Nicotine Exacerbates Brain Edema during in vitro and in vivo Focal Ischemic Conditions

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Nonstandard abbreviations used in the paper: N-CSE: Nicotine-containing Cigarette Smoke Extract, NF-CSE: Nicotine-free Cigarette Smoke Extract, BBMEC: Bovine Brain Microvessel Endothelial Cells, NKCC: Na,K,2Cl-cotransporter, BBB: Blood-Brain Barrier, aCSF: artificial Cerebrospinal Fluid, MCAO: middle cerebral artery occlusion, OGD: oxygen glucose deprivation, nAChR: nicotinic acetylcholine receptor; TTC: 2,3,5-Triphenyltetrazolium chloride

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Abstract

We have previously shown that nicotine, the addictive component of tobacco products, alters the blood-brain barrier (BBB) Na⁺, K⁺, 2 Cl⁻ cotransporter (NKCC) during in vitro hypoxia-aglycemia exposure. Attenuation of abluminal NKCC suggests that accumulation of ions in the brain extracellular fluid would result in an increase of fluid or cytotoxic edema in the brain during hypoxia-aglycemia or stroke conditions. To further investigate if nicotine products have the potential to worsen stroke outcome by increasing edema formation, two separate models to mimic stroke conditions were utilized to decipher the effects of acute and chronic administrations of nicotine products on brain edema following stroke. Oxygen Glucose Deprivation (OGD) was studied in rat hippocampal slices with acute or chronic exposure to nicotine and cigarette smoke constituents. During acute exposure the presence of nicotine at a concentration mimicking heavy smokers increased water content of hippocampal slices during OGD. Additionally, chronic 1 week administration of nicotine increased water content in hippocampal slices, that could be attenuated with nicotine acetylcholine receptor (nAChR) antagonists, suggesting nicotine increase edema during OGD via nAChRs. A second model of focal ischemia, middle cerebral artery occlusion (MCAO), showed an increase of infarct size during acute exposure to nicotine and an increase of edema during both acute and chronic administration of nicotine, compared to saline controls. These findings support the paradigm that nicotine products not only increase the incidence of stroke but also have the potential to worsen stroke outcome by increased edema formation.
Introduction

Stroke has the third highest mortality rate of 160,000 per year and is a leading cause of neurological disease resulting in long term disability with 3.5 million survivors in the United States (Hankey, 1999; Carandang et al., 2006). Cigarette smoking and even second hand smoke has been associated with increased incidence of stroke in both men and women (Bonita et al., 1999). Smoking cigarettes clearly have been shown to be a risk factor for stroke (Bonita et al., 1999; Ueshima et al., 2004). One quarter of strokes are associated with smoking (Hankey, 1999). The risk of stroke is dose dependent and increases from 3.57 to 4.65 with 5-15 cigarettes per day (Bonita et al., 1999). In addition to enhanced risk factors for brain ischemia, there is a growing body of evidence that nicotine alters BBB permeability characteristics that have a direct influence on stroke outcome and the pathophysiology of brain ischemia (Abbruscato et al., 2002; Hawkins et al., 2002; Abbruscato et al., 2004; Hawkins et al., 2004). More importantly, stroke outcome has been shown to be worsened by increased edema observed in a two week exposure to nicotine after focal ischemia in rats (Wang et al., 1997).

The controlled water movement and maintenance of ion balance in the brain extracellular fluid (ECF) are imperative for the reduction of edema and neuronal survival during stroke conditions. Other diseases also have brain water balance as a central point of damage including head trauma, brain cancer, and certain types of epilepsy (Agre et al., 2004). Edema is thought to play a central role at all levels of the neurovascular unit during neuronal damage associated with ischemia. Cellular swelling can be seen at the endothelial cell, pericytes, and astrocytic foot processes within the first 90 minutes of focal occlusion (Liu et al., 2001). Edema formation contributes to stroke morbidity and
mortality and Na$^+$ accumulation in the brain occurs during ischemic assault prior to blood brain barrier (BBB) breakdown (Dzialowski et al., 2004).

Two types of edema are associated with stroke including cytotoxic cellular edema and vasogenic edema (Unterberg et al., 2004). Cytotoxic edema, experienced at the beginning of an ischemic incident, is associated with increased extracellular ion accumulation resulting in increased water content in cells including glia and neurons (Unterberg et al., 2004). Vasogenic edema later amplifies the damage done by cellular edema and is contributed by blood-brain barrier breakdown (Unterberg et al., 2004). Brain water content begins to immediately accumulate before blood-brain barrier (BBB) break down and has been monitored with CT scan in a rat middle cerebral artery occlusion (MCAO) model (Dzialowski et al., 2004). Accumulation of water in astrocytes has been suggested to produce the most damage due to the volume of astrocytes versus neurons in the brain (20:1 in humans and 10:1 in rats) (Kimelberg et al., 1995; Unterberg et al., 2004).

Previously we have shown that nicotine, the addictive component of tobacco products, is responsible for alteration of the Na$^+$, K$^+$, 2 Cl$^-$ cotransporter 1 (NKCC1) on the abluminal (brain facing) surface of the BBB during in vitro hypoxia/aglycemia conditions used to model stroke. This could result in the accumulation of ions including Na$^+$, K$^+$, and Cl$^-$ in the brain extracellular fluid, resulting in increased fluid or brain edema during hypoxic or stroke conditions with or without reperfusion (Abbruscato et al., 2004; Paulson et al., 2006). However, there is no further information on how cigarette smoke constituents, not only nicotine, will affect edema formation in animal models of stroke, especially under chronic exposure.
In order to further investigate the possibility that nicotine products have the potential to worsen stroke outcome by increased edema formation, two separate models to mimic stroke conditions were utilized to decipher the effects of acute and chronic administration of cigarette smoke constituents or nicotine on brain edema following stroke. The first model, Oxygen Glucose Deprivation (OGD), was studied in rat hippocampal slices with acute exposure to nicotine or chronic exposure to cigarette smoke constituents. A second model of focal ischemia, middle cerebral artery occlusion (MCAO), was used to evaluate infarct size and edema during acute and chronic exposure to nicotine. Knowledge gained from these studies could lead to better therapeutic approaches to protect the central nervous system from neurological damage associated with nicotine and/or stroke insults.
Methods

Preparation of smoke condensates

Cigarettes (Marlboro® filter cigarettes, Philip Morris Inc., VA, USA and Quest® #3, Vector Tobacco Inc., NC, USA) were obtained through commercial sources. Marlboro® filtered cigarettes contain 1.1 mg nicotine per cigarette and Quest® #3 contains no more than 0.05 mg nicotine per cigarette and is therefore regarded to be nicotine-free for these studies. Mainstream smoke was bubbled through 50 ml acetone by a slight vacuum drawn repetitiously through a custom manufactured apparatus. A cigarette smoke extract (CSE)-acetone solution was generated which was concentrated under vacuum. The residue was subsequently re-dissolved in 1.626 ml propylene glycol: dimethyl sulfoxide (PG/DMSO, 1:1). The products of the Marlboro® and Quest® cigarette extraction were designated as N-CSE (Nicotine-containing Cigarette Smoke Extract) and NF-CSE (Nicotine-free Cigarette Smoke Extract) respectively. These methods have successfully been used in vitro to model exposure to cigarette smoke chemicals (Sherratt et al., 1988; Paulson et al., 2006).

Hippocampal slice oxygen glucose deprivation

Experimental design was formed by varying a method of hippocampal brain slicing and subsequent exposure to oxygen glucose deprived artificial CSF (MacGregor et al., 2003). Female Spargue Dawley rats were anesthetized and euthanized through cervical dislocation. The brain was extracted and the hippocampus identified by stereotaxic coordinator (Franklin and Paxinos, 2001) was sliced with a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd.) at 400 microns. Hippocampal slices were pre-equilibrated in a solution of ice cold artificial cerebrospinal fluid (aCSF, 137
mM NaCl, 2.7 mM KCl, 0.2 mM CaCl₂, 1.2 mM MgCl₂, 0.2 mM NaH₂PO₄, 11.9 mM NaHCO₃, and 5.6 mM Glucose) with the addition of 1 mM ascorbic acid. Pre-incubation with ascorbic acid prevents artificial edematous conditions produced by *in-vitro* conditions (Brahma et al., 2000). To ensure cellular survival, the time period from brain extraction to placement in artificial CSF was consistently kept under 3 minutes.

Hippocampal slices were treated under normoxic or OGD conditions in a PermeGear permeation chamber (AMIE Systems) with the slices placed between thin nylon 200 mesh screen (Ted Pella Inc.). Artificial CSF flowed through the chamber at a constant rate of 0.7 mL/min at a temperature of 37°C. Normoxic conditions were maintained through control aCSF with the presence of glucose being bubbled with oxygen (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 0.2 mM NaH₂PO₄, 11.9 mM NaHCO₃, 5.6 mM Glucose). OGD was maintained through aCSF with the absence of glucose and bubbled with 95% Nitrogen and 5% CO₂ and kept at a temperature of 37°C.

Acute and chronic treatments were tested in this study. Acute treatment of nicotine/cotinine was administered through the aCSF at varying concentrations ranging from 1/10, 10/100, 100/1000, and 1000/10000 ng/mL of nicotine and cotinine respectively. Chronic 1 week and 3 weeks administration of vehicle control, nicotine, N-CSE, or NF-CSE was administered to rats using alzet 2ML4 osmotic pumps at a concentration to mimic heavy smokers (4.5 mg/kg/day) in accordance to previous studies (Murrin et al., 1987; Wang et al., 1997). Nicotine and cotinine plasma levels were verified by RIA and HPLC to confirm comparable plasma levels within groups.
In addition, hippocampal slices subjected to OGD with N/C and nAChR antagonists (25, 125, 250 µM mecamylamine or 10 nM α-bungarotoxin) were tested to evaluate the edematous effects involved with nAChRs.

Edema formation was evaluated by determining the percentage of water content of each slice. Hippocampal slices were initially weighed on a Cahn microbalance immediately following the treatment incubation on pre-weighed aluminum foil and considered “wet weight.” After 24 hr period of desiccation at 95°C the slices were again weighed and recorded as “dry weight.” Water content was then calculated as \[(\text{wet weight} – \text{dry weight})/\text{wet weight}] \times 100.

**In-vivo middle cerebral artery occlusion (MCAO) model**

The methods used here were a slight modification of previously published procedures (Mdzinarishvili et al., 2005). CD-1 female mice were pretreated with nicotine, N-CSE, or NF-CSE in an acute 1, 3 or 6 hour dosage or chronic 1 week or 3 weeks dosage (nicotine in 0.9% NaCl, N-CSE and NF-CSE in PG/DMSO). Acute injections of nicotine or CSEs were equivalent to 4.5 mg/kg/day calculated from the known nicotine yield. For acute 1hr, 3hr, and 6hr nicotine dosage, i.p injection of 187.5µg/kg, 562.5µg/kg, and 1125µg/kg respectively of nicotine is given. The doses are calculated as for 1hr, 4.5mg/kg divided by 24 hours=187.5 µg/kg and so on for 3hr and 6hr doses. This dose was selected because it does not result in gross signs of toxicity, the resulting nicotine and cotinine plasma concentrations reached are similar to that found in heavy smokers (80-100ng/ml) (Murrin LC et al., 1987; Lockman PR et al., 2005). Data from our previous studies (Lockman PR et al., 2005a; Lockman PR et al., 2005b) demonstrate that this dosage of nicotine does not alter regional BBB integrity or regional cerebral
perfusion flow. Chronic administration of nicotine was delivered through subcutaneously placed Alzet 2001 mini-osmotic pumps at a concentration to mimic heavy smokers (4.5 mg/kg/day) in accordance to previous studies (Murrin et al., 1987; Wang et al., 1997). Nicotine and cotinine plasma levels were verified by RIA and HPLC to confirm comparable plasma levels within groups.

Animals were induced with 4% isofluorane, and maintained on anesthesia with 1% isofluorane in 30% O₂/70% N₂O using a face mask and a Surgivet vaporizer. Surgery was performed using a Zeiss OPMI pico surgical microscope. Local cortical blood flow in the left middle cerebral artery territory was monitored with laser-Doppler flowmetry and observed online in real time on computer using Moor Instruments’ software. After occlusion of the common carotid artery by a microclip, the left external carotid artery was ligated, coagulated and cut distally to the cranial thyroid artery. A small incision in the ECA was made, and a 20 mm monofilament nylon suture (5-0, Harvard Apparatus) which had been rounded at the tip by heat (final diameter: 0.2–0.3 mm) was inserted into the ECA and gently advanced through the internal carotid artery until its tip occluded the origin of the MCA. Correct placement of the suture was indicated by a sudden drop of the local cortical blood flow in the left MCA territory to 10–15% of baseline as seen by laser Doppler flowmetry. After successful occlusion, the monofilament was secured in place with ligature, and the incision was closed by microsurgical clips.

MCAO was sustained for a period of 24 hours, after which the animals were deeply anesthetized with isofluorane and euthanized by decapitation. It has been reported that permanent MCAO (24 hours) creates predictable neuronal injury that is caused by both cytotoxic and vasogenic edema (Kumar et al., 2006; Mdzinashvili et al., 2007).
The brains were quickly removed and sectioned coronally into slices of 1 mm thickness using McIlwain Tissue Chopper. Slices were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffered saline at 37°C for 15 min and fixed by immersion in 4% buffered formaldehyde solution. TTC stains viable brain tissue dark red based on intact mitochondrial function whereas infarcted tissue areas remain unstained (white) (Gorgulu et al., 2000). Images were acquired by digital camera (Nikon), and areas of both hemispheres and the infarcted regions were quantified for each slice using image analysis software (Image J 1.30 and Scion Image version Beta 4.0.2, NIH, Rockville, MD, USA). We elected to measure the areas of damage, and brain swelling by area increase in the ipsilateral (ischemic) hemisphere, and compare these with the contralateral (nonischemic) hemisphere (Elliot and Jasper, 1949; Sydserff et al., 1996). This allowed direct comparison of areas of damage with brain swelling, and allowed us to use each animal as its own control in addition to inter-experimental comparisons (Sydserff et al., 1996).

Five animals were used for each experiment and three measurements were made on each slice to calculate the size of the lesion and to correct for overestimation due to the effects of brain swelling. The calculations are: $a = \text{area of infarct (mm}^2\text{)}$, $b = \text{area of the infarcted (ipsilateral) hemisphere slice (mm}^2\text{)}$, $c = \text{area of the non-infarcted (contralateral) hemisphere slice (mm}^2\text{)}$, $d = \text{brain swelling (mm}^2\text{)} = (b−c)$ (Park and Kang, 2000). The area ($A_l$) of the lesion (mm$^2$), corrected for swelling, was derived from the equation $A_l = a−d$. The swelling area was designated $A_e$ and quantified by determining the ratio between the areas of the infarcted and non-infarcted hemisphere slices, thus: $A_e = b−c$. Infarct and edema ratios of hemispheric areas were expressed as mean±standard
error of the mean (SEM) and compared using Student’s t test. Values of $P < 0.05$ were considered statistically significant.

All the animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Animal Care and Use Committee at TTUHSC.

**Locomotor Activity Measurements**

Six groups of female CD-1 mice namely, naïve mice control; mice with 1 hour exposure of nicotine 4.5 mg/kg/day; mice with MCAO for 6 hours; mice with MCAO for 6 hours plus 1 hour prior exposure to nicotine 4.5 mg/kg/day; mice prior exposure to nicotine 4.5 mg/kg/day for 3 weeks; mice with MCAO for 6 hours plus 3 weeks prior exposure to nicotine 4.5 mg/kg/day were included in this study. Chronic administration of nicotine (4.5 mg/kg/day for 3 weeks) was delivered through subcutaneously placed Alzet 2001 mini-osmotic pumps at a concentration to mimic heavy smokers (4.5 mg/kg/day) in accordance to previous studies (Murrin et al., 1987; Wang et al., 1997). Nicotine (45 ng/ml) and cotinine (262.2ng/ml) plasma levels were verified by RIA and HPLC to confirm comparable plasma levels within groups.

After the treatments, behavioral data were collected using VersaMax animal monitors (model Accuscan Instruments Inc., Columbus, OH) which has been used to monitor stroke outcome using several locomotor parameters and behavioral deficits predictive of stroke injury (Vendrame et al., 2004). The chamber cage was 42 cm × 42 cm × 30 cm, made of clear Plexiglas, and covered with a Plexiglas lid with holes for ventilation. Infrared monitoring sensors were located every 2.5 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16
sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax analyzer (Accuscan Instruments Inc.), which in turn sent information to a computer that ran the VersaMax and Versadat programs. These programs tabulated and processed a number of variables related to locomotor behavior. For the present experiments 4 different locomotor parameters were measured every 4 minutes for 20 minutes which has been shown previously to reflect behavioral changes associated with neurological damage induced by MCAO (Vendrame et al., 2004). Prior to the experiments mice were acclimatized to the behavioral procedure. All testings occurred between 12: 00 to 5: 00 pm. Behavioral tests were performed in all six groups of animals.

**Statistical analysis**

All data are expressed as the mean ± SD and values were compared by ANOVA. When the differences in the means were significant, post-hoc pair wise comparisons were conducted using Newman-Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA). Differences in p-values less than 0.05 were considered statistically significant.
Results

Effects of Nicotine on blood gases and temperature

Studies were carried out to determine if the nicotine administration altered physiologic parameters (temperature and blood gas) during MCAO. Arterial blood samples were drawn (100 µL/sample) under anesthesia from groups of mice that were exposed to 24 hour of MCAO and or 3 hours of nicotine dosage (0.56525mg/kg). Blood samples were drawn 1 hour after induction of MCAO as described above. Blood gases and serum electrolytes were analyzed on a RapidLab 348 blood gas analyzer (Bayer Diagnostics, Tarrytown, NY, USA). We found nicotine exposure didn’t change pO2, pCO2 or pH parameters when compared to control. Interestingly, we also found that nicotine exposure prior to 24 hour MCAO didn’t change these parameters when compared to the 24 hour MCAO alone. Higher PO2 values are explained by the slightly higher oxygen content of the gas mixture (30% compared to room air) used for MCAO (Table 2).

Hippocampal slices subjected to acute nicotine exposure

Each cigarette smoking results in absorption of 1 to 1.5 mg of nicotine by the smoker (Hukkanen et al., 2005). Nicotine is metabolized in the liver resulting in six metabolites of which cotinine is the primary metabolite averaging 72% (Benowitz and Jacob, 1994). Plasma levels of nicotine in smokers ranges from 10-50 ng/mL and cotinine levels predominately ranges from 250 to 300 ng/mL yet can be variable up to 900 ng/mL (Hukkanen et al., 2005). We used levels of nicotine/cotinine respectively as 1/10, 10/100, 100/1000 and 1000/10,000 ng/mL for the dosing paradigm in these studies.
During normoxic conditions nicotine exposure at the highest dose of 1000 ng/mL nicotine and 10,000 ng/mL cotinine showed an increase in water content compared to control (P < 0.05)(Figure 1). OGD exposed slices showed greater sensitivity to nicotine/cotinine exposure with significant increase in water content at doses of nicotine/cotinine including 10/100 (p < 0.01), 100/1000 (p < 0.01) and 1000/10,000 (p < 0.01) (Figure 1).

**Hippocampal slices subjected to chronic cigarette smoke constituent exposure**

Our chronic nicotine exposure through an alzet minipump delivery system for 1 week (4.5mg/kg/day) revealed that during normoxic conditions N-CSE (Nicotine-containing Cigarette Smoke Extract) significantly increased water content of hippocampal slices compared to the vehicle control PG/DMSO and compared to NF-CSE (Nicotine-free Cigarette Smoke Extract) (P < 0.05) (Figure 2A). There is no significant difference in water content of hippocampal slices between vehicle control (PG/DMSO) and saline control (data not shown). Interestingly, nicotine and N-CSE at 4.5 mg/kg/day did not show a significant difference in water content. During OGD conditions, a 1 week exposure to nicotine 4.5 mg/kg/day increased water content compared to control (p < 0.01) and NF-CSE (p < 0.05) (Fig 2A).

Normoxic and OGD exposure following 3 week minipump delivery of all cigarette smoke constituents resulted in no significant alteration of hippocampal slice water content (Figure 2B).
Hippocampal slices subjected to OGD with Nicotine/Cotinine (N/C) and nAChR antagonists

In OGD exposed hippocampal brain slices N/C exposure at 100/1000 ng/mL significantly increased water content (p < 0.01) compared to control OGD conditions (Figure 3). In addition, nAChR antagonists abolished the findings of N/C exposure by significantly decreasing water content in hippocampal slices even in the presence of nicotine/cotinine. The increase of water content with N/C (100/1000) exposure is reduced with various concentration of nAChR antagonists 25-250 µM mecamylamine present in the aCSF: 25 µM mecamylamine (p < 0.01), 125 µM mecamylamine (p < 0.001) and 250 µM mecamylamine (p < 0.001) (Figure 3). Also, the presence of 10 µM α-bungarotoxin in the presence of N/C (100/1000) inhibits the water gain induced by N/C (p < 0.05) but remained higher than 125 µM mecamylamine exposure (p < 0.05) (Figure 3). All nAChR antagonist exposures alone did not significantly differ in water gain compared to OGD control (data not shown), suggesting that these antagonists are acting through a mechanism involving the nAChR.

Acute 1 hour exposure to cigarette smoke constituents in MCAO model

Infarct ratio increases with 1 hour dosage of nicotine 187.5µg/kg (4.5mg/kg/day, 4.5mg/24hr=187.5µg/kg/1hr) (P< 0.001) and N-CSE (P<0.05) at concentrations that mimic plasma levels of a heavy smoker (Figure 4). Exposure to NF-CSE did not significantly differentiate infarct ratio from vehicle control. Edema ratio also increased for 1 hour exposure to nicotine (P<0.05) and N-CSE (P<0.05) compared to control (Figure 4). Again NF-CSE did not differ from control in edema ratio same as with infarct ratio.
Acute 1, 3 and 6 hour exposure to nicotine in MCAO model

All acute i.p. injections of nicotine equivalent to total dosage of 4.5 mg/kg/day, 1 hour (4.5mg/24hr=187.5µg/kg) (p<0.01), 3 hours (4.5mg/8hr=562.5µg/kg) (p<0.05), and 6 hours (4.5mg/4hr=1125µg/kg) (p<0.001) dosage prior to MCAO resulted in significant increase of edema. Additionally, infarct ratio was significantly increased for all three acute nicotine 4.5 mg/kg/day injections: 1 hour (p<0.001) (Figure 5A), 3 hours (p<0.05) (Figure 5B) and 6 hours (p<0.001) (Figure 5C).

Chronic exposure to nicotine in MCAO model

Chronic administration of nicotine was delivered through subcutaneously placed Alzet 2001 mini-osmotic pumps at a concentration to mimic heavy smokers (4.5 mg/kg/day) in accordance to previous studies (Murrin et al., 1987; Wang et al., 1997). Nicotine and cotinine plasma levels were verified by RIA and HPLC to confirm comparable plasma levels within groups. Chronic exposure to nicotine 4.5 mg/kg/day did not alter infarct ratio for 1 day, 1 week, or 3 week minipump administration (Figure 6). Whereas edema ratio was significantly altered compared to control including 1 day (P<0.001), 1 week (P<0.001), and 3 week (P<0.01) (Figure 6).

Locomotor Activity Measurements

A series of locomotor activity parameters were monitored in this set of experiments, including vertical activity, total distance, movement time and stereotypy time. The results showed that for parameters, such as vertical activity, total distance, movement time, stereotypy time, there was no significant difference between control group and nicotine (1 hour) treated group (Table 3). Vehicle control had no effect on these locomotor parameters (data not shown). However, these locomotor parameters were
significantly decreased with animals subjected to MCAO, compared to the control (p<0.05). Combining treatment of nicotine (1 hour) worsened the stroke outcome by decreasing these locomotor activity parameters when compared to the MCAO group (Table 3). For long-term nicotine use (3 weeks), nicotine itself did not affect these selected locomotor activity parameters compared to the control. Animals subjected to both nicotine (3 weeks) and MCAO significantly decrease the locomotor parameters, compared to the control (Table 3) (p<0.05). However, a statistically significant difference could not be detected between MCAO group and MCAO/nicotine 3 weeks group (p>0.05). Interestingly, there was no significant difference in those parameters between nicotine (1 hour exposure)/MCAO group and nicotine (3 week exposure)/MCAO group (Table 3).
Discussion

Studies have shown that nicotine has detrimental effect at the cerebral microcirculation involving: tissue plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1), nitric oxide synthase (NOS), leukocyte migration and BBB dysfunction of tight junctional proteins (Hawkins et al., 2002). In our studies using in-situ and in-vivo models we have demonstrated that nicotine can increase cytotoxic and vasogenic edema after stroke conditions and therefore worsen the stroke outcome.

Hippocampal slice OGD is a model which represents cellular edema associated with the accumulation of water content and has previously been utilized to measure bilobalide’s neuroprotective effects (Mdzinarishvili et al., 2007). Therefore, in-situ one hour incubation of hippocampal slices in either normoxic or OGD media with or without the presence of nicotine was used to investigate cytotoxic cellular edema and in-vivo 24 hour murine MCAO pretreated with nicotine or vehicle control was used to investigate both cellular and vasogenic edema. With acute exposure of nicotine/cotinine at concentrations ranging 10/100 ng/mL N/C to 1000/10,000 ng/mL N/C, there is a significant increase of water content during OGD conditions when compared to OGD control (Figure 1). This shows that the concentration of 10/100 ng/mL N/C similar to plasma levels found in smokers can increase cellular edema during stroke conditions, which is consistent with previous studies (Hukkanen et al., 2005). During normoxic conditions only the highest concentration of 1000/10,000 ng/mL N/C, 100 fold higher than concentrations applicable to heavy smokers, showed a significant effect of increasing water content compared to control (Figure 1). Exposure to nicotine and its metabolite cotinine in this model at concentrations similar to cigarette smokers appears to
be detrimental only during OGD and does not produce edematous conditions during normoxia. This could be explained by the fact that this hippocampal brain slice model mimics cellular edema only during OGD conditions (Mdzinarishvili et al., 2007).

Effective administration in the 1 week and 3 week chronic dosage of nicotine through osmotic minipumps was verified via an RIA kit (Cozart Bioscience U.K) for detection of cotinine in plasma and were detected as a positive or negative reading (Table 1A). Further HPLC analysis of plasma from plasma of rats administered 4.5 mg/kg/day of nicotine for 1 day, 1 week and 2 weeks through alzet™ minipumps detected levels of nicotine and the major metabolite cotinine at levels similar to a heavy smoker (Lockman et al., 2005b) (Table 1B). All values correlate well with values reported in the literature (Benowitz and Jacob, 1994; Hukkanen et al., 2005). In addition, brain uptake of nicotine and cotinine in rats delivered through nicotine loaded alzet™ minipumps at 4.5 mg/kg/day for 28 days demonstrated a unidirectional influx across the BBB for nicotine at a rate of 114 to 143 µg/g/day and cotinine at a rate of 43 to 61 µg/g/day (Lockman et al., 2005a).

Water content representing cytotoxic edema increases after 1 week exposure to nicotine when hippocampal slices were exposed to OGD compared to control and NF-CSE exposures. Interestingly NF-CSE exposure resulted in significantly less water gain compared to administration of nicotine alone, suggesting that the remaining components of cigarette smoke are not responsible for increased cytotoxic edema during 1 wk administration (Figure 2A). Additionally, when hippocampal slices were exposed to normoxic conditions, only a 1 week N-CSE exposure resulted in an increase of water content (Figure 2A). This suggests that nicotine is the main component of cigarette
smoke which is detrimental both during normal conditions and also during OGD conditions exacerbating water gain in the hippocampal slice.

The edematous effects of N/C 100ng/mL / 1000ng/mL during OGD were returned back to control levels with nicotine acetylcholine receptor (nAChR) antagonists mecamylamine (25 µM, 125 µM and 250 µM) and α-bungarotoxin (10 nM) (Figure 3). Mecamylamine inhibits nicotine’s ability to induce the influx of calcium through the open position at the ion channel site of the nAChR (Zevin et al., 2000). The nAChR’s ion channel properties of inward Ca+ and Na+ movements could facilitate edema formation when the agonist nicotine is present, and this action is abolished in the presence of nAChR antagonist mecamylamine and more selective α7-antagonist α-bungarotoxin (Colquhoun and Patrick, 1997). Our studies suggest that nicotine increases edema of the hippocampus during OGD via nAChRs evidenced by mecamylamine and α-bungarotoxin’s ability to reduce water content.

All cellular components of the neurovascular unit have nAChR subunit expression including neurons, astrocytes, and endothelial cells (Abbruscato et al., 2002; Xiu et al., 2005; Brody et al., 2006). Chronic smoking has been associated with an alteration of nAChR expression in the brain with α4 subunit expression increased in neurons and dendritic processes (Teaktong et al., 2004) and decrease in α7 subunit expression in astrocytes and hippocampal regions (Teaktong et al., 2004). Previously we have demonstrated that nicotine decreases NKCC activity in bovine brain microvessel cells (BBMEC’s) which also express α-3, α-5, α-7, β-2, and β-3 nAChR subunit proteins (Abbruscato et al., 2004). The decrease of α7 subunit expression in astrocytes of the hippocampus and a possible modulation of NKCC activity and movement of ions may be
responsible for the lack of edema observed in 3 week chronic administration of nicotine in the hippocampal OGD model. Therefore, we investigated these results using a second model of focal ischemia, MCAO, to evaluate infarct size and both forms of vasogenic and cellular edema during acute and chronic exposure to nicotine.

MCAO (24 hours) creates predictable neuronal injury that is caused by both cytotoxic and vasogenic edema due to the ability of this model to replicate damage to the neurovascular unit (Mdzinarishvili et al., 2007). Cytotoxic edema has been observed within 12 hours of occlusion and water content and ionic disturbances has been observed to increase for 72 hours following rat brain MCAO (Gotoh et al., 1985). Vasogenic edema has been observed to peak at 6 hours following photochemical MCAO and measured through contrast-enhanced T1-weighted imaging (Chen et al., 2007). This combination of edema gives a more predictable model of damage from a chronic focal stroke. Acute 1 hour exposure to nicotine and N-CSE resulted in a significant increase in infarct ratio and edema (p < 0.001 and p < 0.05), respectively, which predicts the size of unrecoverable brain cell loss in the ischemic core (Figure 4). Interestingly exposure to NF-CSE did not result in a change of infarct or edema ratio compared to control which is similar to the hippocampal 1 week OGD experiments suggesting nicotine alone is responsible for the infarct volume changes. We then investigated the effects of nicotine alone on infarct and edema ratio with acute administration (1, 3, 6 hours) prior to 24 hour MCAO (Figure 5). All acute i.p. injections of nicotine equivalent to 4.5 mg/kg/day prior to MCAO resulted in significant increase of edema at various time points: 1 hour (p<0.01), 3 hours (p<0.05), and 6 hours (p<0.001) and also significant increase in infarct ratio: 1 hour (p<0.001) (Figure 5A), 3 hours
(p<0.05) (Figure 5B) and 6 hours (p<0.001) (Figure 5C). We did not observe any increase in infarct area 24 hours following MCAO with chronic dosages of nicotine (4.5 mg/kg/day, SC: 1 day, 1 week and 3 weeks). Yet, edema ratio significantly increases with nicotine exposure for chronic (1 day, 1 week, and 3 weeks) exposures (Figure 6).

In order to further explore the nicotine effect of the functional outcome of the stroke, locomotor studies were designed. This study shows that activity parameter data were decreased significantly after MCAO surgery compared to that of control. Parameters from nicotine plus MCAO treatment group were statistically lower compared to the MCAO group, which supports our conclusion that nicotine worsens stroke outcome (Table 3). Interestingly, we did not observe any differences of locomotor activity parameters between MCAO group and MCAO + nicotine 3 weeks group (Table 3). It is apparent that some level of tolerance develops to the worsened infarct size effects of nicotine with chronic exposure. This may be due to nAChR receptor desensitization in the hippocampus slice. Desensitization of $\alpha_4\beta_2$ nAChR, the predominant receptor in the brain, has recently been elucidated in cigarette smokers through positron emission tomography (PET) scanning (Brody et al., 2006). Plasma levels at 1/25th the level of typical smokers resulted in 50% occupancy of $\alpha_4\beta_2$ nAChR and tobacco dependent smokers with plasma levels ranging from 10-50 ng/mL of nicotine maintain 96% to 98% receptor occupancy/desensitization throughout the day. Future studies will investigate nAChR expressions in brain regions susceptible to stroke damage.

In conclusion, this study shows that the nicotine produces an increase of both cytotoxic and vasogenic edema as seen in the hippocampal slice OGD and MCAO models which simulate brain ischemia. Increased edema in the ischemic hemisphere may
negatively affect penumbral region recovery and eventually lead to increased neuronal damage that may otherwise have been recoverable or responsive to neuron-protective therapy. Prevention of increased edematous conditions during stroke could be afforded through the avoidance of nicotine products in stroke prone individuals. Additionally, clinicians should be aware of the propensity for edema in nicotine using or cigarette smoking patients experiencing cerebrovascular accidents.
References


Footnotes

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* Jennifer R. Paulson and Tianzhi Yang have equal contribution to the manuscript.
Legends for Figures

Figure 1

Water content of hippocampal slices subjected to normoxia or oxygen glucose deprivation with acute nicotine/cotinine exposure

Effect of acute 1 hour exposure to aCSF control, nicotine (1ng/mL)/cotinine (10 ng/mL) (1/10 NC), nicotine (10 ng/mL)/cotinine (100 ng/mL) (10/100 NC), nicotine (100 ng/mL)/cotinine (1000 ng/mL) (100/1000 NC), and nicotine (1000 ng/mL)/cotinine (10,000 ng/mL) (1000/10,000 NC) on water content of brain hippocampal slices following normoxic or oxygen glucose deprivation conditions. Data represent mean ± SEM of 9-17 independent determinations. (* denotes significance of p < 0.05 , ** denotes significance of p < 0.01 and *** denotes significance of p < 0.001 using one-way ANOVA with Newman-Keuls post hoc analysis.)

Figure 2

Water content of hippocampal slices subjected to normoxia or oxygen glucose deprivation with chronic 1 and 3 week cigarette smoke constituent exposure

Effect of 1 week exposure to vehicle control, nicotine (4.5 mg/kg), nicotine-containing cigarette smoke extract (N-CSE), and nicotine free cigarette smoke extract (NF-CSE) on water content of brain hippocampal slices following normoxic or oxygen glucose deprivation conditions. Data represent mean ± SEM of 9-20 independent determinations. (* denotes significance of p < 0.05, and ** denotes significance of p < 0.01 using one-way ANOVA with Newman-Keuls post hoc analysis.)
Figure 3

Water content of hippocampal slices subjected to nicotine/cotinine and nicotinic acetylcholine receptor antagonists during oxygen glucose deprivation

Effects of acute exposure of nicotine 100 ng/mL and cotinine 1000 ng/mL with or without nicotinic acetylcholine receptor (nAChR) antagonists on water content of hippocampal slices subjected to oxygen glucose deprivation (OGD). Control, N/C, or N/C along with 25, 125, 250 µM of Mecamylamine or 10 nM of α-Bungarotoxin in aCSF. Data represent mean ± SEM of 9-20 independent determinations. (* denotes significance of p < 0.05, and ** denotes significance of p < 0.01 using one-way ANOVA with Newman-Keuls post hoc analysis.)

Figure 4

Effect of 1 hour exposure to cigarette smoke constituents on infarct and edema ratios following MCAO

Effect of parenteral injection 1 hour prior to 24 hour MCAO of vehicle control, nicotine (4.5 mg/kg/day), nicotine-containing cigarette smoke extract (N-CSE), or nicotine free cigarette smoke extract (NF-CSE) on infarct ratio (infarct area/brain slice area) and edema ratio (ipsilateral/contralateral hemisphere slice area). Data represent mean ± SEM of 5 independent determinations containing 5-6 slices each. (* denotes significance of p < 0.05 and *** denotes significance of p < 0.001 using one-way ANOVA with Newman-Keuls post hoc analysis.)
Figure 5

Effect of 1, 3, and 6 hour i.p. injection of nicotine on edema and infarct ratios following MCAO.

Effect of parenteral injection 1, 3 and 6 hour of nicotine (4.5 mg/kg/day) on infarct ratio (infarct area/brain slice area) and edema ratio (ipsilateral/contralateral hemisphere slice area) following 24 hour MCAO. Data represent mean ± SEM of 5 independent determinations containing 5-6 slices each. (* denotes significance of p < 0.05, ** denotes significance of p < 0.01 and *** denotes significance of p < 0.001 using one-way ANOVA with Newman-Keuls post hoc analysis).

Figure 6

Effect of chronic exposure to nicotine on infarct and edema ratios following MCAO

Effect of chronic administration of vehicle control, nicotine (4.5 mg/kg) 1 day, nicotine (4.5 mg/kg) 1 week, and nicotine (4.5 mg/kg) 3 weeks, on infarct ratio (infarct area/brain slice area) and edema ratio (ipsilateral/contralateral hemisphere slice area) following 24 hour MCAO. Data represent mean ± SEM of 5 independent determinations containing 5-6 slices each. (** denotes significance of p < 0.01 and *** denotes significance of p < 0.001 using one-way ANOVA with Newman-Keuls post hoc analysis.)
Tables

Table 1A Plasma Cotinine Presence in 1 Wk and 3 Wk Osmotic Minipump Administration via RIA Cotinine Analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 Week</th>
<th>3 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nicotine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NF-CSE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N-CSE</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1B Nicotine and Cotinine Plasma Concentrations via HPLC Analysis

<table>
<thead>
<tr>
<th></th>
<th>1 Day</th>
<th>1 Week</th>
<th>2 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Nicotine</td>
<td>45.0 ng/mL</td>
<td>71.7 ng/mL</td>
<td>60.42 ng/mL</td>
</tr>
<tr>
<td>Plasma Cotinine</td>
<td>269.2 ng/mL</td>
<td>261.6 ng/mL</td>
<td>240.8 ng/mL</td>
</tr>
</tbody>
</table>
Table 2:

Blood Gas Values and Temperature

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 Hour Nicotine</th>
<th>24 hour MCAO</th>
<th>24 hour MCAO + 3 hour nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO2 mmHg</td>
<td>86.3 ± 4.3</td>
<td>85.4 ± 3.8</td>
<td>92.1 ± 2.9</td>
<td>95.6 ± 3.1</td>
</tr>
<tr>
<td>pCO2 mmHg</td>
<td>32.1 ± 3.8</td>
<td>32.7 ± 3.9</td>
<td>29.8 ± 2.6</td>
<td>28.3 ± 2.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.41 ± 0.18</td>
<td>7.43 ± 0.25</td>
<td>7.39 ± 2.0</td>
<td>7.41 ± 0.8</td>
</tr>
<tr>
<td>Temp C</td>
<td>37.1</td>
<td>37.0</td>
<td>37.3</td>
<td>37.1</td>
</tr>
</tbody>
</table>

Studies were carried out to determine if the nicotine administration altered physiologic parameters (temperature and blood gas) during MCAO. Arterial blood samples were drawn (100 µL/sample) under anesthesia from groups of mice that were exposed to 24 hour of MCAO and or 3 hours of nicotine (0.56525mg/kg). Blood samples were drawn 1 hour after induction of MCAO as described above. Blood gases and serum electrolytes were analyzed on a RapidLab 348 blood gas analyzer (Bayer Diagnostics, Tarrytown, NY, USA). Higher PO2 values are explained by the slightly higher oxygen content of the gas mixture (30% compared to room air) used for MCAO.
Table 3

Effects of stroke and nicotine treatment on the mouse locomotor activity. Data represent mean ± SD of three to four independent determinations (*p<0.05 compared to the control group, #p<0.05 compared to the MCAO group).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Nic 1h</th>
<th>MCAO</th>
<th>MCAO + Nic 1h</th>
<th>Nic 3wks</th>
<th>MCAO + Nic 3wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertical activity</td>
<td>4859±872</td>
<td>4098±1018</td>
<td>640±209*</td>
<td>140±52#</td>
<td>4414±1214</td>
<td>578±262*</td>
</tr>
<tr>
<td>Total distance (cm)</td>
<td>3558±934</td>
<td>2993±817</td>
<td>397±247#</td>
<td>244±82#</td>
<td>4337±627</td>
<td>216±225*</td>
</tr>
<tr>
<td>Movement time (s)</td>
<td>278±79</td>
<td>217±69</td>
<td>40±16*</td>
<td>22±10#</td>
<td>309±32</td>
<td>34±16*</td>
</tr>
<tr>
<td>Stereotypy time (s)</td>
<td>178±24</td>
<td>181±38</td>
<td>25±3*</td>
<td>16±6#</td>
<td>167±98</td>
<td>24±16*</td>
</tr>
</tbody>
</table>
Fig 3
Fig 4

Edema Ratio
(pallidal/contralateral hemisphere slice area)

Control Nicotine N-CSE NF-CSE

Infarct Ratio
(Infarct Area/Brain Slice Area)

Control Nicotine N-CSE NF-CSE

* Significant difference
*** Highly significant difference
Fig 6