tremor, or diarrhea even in the experimental group, which received the high dose of the nicotine. For most of our further experiments we primarily focused on the 2 mg/kg nicotine group.

Proinflammatory effects of nicotine at the BBB. To examine the potential proinflammatory action of nicotine at the BBB, mice were divided into two groups that received either nicotine (2 mg/kg sc) or vehicle (0.9% NaCl) for 14 days. The effect of nicotine on the BBB inflammatory phenotype was examined at the gene and protein levels. Pro- and anti-inflammatory gene expression was evaluated using a Real-Time Profile PCR Array to analyze expression of 87 cytokines, cytokines receptors, chemokines, chemokines receptor, and other genes involved in the inflammatory response. Three independent samples of isolated microvessels or whole brain tissue from nicotine-treated mice ($n = 3$) were compared with control (nonexposed) mice. Isolated microvessels were not additionally trypsin digested to preserve the BBB in situ. Thus samples contained astrocyte foot processes as well as perivascular macrophages and pericytes, as confirmed by immunohistochemistry (data not shown).

Genes were evaluated on the basis of the criteria of at least a 2.5-fold up- or downregulation compared with control and that they were regulated in more than 70% of comparisons. Using these stringent selection criteria, we identified 36 genes that were upregulated (2.9- to 15.8-fold) and 21 genes that were downregulated (2.7- to 12-fold) in microvessels (Fig. 1*A*), and 37 genes upregulated (2.7- to 36-fold) and 21 genes downregulated (2.6- to 20-fold) in brain tissue (Fig. 1*A*). The significantly upregulated genes were cytokines IL-18, IL-1β, TNF-α, IL-1α (13-, 13-, 12-, and 6-fold increases, respectively) and chemokines CX_3CL1, CCL2, CXCL5, CCL8, and CXCL1 (14-, 8-, 6-, 5-, and 5-fold increases, respectively), indicating a strong proinflammatory response at the BBB in the presence of nicotine. In addition, there was higher expression of caspase-1 (12-fold increase), complement C3 and CD40 ligand (4.5-fold), suggesting not only a proinflammatory but also a proatherogenic effect of nicotine. There was also a significant downregulation of some anti-inflammatory cytokines and signaling molecules such as Bcl6, CCL25, CCL6, IL-13, IL-10, and Toll interacting protein (Tollip) (12-, 9-, 26-, 12-, and 17-fold decreases, respectively) in the brain as well as the BBB of mice exposed to nicotine. Thus nicotine causes an imbalance in pro- and anti-inflammatory responses that may induce vascular injury (Fig. 1*A*).

To confirm the PCR array findings, single real-time RT-PCR analyses were performed for select proinflammatory mediators. IL-1β, TNF-α, IL-18, CX_3CL1, CCL2, CXCL5, and CD40L all showed significant increases in expression after nicotine treatment compared with vehicle treatment (Fig. 1, *B* and *C*).

In addition to gene expression, the protein levels of proinflammatory mediators were analyzed. The analysis included total secreted amount of cytokines/chemokines (observed by ELISA) and protein expression of microvessels and brain tissue (antibody based protein array). Following the pattern of gene expression, there was significant level of secreted proinflammatory cytokines/chemokines [e.g., IL-1α, IL-1β, IL-12, TNF-α, CCL2, IL-6, INF-γ, granulocyte/macrophage colony-stimulating factor (GM-CSF), G-CSF, and CXCL12] in brain tissue and BBB after exposure to nicotine ($P < 0.001$) compared with controls (Fig. 2*B*). In response to nicotine, brain tissue and the BBB in situ also had 5- to 15-fold increases in a set of platelet-dependent chemokines/cytokines and proteins like CCL17, CXCL12, PF4, CCL3, CXC3CL1, and CD40, as well as the adhesion molecules ICAM-1, P-selectin, and VCAM-1 (Fig. 2*A*). This strongly supports a proatherogenic effect of nicotine at the level of the BBB. Similar pattern of pro- and anti-inflammatory cytokines expression was also presented in brain microvessels and brain tissue of mice exposed to low and high doses of nicotine for 14 days although that magnitude of cytokine expression was directly correlated to the dose of nicotine (data not shown).

Effect of nicotine on brain I/R injury. To investigate the effects of nicotine on ischemic brain damage, we first examined infarct volume and neurological deficits in nicotine- and vehicle-treated mice (exposure time was 14 days) subjected to transient MCAO with reperfusion times lasting up to 10 days. Physiological parameters (pH, P_{O_2}, P_{CO_2}, glucose level, and regional CBF) before MCAO and after 30 min of reperfusion were not significantly different between nicotine- and vehicle-treated groups (Supplemental Table S1). However, there were marked differences in neurological outcome and survival (Fig. 3, *A* and *B*). Nicotine-treated mice had worse neurological deficits. On a scale of 0–4, 80% of the mice showed no spontaneous locomotor activity (score 4) or circling to the affected side (score 3) evaluated from *days 0–5* of reperfusion. In contrast, a score of 0–2 was found in 80% of vehicle-treated mice. There was also a lower survival rate in nicotine-treated mice. Forty percent of nicotine-treated mice survived until *day 3* of reperfusion, and none survived to *day 5*, compared with 100% survival in vehicle-treated mice at *days 3* and *5*. Furthermore, infarct volume was greater with nicotine treatment compared with vehicle-treated mice in dose-dependent manner [*day 3*: vehicle-treated mice 113.9 ± 7.6 mm^3 vs. nicotine-treated mice 132.7 ± 16.3 mm^3, $P < 0.001$ (2 mg/kg group) and 137.2 ± 13.8 mm^3, $P < 0.001$ (5 mg/kg group); Fig. 3*C*]. Analyzing the regional distribution of the infarct, we found that in all experimental groups infarct lesion was present in cortex and striatum. However, the nicotine-treated mice had bigger striatal and cortical infarcts compared with vehicle-treated mice (Fig. 3*D*). Again, there was a close correlation with the dose of nicotine and infract size in nicotine-treated mice. Mice exposed to average (2 mg/kg group) and high (5 mg/kg group) doses of nicotine had significantly increased striatal ($P < 0.001$) and cortical ($P < 0.05$ and $P < 0.001$, respectively). In addition, nicotine-treated mice had increased brain water content (edema) in the ischemic (but not nonischemic) hemisphere compared with vehicle-treated mice at *day 3* of reperfusion in a dose-dependent manner (Fig. 3*E*). Because of the potential confounding effects of edema and infarct resolution, an indirect measure of infarct volume was also used. Using this

A

			Nicotine (isolated brain microvessels)	Nicotine brain tissue
Refseq	Symbol	Description	fold increase	fold increase
NM_007551	Cxcr5	Chemochine (C-X-C motif) receptor 5	10.4	11.1
NM_009778	C3	Complement component 3	3	5.62
NM_009807	Casp1	Caspase 1	12.4	16.3
NM_011329	Ccl1	Chemokine (C-C motif) ligand 1	2.5	3.69
NM_011330	Ccl11	Chemokine (C-C motif) ligand 11	2.6	5.54
NM_011331	Ccl12	Chemokine (C-C motif) ligand 12	4	6.6
NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	2.9	3.1
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	8.0	11.3
NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	3	6
NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	3.1	7.5
NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	5.2	8
NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	6.9	9.9
NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	4.5	7.1
NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	5.8	9
NM_009913	Ccr9	Chemokine (C-C motif) receptor 9	4.5	6.3
NM_007768	Crp	C-reactive protein, pentraxin-related	6.1	9.6
NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	14	19
NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	5.1	9.6
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	4.2	8
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	5	6.3)
NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	4	8.3
NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	6	8
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	15.8	21
NM_008357	Il15	Interleukin 15	4.6	9
NM_008360	Il18	Interleukin 18	13.3	10
NM_010554	Il1a	Interleukin 1 alpha	6.3	9.3
NM_008361	Il1b	Interleukin 1 beta	13.2	15
NM_008362	Il1r1	Interleukin 1 receptor, type I	4	5
NM_019450	Il1f6	Interleukin 1 family, member 6	2.3	3.9
NM_008368	Il2rb	Interleukin 2 receptor, beta chain	3.4	6.3
NM_013563	Il2rg	Interleukin 2 receptor, gamma chain	4	5.3
NM_010556	Il3	Interleukin 3	8	7.3
NM_010559	Il6ra	Interleukin 6 receptor, alpha	3.2	3
NM_009909	Il8rb	Interleukin 8 receptor, beta	2.2	2
NM_013693	Tnf	Tumor necrosis factor	12	14
NM_011616	Cd40lg	CD40 ligand	4.5	3
Refseq	Symbol	Description	fold decrease	fold decrease
NM_009744	Bcl6	B-cell leukemia/lymphoma 6	-12	-9
NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	-3	-2.1
NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	-7	-9
NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	-19	-23
NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	-12	-17
NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	-2	-2
NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	-2	-2.9
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	-2	-3.3
NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	-3.5	-3.6
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	-4.5	-6.6
NM_008337	Ifng	Interferon gamma	-2	-3
NM_010548	Il10	Interleukin 10	-26	-32
NM_008355	Il13	Interleukin 13	-12	-14
NM_027163	Il1f8	Interleukin 1 family, member 8	-2	-3.1
NM_008362	Il1r1	Interleukin 1 receptor, type I	-10	-12
NM_013563	Il2rg	Interleukin 2 receptor, gamma chain	-4	-5
NM_021283	Il4	Interleukin 4	-4	-6.3
NM_009263	Spp1	Secreted phosphoprotein 1	-2	-3
NM_023764	Tollip	Toll interacting protein	-17	-14
NM_011577	Tgfb1	Transforming growth factor, beta 1	-3.6	-4
NM_011609	Tnfrsf1a	TNF receptor superfamily, member 1a	-3.6	-5.3
NM_023764	Tollip	Toll interacting protein	-2.3	-3

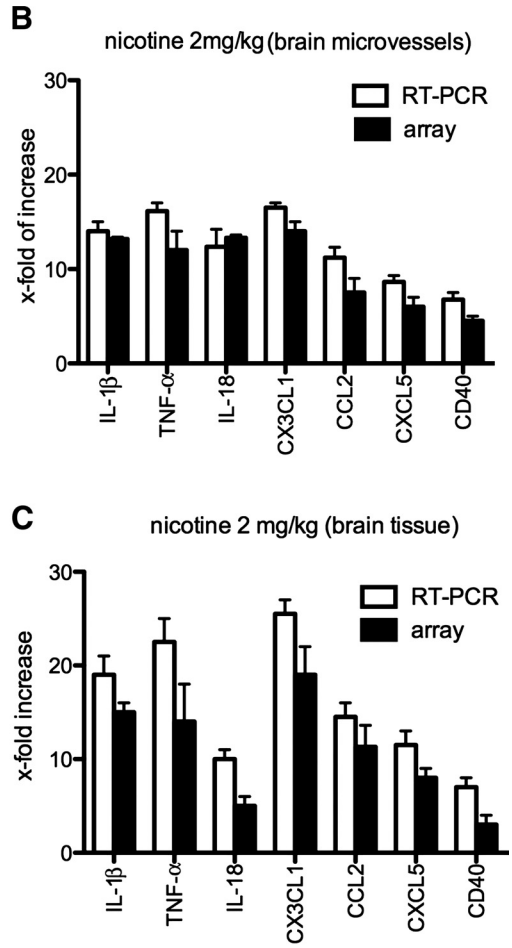


Fig. 1. *A*: list of the transcripts modulated by nicotine in brain tissue and isolated brain microvessels from mice treated with nicotine (2 mg/kg) for 14 days. Fold increase/decrease indicate the level of up- or downregulation of transcripts compared with control (vehicle-treated mice). Three independent samples were analyzed by RT^2 real-time PCR array. *B* and *C*: quantitative real-time PCR for IL-1β, TNF-α, IL-18, CX_3CL1, CCL2, CXCL5, and CD40 was carried out on RNA from isolated brain microvessels (*B*) and brain tissue (*C*) from nicotine-treated ($n = 3$) and vehicle-treated mice ($n = 3$). Expression of target genes was normalized to control vehicle-treated mice. Values are presented as means ± SD. The fold changes obtained with RT-PCR were similar to those obtained by the PCR array.

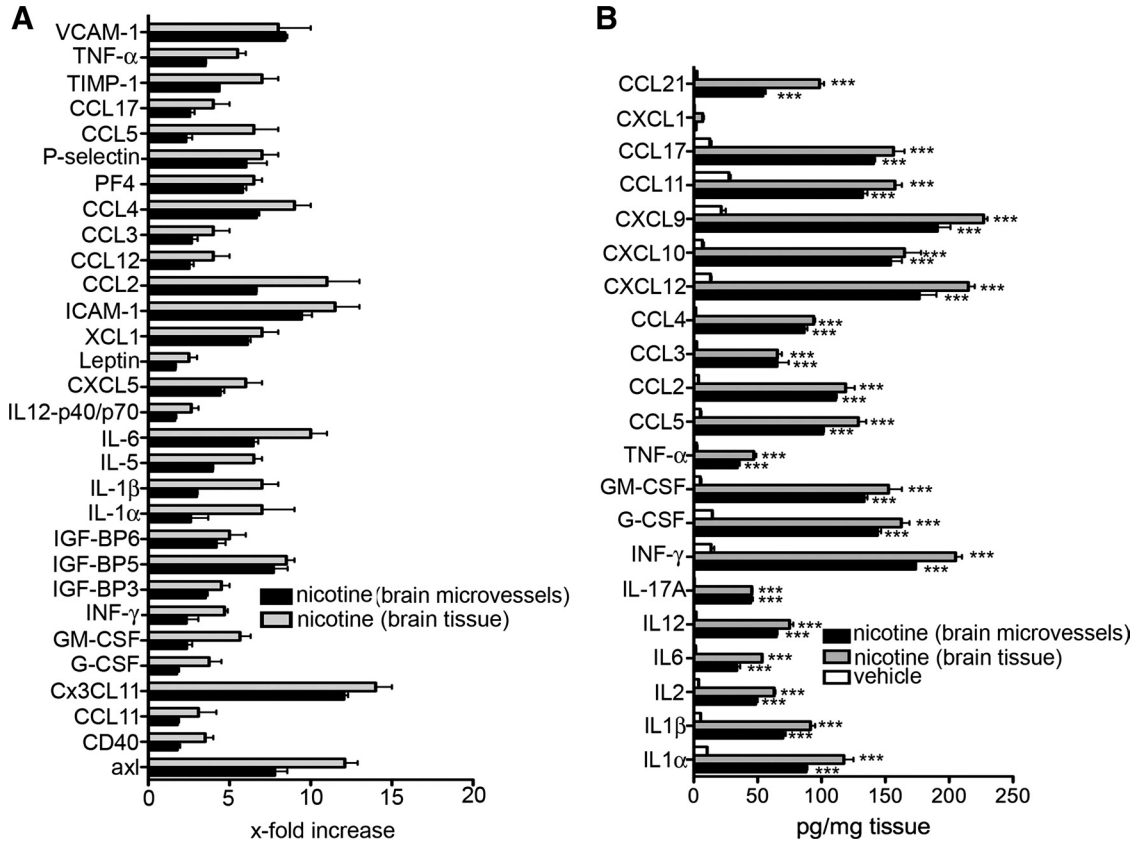


Fig. 2. Cytokine antibody array (*A*) and ELISA (*B*) analysis of isolated brain microvessels and brain. For the cytokine array, 62 cytokines, adhesion molecules, and chemokines were assayed in 3 samples. Values after nicotine treatment (2 mg/kg; 14 days) were normalized to brain microvessels or brain tissue isolated from vehicle-treated mice. For the ELISA, samples (brain microvessels or brain tissue) were taken after mice had been treated with nicotine (2 mg/kg) or vehicle for 14 days. Values represent means ± SD from 3 independent brain samples. ***$P < 0.001$. GM-CSF, granulocyte/macrophage colony-stimulating factor; TIMP, tissue inhibitor of matrix metalloproteinase; INF, interferon.

measure, average and high dose of nicotine-treated mice still showed significantly greater infarct volumes compared with vehicle-treated mice ($P < 0.001$; Fig. 3*F*). Taken together, these data demonstrate that nicotine worsens neurological deficits and increases infarct volume, contributing to the progression of postischemic injury.

Effects of nicotine on the expression of inflammatory mediator after ischemia. Transient focal ischemia leads to increased expression of several proinflammatory genes, and the resulting inflammation significantly contributes to stroke outcome (10). Using a Proinflammatory Cytokines & Receptors GeneArray, we analyzed the profile of proinflammatory gene expression in the core of ischemic lesion and in surrounding penumbra as well as in the isolated microvessels from the penumbra at *day 3* of reperfusion in nicotine (2 mg/kg group)- and vehicle-treated mice. The following groups were examined: *1*) chronically nicotine-treated MCAO mice (nicotine + I/R); *2*) vehicle-treated MCAO mice (vehicle + I/R), and *3*) vehicle-treated sham-operated mice treated (vehicle). In general, nicotine acted as strong proinflammatory agent with an increase (2- to 35-fold) in mRNA levels of 46 proinflammatory cytokines/chemokines during reperfusion compared with vehicle-treated MCAO mice (Fig. 4*A*). For example, nicotine-treated MCAO mice had increased RNA levels of chemokines CCL2, CCL7, CCL9, CXCL13, CX_3CL1, and CXCL5 (6- to 10-fold increase in brain microvessels and 10- to 22-fold increase in brain tissue) compared with vehicle-treated MCAO mice (Fig. 4*A*). Nicotine-treated MCAO mice also had increased levels of the cytokines IL-15, IL-18, IL-1β and TNF-α (10- to 33-fold increase in brain microvessels and 12- to 35-fold increase in brain tissue), as well as complement C3, caspase 1, and CD40L (up to 25-fold increase in microvessels and brain tissue) compared with vehicle-treated MCAO mice. There were also significant decreases in the expression of some anti-inflammatory cytokines or receptors in nicotine-treated MCAO mice, including IL-10, IL-1r1, TGF-β1, and Tollip (up to 12-fold decrease in brain microvessels and up to 16-fold decrease in brain tissue). These results were confirmed by single real-time RT-PCR analysis (Fig. 4).

Further analysis was focused on the protein levels of secreted or expressed proinflammatory mediators in the area of penumbra as well as in the isolated brain microvessels. Nicotine had a profound effect on microvessel and brain tissue levels as manifested by 2- to 25-fold increases in proinflammatory mediators compared with vehicle-treated MCAO mice (Fig. 5*A*). In particular, adhesion molecules (such as VCAM-1, ICAM-1, P-selectin, and CX_3CL1) and cytokines/chemokines (such as GM-CSF, G-CSF, cytokine responsive gene (CRG), CCL2, CXCL4, and CXCL10) had enhanced protein levels in nicotine-treated MCAO mice. There were significant increases ($P < 0.001$) in the production of cytokines (IL-6, IL-17, IL-1β, and TNF-α) and chemokines (CCL2, CCL5, CXCL10,

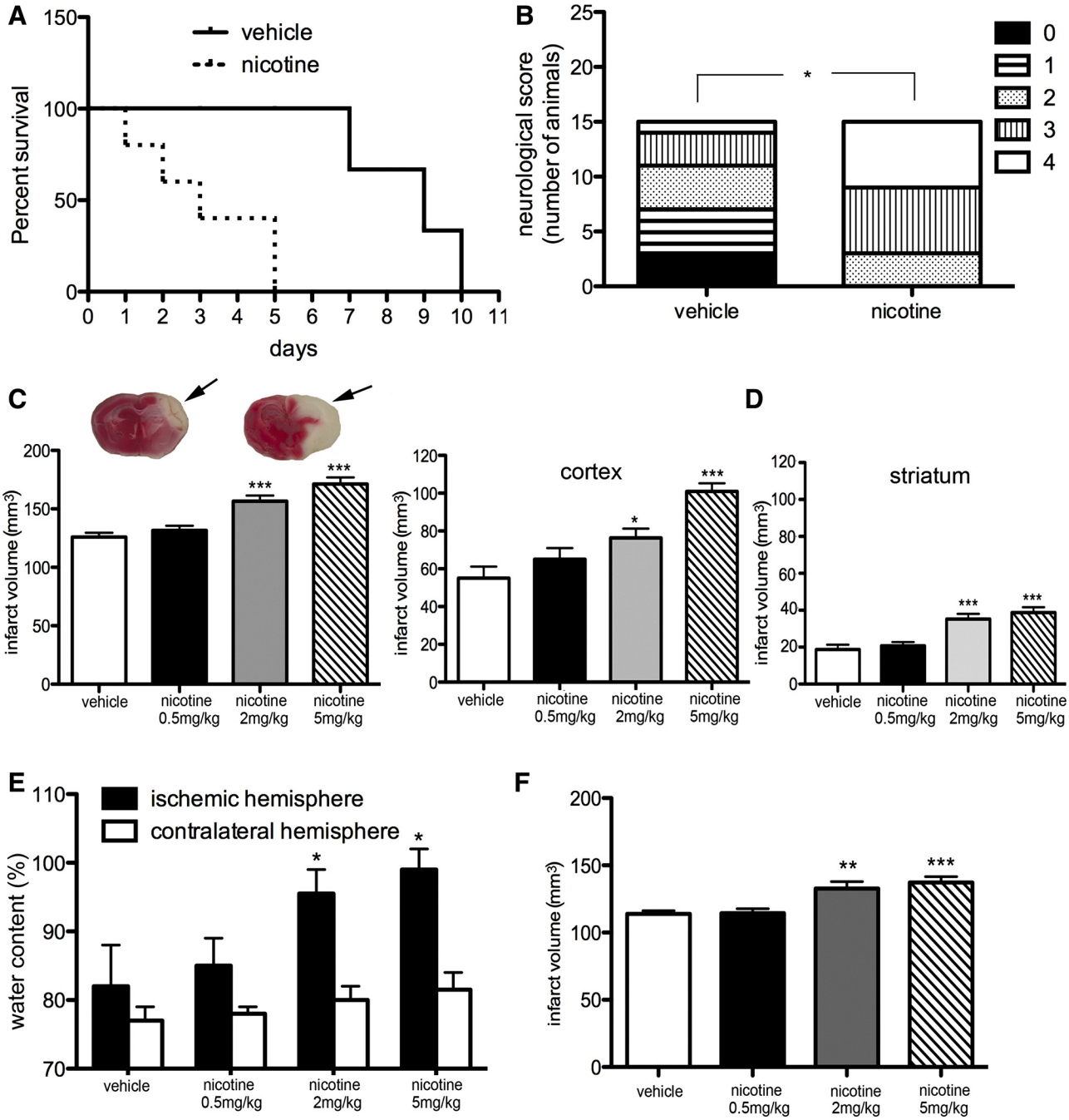


Fig. 3. *A*: Kaplan-Meier survival curve in mice exposed to either nicotine (2 mg/kg) or vehicle (0.9% NaCl) for 14 days followed by induction of middle cerebral artery occlusion (MCAO). *B*: summary of neurological scores in nicotine- (2 mg/kg) and vehicle-treated mice at *day 3* after transient MCAO. No neurological deficit scores are 0; maximal deficit score is 4. Vehicle ($n = 15$) and nicotine ($n = 15$), $*P < 0.05$ by Chi-squared test. *C*: 2,3,5-triphenyltetrazolium chloride-stained coronal sections of brain illustrating typical infarcts (arrows) 3 days after reperfusion in nicotine- and vehicle-treated mice. Bar graph showing infarct volumes at *day 3* after transient MCAO in vehicle ($n = 10$) and nicotine (0.5, 2.0, and 5.0 mg/kg) ($n = 7$) mice. *D*: cortical and striatal infarct volume in same experimental animals as in *C*. Values are means ± SD. $***P < 0.001$. *E*: brain edema formation after MCAO was evaluated by measuring the brain water content in ischemic and contralateral hemispheres at *day 3* after transient MCAO. Values are means ± SD for nicotine-treated mice (0.5, 2.0, and 5.0 mg/kg; $n = 7$) vehicle-treated mice ($n = 7$). *F*: indirect measure (to correct for edema/infarct resolution) of total infarct volume at *day 3* after transient MCAO in vehicle- and nicotine-treated (0.5, 2.0, and 5.0 mg/kg) mice. $**P < 0.01$.

CCL17, and CCL11) after brain I/R injury in the mice exposed to nicotine compared with vehicle-exposed MCAO mice (Fig. 5*B*). Thus nicotine has a proinflammatory effect, and this, in the case of brain I/R injury, aggravates the expression of most proinflammatory mediators, which in turn may contribute to the enhanced brain I/R injury in nicotine-treated mice.

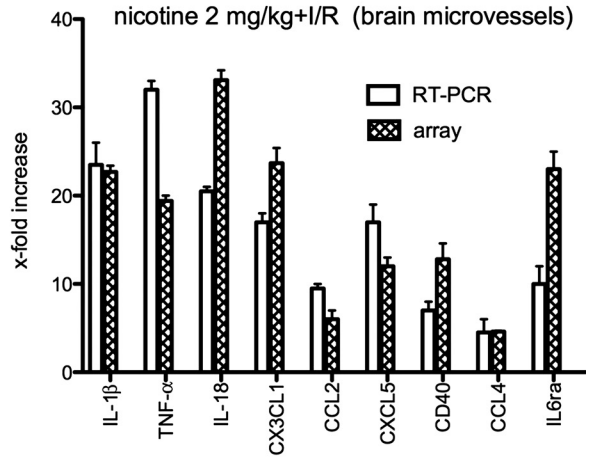
Effects of nicotine on leukocyte infiltration after ischemia. Immunohistochemical analysis of brain tissues from mice exposed to nicotine (2 mg/kg group) or vehicle in vivo did not show significant infiltration of neutrophils (MPO+ cells) or monocytes (Ly6G+) in the absence of ischemia (data not shown). However, the ischemia-induced infiltration of neutro-

A

			Nicotine+I/R (Isolated brain microvessels)	Nicotine+I/R (brain tissue)
Refseq	**Symbol**	**Description**	**fold increase**	**fold increase**
NM_007551	Cxcr5	Chemochine (C-X-C motif) receptor 5	16	22
NM_009778	C3	Complement component 3	25	16
NM_009807	Casp1	Caspase 1	14	20.3
NM_011329	Ccl1	Chemokine (C-C motif) ligand 1	10	10
NM_011330	Ccl11	Chemokine (C-C motif) ligand 11	4	11
NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	8.4	12.2
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	6	17
NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	3.7	6.2
NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	3.6	7.3
NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	4.6	7.2
NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	5.5	12
NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	6	10.6
NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	7.8	10
NM_011338	Ccl9	Chemokine (C-C motif) ligand 9	10.6	23
NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	6	12
NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	6	11.4
NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	4	6.8
NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	2.6	11
NM_007719	Ccr7	Chemokine (C-C motif) receptor 7	2.8	6.3
NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	3.1	8.4
NM_009913	Ccr9	Chemokine (C-C motif) receptor 9	3.9	7.2
NM_007768	Crp	C-reactive protein, pentraxin-related	35	20
NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	8.5	14
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	6	6.3
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	7	15.3
NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	6	11
NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13	10.2	15
NM_019932	Pf4	Platelet factor 4	17	18
NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	23.7	26
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	2	3.2
NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	12	22
NM_007721	Ccr10	Chemokine (C-C motif) receptor 10	6.2	16
NM_008357	Il15	Interleukin 15	10.7	12
NM_010551	Il16	Interleukin 16	4.5	6
NM_008360	Il18	Interleukin 18	33.1	35
NM_008361	Il1b	Interleukin 1 beta	22.7	26
NM_019450	Il1f6	Interleukin 1 family, member 6	5	5
NM_013563	Il2rg	Interleukin 2 receptor, gamma chain	2.5	3
NM_008370	Il5ra	Interleukin 5 receptor, alpha	6.5	6
NM_010559	Il6ra	Interleukin 6 receptor, alpha	23	26
NM_010560	Il6st	Interleukin 6 signal transducer	3.8	5
NM_009909	Il8rb	Interleukin 8 receptor, beta	6.2	6.9
NM_008401	ItgM	Integrin alpha M	13	9
NM_013693	Tnfa	Tumor necrosis factor	19.4	26
NM_011609	Tnfsf1a	TNF receptor superfamily, member 1a	14	16
NM_011616	Cd40Lg	CD40 ligand	12.8	13

Refseq	**Symbol**	**Description**	**fold decrease**	**fold decrease**
NM_009744	Bcl6	B-cell leukemia/lymphoma 6	-6	--9
NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	-2	-3
NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	-3.6	-5.6
NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	-3.7	-6
NM_010548	Il10	Interleukin 10	-3.5	-16
NM_008337	Ifng	Interferon gamma	-4	-6
NM_008355	Il13	Interleukin 13	-13	-12
NM_010556	Il3	Interleukin 3	-2	-3
NM_021283	Il4	Interleukin 4	-6	-6
NM_008362	Il1r1	Interleukin 1 receptor, type I	-12.3	-10
NM_008368	Il2rb	Interleukin 2 receptor, beta chain	-17	-23
NM_009263	Spp1	Secreted phosphoprotein 1	-2	-6
NM_011577	Tgfb1	Transforming growth factor, beta 1	-3.7	-11
NM_023764	Tollip	Toll interacting protein	-2.6	-5
NM_011798	Xcr1	Chemokine (C motif) receptor 1	-10	-15

B



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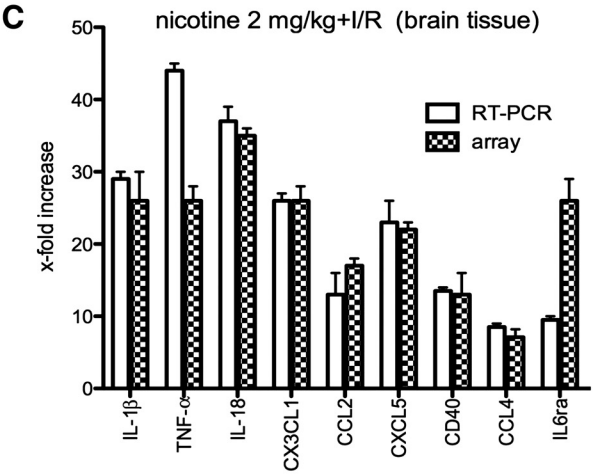

Fig. 4. *A*: list of the genes modulated by nicotine in isolated brain microvessels and brain tissue from around the ischemic area (penumbra) of mice treated with nicotine (2 mg/kg) for 14 days followed by transient MCAO and reperfusion for 3 days. Fold increase/decrease indicates level of up- or downregulation of genes compared with control (mice treated with vehicle for 14 days, followed by transient MCAO and reperfusion for 3 days). Three independent samples were analyzed by real-time RT-PCR array. *B* and *C*: quantitative real-time PCR for IL-1β, TNF-α, IL-18, CX_3CL1, CCL2, CXCL5, CD40, CCL4, and IL-6ra was carried out on RNA of isolated brain microvessels (*B*) or brain tissue (*C*) from nicotine-treated mice with brain ischemia/reperfusion (I/R) injury ($n = 3$) or vehicle-treated mice with brain I/R injury ($n = 3$). Expression of target genes was normalized to control (vehicle-treated mice with brain I/R injury). Values are presented as means ± SD. The real-time PCR confirmed the changes in these genes detected by the PCR array although there were some differences in the absolute level of upregulation with the two techniques.

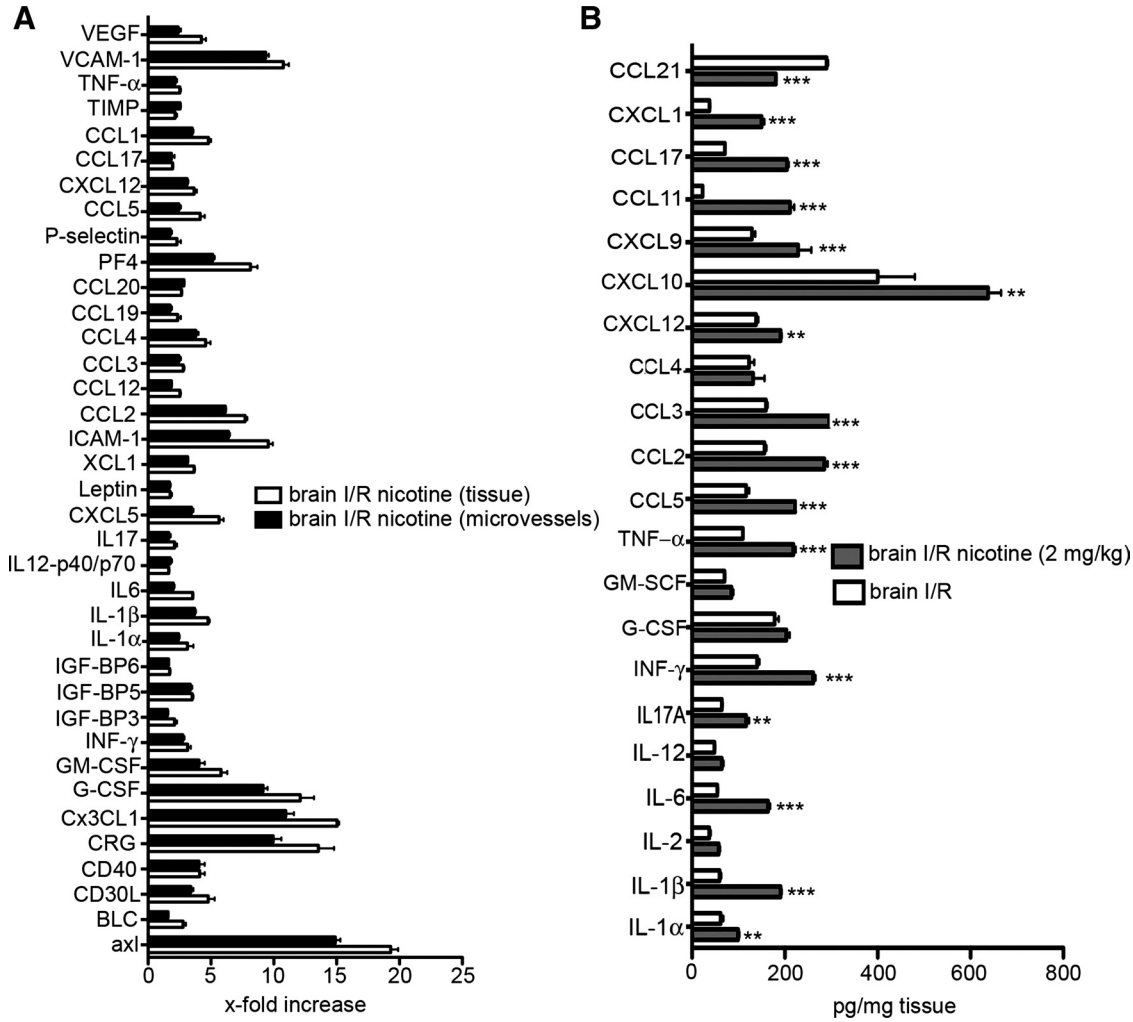

Fig. 5. *A*: cytokine antibody array of brain microvessels and brain tissue from mice exposed to nicotine (2 mg/kg) for 14 days followed by a MCAO for 3 days. For the cytokine array, 62 cytokines, adhesion molecules, and chemokines were assayed in 3 samples. The values were normalized to brain microvessels or brain tissue from the ischemic penumbra from vehicle-treated mice that underwent MCAO with reperfusion. *B*: ELISA analysis of penumbral brain samples collected from mice treated with nicotine or vehicle for 14 days followed by a MCAO for 3 days. Values represent means ± SD from $n = 3$ independent experiments of brain samples or brain microvessels. $^{**}P < 0.01$, $^{***}P < 0.001$ comparing vehicle-treated and nicotine-treated mice that underwent MCAO with reperfusion. BMEC, brain microvascular endothelial cell.

phils and monocytes in brain parenchyma was significantly increased in mice exposed to nicotine vs. vehicle ($P < 0.001$) evaluated at *days 3* and *5* after MCAO (Fig. 6). These data further confirm that nicotine has a profound effect on reperfusion injury and the postischemic inflammatory response.

DISCUSSION

Little is known about the effects of nicotine on the BBB, particularly in the setting of postischemic inflammation. The present study analyzed those potential effects and found that *1*) nicotine altered the BBB phenotype to a proinflammatory one; *2*) this alteration is associated with worsening of I/R injury (increased infarct volume, mortality, neurological deficits, and inflammation); and *3*) the nicotine-induced proinflammatory response is characterized by significant increases in proinflammatory cytokine/chemokine expression and production although it did not alter the specific type (e.g., neutrophil vs. monocyte) of the postischemic inflammatory response. These findings are discussed below.

We would like first to address issues regarding our model system. We are aware that nicotine is just one component of cigarettes, there being ~4,000 compounds detectable in tobacco tar. However, there are some obstacles to study those components. There are still limitations in detection assays for most of the compounds, affecting our ability to determine their effects. On the other hand, several epidemiological and biochemical studies on the basis of the habit of smokers have determined plasma nicotine levels, showing that nonsmokers or passive smokers have less than 10 ng/ml, that modest or average smokers have nicotine levels between 10–100 ng/ml, whereas heavily smokers have nicotine levels of more than 100 ng/ml (6, 20). Using this "empirical" classification, we chose nicotine doses of 0.5, 2.0, and 5.0 mg/kg, which achieved plasma nicotine concentrations corresponding to passive, average, and heavy smokers. Prolonged exposure to these selected doses may best represent the effects that nicotine, one of the major compounds in tobacco, could have on cerebral endothelium and brain I/R injury in smokers. It should be

noted, however, that other compounds in cigarette smoke might potentially modulate the effects of nicotine. Nicotine replacement therapies, (i.e., nicotine chewing gum, inhaler, or skin patches) utilize slightly lower doses of pure nicotine in 6-wk cigarette cessation programs (5, 6). However, there is an increasing body of evidence that they have an effect similar to the one described for nicotine. Although there are lower doses of nicotine in plasma than average smokers, clinical and epidemiological studies indicate that pure nicotine usage may have severe effects on patients with cardiovascular disease and may cause some inflammatory reactions (24). This suggests our model may in part represent the potential changes, which can occur during nicotine replacement therapy and offer some new directions regarding dosage and time of exposure. It is also important to take into account that under certain conditions nicotine can have more anti-inflammatory and protective effects as indicated in several recent studies (20, 28), and this study aimed to clarify whether absorbed nicotine would have damaging or beneficial effects.

The miniosmotic pump delivery system was chosen because it provides a stable level of nicotine in plasma, as can also be seen in smokers. In contrast, other systems (nicotine delivered though water, inhalation device, etc.) show very unstable levels of nicotine in plasma and are often associated with hypoxic episodes and stress of repeated application, effects eliminated with the miniosmotic delivery system (40, 41). It is important also to pinpoint that, via the miniosmotic delivery of nicotine, mice did not exhibit any signs of intoxication (behavioral and pathophysiological symptoms) for 14 days. However, prolonging exposure more than 14 days did have an impact on some physiological symptoms as well as on the rate of survival after MCAO. Finally, it is also important to address that in our experimental groups we did not find evidence of alterations in blood flow during nicotine treatment. This could be the result of the young age of mice and the relatively short duration (14 days) of nicotine exposure. Future analysis of the cerebral blood vessel wall with nicotine exposure should address this point.

The pro- or anti-inflammatory effects of nicotine have been the subject of controversy and discussion. Nicotine as the major component of tobacco is, on the one side, denoted as a strong proinflammatory mediator enhancing the inflammatory responses by regulating monocyte, interacting with endothelial cells, controlling leukocyte rolling and adhesion, inducing massive leukocyte infiltration, and upregulating proinflammatory factors (IL-8, IL-1β, TNF-α, ICAM-1, and P-selectins) (26, 39, 43, 47). However, there is also compelling evidence that nicotine can display opposite effects. Acting through nicotinic acetylcholine receptors on neurons, nicotine can have anti-inflammatory effects protecting, for example, against neural damage during inflammation associated with Parkinson's disease or traumatic brain injury (19, 37, 31, 32). Obviously, the microenvironment, types of additive stimuli, as well as the targeted cells (endothelial cells, neurons, glial cells, or leukocytes) significantly impact upon the effects of nicotine, adding complexity to any analysis of the contribution of nicotine as an inflammatory factor.

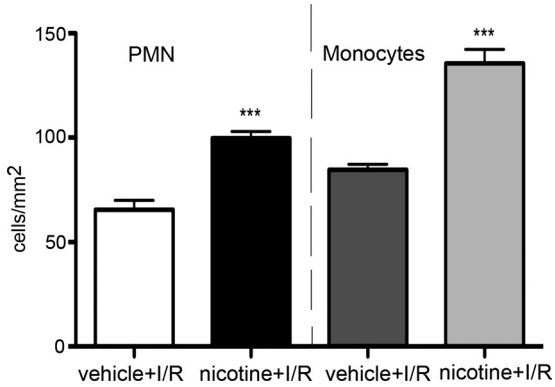

Fig. 6. Immunohistochemical analysis of polymorphonuclear leukocytes (PMNs) and monocytes infiltration into brain of mice exposed to nicotine (2 mg/kg) or vehicle for 14 days before MCAO. Analysis was performed at *day 3* of reperfusion. Anti-MPO (PMN) and anti-Ly6G (monocytes) antibodies were used. ***$P < 0.001$ vs. vehicle-treated group.

At the cerebrovascular level, several proinflammatory factors including cytokines (IL-6, TNF-α, and IL-1β), matrix metalloproteinases (MMP-2, MMP-9, and MMP-13), inducible NO synthase, adhesion molecules (ICAM-1, VCAM-1, and selectins), and angiotensin I and II receptors are indicated as being involved in the inflammatory response triggered by tobacco smoking (18, 25, 42). These data point to the ability of cigarette smoke and nicotine to modulate the complex interplay of signaling, adhesion molecules, and extracellular matrix remodeling that control the vascular inflammatory response and, therefore, increase the risk for the pathogenesis and progression of atherogenesis and vascular impairments. Our results extend previous findings and highlight a very similar proinflammatory pattern after administration of nicotine alone at a dose found in the plasma of average to heavy smokers. So as not to focus on a specific group of proinflammatory mediators, we analyzed a broad set of different pro- and anti-inflammatory cytokines, chemokines, and adhesion molecules (87 at the gene level and 62 at the protein level) in isolated cerebral microvessels (BBB in situ) and brain tissue.

The presence of nicotine induced in the BBB and brain parenchyma mostly a nonspecific, acute inflammatory response mirrored in expression of cytokines IL-1β, TNF-α, IL-6, and IL-6Ra. This is in strong agreement with recently published studies by two laboratories, which studied the direct effect of cigarette particles on cerebral blood vessels and brain endothelial cells (20, 42). Expression of these cytokines could imply higher sensitivity of the cerebrovascular endothelium on cytokine stimulation and may be responsible for triggering and supporting the expression of other proinflammatory mediators. Our study implicated a variety of other cytokines (e.g., IL-2, IL-12, GM-CSF, and G-SCF), chemokines (e.g., CCL2, CCL9, CCL11, CCL17, and CXCL5), adhesion molecules (ICAM-1, VCAM-1, P-selectins, and CX_3CL1), and molecules such as caspase-1, CD40L, and C3, which could play role in further promoting the development of a proinflammatory and proatherogenic phenotype of brain endothelial cells by nicotine. In addition, nicotine caused a significant downregulation of anti-inflammatory mediators, creating a proanti-inflammatory imbalance in the cells, which may have a profound effect on vascular function. A possible result of these effects could be activation of various pathophysiological programs such as matrix remodeling, apoptosis, changes in vascular hemodynamics (sheer stress, flow pattern) at the vascular interface, and an alteration in the intravascular environment from a hemodynamically stable state to a procoagulant and prooxidant state favoring an exaggerated response to

vascular injury and prompting the development of ischemic events. Support for these observations is found in a recent study by Vikman and colleagues, which clearly pinpointed that lipid-soluble cigarette-smoking particles may induce the upregulation of MMP-9 and MMP-13, important in remodeling of the extracellular arterial wall (42). Taking into consideration that the studied dose of nicotine corresponds to the level found in average to heavy smokers and the proinflammatory response of brain endothelial cells in vivo, nicotine should be considered as a severe factor for developing the biological phenomena known as vascular aging, fueling the development of stroke. In the support of this concept is our finding of significant upregulation of proatherogenic factors such as caspase-1, C3, and CD40L, which in combination with other proinflammatory mediators make substrate for the aging type of vascular dysfunction.

Besides affecting the brain endothelium under resting conditions, nicotine also affected the development of brain I/R injury. Although it is well known that cigarette smoking/nicotine is a major risk factor for stroke, extensive analysis on the detrimental effects of nicotine on ischemic injury is still lacking. Using a two-pronged approach examining infarct volume and neurological deficits after MCAO, we found that nicotine enhanced infarct size and worsened neurological status. Furthermore, the brain infarct size was closely associated with the exposed dose of nicotine, pinpointing that increased levels of nicotine directly aggravate brain injury. The potential reason for the enhanced ischemic injury may be that prior nicotine treatment induces a low inflammatory response, which can be a solid substrate for a profound response to ischemic injury at the BBB and in brain parenchyma. Analyzing the changes in brain tissue, our results indicated a significant upregulation in expression of IL-1β, TNF-α, and IL-6 at the mRNA and protein levels after nicotine exposure, whereas anti-inflammatory cytokines IL-10 or IL-1ra were significantly downregulated. Taking into consideration that IL-10 and IL-1ra act as neuroprotective mediators (prevent apoptotic events and glutamate excitotoxicity), particularly after brain I/R injury, the imbalance in pro- and anti-inflammatory mediators generated by nicotine may affect the susceptibility of neurons and glial cells to injury, and this could play a role in expanding infarct size in nicotine-treated animals. The adverse effects on brain parenchyma may be enhanced by effects at the level of the BBB, the first defense against noxic stimuli. Alterations in the BBB proinflammatory phenotype even before ischemic onset, as well as the profound effect during the reperfusion injury, may facilitate potential stroke onset and enhance the final ischemic outcome. Therefore, the actions of nicotine could be defined as "breaking the system of defense" at the level of BBB and brain tissue, which in turn affects neuronal viability and worsens the outcome. The increased infarct size and worse neurological deficits in nicotine-treated mice was not a surprising outcome considering its profound proinflammatory effects.

Nicotine has been implicated in BBB changes leading to brain edema formation. It is known that nicotine alters the Na^+, K^+, 2Cl cotransporter 1 (NKCC1) on the abluminal (brain facing) surface of the BBB during in vitro hypoxia/aglycemia conditions, affecting the development of both cytotoxic and vasogenic brain edema (1, 16, 29, 49). Nicotine also affects the tight-junction complexes between brain endothelial cells, which may contribute to vasogenic brain edema (16). Although our study did not focus on BBB permeability, the increased water content in the injured hemisphere of nicotine-treated mice supports these findings. In addition, our evidence regarding the proinflammatory alterations in BBB with nicotine treatment and the known effects of proinflammatory mediators on vasogenic edema suggest that the extensive brain edema in nicotine-treated mice may result from the enhanced postischemic inflammatory response (36, 45). The exacerbation of brain edema may contribute to worsening of stroke outcome by nicotine, particularly to the neurological deficit and high mortality rate.

Nicotine also affects reperfusion injury, and our study pinpoints a major effect on the postischemic inflammatory response. That response is a critical event in reperfusion injury after ischemic stroke. It is designated as an acute inflammatory response manifested by significant upregulation of inflammatory molecules. In patients, cytokines (TNF-α, IL-1β, IL-8, and IL-18) and soluble adhesion molecules (L-, E-, and P-selectin, sICAM-1, and sVCAM-1) are elevated in blood and CSF from the first day of stroke. In animals, there is upregulation of cytokines (IL-1β, TNF-α, and IL-6) and chemokines (CCL2, CCL3, CCL5, and CCL4) after MCAO, and this is associated with significant infiltration of neutrophils and monocytes into brain parenchyma (10, 30). Analyzing the expression of proinflammatory mediators in our ischemic model, we found expression of a variety of cytokines (i.e., IL-1β, TNF-α, CCL2, CXCL5, and CX_3CL1) not only in the brain parenchyma but also in the BBB in situ (isolated microvessels). This pinpoints that the inflammatory response develops at the level of the BBB and vascular interface as well as the parenchyma. The inflammatory events at the BBB and brain parenchyma cause further aggravation of ischemic lesion by promoting infiltration of neutrophils and monocytes into the area around the ischemic lesion. Nicotine actions under these ischemia/reperfusion conditions can be characterized as detrimental. Whereas mice exposed chronically to nicotine did not have changes in the pattern of cytokines/chemokines and adhesion molecules expression, there were marked changes in magnitude of the response, with some cytokines and chemokines reaching three- to fivefold increases. A similar pattern was also found for infiltrating leukocytes where nicotine-treated animals had more infiltrating cells (both neutrophils and monocytes) compared with vehicle-treated mice. Thus, although the effect of nicotine on the postischemic inflammatory response is marked, it can be characterized as affecting the magnitude and not the type of response.

It is also important to address the issue that the delivery of nicotine was stopped during I/R injury, mimicking what is expected in patients with a stroke. Under in vivo conditions, we expect that some nicotine will remain in the circulation for several days after miniosmotic pump removal, but it will not be at as high a concentration. However, even with this reduced level of exposure during I/R, prior nicotine treatment still exacerbated the inflammatory response during I/R injury. Presumably, alterations in the proinflammatory phenotype at the brain vascular interface attributable to prior nicotine treatment continue to aggravate the inflammatory response and I/R injury. However, future studies should address whether high concentrations of nicotine during I/R affect injury. The dose dependency of the actions of nicotine and the mechanism by which nicotine has proinflammatory effects also need further study.

In summary, nicotine exerts marked effects on the expression of inflammatory mediators at the level of the BBB, changing the brain endothelium to a proinflammatory phenotype. This phenotype change may affect stroke occurrence and our results show that it does enhance ischemia-induced brain injury in a dose-dependent manner. We did not find any sign of an anti-inflammatory or protective role of nicotine at the level of BBB, and our conclusion is that nicotine at the BBB has an exclusively proinflammatory role. This study provides new insights into how to develop new therapeutic strategies for stroke in smokers. In addition, although this study focuses on stroke, the results have implications for other neurological disorders involving an inflammatory component and for the postischemic inflammatory response that occurs in tissues other than brain.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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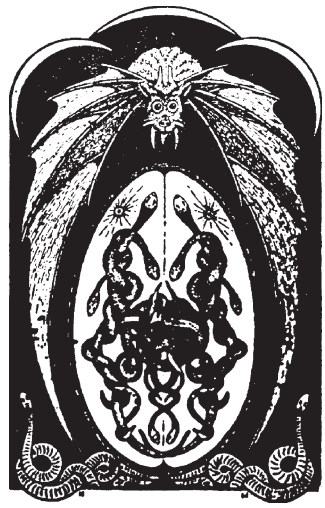

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Novel role of Egr-1 in nicotine-related neointimal formation

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Aims The aim of this study was to investigate the mechanisms by which nicotine increases vascular smooth muscle cell (VSMC) proliferation and post-injury neointimal formation.

Methods and results Vascular injury was inflicted in the right iliac artery of nicotine-treated and control rats. Nicotine increased post-injury VSMC proliferation ($Ki67^+$ cells) and neointimal formation (neointima/media ratio, 0.42 ± 0.23 vs. 0.14 ± 0.07, $P = 0.02$). To determine the mechanisms by which nicotine exacerbates VSMC proliferation, cultured cells were exposed to nicotine, and signalling pathways leading to cell proliferation were studied. Nicotine activated extracellular signal-regulated kinase (ERK) 1/2 in a dose- and time-dependent manner. The blockade of this signalling axis abolished nicotine-mediated proliferation. Functional nicotinic acetylcholine receptors and Ca^{2+} influx were necessary for ERK1/2 activation and nicotine-induced mitogenesis in VSMCs. Downstream to ERK1/2, nicotine induced the phosphorylation of Ets-like gene 1 in a timely co-ordinated manner with the up-regulation of the atherogenic transcription factor, early growth response 1 (Egr-1). The treatment of balloon-injured arteries with a lentivirus vector carrying a short hairpin RNA against Egr-1 abolished the deleterious effect of nicotine on vascular remodelling.

Conclusion Nicotine acts through its receptors in VSMC to activate the ERK–Egr-1 signaling cascade that induces cell proliferation and exacerbates post-injury neointimal development.

Keywords Vascular smooth muscle cell • Extracellular signal-regulated kinase • Neointima • Egr-1 • Nicotine

1. Introduction

Extensive epidemiological evidence indicates that cigarette smoking increases the risk of cardiovascular diseases.[1] Smokers are estimated to have a two- to three-fold higher risk of developing coronary disease than non-smokers.[2] Understanding the mechanisms by which cigarette smoking modifies the normal healing of the vasculature is essential to design effective therapeutic strategies to lessen the impact of smoking on cardiovascular diseases. Nicotine, the addictive substance of cigarettes, stimulates the sympathetic nervous system, increases cardiac output and causes endothelial injury.[3] It also exaggerates post-injury neointimal hyperplasia in preclinical animal models of balloon angioplasty[4–6] and increases atherosclerosis burden in Apo null mice.[7,8] Despite compelling evidence of the harmful effect of nicotine on the vasculature, the mechanisms by which nicotine induces vascular smooth muscle cell (VSMC) proliferation and exaggerates neointimal development remain unknown.

Previous studies have suggested that nicotine modifies the biology of VSMCs to make them more atherogenic. Thyberg and associates demonstrated for first time that nicotine had a mitogenic effect on rat arterial VSMCs *in vitro*.[9] They also showed that nicotine induced a phenotypic change in VSMCs, converting them from a contractile to a synthetic phenotype. These findings suggest the important role of nicotine in vascular disease, because recruitment and proliferation of synthetic VSMCs within the tunica intima of injured vessels are key events in the pathogenesis of vascular occlusive diseases.[10] Subsequently, using human arterial VSMCs, Carty and associates confirmed the mitogenic effect of nicotine.[11] More recently, we and others have demonstrated that nicotine induces human aortic VSMC proliferation through its interaction with the non-neuronal nicotinic acetylcholine receptors (nAChRs) to increase the secretion of platelet-derived growth factor (PDGF) and to up-regulate the expression of the latter's receptors. The mechanisms by which nicotine induces mitogenesis in VSMCs are, however, not fully understood.[12,13]

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This study sought to elucidate the molecular mechanism(s) by which nicotine increases VSMC proliferation and neointimal formation after vascular injury. We have demonstrated that direct action of nicotine on VSMC nAChRs leads to the activation of the mitogen-activated kinase extracellular signal-regulated kinase 1 and 2 (ERK1/2) and the up-regulation of the atherogenic transcription factor early growth response 1 (Egr-1). We have also demonstrated that by targeting Egr-1 with specific short hairpin (sh) RNAs it is possible to prevent the harmful effects of nicotine on post-injury neointimal formation.

2. Methods

2.1 Animal and surgical procedures

Sprague–Dawley rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Nicotine was given in the drinking water (100 mg/L) for 3 months before surgery until the animals were killed. Cotinine was measured in plasma by ELISA (Calbiotech, Spring Valley, CA, USA). Enzymatic determination of total serum cholesterol was performed according to Allain *et al.*[14] Serum triglycerides were determined by quantitative enzymatic measurement of glycerol (Sigma-Aldrich, St Louis, MO, USA). Tail-cuff blood pressure was measured with a CODA non-invasive blood pressure system for rodents (Kent Scientific Corporation, Torrington, CT, USA). Plasma levels of interleukin (IL)-6, IL-1α and IL-1β were quantified using a LINCOplex bead assay kit (Millipore, Billerica, MA, USA).

All surgeries were performed under isoflurane anaesthesia. Vascular injury was inflicted in the right iliac artery.[15] An aortotomy was made in the abdominal aorta to insert a 2 French Fogarty embolectomy catheter to the level of the right iliac artery. The balloon was inflated to 1.5 atmospheres and retracted to the aortotomy site. This was repeated three times to assure a good vascular injury. The aortic incision was repaired with 8–0 nylon sutures (0.4 metric, Ethicon, San Lorenzo, PR, USA). Rats were killed 3 weeks after surgery using an overdose of isoflurane by inhalation, and the injured arteries were harvested and formalin fixed for histology.

Animal care was in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Institutes of Health (NIH publication No. 85-23, revised 1996). All animal procedures were revised and approved by the University of Miami Institutional Animal Care & Use Committee, protocols 06-078 and 09-074.

2.2 Histopathology and immunohistochemistry

Paraffin embedding and sectioning were performed by American Histolabs, SA (Bethestha, MD, USA). Morphometric analysis was performed on elastic Van Gieson or haematoxylin and eosin stained slides. The area of each vascular layer was measured to calculate the neointima-to-media ratio (N/M), where $N/M = N/(M + N)$. Morphometric measurements and cell countings were performed on digital images using Image Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA).

For immunohistochemistry, sections were deparaffinized and rehydrated by serially immersing them in xylene, alcohol and water. After tissue rehydration, endogenous peroxidase was blocked with 3% hydrogen peroxide. Epitope retrieval was performed by boiling slides in citrate buffer (10 mM sodium citrate, pH 6.0) for 25 min. Non-specific binding was blocked with 0.5% blocking solution (DAKO, Carpinteria, CA, USA). Rabbit anti-Ki67 polyclonal antibodies (DAKO) were added for 1 h at room temperature. Bound primary antibodies were detected using the DAKO Universal link kit (DAKO). Colour was developed with a DAB chromogenic solution (DAKO). Nuclei were counterstained with Meyer's haematoxylin and mounted in Entellan mounting medium (EMD, Gibbstown, NJ, USA). Images were obtained with an Olympus 1X71 camera fitted to an Olympus BX 40 microscope (Olympus America Inc., Center Valley, PA, USA).

2.3 Cell culture and transfections

Rat VSMCs up to passage 22 were grown in serum-rich medium, Dulbecco's modified Eagle's medium–F12–fetal bovine serum (50:30:20).[13] Cells were serum starved in 0.1% fetal bovine serum medium for 24 h to synchronize all cells at the G0 phase of the cell cycle. Nicotine hydrogen tartrate salt (Sigma-Aldrich, St Louis, MO, USA) was added to cells in 2% fetal bovine serum medium as indicated. Vascular smooth muscle cells were transfected by Amaxa's Nucleofector technology as described by the manufacturer (Lonza Cologne AG, Germany). Transfection efficiency was around 30% (Supplementary material online, *Figure S1*).

2.4 Proliferation

Proliferation was assessed by direct counting of cells after nicotine stimulation. Cells were seeded into six-well plates at a concentration of 1×10^5 cells per well in serum-rich medium for 24 h. After starvation, cells were stimulated with nicotine (1 μM) for an additional 24 h. Pharmacological agents were used as indicated and added to cultures 2 h before nicotine stimulation. Cell counts were performed after trypsinization with a single threshold Coulter counter (Model ZF, Coulter Electronics, Miami, FL, USA).

2.5 Western blot analysis

Vascular smooth muscle cells (2×10^5 cells per well) seeded in six-well plates were incubated with or without pharmacological agents for 2 h before nicotine stimulation. Cells were rinsed twice with ice-cold phosphate-buffered saline and lysed in RIPA buffer (Millipore) supplemented with leupeptin (10 μg/mL), aprotinin (20 mU/mL), phenylmethylsulphonyl fluoride (10 μM), NaF (10 μM), and $NaVO_4$ (10 μM). Cell lysates were loaded onto the QIAshredder homogenizer columns (Qiagen, Valencia, CA, USA) and centrifugated at 9 000 *g* for 3 min. Total proteins were quantified with the Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein extracts were diluted 1:1 (vol/vol) in Laemmli buffer, and 15 μg of protein was loaded on each lane of a 4–12% Tris–glycine gel (Invitrogen, Carlsbad, CA, USA). The electrophoresed proteins were transferred to Amersham Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Blots were developed with the WesternBreeze Chemiluminescent kit (Invitrogen). The integrated optical density of each band and gel background was measured with the ImageJ NIH image software. The amount of each phosphorylated kinase was normalized with respect to the amount of the total kinase determined in a parallel blot.

Primary antibodies were: rabbit anti-MEK1/2 (no. 9122), rabbit anti-pMEK1/2 (Ser217/221, no. 9121), rabbit anti-p44/42 ERK1/2 (no. 9102), mouse anti-pp44/42 ERK1/2 (Thr202/Tyr204, no. 9106), rabbit anti-p38 MAPK (no. 9212), mouse anti-pp38 MAPK (Thr180/Tyr182, no. 9216), rabbit anti-stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) (no. 9252), mouse anti-pSAPK/JNK (Thr183/Tyr185, no. 9255), rabbit anti-ERK5 (no. 3372), rabbit anti-pERK5 (Thr218/Tyr220, no. 3371), rabbit anti-Ets-like gene 1 (Elk-1) (no. 9182), rabbit anti-pElk-1 (Ser 383, no. sc-135646), and rabbit anti-Egr-1 (no. 4152). All antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA) except for the one against p-Elk-1, which was from SantaCruz Biotechnology (SantaCruz, CA, USA).

2.6 Egr-1 promoter activity

Cells co-transfected with the pEgr-1 and pRL-TK (Promega, Madison, WI, USA) plasmids were seeded in six-well plates at a concentration of 2×10^5 cells per well. pEgr-1 carries the firefly luciferase gene under the control of the rat Egr-1 promoter,[16] while pRL-TK, the transfection control plasmid, carries the Renilla luciferase gene under the HSV TK promoter. After starvation and nicotine stimulation for 30 min, cells were

lysed in 500 μL of passive lysed buffer (Promega) at room temperature for 15 min. Control cells were treated as described but omitting the nicotine. Luciferase activities were determined using the double luciferase reporter assay system (Promega) in a Tuner Biosystems Lumminometer model TD 20/20 (Mountain View, CA, USA). Luciferase activity was normalized based on the Renilla luciferase activity of the transient transfection control vector. Promoter activity was expressed as multiples of the control values.

2.7 RT–PCR and TaqMan real time PCR

Total RNA was purified using the TRIzol reagent (Invitrogen). RT–PCRs for detection of 15 rat nAChR subunits were performed as previously described.[17,18]

Rat *egr-1*, *pdgf-B*, and *β-actin* mRNAs were quantified using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on an ABI Prism 7500 Fast Real-Time PCR System (96-well plate). Relative gene expression was determined using the ΔΔCT method.[19]

2.8 Gene knockdown of *egr-1*

SureSilencing™ shRNA Plasmids (SABiosciences, Frederick, MD, USA) were used to specifically knockdown the expression of the rat *egr-1* gene by RNA interference in cultured VSMCs. This vector carries a puromycin-resistant gene and expresses the shRNA under control of the U1 promoter. Only one of the four tested plasmids effectively silenced *egr-1* gene expression. This functional sequence was 5′-CTACTCCCAACACTGACATTT-3′. Vascular smooth muscle cells were selected in serum-rich medium supplemented with 6 μg/mL puromycin. Knockdown of *egr-1* gene in VSMCs was confirmed by Western blot.

Post-injury *egr-1* gene expression was also silenced via shRNA. A human micro RNA 30[20] containing the above functional sequence was chemically synthesized and inserted between the *Xho*I and *Eco*RI restriction sites into the pGIPZ lentiviral vector (Thermo Scientific Open Biosystems, Huntsville, AL, USA). The viral stocks were generated from 293T cells previously co-transfested with the shRNA lentiviral vector and the packaging and envelope plasmids psPAX2 and pMD2G (Addgene Inc., Cambridge, MA, USA) as previously described.[21] Cell supernatants were passed through 0.45 μm filters and viruses concentrated using PEG-it precipitation solution (System Biosciences, Mountain View, CA, USA). Viral stocks contained at least 10^8 transducing units per millilitre titrated on rat VSMCs. Lentiviruses were applied perivascularly at the time of surgery in 100 μL Pluronic-127 gel containing 30 μL of the viral stock.[22] This formulation forms a gel after contact with the vessel and releases the viruses for approximately 2 days.

2.9 Statistics

Data were expressed as means ± SEM of at least three independent experiments. Two-group comparison was performed using Student's *t*-test for independent samples. Multiple group statistical analyses were performed by one-way ANOVA followed by Bonferroni's correction for multiple comparisons. Statistics were calculated with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Nicotine increases intimal thickness and VSMC proliferation after balloon injury in rats

To study the effect of nicotine on post-injury VSMC proliferation and neointimal formation, Sprague–Dawley rats were chronically exposed to nicotine for 3 months before undergoing vascular injury. Nicotine intake was determined by measuring cotinine blood levels. The blood cotinine in treated rats was 578.27 ± 149.94 ng/mL, a level similar to the one found in heavy smokers.[23] There were no differences in blood cholesterol and triglycerides between treated and control rats. The inflammatory markers IL-6, IL-1α, and IL-1β were similar between the two groups. Rats drinking nicotine were slightly more hypotensive than control animals (mean arterial blood pressure = 99.68 ± 6.89 vs. 109.99 ± 4.42 mmHg, $P = 0.02$; Supplementary material online *Table S1*), though both treated and control rats had similar heart rates.

The effect of nicotine on neointimal formation was examined in animals that underwent balloon injury in the right iliac artery. The intima-to-media ratios in nicotine-treated and control animals were 0.22 ± 0.04 and 0.12 ± 0.03, respectively ($P = 0.014$, *Figure 1A*). Neointimas of treated animals had 1.8 times more $Ki67^+$ cells than those of control animals ($P = 0.043$, *Figure 1B*). In agreement with previous studies,[5] these data confirm the pro-stenotic property of nicotine in the injured vessels.

3.2 Nicotine induces a rapid phosphorylation of ERK1/2 in VSMCs

To elucidate the molecular mechanism by which nicotine induces mitogenesis in VSMCs four major signalling pathways leading to cell proliferation were studied. Nicotine induced a rapid activation of ERK1/2 (p44/p42) in a dose-dependent manner (*Figure 2A*). It occurred within 5 min after exposure and returned to basal levels 20 min later (*Figure 2B*). The nuclear translocation of ERK in treated VSMCs was further confirmed by immunofluorescence microscopy (Supplementary material online, *Figure S2*). The effect of nicotine on ERK phosphorylation was faster and less lasting than that produced by PDGF. Nicotine caused only little activation of p38MAPK and had no effect on SAPK/JNK and ERK5 activation (Supplementary material online, *Figure S3*).

3.3 MAP kinase (MEK1/2) inhibitors block nicotine-induced ERK phosphorylation in VSMCs

We also investigated whether inhibition of MEK1/2 kinases, the upstream components of the ERK signalling axis, would prevent nicotine-mediated VSMC proliferation. Nicotine induced a rapid phosphorylation of MEK1/2 as shown by Western blot with phospho-specific antibodies (*Figure 2C*). The incubation of VSMCs with MEK inhibitors U0126 and PD98059 prior to nicotine exposure completely abolished the activation of ERK1/2 and cell proliferation (*Figures 2D* and *E*).

3.4 nAChRs are essential for nicotine-mediated activation of the ERK1/2 pathway in VSMCs

The expression of nAChR subunits in rat VSMCs was analysed by RT–PCR. This VSMC line was free of any endothelial or fibroblast contamination as previously shown.[24] VSMCs contained abundant mRNAs for α2, α5, α7, β1, and β2 subunits (*Figure 3A*). The role of these receptors on nicotine-mediated proliferation was further confirmed with pharmacological agents. The nAChR agonist epibatidine induced a rapid activation of ERK1/2 in doses ranging from 100 to 0.1 nM (data not shown). Nicotinic AChRs on VSMCs were blocked with two different antagonists, mecamylamine, and hexamethonium dichloride. These two blockers inhibited activation by nicotine of

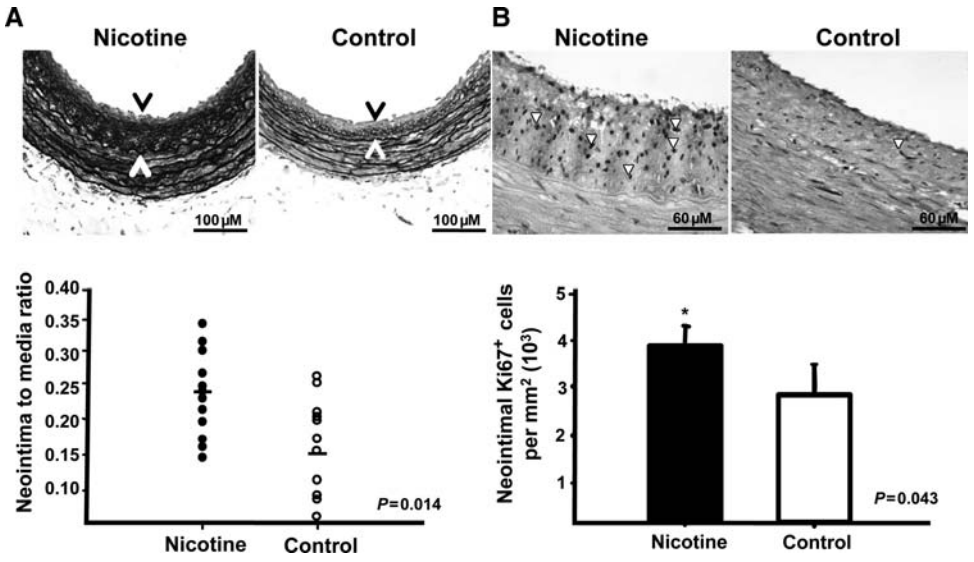

Figure 1 Nicotine exacerbates neointimal formation and increases VSMC proliferation after vascular injury in rats. (*A*) Vascular injury was inflicted in the iliac artery of nicotine-treated ($n = 12$) and control rats ($n = 13$). Animals were killed 3 weeks after injury, and the intima-to-media ratios (by area) were measured in elastic Van Gieson stained sections. Neointima is indicated between arrowheads. (*B*) Cell proliferation in the neointima was detected by immunohistochemistry with an anti-Ki67 monoclonal antibody. Representative $Ki67^+$ cells (dark spots) are indicated with arrowheads. Each bar represents the mean ± SEM. Significance of the difference between the two groups was calculated by a Student's two tailed *t*-test with unequal variances.

ERK at concentrations ranging from 0.05 to 50 μM (*Figure 3B*). These same antagonists blocked nicotine-induced proliferation of VSMCs (*Figure 3C*). These data demonstrate the presence of operational non-neuronal nAChRs on rat VSMCs that mediate nicotine-induced proliferation.

3.5 Calcium is the early intracellular mediator of nicotine signal in VSMCs

Next, we looked for the early intracellular mediators of nicotine signalling in VSMCs. It has been reported that nAChRs on VSMCs have high Ca^{2+} permeability.[25] To test whether calcium mediated nicotine signalling in VSMCs, we pretreated cultured VSMCs with the intracellular Ca^{2+} chelator BAPTA (between 10 and 0.1 μM) before pulsing them with nicotine for 3 min. BAPTA totally inhibited the nicotine-induced activation of ERK, demonstrating the role of calcium influx in nicotine-mediated mitogenesis in VSMCs (*Figure 3D*).

3.6 Egr-1 is an essential component in nicotine-induced VSMC proliferation and neointimal formation

As part of this study, we also searched for the components that are downstream of ERK1/2 in the transduction of nicotine signalling in VSMCs. Elk-1 is directly phosphorylated by ERK1/2 at multiple sites. Elk-1 is a strong transactivator of serum-responsive element, an element that resides within the *egr-1* gene promoter.[26] We found that nicotine induced a tightly co-ordinated phosphorylation of Elk-1 in the Ser383 as shown by Western blot with phosphor-specific antibodies (*Figure 4A*). Phosphorylated ELK-1 was detected within 5 min after ERK1/2 activation and 12 min after exposure to nicotine (*Figure 4B*).

The ELK-1 transcriptional activity was further confirmed by measuring the Egr-1 promoter activity. For this, VSMCs were transfected with pEgr-1, a plasmid that carries the firefly luciferase gene under the control of the rat Egr-1 promoter.[16] Nicotine increased Egr-1 promoter activity by 50% with respect to untreated cells (*Figure 5A*). In agreement with these data, nicotine up-regulated *egr-1* gene expression in VSMCs by 15-fold, and Egr-1 protein content by more than six-fold (*Figure 5B*). The *egr-1* gene expression increased 1 h after nicotine exposure and returned to baseline levels after 4 h (*Figure 5B*). The activity of the Egr-1 transcription factor was indirectly measured through the PDGF-β mRNA levels. It is known that the binding of Egr-1 to this gene promoter is sufficient to initiate transcription.[27] The PDGF gene expression was temporally co-ordinated with the Egr-1 protein production. The PDGF-β mRNA level in rat VSMCs reached its zenith 2 h after nicotine exposure, which was 1 h later than the peak expression of the *egr-1* gene (*Figure 5B*). The nicotine-mediated up-regulation of Egr-1 was significantly blocked by hexamethonium dichloride, a nAChR antagonist, and by the MEK inhibitors U0126 and PD98059 (*Figure 5C*).

The role of Egr-1 in nicotine-induced proliferation in VSMCs was further confirmed by knocking down the *egr-1* gene expression with a specific shRNA. *Figure 5D* shows a significant reduction (by 75%) of the level of the Egr-1 protein in nicotine-treated VSMCs whose *egr-1* gene expression had been blocked with a specific shRNA (*Figure 5D*). The nicotine-induced proliferative response of Egr-1-deficient VSMCs was 80% less than that of cells transfected with the mock shRNA (*Figure 5E*).

Finally, we examined the effect of *egr-1* gene silencing on nicotine-mediated neointimal formation. The *egr-1* gene expression was targeted using a lentiviral vector carrying a specific shRNA. Lentiviruses were embedded in pluronic gels and delivered perivascularly around the iliac artery at the time of surgery. The knockdown of *egr-1* gene

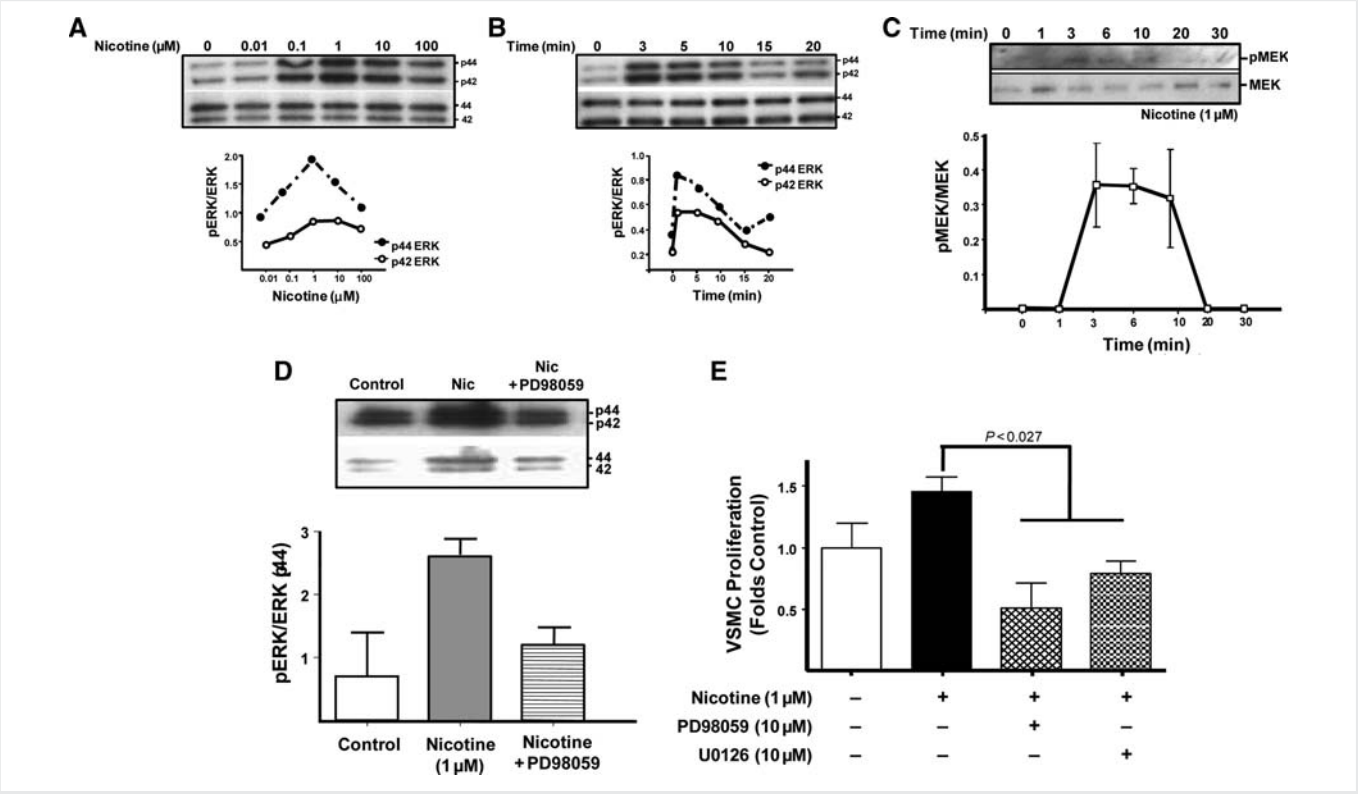

Figure 2 Nicotine induces a rapid activation of ERK1/2 in VSMCs. (*A*) Nicotine induces ERK1/2 (p44/p42) phosphorylation in VSMCs. Treated cells were harvested 3 min after exposure to the indicated nicotine concentration. (*B*) Activation of ERK1/2 by nicotine (1 μM) was rapid and reversible after 10 min. Total (ERK) and phosphorylated ERK1/2 (pERK) were detected by Western blot. (*C*) Nicotine rapidly activates MEK1/2, the main ERK upstream kinase. (*D*) Inhibition of MEK1 with PD98059 blocks nicotine activation of ERK1/2. (*E*) Inhibition of MEK1/2 inhibits nicotine-induced mitogenesis in VSMCs. Each bar represents the mean ± SEM, $n = 5–7$.

expression inhibited the neointimal formation after balloon injury (*Figure 6*). The neointimas of injured arteries treated with Egr-1 shRNA were significantly thinner than those of control arteries (0.44 ± 0.12 vs. 0.84 ± 0.06, $P < 0.01$).

4. Discussion

Nicotine, the main addictive component of cigarette smoke, is a pro-restenotic[4–6] and pro-atherosclerotic[7] substance. Herein, we describe a comprehensive mechanism of action for nicotine-induced mitogenesis in VSMCs. We identify the atherogenic transcription factor Egr-1 as the main molecular player that translates nicotine extracellular signal into a proliferative response. Most importantly, we provide a mechanism for previous descriptive studies indicating that nicotine exposure increases the severity of neointimal formation after vascular injury.[4–6]

The mitogenic property of nicotine on vascular cells is well documented.[12,13,28] Our results reported herein demonstrate that nicotine acts as an agonist of the nAChR on VSMCs to trigger an intracellular Ca^{2+} influx, which in turn activates the ERK proliferative pathway. The activated ERK1/2 in nicotine-treated cells phosphorylates Elk-1, which in turn binds to the Egr-1 promoter to up-regulate this gene expression. Egr-1 is a known atherogenic transcription factor that controls the production of VSMC mitogenic cytokines, such as PDGF-BB.[27]

In agreement with other studies,[17,18] we found multiple nAChR subunits in rat VSMCs, suggesting that the effects of nicotine on VSMCs may depend upon the action of more than one nAChR receptor type. Nicotinic AChRs are pentameric ligand-gated ion channels made up of various subunits.[29,30] The subunit composition of nAChRs determines ligand specificity, ligand affinity, cation permeability, and channel kinetics. Among the nAChR subtypes present in VSMCs is the homomeric α7-nAChR that possesses high Ca^{2+} permeability and rapid onset of desensitization.[31] It has been shown that the α7-nAChR mediates nicotine-induced angiogenesis[8] and is an essential regulator of inflammation.[32] The presence of the α7subunit in VSMCs reported herein (*Figure 3*) suggests that the α7-nAChR also participates in VSMC proliferation and post-injury vascular remodelling.

Our results also extended previous findings that demonstrated the role of ERK signalling in the nicotine-mediated proliferation of VSMCs and fibroblasts.[18,33,34] The activation of ERKs, also called mitogen-activated protein kinases (MAPK), plays a critical role in signal transduction cascades from the cell surface to the nucleus, promoting gene expression, cell proliferation, and cell survival.[35] We demonstrated that nicotine activates ERKs in VSMCs via a Ca^{2+}-dependent mechanism that may involve a variety of upstream effectors, including MEK1/2 kinases. The increase in ERK activity after exposure to nicotine confers a proliferative advantage to VSMCs and may therefore exacerbate neointimal formation after an acute vascular injury. We also sought to identify the molecules responsible for transduction of nicotine signalling into the VSMC nucleus. We found that ERK1/2

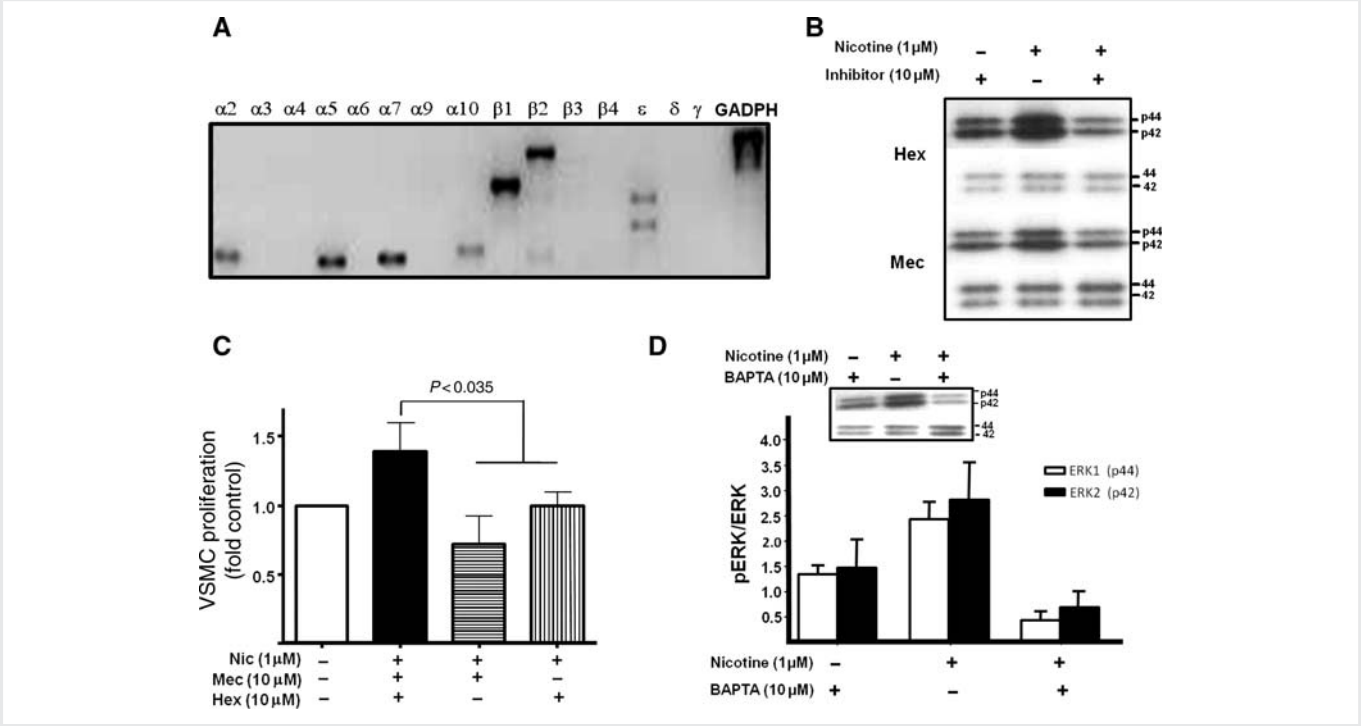

Figure 3 Multiple nAChR subunits are expressed on VSMCs. (*A*) The expression of 15 nAChR subunits in VSMCs was assessed by RT–PCR. (*B* and *C*) Blockade of non-neuronal nAChRs with 0.1 μM of either mecamylamine (Mec) or hexamethonium dichloride (Hex) inhibits nicotine-mediated phosphorylation of ERK1/2 (*B*) and proliferation (*C*). (*D*) Calcium mediates nicotine signalling in VSMCs. Pretreatment of VSMCs with BAPTA, an intracellular Ca^{2+} chelator, prevented the activation of ERK1/2 by nicotine. Each bar represents the average of six independent experiments.

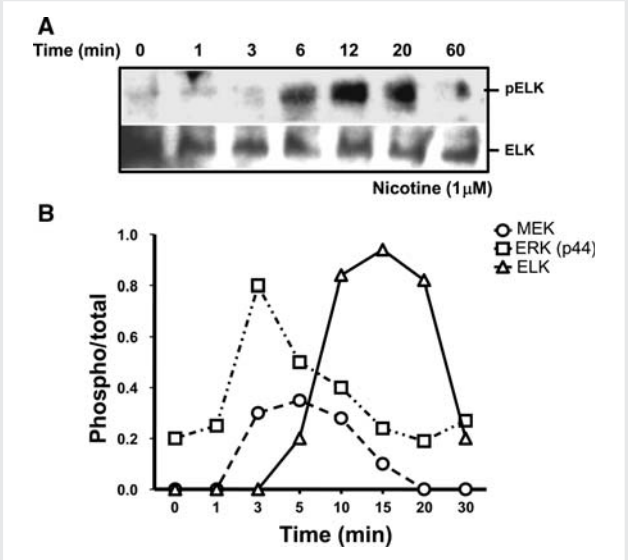

Figure 4 Nicotine activates the transcription factor Elk-1. (*A*) Phosphorylated Elk (pElk) was detected by Western blot in VSMCs after nicotine exposure (1 μM) at different time points. (*B*) Time course of nicotine-mediated activation of MEK1, ERK1/2 and Elk-1 in VSMCs. Each point represents the average of five independent experiments

is translocated to nucleus of VSMCs after nicotine exposure to target the transcription factor Elk-1, which in turn up-regulates the expression of Egr-1. Egr-1 is a key player in atherosclerosis progression[36] and post-injury neointimal formation.[37] We demonstrated that the silencing of *egr-1* gene expression with specific shRNA prevented the proliferative effects of nicotine on VSMCs, and thus inhibited the exaggerated neointimal formation after vascular injury. The latter data agree with previous results showing that the inhibition of Egr-1 activity with a DNA enzyme prevents the neointimal formation in response to balloon injury in the rat.[37] Egr-1 seems to be a potential target for the development of new therapies to prevent the deleterious effects of nicotine on the blood vessels.

Finally, we would like to point out a difference in the mechanisms of nicotine-induced proliferation in VSMCs and tumour cells. In both types of cells, nicotine-mediated proliferation depends on the activation of ERK signalling. In tumours, however, the action of nicotine relies on the physical interaction between nAChRs and β-arrestin to initiate ERK signalling.[38] In contrast, nicotine-mediated activation of ERK signalling in VSMCs depends more on nAChR permeability. Results from the present study do not rule out the existence of other mechanisms that involve β-arrestin in nicotine-induced mitogenesis in VSMCs.

4.1 Limitations of the study

Using nicotine alone vs. cigarette smoking had the advantage of isolating the biological effects of nicotine from those of other noxious com-

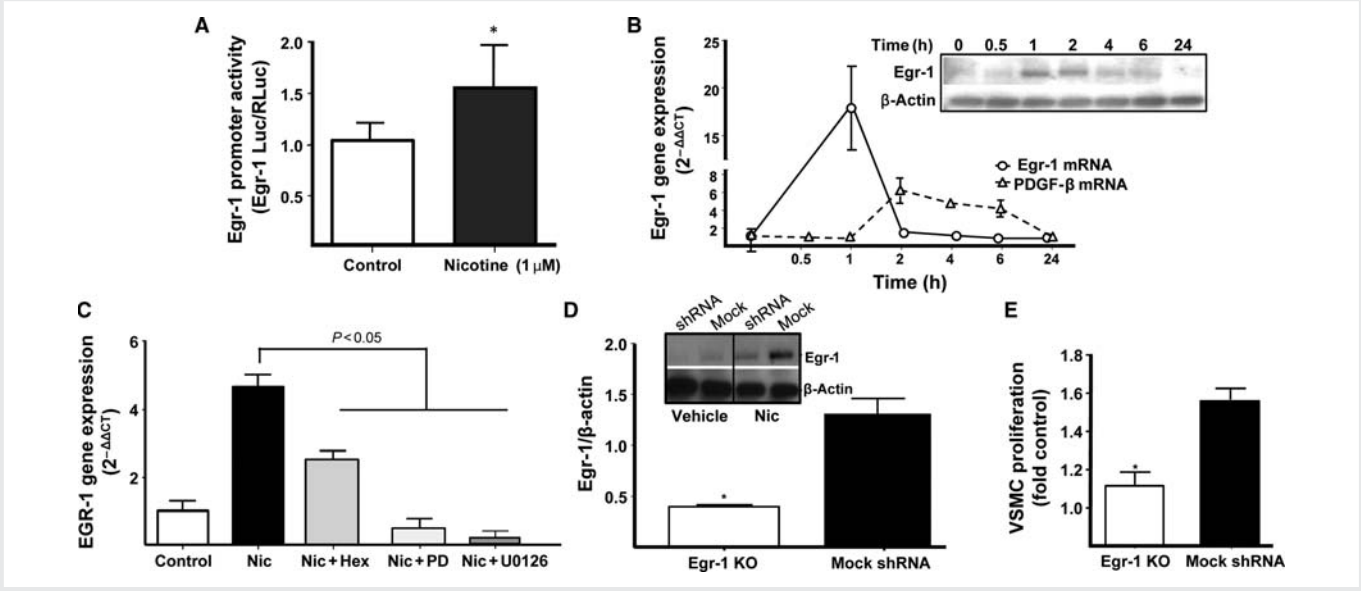

Figure 5 Egr-1 transcription factor is essential for nicotine-induced mitogenesis in VSMCs. (*A*) Nicotine activates Egr-1 promoter in VSMCs. Luciferase activity was determined after nicotine exposure in VSMCs carrying the luciferase gene driven by the rat Egr-1 promoter. Promoter activity is expressed as muliples of activity in control cells. (*B*) Nicotine up-regulates, in a timely, co-ordinated manner, the *egr-1* and the *PDGF*-β gene expression in VSMCs. The mRNAs were quantified by TaqMan quantitative RT–PCR. The Western blot (top right) shows Egr-1 protein levels at different time points after exposure to nicotine. (*C*) The nAChR antagonist hexametonium (Hex) and MEK blockers (U0126 and PD 98059) inhibited nicotine (Nic)-mediated up-regulation of the *egr-1* gene in VSMCs. (*D*) *Egr-1* gene knockdown in VSMCs using a SureSilent shRNA. Egr-1 production in knockdown VSMCs was measured by Western blot in the presence and absence (vehicle) of nicotine. (*E*) Nicotine-mediated proliferation is blunted in Egr-1-deficient VSMCs. Values are expressed as multiples of the values for control, untreated cells. Each bar represents the mean ± SEM of $n = 5–7$. Significance of the difference between the two groups was calculated by Student's two-tailed *t*-test with unequal variances.

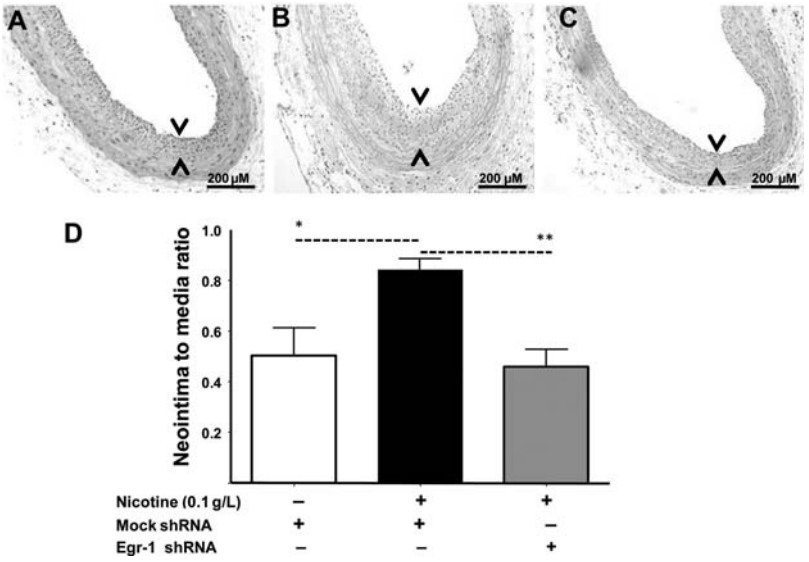

Figure 6 Silencing of the *egr-1* gene prevents nicotine-enhanced neointimal formation in the rat balloon injury model. (*A–C*) Vascular injury was inflicted in the iliac artery of nicotine-treated and untreated rats. Animals received a lentiviral vector carrying either the Egr-1 specific ($n = 7$, *C*) or the scrambled shRNA ($n = 6$, *A* and *B*). Lentiviruses were embedded in pluronic gels and delivered perivascularly around the iliac artery at the time of surgery. This formulation forms a gel after contact with the vessel and releases the viruses for approximately 2 days. Animals were killed 3 weeks after injury, and the intima-to-media ratios (by area) were quantified. Neointima is indicated between arrowheads. (*D*) Neointima-to-media ratios (by area). Each bar represents the mean ± SEM of $n = 5–7$. Significance of the difference among groups was calculated by a one-way ANOVA, and comparisons were made using Boferroni's correction. *$P < 0.05$ and **$P < 0.01$.

ponents, such as ammonia, tar, and carbon monoxide, that may also have effects on post-injury vascular remodelling. Therefore, the mechanisms by which nicotine modifies vascular repair may be modified by the presence of other active components of tobacco. The rat balloon injury model also carries important limitations that need to be taken into account when interpreting the data. In this model, injury is induced in normal elastic artery with rare intimal cells, and little vasa vasorum. In contrast, arterial injury leading to restenosis in humans is mainly produced during percutaneus coronary interventions in atherosclerotic muscular coronary arteries. However, the rat balloon injury model is perhaps the most commonly used and has the advantages of availability, low cost, and the ability to develop a rapid, reproducible response to balloon injury.

4.2 Clinical significance

Nicotine may contribute to cardiovascular disease either by activating the sympathetic nervous system[3] or by accelerating atherosclerosis[7] and restenosis.[4–6] This study reveals new insights into the pathobiology of smoking-related cardiovascular diseases, specifically the mechanisms by which nicotine exacerbates post-injury VSMC proliferation. Most importantly, this study suggests that the atherogenic transcription factor Egr-1 is a plausible therapeutic target to ameliorate the deleterious effects of nicotine on vascular occlusive diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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EDITORIAL

Cigarette smoking induces vascular proliferative disease through the activation of Egr-1

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This editorial refers to 'Novel role of Egr-1 in nicotine related neointimal formation' by R.I. Vazquez-Padron *et al.*, pp. 296–303, this issue.

Vascular proliferative diseases such as atherosclerosis, restenosis after arterial injury, and hypertensive vascular diseases are commonly associated with the proliferation of vascular smooth muscle cells (VSMCs). Multiple stimuli such as shear stress,[1] hypoxia,[2] arterial injury, and angiotensin II (Ang II)[3] are associated with the development of vascular proliferative diseases by the activation of early growth response (Egr)-1 as a master regulator for the proliferation of VSMCs.

The zinc finger transcription factor Egr-1 is an 80 to 82 kDa nuclear phosphoprotein consisting of 533 amino acids. The DNA-binding domain of Egr-1 consists of three zinc finger motifs located between amino acids 332 and 416 towards the carboxy-terminal region of the protein. Via these zinc finger motifs, Egr-1 binds to GC-rich DNA sequences to activate or repress gene transcription.[4] Egr-1 activated by shear stress, mechanical injury, or Ang II, for example, stimulates transcription of several proinflammatory genes, including TNF-α,[2] IL-2,[5] MCP-1, and ICAM-1,[6] to induce atherosclerosis. Egr-1 also stimulates expression of growth factors such as PDGF, TGF-β,[3] bFGF,[7] and tissue factor[8] to induce arterial proliferation. Many of these genes also stimulate the expression of Egr-1 in vascular cells. These positive feedback loops amplify and sustain gene transcription through Egr-1-mediated mechanisms in vascular proliferative diseases.

Cigarette smoking is well known to increase the risk of cardiovascular diseases. Nicotine, the addictive substance in cigarettes, stimulates the sympathetic nervous system and causes endothelial injury.[9] Nicotine also exaggerates arterial neointimal hyperplasia after injury and atherosclerosis in the ApoE knockout mouse.[1] Vazquez-Padron *et al.*[10] report on their investigation of the mechanisms by which nicotine increases VSMC proliferation and neointimal formation after arterial injury. They showed that nicotine activates the extracellular signal-regulated kinase ERK1/2 through nicotinic acetylcholine receptors to induce mitogenesis in VSMCs. Downstream of ERK1/2, nicotine induces phosphorylation of Elk-1 by the up-regulation of Egr-1. Treatment of balloon-injured arteries with a lentivirus vector carrying an shRNA against Egr-1 abolished the deleterious effect of nicotine on vascular remodelling. Thus, nicotine acts through its receptors in VSMCs to activate the ERK/Egr-1 signalling cascade that induces cell proliferation and exacerbates neointimal formation after arterial injury. This study provides new insights into cigarette smoking-related cardiovascular diseases, including the finding that restenosis is accelerated by the activation of Egr-1 by nicotine.

Cigarette smoking is also well known to lower levels of HDL cholesterol, one independent risk factor for atherosclerotic vascular disease. HDL cholesterol promotes reverse cholesterol transport to prevent the formation of atherosclerotic plaque by high levels of LDL cholesterol and acts as an anti-inflammatory and antioxidant.[11] Previous studies have shown that cigarette smoke contains reactive oxidants that can enter the bloodstream and cause macromolecular damage in endothelial cells. Cigarette smoking also elicits a marked activation of leucocytes and platelets, which can additionally contribute to the oxidative vascular damage seen in smokers. Furthermore, constituents of cigarette smoke circulating in the blood activate radical oxygen species that induce enzyme systems within the vascular wall and lead to vascular inflammation and development of atherosclerosis.[12] However, stem cells hold great promise for tissue repair and regenerative medicine, and endothelial progenitor cells (EPCs) play a significant role in endothelial repair. Recent studies have shown that cardiovascular risk factors such as hypertension, hypercholesterolaemia, diabetes, and cigarette smoking are inversely correlated with EPC number and function. We found that EPC colony numbers in cigarette smokers are quite low, indicating that cigarette smoking impairs the repair by EPCs of endothelial damage that eventually induces atherosclerotic vascular disease.[13]

The mechanisms underlying the vascular diseases related to cigarette smoking are complex. The findings by Vazquez-Padron *et al.*,[10] which reveal that the activation of Egr-1 by nicotine is one possible mechanism by which cigarette smoking induces vascular proliferative disease, may aid in developing a potential treatment for vascular disease in cigarette smokers in the future.

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Review Article

Epigenetic Effects and Molecular Mechanisms of Tumorigenesis Induced by Cigarette Smoke: An Overview

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Cigarette smoking is one of the major causes of carcinogenesis. Direct genotoxicity induced by cigarette smoke leads to initiation of carcinogenesis. Nongenotoxic (epigenetic) effects of cigarette smoke also act as modulators altering cellular functions. These two effects underlie the mechanisms of tumor promotion and progression. While there is no lack of general reviews on the genotoxic and carcinogenic potentials of cigarette smoke in lung carcinogenesis, updated review on the epigenetic effects and molecular mechanisms of cigarette smoke and carcinogenesis, not limited to lung, is lacking. We are presenting a comprehensive review of recent investigations on cigarette smoke, with special attentions to nicotine, NNK, and PAHs. The current understanding on their molecular mechanisms include (1) receptors, (2) cell cycle regulators, (3) signaling pathways, (4) apoptosis mediators, (5) angiogenic factors, and (6) invasive and metastasis mediators. This review highlighted the complexity biological responses to cigarette smoke components and their involvements in tumorigenesis.

1. Introduction

It is known that 90–95% of all cancers are caused by or closely associated with environmental factors and lifestyle. This includes diet (30–35%), cigarette smoking (25–30%), and alcohol consumption (4–6%) [1]. Cigarette smoking is an important risk factor for heart disease, chronic obstructive pulmonary disease, stroke, and acute respiratory diseases. In addition to all these noncancer diseases, it is also highly associated with human cancer development. The International Agency for Research on Cancer (IARC) identified cigarette smoking as the cause of cancer in more organ sites than any other human carcinogens. These include cancers of the lungs, oral cavity, larynx, nasal cavity, esophagus, stomach, pancreas, liver, kidney, urinary bladder, uterine cervix, and bone marrow [2]. There are over 5000 chemical compounds identified in tobacco and 62 of these have been evaluated by IARC as showing "sufficient evidence for carcinogenicity" in either animals or in humans [2, 3]. The major carcinogenic compounds include, but not limited to, radioactive polonium, N-nitrosamines such as 4-(methylnitrosaminao)-1-(3-pyridyl)-1-butanone (NNK), polycyclic aromatic hydrocarbons (PAHs) (e.g., benzo[a]pyrene (BaP)), and benzene [4]. A fine review on this aspect has been presented by Hecht in 2006 [5].

The carcinogenesis process is complex. Multistep processes of genetic and molecular defects have taken place before the manifestation of cancer [6]. Traditionally, there are three basic stages of carcinogenesis: initiation, promotion, and progression [7]. Carcinogenesis process is usually

accompanied by changes in structure and function of central genomic information coded in the DNA leading to various oncogene activations and tumor suppressor gene inactivations [8]. In addition, multiple signaling pathways may also be deregulated during the process of cancer development. Cancer growth also requires molecular changes that either affect the tumor cells themselves or alter the interaction between tumor cells and their surrounding stromal environment or the immune system. These events may eventually lead to tumor growth, invasion, and metastasis.

Cigarette smoke components have been reported to promote tumorigenesis by several mechanisms involving all three stages of carcinogenesis [5]. Genotoxic agents in cigarette smoke induce DNA damage through several mechanisms including gene point mutation, deletions, insertions, recombinations, rearrangements, and chromosomal aberrations. PAHs and nitrosamines are two of the most abundant genotoxic components in cigarette smoke. In addition to genotoxic effects, nongenotoxic effects of cigarette smoke are also extremely important. These effects can also act as modulators which alter cellular functions including cell proliferation and cell death. While synergistic effects of genotoxic carcinogens are known to occur, interaction between nongenotoxic (epigenetic) factors and genotoxic agents may also synergistically increase the risk for carcinogenesis [9]. The genotoxicity leading to carcinogenesis has been extensively reviewed in recent years [9–11]. In this present review, aside from a brief overview on the genotoxic effects of cigarette smoke components, we will provide a more extensive review on the non-genotoxic mechanisms of carcinogenesis by cigarette smoke or its components.

2. The Three Carcinogensis Steps Affected by Cigarette Smoke

Step 1 (Initiation of Carcinogenesis). Carcinogenesis may be the result of chemical or biological insults to normal cells through multistep processes that involves genomic changes (initiation of cancer development). Such changes eventually may also lead to cancer promotion and progression [12]. Some of the cigarette smoke components can act directly on DNA, but many require enzyme conversion before becoming carcinogenic [10, 11]. Most of such "conversions" involve metabolic changes via cytochrome *p*450s (*P*450s) such as *P*450s 1A2, 2A13, 2E1, and 3A4 to form the electrophilic entities that can bind to DNA to form DNA adducts. Such adduct formation is usually at the adenine or guanine sites of the DNA and lead to mutations such as those observed in the *KRAS* oncogene in lung cancer or those in the *TP53* gene in a variety of cigarette smoke-induced cancers [13, 14]. These mutation represent the so-called initiation step of carcinogenesis [15].

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are the most potent tobacco-specific nitrosamines in tobacco products and cigarette smoke. These compounds are formed from tobacco alkaloids like nicotine during the curing process of tobacco and are important tobacco carcinogens that can affect different tissues depending on the specific nitrosamines or their metabolites involved [5, 10]. NNK is a potent lung carcinogen but can also induce liver and nasal cancers. NNN has been shown to be carcinogenic to esophagus, nasal cavity, and respiratory tract in laboratory animals [16]. In humans, metabolites derived from NNK and the metabolites of NNK can also be identified in the smoker's urine [17].

Benzo[a]pyrene (BaP), one of the PAHs, is classified as a Group 1 carcinogen to humans [3]. It has been shown to have strong association and tumor-induction potentials in lungs, trachea, and mammary glands [5]. The carcinogenic potency of BaP has been demonstrated to be related to its metabolites which form DNA adducts with site-specific hotspot mutation in the *p53* tumor suppressor gene. Positive correlations of such adduct formation and tumor are indeed found in the lung cancer tissues of cigarette smokers [18].

These findings indicate that DNA mutations are increased in both tumor and nontumor bearing tissues of smokers. However, it must be pointed out that DNA adduct formations induced by cigarette smoke still cannot fully represent all the risk factors for cancer development in cigarette smokers [19]. For example, while there is higher incidence of pancreatic cancer in cigarette smokers than nonsmokers [20]. Assays for NNK metabolites in pancreatic cancer tissues in humans showed no significant difference between smokers and nonsmokers [21]. Thus, it is apparent that NNK-induced DNA adducts alone are not solely responsible for the pancreatic cancers in cigarette smokers. Nevertheless, NNK and its metabolite, NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), are the only environmental carcinogens known to induce pancreatic cancer in animal models [22]. Thus, the contribution of NNK to pancreatic cancer in cigarette smokers still cannot be ignored. Furthermore, it is suggested that, in addition to DNA damage, synergistic interactions between DNA reactivity and epigenetic actions such as increased cell proliferation induced by NNK or by other chemicals in cigarette smoke may be needed for actual cancer development in such patients [23, 24]. There is indication that cigarette smoke carcinogens or cocarcinogen, such as nicotine, may also play a direct role to enhance cancer promotion and progression in human cancers after cancer development [25]. Such genotoxic mechanisms for cancer initiation and carcinogenesis by cigarette smoke components are well covered and discussed in several excellent reviews [5, 10, 11, 26–28]. Readers are encouraged referring to them. For the remaining portion of this article, we will provide more information on the non-genotoxic (epigenetic) mechanisms involved in cancer promotion and progression via cigarette smoke.

Step 2 (Cancer Promotion). Cancer promotion is characterized by deregulation of signaling pathways which control cell proliferation, apoptosis, and so forth, [29]. It is believed that although there are various genetic pathways which may lead to cancer development or cancer behaviors, there are certain hallmark capabilities or mechanisms which are commonly shared by all tumors. In the following discussion, we will describe each mechanism with illustrated examples.

2.1. Effects of Cigarette Smoke on Self-Sufficiency in Growth Signals. Normal cells need mitogenic growth signals to induce proliferation. These signals are transmitted into cells by receptors that bind distinct signaling molecules. In cancer cells, the receptors which transduce growth signals into cells are targets of deregulation during tumorigenesis. Receptor overexpression allows cancer cells to become hyperresponsive to low levels of growth factors that generally are not sufficient to trigger proliferation in normal cells [29]. Nicotine, a major component of cigarette smoke, is known to be a chemical that plays an important role in carcinogenesis in cigarette smokers [30]. Nicotine behaves like those growth factors which exert their biological functions mainly through the nicotinic acetylcholine receptors (nAChR) [31], β-adrenoceptors (β-AR) [32] or epidermal growth factor receptor (EGFR) [33]. The functions of these receptors are cell-type specific and the expression level and receptor sensitivity can be modified by nicotine. Obviously, alterations in either the receptor expressions or sensitivity play an important role in cigarette smoke-induced carcinogenesis [34–36].

Recent study by Lee et al. reported that α9 nAChR expression in human breast tumors is elevated in advanced stages of breast cancer and plays important roles in human breast carcinogenesis [37]. Nicotine has been shown to mediate α9 nAChR signaling and upregulate cyclin D3 expression in breast caner cells and breast cancer tissues [38]. Furthermore, it is also found that activation of the expression of α9 nAChR by nicotine is through AKT signaling [39] and activation of α9 nAChR signaling would elevate the phosphorylation status of adhesion molecule which plays a role in cancer metastasis [40]. Proliferation of mesothelioma cells is also found to be enhanced by nicotine [41]. This enhancement has been shown to be via α7 nAChR with activation of ERK1/2 cascade as well as induction of NF-κB and Bad phosphorylation. All these events eventually lead to inhibition of apoptosis [41] and increase of cancer risk. These findings were further supported by Wada et al. [42] who observed that nicotine promoted cell proliferation via α7 nAChR mediated p44/p42-MAPK activation. Moreover, in our own study, we also reported that nicotine induced human bladder cells proliferation through ERK1/2 and Stat3 signaling downstream of α7 nAChR and β-adrenoceptors (β-AR) [43]. In sum, all these studies indicate that nicotine, an important ingredient of cigarette smoke, promotes cellular proliferation which plays a critical role in carcinogenesis.

Other than nicotine, nitrosamines, such as NNK and NNN, also induced cancer cells growth through nAChR. NNK induced carcinogenesis by binding to nAChR especially for α7 nAChR, whereas the biological impact of NNN is mainly modulated by α4/β2 nAChR [8, 44–46]. It has been demonstrated that nicotine or NNK stimulated lung cancer cell proliferation via α7 nAChR with activations of PKC, RAF1, AKT, ERK1/2, and transcription factors such as JUN, FOS, and MYC [47–49]. Question has been raised concerning the possibility that specific nAChR subunit upregulated by nicotine or NNK may be tissue specific or dependent. For instance, with nicotine or NNK, α7 nAChR is the primary nAChR subunit which mediates tumorigenesis in lungs giving rise to pulmonary squamous cell carcinoma and mesothelioma [36]. On the other hand, α9 nAChR is more associated with breast cancer [37]. Thus, the specific types of nAChR expressed in cancer cells may be considered as useful molecular targets for potential clinical therapy [50]. However, most of the nAChR present in cancer cells are still not functionally characterized yet. Future study will be needed to understand the functions of different nAChR subtype in cancer cells and the downstream signal pathways involved in tumorigenesis.

In addition to nAChR, a number of studies indicated that nicotine and NNK might also exert their biological activities through activation of receptors such as β-adrenoceptors (β-AR), EGFR, or insulin-like growth factor receptor (IGFR) or transactivation by nAChR signaling. It has been demonstrated that β-AR activation promotes the growths of various adenocarcinoma. For example, NNK can stimulate HT-29 cell proliferation through β-AR followed by cyclin AMP elevation and COX-2 expression [51]. Consistently, NNK stimulates the growth of pulmonary adenocarcinoma *in vitro* and *in vivo* via the release of arachidonic acid through COX-2 and 5-lipoxygenase (5-LOX) pathways that are mainly regulated by β-AR [52]. In another study by Schuller and Cekanova, NNK is reported to stimulate β2-AR receptor pathway (including PKA, cAMP, CREB) and transactivate EGFR pathway (such as Raf-1/ERK1/2 signaling) in the development of lung cancer [53]. It has also been reported that antagonists of β-AR can inhibit the development of NNK-induced lung adenocarcinoma [52]. Such antagonists are also found to be effective in reducing the stimulatory effects of nicotine on PKC, ERK1/2 activations, COX-2 expression, and gastric cancer cell proliferation [54]. Elevation of noradrenaline by nicotine via α7 nAChR upregulation leading to significantly enhanced growth and angiogenesis in both gastric cancer and colon cancer has also been demonstrated [55]. Various investigators have also shown increases in neurotransmitters lead to β-AR activation, transactivation of EGFR, and the release of EGF [32, 54, 56]. Thus, an interrelationship between nAChR and neurotransmitter is apparent. Indeed, our recent investigation provided compelling evidence that chronic nicotine exposure induced release of noradrenaline via α4/β2 nAChR activation followed by β-AR transactivation. Our study further demonstrated that blocking of β-AR with antagonist reversed the nicotine-induced cellular proliferative and chemoresistance [57].

Al-Wadei et al. first reported that nicotine contributes to the development of smoking-related pancreatic ductal adenocarcinoma (PDAC) with elevated levels of stress neurotransmitters (adrenaline and noradrenaline) and induction of cAMP, pCREB, and pERK1/2, and inhibition of γ-aminobutyric acid (GABA) [58]. GABA has been reported to possess tumor suppressor function suppressing both β-AR stimulated PDAC growth and migration *in vitro* [59]. However, while GABA is suppressed in PDACs, noradrenaline, PKA, p-CREB, and pERK1/2 in these tissues are overexpressed. A reduction of GABA by NNK is observed in lung adenocarcinoma [60]. These authors suggested that nicotine and NNK may contribute to the development of

PDAC in smokers by suppression of GABA with induction of stress neurotransmitters [61]. Schuller et al. further proposed that nicotine induces the release of stress neurotransmitters through activation of $\alpha 7$ nAChR and inhibits release of GABA via inhibition of $\alpha 4/\beta 2$ nAChR [61]. It is now believed that the stress neurotransmitter released via nAChR activation plays an important role in smoking-associated tumorigenesis [62]. However, the precise mechanisms involved in the regulation and the function of neurotransmitter released by nicotine and NNK are still uncertain. Future research on this area is encouraged.

It has also been shown that NNK can promote β-AR-mediated transactivation of EGFR followed by ERK1/2 phosphorylation leading to an increased proliferation in pancreatic cancer cells [63]. NNK is also reported to induce endogenous IGFR which is associated with the development of lung tumors [64]. Huang et al. also indicated that both activation of thromboxane A2 (TxA2) receptor and synthesis of TxA2 play critical roles in NNK-promoted lung cancer cell proliferation. TxA2 activates the transcriptional factor CREB through both ERK and PI3K/AKT pathways, which may also lead to PCNA and Bcl-2 overexpressions and cell proliferation [65]. These studies provide valuable information on the mechanisms which involve in proliferative signaling stimulated by nicotine and NNK through activation of nAChR, β-AR and other growth factor receptors in cancer cells. Triggering such receptors by cigarette smoke would further lead to rapid cell proliferation, cellular migration, invasion, and metastasis. In short, these investigations on the nAChR, and nAChR transactivated with other receptors represent the pivotal role in regulating multiple cellular cascades in general cell functions and in carcinogenesis.

Nicotine is also known to influence signal transducers and activators of transcription 3 (Stat 3) which is an important signal transducer mediating signaling by numerous cytokines, growth factors, and oncoproteins [66]. Findings from our own laboratories indicate that nicotine induces bladder cancer cells proliferation through $\alpha 7$ nAChR, $\alpha 4\beta 2$ nAChR, and β-AR followed by activation of ERK1/2 and Stat 3 [43]. Stat3 signaling further enhanced NF-κB activation, cyclin D1 overexpression, and cell cycle progression [43]. Moreover, we also revealed that prolonged stimulation by nicotine upregulated $\alpha 4/\beta 2$ nAChR and β-AR followed with activation of Stat 3 leading to significant increase in chemoresistance in cells from bladder cancers [57].

In recent years, nongenotoxic actions of PAHs have gained increasing attentions. The biological effects of PAHs are mainly mediated via aryl hydrocarbone receptor (AhR). Through AhR, PAHs can then trigger ERK1/2 activation and signaling in hepatic "stem cell-like" epithelial cells [67, 68]. Other PAHs, such as benz(a)anthracene (BaA), has also been found to increase DNA synthesis and promote G1-S progression in serum deprived MCF-7 cells [69]. BaP has been shown to increase incidence of tumors in estrogen-responsive rodents, suggesting that it may also affect ER-mediated signaling [70]. PAHs can have actions which mimic those of estrogen. Some investigators believed that the estrogenic property of PAHs may be responsible for the induction of cell proliferation. BaP and BaA have been reported to act as estrogens that stimulate and initiate the ER-mediated transcription and cell cycle progression and enhance ERα phosphorylation [70]. On the other hand, there is also indication that the estradiol-dependent cell growth of MCF-7 cells can be inhibited by BaP and BaA [71, 72]. Thus, the actions of PAHs on estrogen-dependent cell proliferation are still controversial. Further studies are needed to elucidate more on the roles of PAHs in carcinogenicity.

2.2. Effects of Cigarette Smoke on Antigrowth Signals. In normal tissues, the antigrowth signals operate to maintain cellular quiescence and tissue homeostasis. Antigrowth signals can block proliferation by forcing the cell cycle progression into the quiescent (G0) state. The cell cycle transition from G1 to S phase is the key regulatory step in the cell cycle and is mainly regulated by CDK4/6-cyclin D and CDK2-cyclin E complexes. These complexes induce Rb phosphorylation and liberate E2Fs allowing cell proliferation to occur [73]. Disruption of the Rb pathway would therefore render cells insensitive to antigrowth factors [29]. Nicotine has been reported to induce binding of Raf-1 to Rb with activation of cyclins and CDKs as well as inactivation of Rb [74]. Via activations of nAChR and β-AR, nicotine and NNK both exhibit mitogenic properties by inducing cyclin D1 overexpression leading to G1/S transition and increasing cell cycle progression [49, 75, 76]. NNK can also stimulate normal human lung epithelial cells proliferation through NF-κB and cyclin D1 upregulation in an ERK1/2-dependent pathway [75]. In our own laboratory, we have also demonstrated that nicotine-induced cyclin D1 overexpression is regulated via Stat3, ERK1/2, and NF-κB-dependent pathways in bladder cancer cells [43].

Other study also shows that PI3K/AKT-dependent cellular proliferation is also enhanced in response to NNK [49]. The PI3K/AKT pathway is critical in cancer cells because it influences tumorigenesis, tumor growth, and therapeutic resistance [77]. The PI3K/AKT activation is documented in both NNK-treated A/J mice and in human lung cancers from smokers [48]. It also plays a role in NNK-induced cell transformation, proliferation, and metastasis [48]. It has been suggested that AKT and NF-κB may serve as key targets for nicotine or NNK stimulation in the development of lung cancer [49]. West et al. also reported that BEAS2B cells treated with NNK for eight-week period increased cellular proliferation through activation of PI3K/AKT pathways [48]. However, PI3K/AKT activation does not always occur in all cancer cells induced by nicotine. Our previous study indicates that nicotine induced bladder cancer cell proliferation through Stat3 and ERK1/2 signalings instead of via AKT pathway [43]. All these investigations suggest that nicotine or NNK can activate ERK1/2, Stat3, or AKT signaling to interrupt the antigrowth signals leading to enhanced cell cycle progression and cancer promotion. It is important to remember that cigarette smoke components other than nicotine or NNK may also impede on antigrowth mechanisms enhancing cancer development and promotion. Such area of research also deserves focus in the future.

2.3. Antiapoptotic Effects of Cigarette Smoke. Apoptosis plays an important role in controlling normal development, homeostasis, and immune defense via elimination of redundant or abnormal cells in the organism [78]. Failure in cell elimination (reduction of apoptosis) may lead to undesirable cell survival and unchecked cell growths. Resistance to apoptosis is often seen in cancers where cancer cells tend to lose their proapoptotic potentials because of gene mutations. The most important gene mutations include tumor suppressor genes such as *p53*. Nicotine has been shown to inhibit apoptosis induced by tumor necrosis factor (TNF), by ultraviolet (UV), radiation, or by chemotherapeutic drugs such as cisplatin, vinblastine, paclitaxel, and doxorubicin [79]. This antiapoptotic action has been shown to be via PI3K/AKT, Raf/MEKK/ERK1/2, NF-κB, Bcl-2, Bax, Bad, or surviving [23, 80–82]. West et al. demonstrated inhibition of apoptosis and promotion of proliferation in human bronchial epithelium cells by NNK are induced via activation of $\alpha 3/\alpha 4$ nAChR followed by upregulation of AKT, mitogen-activated protein kinase (MAPK), and PKC pathways [48]. Similar results are also observed by Xu and coworkers showing that both AKT and survivin pathways are involved in anticisplatin-induced apoptosis by nicotine [79]. Indeed, drug-induced enhancements of p53 and p21 expressions are shown to be suppressed by nicotine. This anti-apoptotic mechanism is mediated through $\alpha 3$ nAChR [83]. Our recent study also indicated that long-term nicotine treatment activated $\alpha 4/\beta 2$ nAChR and β-AR leading to reduction of apoptosis induced by cisplatin or paclitaxol [57]. Consistently, Zhao et al. also reported that nicotine induced up-regulation of Mcl-1 phosphorylation though ERK1/2 via β-AR activation with increased chemoresistance (anti-apoptosis) of human lung cancer cells [84]. Other investigators also indicate that NNK can prevent cell apoptosis by modulating the anti-apoptotic Bcl-2 and c-Myc proteins [23]. Heme oxygenase-1 (HO-1) is a protein induced during oxidative stress. It is found to be associated with cellular proliferation and is elevated during the developments of certain malignant tumors such as gastric and thyroid cancers [11–13]. Comparing the HO-1 in lung tissues of smokers and nonsmokers, Li et al. noticed that the expression of HO-1 is significantly increased in both tumor and nontumor tissues of smokers. These studies further revealed that NNK or its metabolites probably induce oxidative stress in lung tissues with elevation on stimulates the expression of HO-1. Such event is through ERK and NF-κB activation and Bad phosphorylation induction leading to eventual apoptosis inhibition [11, 85].

Cell proliferation and apoptosis can also be modulated by the peroxisome proliferator-activated receptors (PPARs). PPARs are members of nuclear hormone receptor superfamily of ligand-dependent transcription factors. The major PPAR isoforms are α, β/δ, and γ [86]. PPARβ/δ is expressed in most tissues and has been reported to be associated with cancer growths, especially those in liver, colon, breast and lungs [87–89]. Sun et al. reported a novel mechanism that nicotine increases PPARβ/δ expression through $\alpha 7$ nAChR follow by PI3K/mTOR activation leading to enhanced lung tumor cells proliferation [90]. In contrast to PPARβ/δ, activation of PPARγ by its ligands induces apoptosis and inhibits cell proliferation [91]. Thus, an intact PPARγ levels or its activation is needed to reduce cancer risk (anti-apoptosis and cell proliferation). Interestingly enough, activation of PPARγ is found to be defective in lung cancers [92]. Furthermore, a significant reduction in the transcriptional activity of PPARγ and its endogenous ligands, including 15-S-Hydroxyeicosatertraenoic acid (15(S)-HETE) and 3-S-hydroxyocatadecadienoic acid (13(S)-HODE), are found reduced in lung tissues of NNK-treated mice. Indeed, lung tumors developed in these mice later. Yuan et al. further suggested that the reduction of 15(S)-HETE and 13(S)-HODE may enable lung cells to be more resistant to apoptosis by NNK and facilitate tumor development in the animals [93].

In contrast to nicotine or NNK, PAHs induce either apoptosis or antiapoptosis in mammalian cells [94, 95]. For instance, BaP is known to induce signaling through IGFR and increases cell survival through PI3K activation in human mammary epithelial cells [68]. Solhaug et al. reported that both AKT and ERK1/2 act as anti-apoptosis signals leading to Bad phosphorylation. However, BaP can also induce apoptosis through p53 and p21 signaling in the same model [96]. The results suggest that BaP is capable in stimulating both apoptosis and anti-apoptosis signals. Teranishi et al. reported that light-irradiated BaP (LBaP) inhibited apoptosis through production of ROS from degraded BaP [97]. This anti-apoptotic signal induced by BaP in combination with DNA damage would increase the possibility of cell survival and producing mutations. Thus, while the apoptotic signal of BaP induces cell death (cytotoxicity), the anti-apoptotic signals of BaP play an important role in cell proliferation and carcinogenesis. The precise factors influencing either apoptotic or anti-apoptotic outcome are still unclear. The anti-apoptosis mechanisms induced by components of cigarette smoke are obviously quite complex. It is evident that evading apoptosis plays a critical role in cigarette smoke-induced tumorigenesis and chemoresistance. Further explorations are very much needed. New understandings on the molecular target regulating the apoptotic and anti-apoptosis machineries by cigarette smoke could provide novel strategies for drug development with substantial therapeutic benefits.

2.4. Effects of Cigarette Smoke on Replicative Lifespan. When a cell population has progressed through a certain number of doublings (replications), they would normally stop growing and enter into a process called "senescence". Tumor cells, however, appeared to have limitless replicative potentials (immortalization) during tumor progression [29]. Telomeres, which define the end segments of chromosomes, consist of short, tandemly repeated DNA sequences $(TTAGGG)n$ together with associated proteins. They represent important devices in controlling cell divisions and proliferations. Small amount of these end DNA sequences may be lost during each cell cycle as a result of incomplete DNA replication. However, de novo additions of TTAGGG repeats by the enzyme telomerase may compensate for this loss [98]. Thus, telomerase plays an important role in the maintenance

of the telomere ends in normal cells. Ectopic expression of telomerase would immortalize the cells.

By using human tissue samples, Yim et al. reported that there are different distributions of the telomerase activity between smokers or ever-smokers and non-smoker. A strong correlation between telomerase activity and the number of packs years smoked can be established among these subjects indicating that there is an association between tobacco exposure and telomerase activity in the human bronchial epithelium. Increased telomerase activity would extend the "lifespan" of cells and put these cells to be at higher risks for malignant transformation and carcinogenesis [99]. Similar finding is reported by Targowski et al. that extensiveness of tobacco smoking correlated positively with increases in telomerase activity in tumor cells from patients with non small cell carcinoma of the lungs [100]. All these studies point to the fact that enhancement of the telomerase activity by cigarette smoke certainly underlies the cancer promotion potentials of cigarette smoke. However, which components in cigarette smoke altered telomerase activity are still not known. Further study in this aspect is very much needed.

2.5. Effects of Cigarette Smoke on Mobilization of Cellular Resources. Tumorigenesis requires adequate ability for protein synthesis and the energy for activating signaling. Indeed, there are indications that certain protein synthesis and mitochondria play central roles in neoplastic transformation. It is well known that mTOR and MAP kinase signaling pathways modulate the phosphorylation of transcriptional factors, stability of mRNAs, and protein synthesis [101]. Jin et al. reported that both nicotine and its metabolite NNK can induce survivin mRNA expression through AKT-mTOR and mediated *de novo* synthesis of survivin protein in normal lung epithelial cell HBE cells. This induced survivin expression has been claimed to play a role in the malignant transformation of HBE cells by stimulating the survival pathways [102].

Cigarette smoke may damage respiratory chain function in mitochondria enhancing oxidative stress leading to mitochondria dysfunction [103, 104]. It has also been reported that nicotine exposure resulted in reduced pancreatic mitochondrial enzyme activity, degranulation of beta cells, elevated islet oxidative stress, and impaired glucose stimulated insulin secretion in rats [105]. Continued exposure to ROS and free radicals from such "mitochondrial stress" may lead to mitochondria DNA (mtDNA) mutation which may play an important role in carcinogenesis [106]. Analyzing clinical samples, Tan et al. demonstrated mtDNA mutation in buccal cells of smokers [107]. Petros et al. also showed that tumor cells with mtDNA mutations grow faster then cells without mitochondrial mutation [108]. Hence, it is apparent that cigarette smoke would induce oxidative damage to the mtDNA leading to more aggressive tumor growths. Impact of cigarette smoke or its components on mitochondrial dysfunction needs further exploration.

Step 3 (Cancer Progression). The "malignancy" of a tumor is usually evaluated by its ability in invasion and metastasis as well as in the associated angiogenesis. There are ample evidence which indicate that cigarette smoke participates in the processes of angiogenesis, invasion, and tumor metastasis. These phenomena are presented and discussed below.

3. Effects of Cigarette Smoke on Sustained Angiogenesis

Angiogenesis, the development of new blood vessels from endothelial cells (ECs), is a critical event which allows the cancer cells to receive adequate nutrients and oxygen. Angiogenesis involves mature vascular changes, including detachment of pericytes, degradation of extracellular matrix, endothelial cells remodeling, proliferation, migration, and formation of new endothelial cells into tubular structures [109]. Survival and proliferation of vascular endothelial cells are often stimulated by tumor-derived mitogens, and vice versa. Tumor cells are known to activate angiogenesis by changing the balance of angiogenic inducers such as VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor), and by countervailing inhibitors such as thrombospondin-1 [29]. VEGF promotes angiogenesis and lymphangiogenesis in tumors, providing routes for dissemination. It has been shown that nicotine can induce angiogenesis both *in vitro* and *in vivo* and contributes to the growth of tumors [30, 110]. Similar to the FGF, nicotine is found to have the ability to promote migration, proliferation, tube formation and nitric oxide (NO) production of endothelial cells [111]. NO is a well-known vasodilator and angiogenesis mediator, and nicotine has been reported to enhance the expression of endothelial nitric oxide synthetase and promote NO release [110].

Nicotine is also found to induce expression of endothelial growth factors such as VEGF, bFGF, PDGF, TGF-α, and TGF-β in endothelial cells and smooth muscle cells [112, 113]. Enhanced bFGF release and increases in metalloproteinase expression with degradation of ECM have been demonstrated with nicotine [114, 115]. Moreover, nicotine is found to induce secretion of prostacyclin which is a vasodilating molecule associated with endothelial cell proliferation, survival and migration [116]. These effects are believed to be associated with cigarette smoke-induced hyperplasia of the intima in the blood vessels and other vascular wall lesions [115].

Tumor angiogenesis can also be modulated by the nAChR [117]. $\alpha 7$ nAChR is important in both physiological and pathological angiogenesis [110, 118]. $\alpha 7$ nAChR in endothelial cells needs to be sensitized or activated by hypoxia or ischemia in order to induce angiogenesis [110]. Indeed, specific antagonist of the $\alpha 7$ nAChR (α-bungaratoxin) is shown to inhibit nicotine-induced angiogenesis (new vascular tube formation from endothelial cells) [25, 114]. Interestingly enough, it is apparent that the AKT pathway is found to be not involved in either angiogenesis or VEGF release induced by nicotine [25]. In contrast, Heeschen et al. suggested that inhibition of ERK1/2, p38 MAPK, and PI3K/AKT can completely block and prevent

endothelial tubule formation induced by nicotine-triggered $\alpha 7$ nAChR activation [110]. Consistent with Heeschen's study, Zhang and coworkers reported that nicotine apparently increases angiogenesis and invasion by activating PKC, PI3K/AKT, ERK1/2, mTOR, and Src in human NSCLC [119]. Excellent reviews on angiogenesis induced by nicotine were recently published [120, 121] and will not be further discussed here.

Interaction between nAChR and the growth factor-mediated angiogenesis occurs at signaling and transcription levels. Nicotine-induced expression of VEGF has been shown to be regulated by EGFR transactivation and via the ERK1/2 pathway in smooth muscle cells [122]. Phosphorylation of the VEGF receptor KDR by nicotine activates VEGF and increases its activity [112]. Additionally, nicotine can also upregulate the expression of VEGF receptor VEGFR2 during angiogenesis in certain cancer cells [123]. Recent study further indicated that nicotine can synergistically promote the proangiogenic effect of estradiol in nonsmall lung cancer [124]. Induction of angiogenesis in colon cancer by nicotine via β-AR followed by arachidonic acid pathway has also been reported [32, 125].

In sum, $\alpha 7$ nAChR subtype has been linked to angiogenic process induced by nicotine leading to tumor vascularity, inflammation, and ischemia. Nevertheless, whether nicotine or NNK acts specifically via nAChR or β-AR receptors or both or whether it is controlled in a cell-specific manner needs further study. Other components present in cigarette smoke that may also contribute to angiogenesis remain to be identified. The significant role of nAChR in various pathogenic angiogenesis is still largely unknown. This information would be critical for the development of new anti-angiogenic therapies. Several excellent reviews on the roles of nicotine and nAChR in angiogenesis exist [117, 120, 121, 126]. Readers are encouraged to refer to them for more detailed information.

4. Effects of Cigarette Smoke on Cancer Invasion and Metastasis

The ability of invasion and metastasis allows cancer cells to escape from the primary tumor mass to new terrains in the body. Metastasis is the final and most devastating consequence in malignancy. The processes of invasion and metastasis are exceeding complex. The genetic and biochemical determinants as well as the molecular mechanisms involved are still poorly understood. Many evidence indicate that cigarette smoking not only increases proliferation of cancer cells but also promotes metastasis [127]. Clinical and epidemiological studies suggest that smokers have more rapidly progressing tumors and cancer metastasis than nonsmokers [128]. These processes are now known to be dependent on cellular and stromal interactions and on extracellular matrix degradation. E-cadherin is a cell-to-cell interaction molecule expressed on epithelial cells. A loss of E-cadherin is seen in epithelial to mesenchymal transition (EMT), which is a major pathologic event in cancer metastasis. Chronic treatment of nicotine downregulated the expression of ECM proteins such as E-cadherin and β-catenin with concomitant increases of fibronectin and vimentin in lung cancer cells [129]. Wei et al. also indicated that NNK enhanced colon cancer cell migration with downregulation of E-cadherin. This author also found that the expressions of Snail and ZEB1, 2 major transcription repressors of E-cadherin, were also induced by NNK in colon cancer cell cultures [44]. Contactin-1 is a glycophosphotidylinositol (GP)-anchored adhesion molecule. Its upregulation is significantly linked with tumor progression, metastasis and poor prognosis in lung cancer patients [130]. It has been shown that NNK can upregulate contactin-1 via $\alpha 7$ nAChR/ERK activation and enhances invasiveness of lung cancer cells [131].

The second general mediators for invasion and metastasis are the extracellular proteases [29]. Breakdown of the extracellular matrix (ECM) through a family of enzyme called matrix metalloproteinases (MMPs) is needed for tumor cells to invade adjacent tissue and to metastasize. Zong et al. reported that nicotine enhanced the invasiveness of esophageal squamous carcinoma cells (TE-13) by up-regulating the expressions and activity of MMP-2, and COX-2 [132]. Nicotine is found to enhance the activity of MMP-2, and MMP-9 as well as activation of plasminogen activators in a COX-2 and VEGF-dependent manner [123]. Osteopontin (OPN) is a proinflammatory and pro-metastatic protein. It can be upregulated by nicotine. It serves as a good marker for PDAC (pancreatic ductal adenocarcinoma) metastasis especially in cigarette smoking population [133]. In a recent investigation, Lazar et al. demonstrated that nicotine contributes to PDAC metastasis through the induction of MMP-9 and VEGF mediated by OPN [134].

PAHs, including BaP, are also found to play a role in the promotion of cancer metastasis. Through augmented COX-2 expression and PGE2 production via activated AhR pathway, BaP induces breast cancer cell invasions [135]. BaP and PAHs mixture has also been demonstrated to induce cancer cell invasions and metastasis through upregulating the expressions of MMPs, proteinase-activated receptor-2, fibronectin, migration stimulating factor, and Bcl-2 protein in lung adenocarcinoma [136]. The importance of FGF-9 and its up-regulation by BaP in lung cancer invasion and metastasis has been proposed. Indeed, recent study by Ueng et al. [137] demonstrated that BaP increases the invasive potential of lung cancer cells *in vitro*. Such process involves the up-regulation of FGF-9 mRNA expression via the p38 and ERK1/2 pathways [137].

During metastasis, the cancer cells co-opt signals that control leukocyte trafficking and chemokines-mediated cell migration [138]. Among these chemokines, CXCR4 and its natural ligand CXCL12 serve as key mediators for tumor migration and metastasis [139]. Nicotine has been shown to increase the expressions of several CXC chemokines receptors such as CXCR2, CXCR3, and CXCR4 as well as CCL12 in SCLC cells [140] suggesting the nicotine would stimulate cancer cell migration and eventual metastasis.

Although epidemiology studies have long demonstrated the relationship between smoking and cancer metastasis, the molecular mechanisms of metastasis influenced by cigarette smoke or its components remain very limited. Further studies in this subject are urgently needed.

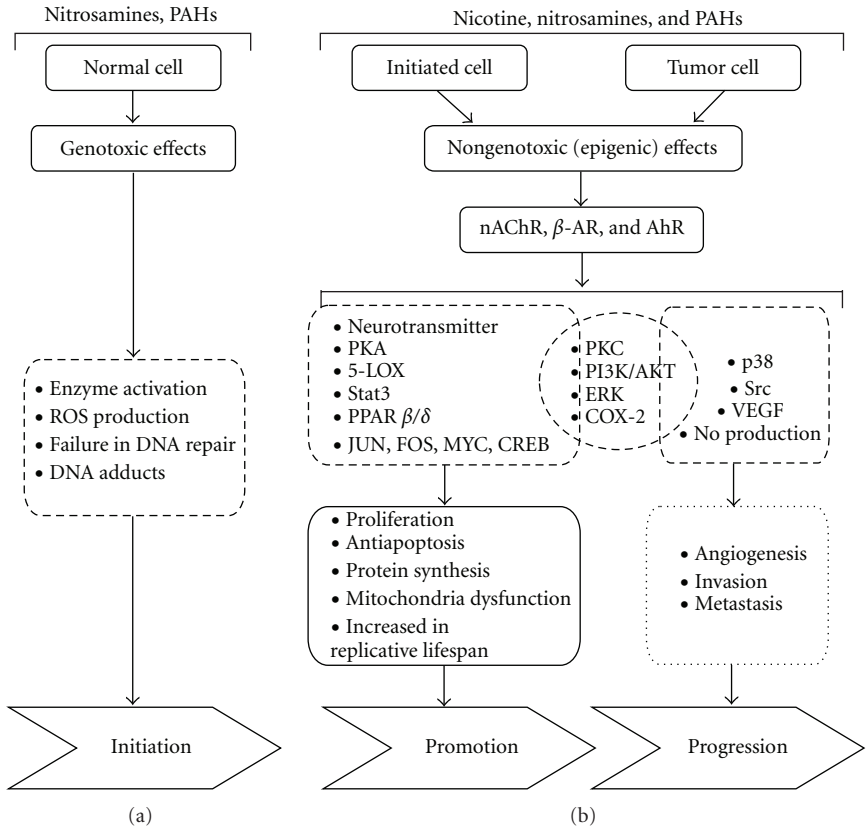

Figure 1: Diagrammatic models summarizing simplified molecular mechanisms of genotoxic and non-genotoxic modes of action in carcinogenesis by cigarette smoke. (a) Nitrosamines and PAHs are carcinogens, inducing genotoxic effects leading to cancer initiation. (b) Non-genotoxic (epigenic) effects of cigarette smoke components (nicotine, nitrosamines, and PAHs) in cancer promotion and progression. Activation of nAChR, β-AR, or AhR, followed by neurotransmitters release, activation of signaling pathways (PKA, 5-LOX, Stat3 and PPARβ/δ), and increased the expression of transcriptional factors (JUN, FOS, MYC, and CREB) regulate cancer promotion by cigarette smoke. PKC, PI3K/AKT, ERK, and COX-2 signaling pathways downstream of receptors play important roles in both promotion and progression stages. p38, Src, VEGF, and NO releasing involve in enhancement of cancer progression by cigarette smoke.

5. Summary, Conclusive Remarks, and Future Perspectives

In this paper, we have reviewed the recent investigations concerning cigarette smoke and cancer development, promotion and progression. While chemicals with carcinogenic potentials in cigarette smoke are many (over 62), most research efforts have been devoted to three components of cigarette smoke: nicotine, NNK, and PAHs. While PAHs are common chemicals in the environment, nicotine and NNK are considered to be tobacco specific. These three important components of cigarette smoke, especially nicotine and NNK, therefore, are targeted as the major compounds of focus in this review. Many previous reviews have devoted to the interrelationship between cigarette smoke and lung carcinogenesis or the genotoxicity of cigarette smoke or its components. In this review, we are focused on the mechanistic information on tumorigenesis, especially those involving epigenetic or non-gentoxic effects. Aside from lung cancer, other tobacco-related cancers are also discussed. It is our hope that this review will summarize the vast information cumulated in the literature and provide valuable reference resource for those who are interested in tobacco-related carcinogenesis.

The overall mechanisms on carcinogenesis cancer promotion and progression are complex involving many molecular targets which include receptors, cell cycle regulators, mitogen-activated protein kinases, apoptosis mediators, angiogenic factors, and invasion, and metastasis mediators. Among the receptors, nAChR, β-AR, and AhR probably are the most important and have the closest association with cigarette smoke-induced carcinogenesis. Overexpression or activation of these receptors may result in the release of neurotransmitters and growth factors that participate in apoptosis inhibition, cell proliferation, angiogenesis, cancer cell invasion and metastasis. It should be noted that the importance of nAChR in cancer may be cell-type-dependent or specific and their sensitivity and expression can be also be modified by various environmental factors such as insecticide organophosphates [141].

As shown in Figure 1, signaling pathways, PI3K/AKT, Stat3, and ERK1/2 play important roles in the carcinogenesis processes. They are also common paths affected by the cigarette smoke components, including nicotine, NNK, and

PAHs. In addition, PKC, AKT, ERK, and COX-2 signaling pathways are involved in both promotion and progression stages by cigarette smoke. It is suggested that these molecules could be utilized the potential targets for future developments in cancer diagnoses or therapies.

Avoidance of cigarette smoke remains to be the best way of prevention for cigarette-related cancer. However, in view that tobacco smoke is legalized and smokers are still abundant, understanding on the health impacts by tobacco smoking still constitutes important public health concern. Understanding the disease process and the mechanisms involved is the first step to solution. The emerging understanding on the molecular mechanisms in the development and progression of caners induced by cigarette smoke provides novel inspirations and approaches for potential measures on early diagnosis, reduction in progression and metastasis, and therapy of cancers. Many dietary supplements, foods, or herbal medicines might significantly attenuate the proliferative effects by cigarette smoke. They may also enhance antigrowth signals to reduce cancer growth. From our own experience, the natural compound pterostilbene could induce apoptosis and autophagy in chemoresistant bladder cancer cells derived from nicotine exposure [142]. Future research on natural compounds may help to provide additional novel chemopreventive or chemotherapeutic possibilities in reducing cancer risks or other health impacts of cigarette smoke. This area of research is still weak and should be explored.

This review has also discussed the various molecular mechanisms and paths involved in carcinogenesis induced by cigarette smoke. However, there are still many mysteries in the carcinogenic process by cigarette smoke. Several recommendations can be offered for future research needs.

(1) In the past, most research efforts were focused on the proliferative and antiapoptosis mechanisms induced by cigarette smoking. As tumors are the results of multiple and interactive genetic abnormalities, study of cancers induced by cigarette smoke should assess more than one or two acquired alterations or paths. Explorations of other "paths" or mechanism other than those "popular" ones are needed.

(2) Those molecular pathways which are significantly activated by cigarette smoke are probably the most important ones involved in cigarette smoke-induced tumorigenesis. These pathways include nAChR signaling (such as α7 nAChR, α9 nAChR, or α4/β2 nAChR), β-AR signaling, PI3K/AKT signaling, ERK1/2 signaling, Stat3 signaling, VEGF, and MMPs pathways, and so on. Targeting to modulate these pathways via dietary factors or therapeutic drugs may reduce cigarette smoking induced tumorigenesis significantly. Studies on the non-genotoxic (epigenetic) effects of cigarette smoke components are few and need more efforts. The epigenetic effects of cigarette component must be evaluated to include both upstream and downstream pathways.

(3) Carcinogenesis is often species or cell-type specific and can be influenced by many factors or cofactors. Proper study of carcinogenicity requires consideration of these different variables. The same factor which is highly oncogenic to certain cell type or individuals may not be oncogenic to others. Moreover, some cell type may become susceptible to a "carcinogen" only in the presence of certain factor(s), cofactor(s), genetic predisposition, or immune depression. Identification of such influencing factors will be important. Specific "mechanism" for carcinogenesis for the same "carcinogen" may also vary in different tissues. Information obtained will be helpful for future cancer prevention, diagnosis and treatment.

(4) Synergistic interaction between cigarette smoke components and other environmental toxicants or carcinogens, such as arsenic or dioxin, on cancer development has been demonstrated both epidemiologically and in animal studies [143–145]. Traditionally, most past investigations focused only on "single" compound or one cigarette smoke component. The synergistic interaction between otherwise "safe" level of environmental chemical and low level of cigarette smoke or its component (via either active or secondhand smoking) for carcinogenesis raised novel public health concerns and challenging questions. This area of research certainly deserves future attentions and efforts.

In conclusion, we have provided an overview on the major concepts and insights on the molecular mechanisms involved in cigarette smoke-induced cancers. It is hoped that these mechanistic insights can be translated into practical applications for the prevention and treatment of cigarette smoke-related cancers. We have also offered several recommendations for future research. We also hope that these suggestions will be helpful to those who are interested in this area of cancer research.

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