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Tobacco Smoke Chemicals Attenuate Brain-to-Blood Potassium Transport Mediated by the Na,K,2Cl-Cotransporter During Hypoxia-Reoxygenation

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Tobacco Smoke Chemicals Influence NKCC1 During Ischemia

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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, ACM: Astrocyte-conditioned Media

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Abstract

Smoking tobacco, including cigarettes, has been associated with an increased incidence and relative risk for cerebral infarction in both men and women. Recently, we have shown that nicotine and cotinine attenuate abluminal (brain facing) K^+ uptake mediated by the Na,K,2Cl-cotransporter (NKCC) in bovine brain microvessel endothelial cells (BBMECs) after hypoxic/aglycemic exposure (stroke conditions). The purpose of the current study was to explore the effects of nicotine and tobacco smoke chemicals on K^+ movement through the blood-brain barrier during both hypoxia/aglycemia and reoxygenation. BBMECs were exposed to nicotine/cotinine, nicotine-containing cigarette smoke extract (N-CSE), or nicotine-free cigarette smoke extract (NF-CSE) in quantities designed to mimic plasma concentrations of smokers. Stroke conditions were mimicked *in vitro* in BBMECs through 6 hr hypoxia/aglycemia with or without 12 hr reoxygenation, after which NKCC-mediated K^+ uptake and paracellular integrity were measured with ^{86}Rb and ^{14}C-sucrose, respectively. In addition, K^+ concentrations in brain extracellular fluid were estimated in ^{86}Rb injected rats that were administered nicotine, N-CSE, or NF-CSE and on whom global ischemia/reperfusion by *in vivo* four vessel occlusion was performed. Both *in vitro* and *in vivo* paradigms showed nicotine, the major alkaloid present in tobacco smoke, to be the determining factor of an inhibited response of abluminal NKCC in BBMECs during and after stroke conditions. This was measured as a decrease in abluminal brain endothelial cell NKCC activity, and as an increase in brain extracellular K^+ concentration measured as the brain extracellular fluid ^{86}Rb-to-plasma ratio after *in vivo* four vessel occlusion with reperfusion.

Introduction

The blood-brain barrier is a selective interface which restricts movement of substances between the brain interstitial fluid and plasma (Reese and Karnovsky, 1967). Endothelial cells of the cerebromicrovasculature form tight junctions which provide a physical barrier between blood and the central nervous system that can regulate transport of ions into and out of the brain (Brightman and Reese, 1969). A properly functioning blood-brain barrier will maintain brain homeostasis of key ions such as Na^+, K^+ and Cl^-, all of which are important for mitigating neuronal action potentials (Keep et al., 1993). Edema associated with ischemia may be caused by two mechanisms, either vasogenic or cellular (Brillault et al., 2002). Increase of intracerebral pressure due to edematous swelling leads to further ischemia and may result in death (MacGregor et al., 2003). During ischemia following brain trauma, neurons undergo cellular swelling due to the depolarizing action of $GABA_A$ receptors and a resulting influx of Cl^- and K^+ (Payne et al., 2003). This damage is referred to as cellular edema and occurs after vasogenic edema which is observed as blood-brain barrier opening (Betz et al., 1994; Kawai et al., 1996)

Two transporters of the blood-brain barrier important in brain K^+ ion homeostasis include Na,K,ATPase and the Na,K,2Cl-cotransporter (NKCC) (Abbruscato et al., 2004). Recently, we demonstrated that hypoxia/aglycemia in bovine brain microvessel endothelial cells (BBMECs) increases the activity of the NKCC by 91% and decreases the activity of Na,K,ATPase by 62% (Abbruscato et al., 2004). NKCC functions as a cation-chloride cotransporter that can be modulated by alterations of the extracellular and intracellular ion gradients. Two NKCC isoforms are believed to exist including NKCC1 and NKCC2, both with unique distributions. NKCC2 is present mostly in the epithelial

cells of the thick ascending limb of the Loop of Henle of the kidneys (Haas and Forbush, 2000). NKCC1 is more ubiquitously expressed in secretory epithelial and endothelial cells (Haas et al., 1995; Yerby et al., 1997). The activity of NKCC1 is important in brain extracellular ion homeostasis and is present not only in brain microvascular endothelial cells but also in neurons and astrocytes (O'Donnell et al., 1995; Payne et al., 2003; Lenart et al., 2004).

Previously, our lab has shown that stroke conditions mimicked in primary cultures of endothelial cells through 6 hr exposure to hypoxia/aglycemia raised K^+ uptake due to increased actions of abluminal NKCC1 expressed at the blood-brain barrier. This alteration of NKCC1 abluminal activity suggests a protective function of the blood-brain barrier during traumatic brain events such as stroke by shuttling Na^+, K^+, and 2 Cl^- away from the brain extracellular space (Abbruscato et al., 2004). A decrease in the amount of K^+ and Cl^- from brain extracellular fluid would be neuroprotective and reduce the ability of $GABA_A$ receptors to continually depolarize and cause cellular swelling (Payne et al., 2003).

Cigarette smoking has been associated with a greater incidence of stroke (Gill et al., 1989; Hawkins et al., 2002). Up to a quarter of the 700,000 strokes in the U.S. can be attributed to cigarette smoking (Hankey, 1999). Smoking alone without any other contributing factors increases the risk of stroke by more than 1.5 times in men and women (Hawkins et al., 2002). Worsening of the outcome of stroke, which is the focus of this work, has been shown after chronic nicotine exposure in a focal middle cerebral artery occlusion (MCAO) model showing an increase in edema and infarct ratio (Wang et al., 1997). Also, we have shown that the normal increase of blood-brain barrier abluminal

NKCC1 activity during hypoxia/aglycemia is hindered by the presence of nicotine (100 ng/mL) and cotinine (1000 ng/mL) (Abbruscato et al., 2004). Although nicotine has been shown to increase the incidence of stroke and negatively impact the outcome of stroke, the effects of nicotine in the presence of the more than 4000 chemicals present in cigarette smoke has not been explored in models of stroke (Brunnemann and Hoffmann, 1991).

To better understand the effects of cigarette smoke constituents on NKCC activity during hypoxia/aglycemia, nicotine/cotinine, nicotine-containing cigarette smoke extract (N-CSE), or nicotine-free cigarette smoke extract (NF-CSE) were administered to BBMECs during paradigms of normoxia, hypoxia/aglycemia, and hypoxia/aglycemia with reoxygenation. These paradigms were designed to mimic the effects of normal state, brain ischemia, and the recovery period after brain ischemia on the activity of blood-brain barrier endothelial cells to move K^+ ions. Additionally, the effects of nicotine, N-CSE, and NF-CSE on an *in vivo* model were explored with a four vessel occlusion model of global ischemia to estimate the acute effects of tobacco smoke chemicals on brain extracellular fluid K^+ (Pulsinelli and Brierley, 1979).

Methods

Cell Culturing. BBMECs were isolated from the grey matter of fresh bovine brains obtained from a local slaughterhouse and cryopreserved (Audus and Borchardt, 1987). First passage cells were cultured on 12-well transwell plates (0.4 μm pore size) that were pretreated with collagen and fibronectin at a cell density of 50,000 cells/cm^2 and grown to confluence in 12 days. To achieve the greatest NKCC activity, a mixture of astrocyte-conditioned media (ACM) was placed in the abluminal chamber 48 hrs pre-confluence (O'Donnell et al., 1995; Abbruscato et al., 2004). BBMECs were treated with ACM consisting of 45% BBMEC growth media, 45% astrocyte media, and 10% fetal bovine serum. Astrocyte media was prepared by culturing C6 cells #CC1-107 (American Type Cell Collection, Rockville, MD) in Ham's F-10 with 10% fetal bovine serum. The C6 cells were seeded at 40,000 cells/cm^2 and grown to confluence in 75 mL flasks. Fresh media in the flasks was replaced every 48 hrs and sterile filtered through a 0.22 μm filter. Media for the BBMECs was maintained at 280 ± 10 mOsM/l (Abbruscato et al., 2004). ^{14}C-Sucrose permeability and K^+ uptake were measured on the BBMEC confluent monolayers on the 12th day.

Hypoxia/Aglycemia ± Reoxygenation. Experimental conditions for the confluent BBMEC monolayers included 6 hrs of hypoxia/aglycemia ± 12 hrs of reoxygenation. Aglycemic media was prepared with RPMI 1640 (without L-glucose) and bubbled for 5 min with 1% O_2-99% N_2. Hypoxic conditions were maintained by placing the confluent BBMEC monolayers in a custom hypoxia polymer glovebox (Coy Laboratories, Grass Lake, MI) infused with 1% O_2-99% N_2 at 37 °C. These methods have previously been used to evaluate effects of hypoxia/aglycemia on blood-brain barrier characteristics

(Abbruscato and Davis, 1999; Abbruscato et al., 2004). Reoxygenation of the 6 hr hypoxia/aglycemia treated cells was achieved by replacing the aglycemic media with BBMEC growth media and kept in an incubator for 12 hrs with 95% room air and 5% CO_2 . The concentration of oxygen in the atmosphere was maintained at 1%, and the PO_2 in the media was < 25 mmHg. Such hypoxia/aglycemia exposure as described above has been used previously to study alterations in blood-brain barrier properties (Abbruscato and Davis, 1999; Mark and Davis, 2002). Reoxygenation produced a rapid return to control PO_2 levels in the medium within 5 min, as previously reported (Mark and Davis, 2002).

Smoke Constituents. BBMECs were treated in the luminal chamber 24 hrs before hypoxia/aglycemia ± reoxygenation with nicotine (100 ng/mL) and cotinine (1000 ng/mL), N-CSE, or NF-CSE. The concentrations of nicotine and cotinine were chosen to mimic plasma concentrations detected in heavy smokers (Henningfield et al., 1993).

Cigarettes (Marlboro® filter cigarettes, Philip Morris Inc., VA, USA and Quest® #3, Vector Tobacco Inc., NC, USA) were obtained through commercial sources. Marlboro® filter contains 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. Mainstream smoke was bubbled through 50 ml acetone by a slight vacuum drawn repetitiously through a custom manufactured apparatus. To simulate a smoker's smoking behavior, each cigarette was lighted and "smoked" for a total of 5 min with alternating increments of smoking (vacuum, 3 sec) and rest (no vacuum, 30 sec). 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 and NF-CSE respectively. The concentration of nicotine in N-CSE was approximated at 0.677 mg/mL for Marlboro® cigarette extract assuming 1.1 mg of nicotine is present per cigarette. These methods have successfully been used *in vitro* to model exposure to cigarette smoke chemicals (Sherratt et al., 1988).

Na, K, 2Cl-cotransporter activity. The abluminal uptake of K^+ into BBMECs was performed through ^{86}Rb uptake studies for 10 min at RT with or without 2 µM ouabain as described previously (Abbruscato et al., 2004). Earlier studies have clearly shown that Rb quantitatively substitutes for K^+ in the NKCC system (Owen and Prastein, 1985). Ouabain-insensitive ^{86}Rb uptake was considered to be Na,K,2Cl-cotransporter activity. Our group has also shown that this ouabain-insensitive ^{86}Rb uptake is both Na^+ and Cl^- dependent, ensuring that we are measuring NKCC activity (Abbruscato et al., 2004).

BBMEC Permeability Coefficient (PC) Calculation. Apical-to-basolateral ^{14}C-sucrose permeability across BBMECs was expressed as a permeability coefficient (PC, cm/min x 10^{-4}). The 12-well transwell plates were allowed to equilibrate in the hypoxia glovebox in HEPES buffered transport medium for 15 min at 37 °C. Permeation of ^{14}C-sucrose was measured across BBMECs using an adaptation of described methods (Dehouck et al., 1992; Rose and Audus, 1998). The flux was determined by dividing the amount (in picomoles) of radioactive drug appearing in the receiver chamber by the time in min (sampling times were 15, 30, 60 min). The apparent permeability coefficient was then calculated using the equation:

$$PC=Flux/(A \times Cd_o) \quad \text{(eq. 1)}$$

Where flux is the slope of the line (picomoles of ^{14}C-sucrose versus min), A is the area of the membrane (1.0 cm^2), and Cd_o is the initial donor concentration.

Smoke Constituent Exposure. The nicotine regimen (4.5 mg/kg/day) maintains nicotine and cotinine plasma concentrations (45.04 ± 20.33 ng/mL and 269.15 ± 74.64 ng/mL, respectively, confirmed by HPLC analysis, data not shown) comparable to those observed in chronic and heavy smokers (2 to 3 packs per day) (Murrin et al., 1987; Wang et al., 1997). We calculated doses for CSEs using the body weight of the rats, to deliver an equivalent amount of nicotine equal to a 4.5 mg/kg/day dose. Vehicle, nicotine (N), N-CSE, and NF-CSE were administered IP for 100 min prior to the 20 min sampling period during reperfusion allowing for a 2 hr experiment. Equivalent doses of N-CSE and NF-CSE were administered to rats. For *in vitro* experiments, we exposed cells to nicotine (100 ng/ml) and cotinine (1000 ng/ml) for 24 hr and extrapolated equivalent doses for the N-CSE and NF-CSE groups.

Global Ischemia in the Rat. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Tech University Health Sciences Center and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Transient global forebrain ischemia was performed to simulate cerebral ischemia, according to the four vessel occlusion model (Pulsinelli and Brierley, 1979). Rats were anesthetized with a cocktail of ketamine HCL (78.3 mg/ml), xylazine (3.1 mg/ml) and acepromazine maleate (0.6 mg/ml) at a volume of 1 ml/kg before both common carotid arteries were isolated via a ventral midline cervical incision. An arterial clasp was placed around each common carotid artery without interrupting blood flow and the incision was closed with a single suture. A second incision was made behind the

occipital bone that overlies the two cervical vertebrae. The right and left alar foramen and both vertebral arteries were then electrocauterized and permanently occluded. These procedures result in consistent bilateral hemispheric ischemia with a high incidence of predictable brain damage in the hippocampus, neocortex and striatum (Pulsinelli and Brierley, 1979).

Rats were allowed to recover from the surgery and control parameters were monitored. Awake rats were restrained, the ventral neck suture removed and the carotid clasps tightened to produce four vessel occlusion. Carotid clasps were removed after 5 min of four vessel occlusion and restoration of carotid blood flow was verified by laser-doppler flowmetry. Animals were then subjected to *in vivo* brain microdialysis as described below.

***In Vivo* Brain Microdialysis.** The methods for *in vivo* microdialysis have been described previously (Allen et al., 1995). Rats were cannulated via femoral vein with PE 50 or 10 tubing to enable peripheral intravenous ^{86}Rb injection with a specific activity of 7.68 mCi/mg. Microdialysis probe implantation into the frontal cortex was conducted in anesthetized rats, the heads of which remained secured in a stereotaxic frame throughout the experiment. Probes were implanted in the frontal cortex according to published coordinates (Paxinos et al., 1980). Ketamine/xylazine was used as the anesthetic agent with supplements as needed to suppress moderate foot pinch. Body temperature was maintained at 37 °C by a rectal probe connected to a feedback device and a heated blanket. Microdialysis samples were collected for 20 min starting immediately after four vessel occlusion. A sample of blood was drawn from the femoral vein cannulae during reperfusion and centrifuged to determine the counts of ^{86}Rb in plasma. The DPMs

detected in the brain extracellular fluid (DPM/mL) were then expressed as a ratio of the DPMs detected in the plasma (DPM/mL) and termed R_{ECF}. Due to the possibility of declining plasma concentration of ^{86}Rb over the course of the experiment there may be a slight overestimation in R_{ECF}, yet the significance is not altered for an endpoint comparison. The timeline of the *in vivo* experimental paradigm is explained in Fig 4.

Statistical Methods. Statistical analysis for all of the experiments were done using one-way analysis of variance (ANOVA) with Newman-Keuls multirange post hoc comparison of the means. The data are presented as the mean ± S.E.M.

Results

In Vitro

Normoxia: Under normoxic conditions the presence of nicotine and cotinine, N-CSE, and NF-CSE did not significantly change the abluminal K^+ uptake through NKCC1 when compared with control (Fig 1A). Sucrose permeability of the blood-brain barrier in the presence of nicotine and cotinine increased significantly ($p < 0.01$) compared to control (Fig 1B). Paracellular permeability was not altered in the presence of either N-CSE or NF-CSE (Fig 1B). Previous experiments have shown that cotinine alone does not alter ^{14}C-sucrose permeability or ^{86}Rb uptake of BBMECs mediated by the NKCC (Abbruscato et al., 2004).

Hypoxia and Aglycemia: BBMECs exposed to 6 hr hypoxia/aglycemia in the absence of smoke constituents had a significant increase in abluminal K^+ uptake mediated by the NKCC compared to control ($p < 0.01$, Fig 2A). Endothelial cells exposed to either nicotine and cotinine or N-CSE for 24 hours prior to 6 hr hypoxia/aglycemia had a significant decrease ($p < 0.01$) in abluminal K^+ uptake when compared to 6 hr hypoxia/aglycemia alone. Interestingly, NF-CSE exposure combined with hypoxia/aglycemia showed no significant change in abluminal K^+ uptake compared to 6 hr hypoxia/aglycemia alone (Fig 2A). Sucrose permeability was found to statistically increase when the cells were exposed to 6 hr hypoxia/aglycemia alone ($p < 0.01$). Additionally, the presence of nicotine and cotinine exacerbates this permeability increase induced by 6 hr hypoxia/aglycemia alone with a statistically significant increase ($p < 0.01$). Under hypoxia/aglycemia conditions, the addition of neither N-CSE nor NF-CSE altered sucrose permeability compared to hypoxia/aglycemia alone (Fig 2B).

Hypoxia and Aglycemia with Reoxygenation: Combining 6 hr hypoxia/aglycemia with 12 hr reoxygenation showed a continued increase in abluminally mediated K^+ uptake compared to control ($p < 0.01$). Both nicotine and cotinine and N-CSE significantly decreased ($p < 0.01$) the abluminal K^+ uptake following 6 hr hypoxia/aglycemia and 12 hr reoxygenation (Fig 3A), whereas the NF-CSE was ineffective. Viability of the cellular monolayer is maintained following six hours of hypoxia/aglycemia combined with 12 hr reoxygenation with a return in sucrose permeability values back to basal levels (no different than normoxic controls). Sucrose permeability was significantly increased only with the presence of nicotine and cotinine during hypoxia/aglycemia combined with reoxygenation. Thus reoxygenation returns the sucrose permeability to the original normoxic control range for all experimental paradigms except for the samples exposed to nicotine and cotinine (Fig 3B).

In Vivo

Four Vessel Occlusion and Cigarette Smoke Constituent Exposure: The R_{ECF} for ^{86}Rb was shown to increase ($p < 0.05$) after four vessel occlusion compared to control, revealed by an increase in the ratio of ^{86}Rb detected in the brain (DPM/mL) compared to the amount detected in the plasma (DPM/mL). Statistically significant increases in R_{ECF} were also observed when animals undergoing four vessel occlusion were administered either nicotine ($p < 0.05$) or N-CSE ($p < 0.05$) at doses approximate to concentrations seen in smokers. Interestingly, R_{ECF} in four vessel occlusion animals administered NF-CSE was not significantly changed compared to four vessel occlusion animals. Control brain perfusion experiments were performed showing that the ratio of ^{14}C-sucrose in the brain compared to the plasma was not increased after 5 minutes of four vessel occlusion

or 2 hour nicotine exposure, ensuring that the integrity of the BBB was maintained. Additionally, our blood gas values correlate well with those of other researchers using this model whereby 4-VO resulted in slight hyperventilation and moderate respiratory alkalosis (data not shown) (Pulsinelli and Brierley, 1979)

Discussion

Stroke increases K^+ concentrations in brain extracellular fluid and results in cellular edema in both neurons and astrocytes (O'Donnell et al., 2004). A model of abluminal NKCC activity at the blood-brain barrier that monitors movement of K^+ from brain extracellular fluid to endothelial cell has previously been reported (Vigne et al., 1994). Conditions which increase NKCC activity include cell shrinkage, lowered intracellular Cl^-, and hypoxia (Haas et al., 1995; Kawai et al., 1996; Lytle, 1997). We have recently shown that hypoxia increases abluminal NKCC activity at the blood-brain barrier through phosphorylation of NKCC by Protein Kinase C (PKC) (Abbruscato et al., 2004). The activity of NKCC at the abluminal (brain facing) side of the blood-brain barrier may provide a protective function during ischemic assaults by facilitating brain-to-blood extrusion of accumulated brain extracellular fluid K^+.

Recent research has shown that NKCC1 overstimulation may play a role in ischemic cell damage at the neuron by increasing Na^+, K^+, and Cl^- influx (O'Donnell et al., 2004). Additionally, NKCC1 has been shown to be one of the ion cotransporters responsible for astrocyte cell swelling when brain extracellular fluid K^+ concentrations are high (Lenart et al., 2004). The contribution of enhanced NKCC1 activity at the brain facing (abluminal surface) of the blood-brain barrier should also be considered, since it has been suggested and demonstrated that NKCC1 activity increases during oxygen/glucose deprivation (Kawai et al., 1996; Abbruscato et al., 2004). Brain endothelial cells may contribute significantly to extracellular fluid removal of key ions locally at the neurovascular unit which includes the site of endothelial cell, astrocytic and neuronal connections in brain.

Tobacco smoking clearly has been shown to be a risk factor for stroke (Bonita et al., 1999). One quarter of stroke incidences are associated with smoking (Hankey, 1999). The increased risk of stroke due to tobacco smoking is dose dependent and increases from 3.57 to 4.65 times the risk compared with non-smoking populations when a smoker increases smoke consumption from 5 to 15 cigarettes per day (Bonita et al., 1999). In addition to being a risk factor for brain ischemia, there is a growing body of evidence that suggests that nicotine alters blood-brain barrier permeability characteristics which have a direct influence on stroke outcome (Abbruscato et al., 2002; Abbruscato et al., 2004). Animal studies have shown that chronic nicotine exposure worsened the outcome of stroke through increased edema as assessed in a murine MCAO model (Wang et al., 1997). During hypoxic conditions, the presence of nicotine attenuated abluminal protein expression and activity of the NKCC in BBMECs (Abbruscato et al., 2004). The present study set out to determine the fate of NKCC activity after *in vitro* and *in vivo* hypoxia coupled to reoxygenation or reperfusion. Our *in vitro* results clearly show that the blood-brain barrier regains structural integrity after periods of hypoxia/aglycemia coupled to reoxygenation, evidenced by return to basal ^{14}C-sucrose permeability values. Yet, hypoxia/aglycemia coupled to reoxygenation resulted in NKCC abluminal activity that remained elevated. These results show that upon reoxygenation, the blood-brain barrier can maintain paracellular barrier integrity while still maintaining enhanced brain-to-blood K^+ transport, possibly aiding in brain extracellular fluid spatial buffering. The addition of both nicotine and cotinine and N-CSE attenuated the normal increase of brain facing NKCC activity during hypoxia/aglycemia and reoxygenation conditions. Interestingly, NF-CSE, did not show an alteration of permeability or K^+ uptake. This finding suggests

that nicotine is the chemical present in cigarette smoke which is responsible for the attenuation of enhanced abluminal NKCC activity both during hypoxia/aglycemia insult and hypoxia/aglycemia insult coupled to reoxygenation. Structural integrity of the blood-brain barrier is also altered in the presence of nicotine and cotinine as shown through increased ^{14}C-sucrose permeability in all the models of normoxia, hypoxia/aglycemia, and hypoxia/aglycemia with reoxygenation. This alteration was not seen in the presence of N-CSE or NF-CSE. Although the permeability was not increased in the presence of N-CSE, the K^+ uptake in hypoxia/aglycemia and hypoxia/aglycemia with reoxygenation was significantly decreased. This suggests that during reperfusion periods, the NKCC at the blood-brain barrier is able to maintain polarity selectivity that would aid in brain-to-blood ion movement which is sensitive to nicotine. Previous experiments showed that cotinine alone did not alter permeability of the BBMECs to ^{14}C-sucrose or alter ^{86}Rb uptake of BBMECs mediated by the NKCC (Abbruscato et al., 2002; Abbruscato et al., 2004). In recent studies we have shown that the NKCC is activated through phosphorylation by PKC during hypoxic conditions. Nicotine attenuates abluminal NKCC expression during hypoxia/aglycemia, possibly through decreased activity of PKC and increased activity of phosphatases (Abbruscato et al., 2004). Future work will explore these cell signaling mechanisms during reoxygenation.

In addition, we confirmed these *in vitro* results with an *in vivo* four vessel occlusion model of global forebrain ischemia coupled with a period of reoxygenation, whereby reperfusion was established. *In vivo* experiments were designed to be short enough in duration (less than 3-6 hrs) to prevent the effects of blood-brain barrier breakdown (Betz et al., 1994). This paradigm eliminates damage associated with

vasogenic edema that follows the ischemic incident. Previous work has shown that K^+ is known to enter into the ventricular cerebral spinal fluid (CSF) rapidly and diffuse from the CSF into the brain tissue (Bradbury and Kleeman, 1967; Smith and Rapoport, 1986). Thus, animals were preloaded with ^{86}Rb 40 min prior to four vessel occlusion to allow sufficient time for ^{86}Rb to reach steady state and enter into the brain extracellular fluid K^+ pool. After four vessel occlusion, we observed a significant increase of brain extracellular fluid K^+ which was estimated by the ratio of ^{86}Rb detected in the brain compared to that detected in plasma. Our data demonstrate that, even after only 5 min of global ischemia, increases in the brain K^+ concentrations can be observed compared to control ($p < 0.05$). Animals subjected to four vessel occlusion with nicotine or N-CSE showed a significantly larger increase ($p < 0.05$) in brain extracellular fluid K^+ concentrations compared to control animals that underwent four vessel occlusion alone. Also, brain extracellular fluid K^+ values in NF-CSE animals were significantly decreased ($p < 0.01$) compared with the N-CSE treated animals. Both our *in vivo* and *in vitro* results suggest that diminished NKCC abluminal activity at the blood-brain barrier may hinder the export of brain extracellular fluid K^+ across the endothelial cell back into the blood. This work also provides convincing evidence that nicotine is most likely the chemical present in cigarette smoke responsible for altering NKCC activity and the movement of K^+ out of the brain during global ischemia coupled to reperfusion.

We suggest that nicotine contributes to an alteration in abluminal NKCC activity at the blood-brain barrier during and after stroke conditions**.** In brain trauma, the presence of ion cotransporters may play an important role by removing the K^+ ions that accumulate during an ischemic insult, from brain extracellular fluid. The movement of K^+ from brain

extracellular fluid and astrocytic endfeet, to endothelial cells has previously been proposed (Vigne et al., 1994). In astrocytes, transporters such as Kir4.1 and aquaporin 4 have been shown to be abundantly expressed in astrocytic endfeet (Higashi et al., 2001). The abundance of these transporters are thought to be responsible for the concept of "potassium spatial buffering," which portends to transport the K^+ from the brain extracellular fluid to the endothelial cells (Kofuji and Newman, 2004). Our research has shown that abluminal NKCC activity is increased during conditions of both hypoxia/aglycemia and hypoxia/aglycemia coupled to reoxygenation, both events that mimic stroke conditions and recovery *in vitro*. Most importantly, paracellular permeability returns to a normal restrictive condition upon reoxygenation which would allow for polarity of ion transporter action at the blood-brain barrier. Brain-to-blood movement of K^+ could return brain extracellular fluid concentrations to 3 mM which is imperative for proper neuronal function (Kofuji and Newman, 2004).

Smoking cigarettes has been known to increase the risk of many diseases including stroke. The alteration of NKCC activity is one mechanism which may contribute to an increase of brain extracellular fluid K^+ during an ischemic assault in individuals using products containing nicotine. Downregulation of blood-brain barrier abluminal NKCC activity during hypoxia/aglycemia in the presence of nicotine may exacerbate ischemic cellular damage. Recovery from ischemic damage may also be hampered due to the continuation of NKCC downregulation following an ischemic assault. Together with an inhibition of the protective mechanism by which K^+ is removed, brain extracellular fluid K^+ could accumulate, leading to increased cellular edema in neurons and astrocytes and impaired neuronal conduction following stroke.

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Footnotes

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Legends for Figures

Figure 1A

Effects of Tobacco Smoke Constituents on Abluminal Na,K,2Cl-Cotransporter-mediated K^+ Uptake after Normoxic Conditions

Ouabain insensitive K^+ uptake using ^{86}Rb as a replacement of K^+ under normoxic conditions. Experimental variables of vehicle control, nicotine(100ng/mL)/cotinine(1000 ng/mL) (N/C), Nicotine-containing Cigarette Smoke Extract (N-CSE), and Nicotine-free Cigarette Smoke Extract (NF-CSE). Data represent mean ± SEM of 6 independent determinations. **$p < 0.01$ all from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 1B

Effects of Tobacco Smoke Constituents on Luminal ^{14}C-Sucrose Permeability after Normoxic Conditions

Exposure to vehicle control, N/C, N-CSE, and NF-CSE under normoxic conditions on the ^{14}C-sucrose permeability coefficient of BBMEC confluent monolayers. Data are expressed as the mean permeability coefficient (PC) (cm/min x 10^{-4}) ± SEM. N = 6 monolayers per treatment from two separate BBMEC isolates. Statistical significance is shown by **$p < 0.01$ from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 2A

Effects of Tobacco Smoke Constituents on Abluminal Na,K,2Cl-Cotransporter-mediated K^+ Uptake after 6 hr Hypoxia/Aglycemia

Ouabain insensitive K^+ uptake using ^{86}Rb as a replacement of K^+ under hypoxia/aglycemia conditions. Experimental variables of vehicle control,

nicotine(100ng/mL)/cotinine(1000 ng/mL) (N/C), Nicotine-containing Cigarette Smoke Extract (N-CSE), and Nicotine-free Cigarette Smoke Extract (NF-CSE). Data represent mean ± SEM of 6 independent determinations **p < 0.01 all from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 2B

Effects of Tobacco Smoke Constituents on Luminal ^{14}C-Sucrose Permeability after 6 hr Hypoxia/Aglycemia

Exposure to vehicle control, N/C, N-CSE, and NF-CSE under hypoxia/aglycemia conditions on the ^{14}C-sucrose permeability coefficient of BBMEC confluent monolayers. Data are expressed as the mean permeability coefficient (PC) (cm/min x 10^{-4}) ± SEM. N = 6 monolayers per treatment from two separate BBMEC isolates. Statistical significance is shown by **p < 0.01 from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 3A

Effects of Tobacco Smoke Constituents on Abluminal Na,K,2Cl-Cotransporter-mediated K^+ Uptake after 6 hr Hypoxia/Aglycemia with 12 hr Reoxygenation

Ouabain insensitive K^+ uptake using ^{86}Rb as a replacement of K^+ under 12 hr reoxygenation. Experimental variables of vehicle control, nicotine(100ng/mL)/cotinine(1000 ng/mL) (N/C), Nicotine-containing Cigarette Smoke Extract (N-CSE), and Nicotine-free Cigarette Smoke Extract (NF-CSE). Data represent mean ± SEM of 6 independent determinations **p < 0.01 all from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 3B

Effects of Tobacco Smoke Constituents on Luminal ^{14}C-Sucrose Permeability after 6 hr Hypoxia/Aglycemia with 12 hr Reoxygenation

Exposure to vehicle control, N/C, N-CSE, and NF-CSE on the ^{14}C-sucrose permeability coefficient of BBMEC confluent monolayers after 12 hour reoxygenation. Data are expressed as the mean permeability coefficient (PC) (cm/min x 10^{-4}) ± SEM. N = 6 monolayers per treatment from two separate BBMEC isolates. Statistical significance is shown by **p < 0.01 from one-way ANOVA using Newman-Keuls post hoc analysis.

Figure 4

***In Vivo* Experimental Paradigm**

Outline of the time points for global ischemia by four vessel occlusion. ^{86}Rb was injected into the femoral vein to reach steady state and enter into the brain extracellular fluid K^+ pool. Global ischemia was maintained for 5 minutes in order to prevent blood-brain barrier breakdown and vasogenic edema.

Figure 5

Effects of Tobacco Smoke Constituents on R_{ECF} of [^{86}Rb] After 5 Min of Four Vessel Occlusion and 20 Min Reperfusion

R_{ECF} is expressed as the ratio of the DPMs detected in the brain extracellular fluid (DPM/mL) compared to the DPMs detected in the plasma (DPM/mL) and termed R_{ECF}. N-CSE doses were calculated to deliver an equivalent amount of nicotine equal to a dose of 4.5 mg/kg, which is a good estimate of plasma equivalents in heavy smokers (N = 3 rats/administration) (* denotes significance of $p < 0.05$ and ** denotes significance of $p < 0.01$ using one-way ANOVA with Newman-Keuls post hoc analysis.)

Normoxia: 24 Hour Exposure to Cigarette Smoke Chemicals

Potassium Uptake

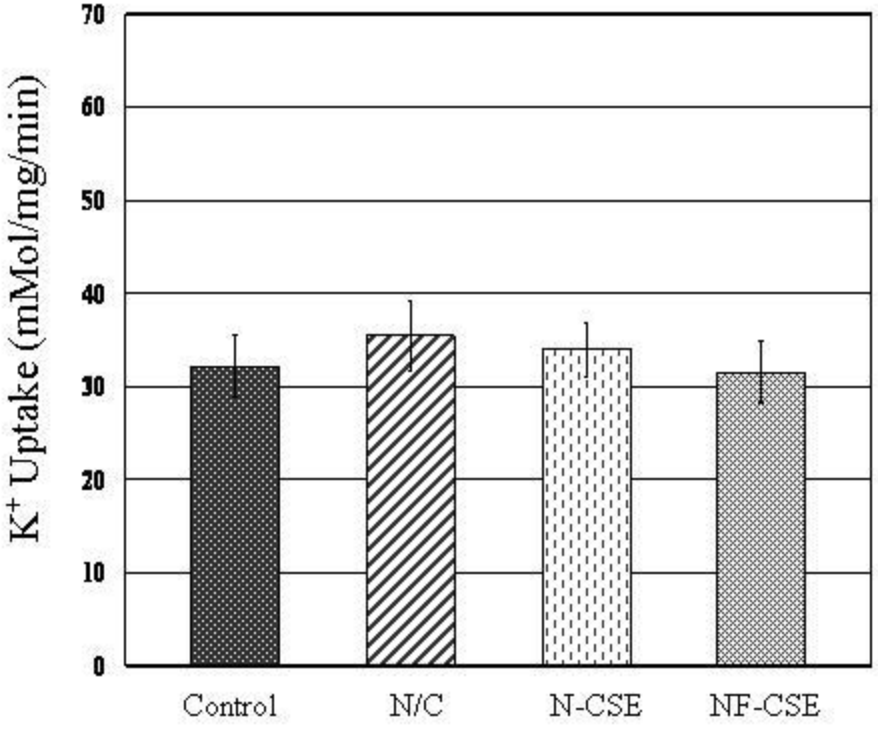

Fig 1 A

Sucrose Permeability

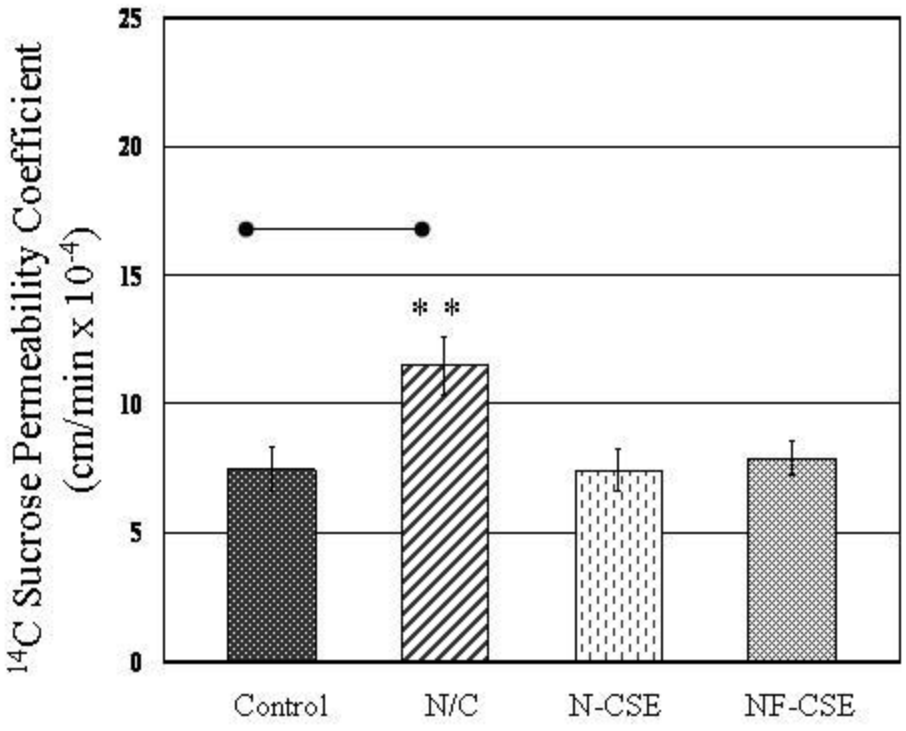

Fig 1 B

6 Hour Hypoxia/Aglycemia

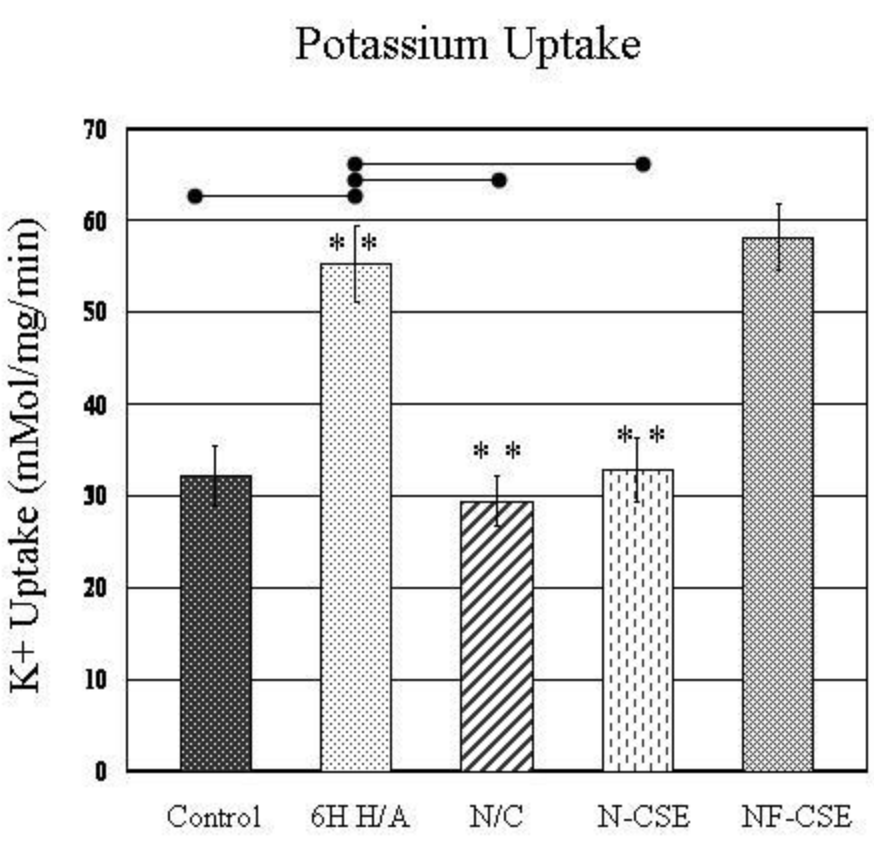

Fig 2 A

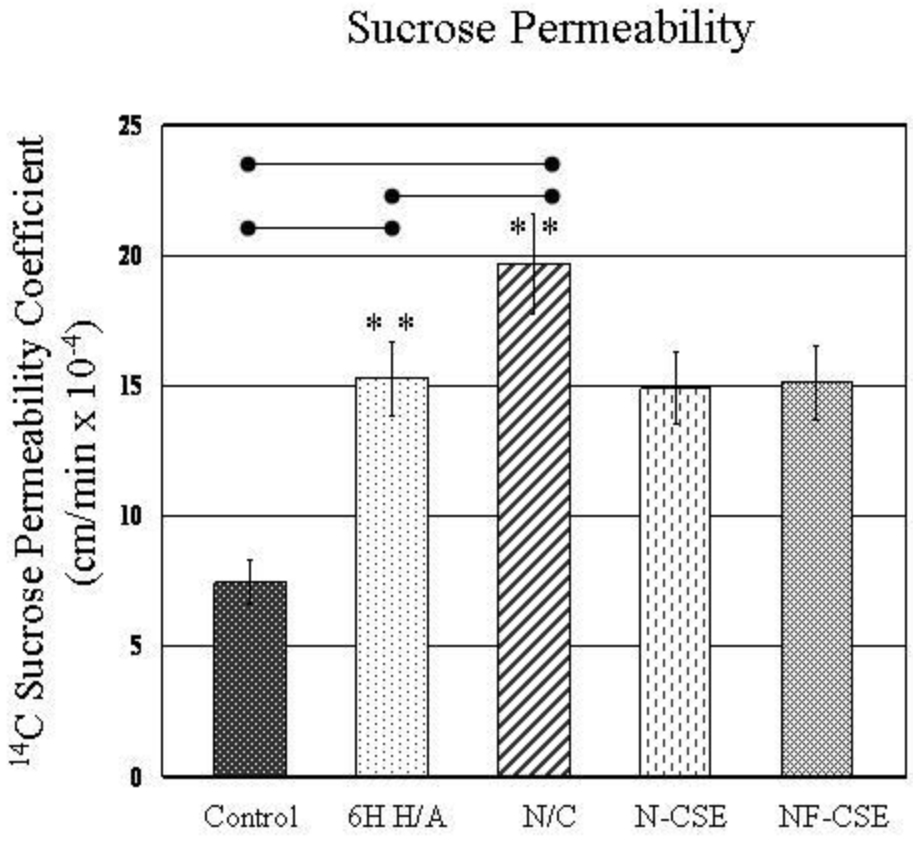

Fig 2 B

6 Hour Hypoxia Aglycemia + 12 Hour Reoxygenation

Potassium Uptake

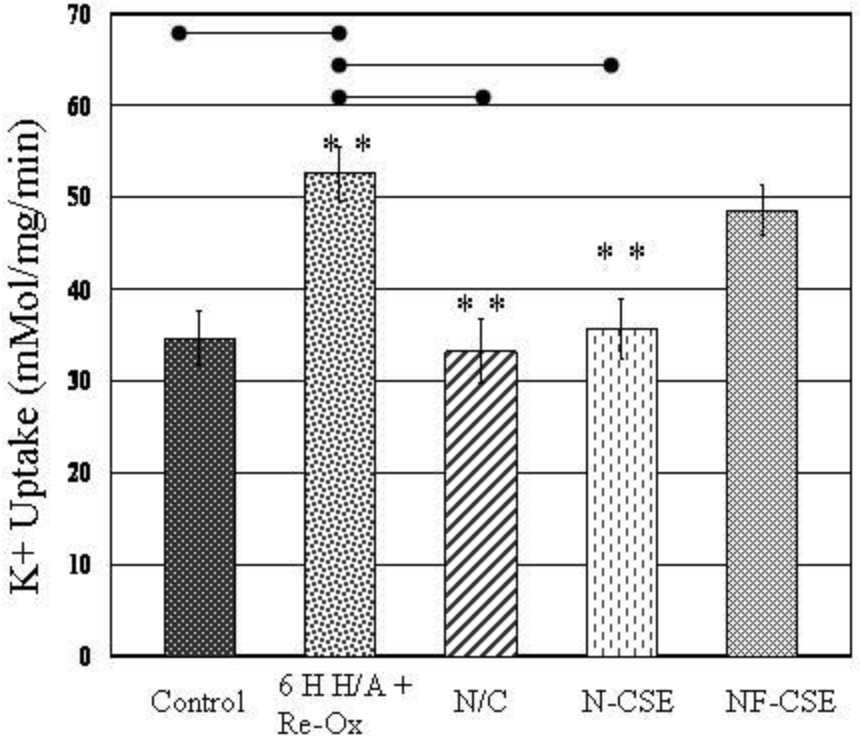

Fig 3 A

Sucrose Permeability

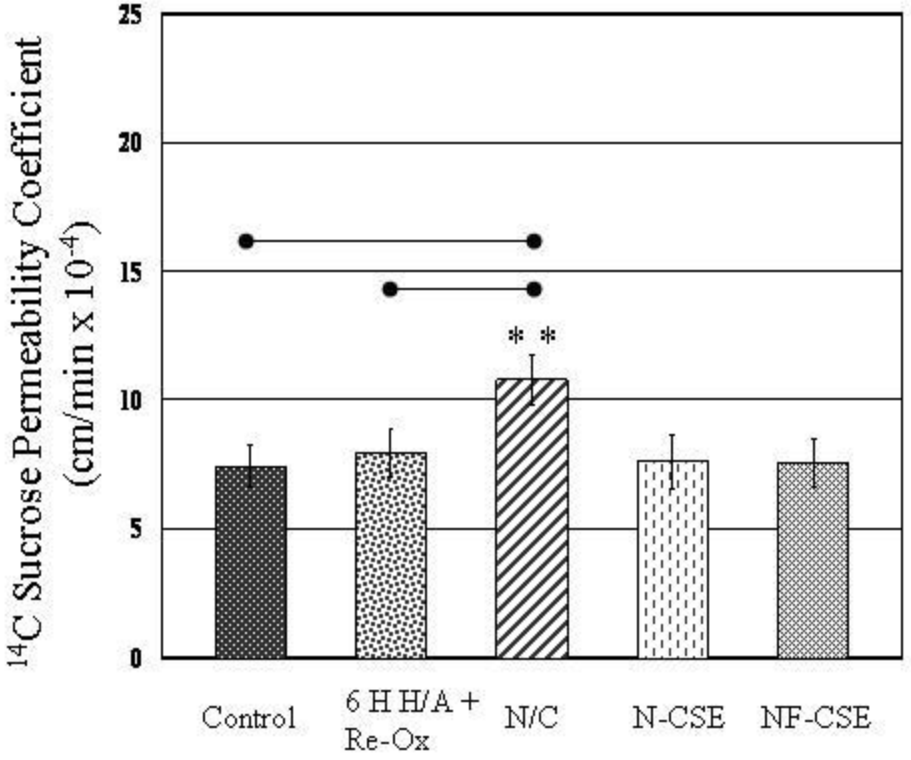

Fig 3 B

In Vivo Experimental Paradigm

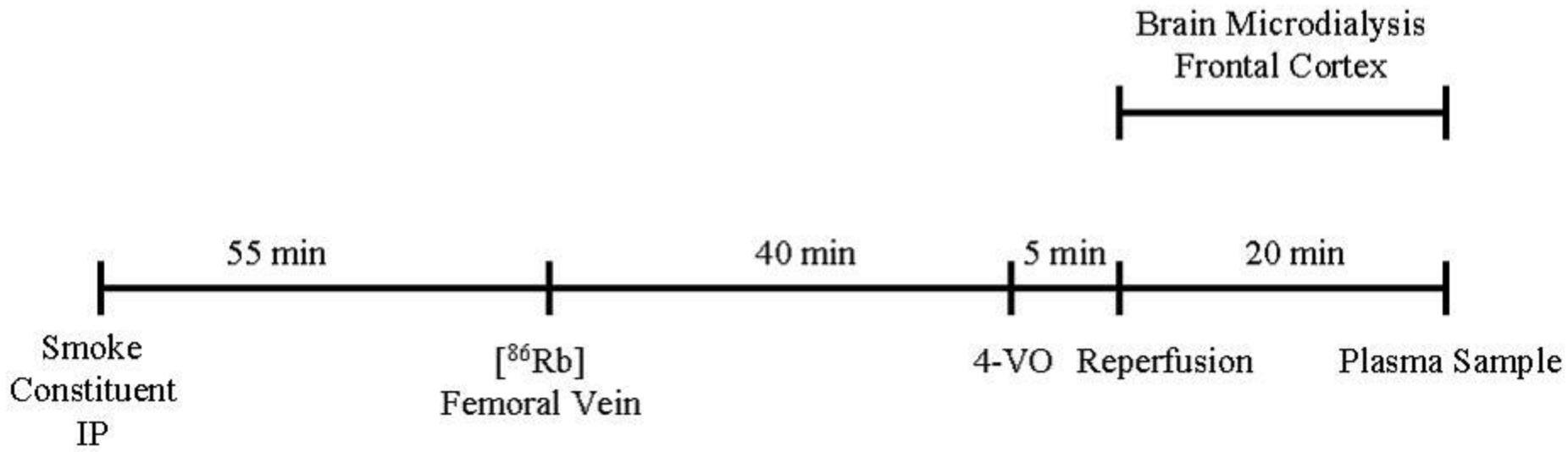

Fig 4

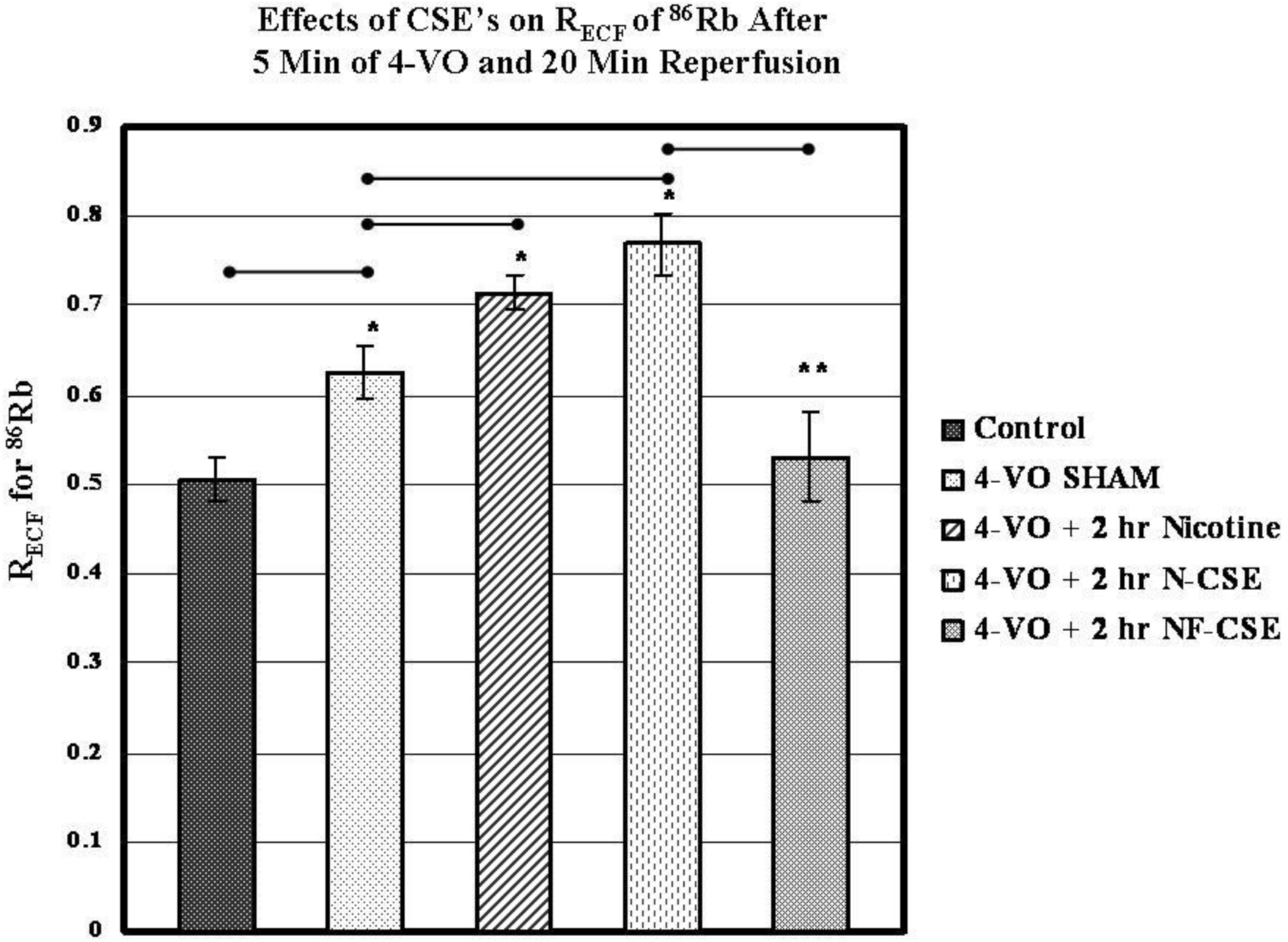
Effects of CSE's on R_{ECF} of ^{86}Rb After
5 Min of 4-VO and 20 Min Reperfusion
R_{ECF} for ^{86}Rb
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
**
Control
4-VO SHAM
4-VO + 2 hr Nicotine
4-VO + 2 hr N-CSE
4-VO + 2 hr NF-CSE

Fig 5