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Research article

Conventional and electronic cigarettes dysregulate the expression of iron transporters and detoxifying enzymes at the brain vascular endothelium: In vivo evidence of a gender-specific cellular response to chronic cigarette smoke exposure

Mohammad A. Kaisar[a], Farzane Sivandzade[a], Aditya Bhalerao[a], Luca Cucullo[a,b,*]

[a] Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, Amarillo, TX 79106, USA
[b] Center for Blood Brain Barrier Research, Texas Tech University Health Sciences Center, Amarillo, TX 79106, USA

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ABSTRACT

It is well established that tobacco smoking is associated with vascular endothelial dysfunction in a causative and dose dependent manner primarily related to the tobacco smoke (**TS**) content of reactive oxygen species (**ROS**), nicotine, and oxidative stress (**OS**) –driven inflammation. Preclinical studies have also shown that nicotine (the principal e-liquid's ingredient used in e-cigarettes (**e-Cigs**) can also cause OS, exacerbation of cerebral ischemia and secondary brain injury. Likewise, chronic e-Cig vaping could be prodromal to vascular endothelial dysfunctions. Herein, we provide direct evidence that similarly to TS, e-Cig promotes mitochondrial depolarization in primary brain vascular endothelial cells as well as the vascular endothelial cell line bEnd3. In addition, both TS and e-Cig exposure upregulated the transmembrane iron exporter Slc40a1 (crucial to maintain cellular iron and redox homeostasis) and that of porphyrin importer Abcb6 (linked to accelerated atherosclerosis). We then investigated in vivo whether gender plays a role in how chronic TS affect vascular endothelial functions. Our results clearly show chronic TS exposure differentially impacts the expression levels of Phase-II enzymes as well as the iron transporters previously investigated in vitro. Although the physiological implications of the gender-specific differential responses to TS are not fully clear, they do demonstrate that gender is a risk factor that needs to be investigated when assessing the potential impact of chronic smoking and perhaps e-Cig vaping.

1. Introduction

TS is accountable for 434,000 deaths each year in United States (US) [1] and has been reported to enhance the risk of stroke [2] and other cerebrovascular/neurological disorders like Alzheimer's [3] and vascular dementia [4]. OS, inflammation and the resulting brain microvascular impairment [5,6] are often the major prodromal factors linking TS to cerebrovascular toxicity. Additionally, in the past decade several alternative vaping products have hit the market, rapidly gaining consumers among adults and adolescents [7]. Electronic cigarettes have become the sought-after product partly due to the belief that they are safe. However, recently published data [8] from side by side experiments investigating the impact of e-Cig (Blu™; 24 mg/mL nicotine) vs. TS (3R4F research cigarettes containing 9.4 mg tar and 0.726 mg nicotine/cigarette and equivalent to full flavor brands; University of Kentucky) on mouse primary brain microvascular endothelial cells (**mBMEC**) clearly show that OS promoted by 24 h exposure to e-Cigs extract was not dissimilar from that induced by cigarette smoke extract from 3R4F. Therefore, we decide to briefly investigate whether both TS and e-Cig can also impact mitochondrial functions as well as iron transporters such as **Slc40a1** (also known as Ferroportin 1) which is heavily implicated in the regulation of cellular iron and redox hemostasis as well as AD brain pathology, infection, and oxidative stress [9,10]. In addition to Slc40a1, **Abcb6** (a mitochondrial ATP-binding cassette porphyrin importer we also investigated) is implicated in the onset of atherosclerosis [11,12].

Previous studies [13,14] suggest that females may respond to oxidative stress stimuli (such as those induced by chronic TS exposure)

Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; OS, oxidative stress; Nrf2, nuclear factor erythroid 2-related factor 2; Nqo1, NAD(P)H: quinone oxidoreductase 1; SFN, sulforaphane; RT-PCR, reverse transcription polymerase chain reaction; mBMEC, mouse primary brain microvascular endothelial cells; Slc, solute carrier; Abc, ATP-binding cassette; VDAC, voltage-dependent anion channels

* Corresponding author at: School of Pharmacy, 1300 S. Coulter Street, Amarillo, TX, 79106, United States.
E-mail addresses: md.a.kaisar@ttuhsc.edu (M.A. Kaisar), farzane.sivandzade@ttuhsc.edu (F. Sivandzade), aditya.bhalerao@ttuhsc.edu (A. Bhalerao), luca.cucullo@ttuhsc.edu (L. Cucullo).

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differently than male. Therefore we also investigated the role of gender in determining the impact of TS on the cerebrovascular cellular responses including the iron transporters previously examined in vitro and 2 major phase2 detoxification enzymes also involved in redox functions (glutathione S-transferase P1 – GSTP1 and glutathione S-transferase A4 (GSTA4). While the extent of the physiological implications are not fully clear, our study strongly suggests that chronic TS exposure has a gender-dependent differential impact on the cerebrovascular system in respect to regulation of phase 2 detoxification enzymes and iron transporters. Thus this study provides a strong baseline work supporting further and more extended studies on to assess gender-specific differential response to TS and other pathological stimuli (possibly including e-cigarettes) as well gender-specific treatments.

2. Methods

2.1. Materials and reagents

Mitoprobe JC-1 kit was purchased from Life Technologies Corporation (Carlsbad, CA); rabbit anti-Slc40a1 (#NBP1-21502), rabbit anti-Abcb6 (#NBP2-58327), mouse anti-Gstp1 (#NB110-60512) and rabbit anti Gsta4 (#H00002941-D01P) were purchased from Novus Biologicals (Littleton, CO). Mini-Protean® TGXTM gels for western blotting were purchased from Bio-rad (Hercules, CA, USA). Donkey anti-rabbit (#NA934) and sheep anti-mouse (#NA931) secondary antibodies were from GE Healthcare (Piscataway, NJ); Alexa Fluor® 488 or 555 conjugated goat anti-rabbit or anti-mouse antibodies were obtained from Invitrogen (Carlsbad, CA). RNeasy Plus Universal Mini Kit (#73404) were purchased from RNAeasy plus mini kit (Qiagen Inc, Germantown, MD). Sterile culture wares were purchased from Fisher Scientific (Pittsburgh, PA, USA) while molecular biology grade reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Bio-rad laboratories (Hercules, CA, USA). RNA Stabilization Reagent (RNA later, cat #76104).

2.2. Cell culture

Mouse bEnd.3 cell line [15,16], was obtained from American Type Culture Collection and cultured in complete DMEM with 10% FBS. Mouse primary brain microvascular endothelial cells (mBMECs, #C57-6023) from Cell Biologics (Chicago, IL, USA) were seeded (p3-6) on gelatin coated cell culture flasks or glass chamber slides, cultured in recommended medium (M1168) and maintained at 37 °C with 5% CO_2 exposure. The culture medium was changed every other day until the cells reached confluency. Phase contrast microscopy and the expression of characteristic phenotypic markers confirmed the monolayer integrity of both mBMEC at confluency. Established cultures were exposed to 5% TS for 72 h.

2.3. Soluble tobacco smoke (TS) and e-cigarette (e-Cig) extract preparation

Soluble TS and e-Cig extracts were prepared according to the FTC standard smoking protocol (35 mL puff volume, 2 s puff duration, 58 s interval, 8 puffs per cigarette) using a Single Cigarette Smoking Machine (**SCSM**, CH Technologies Inc., Westwood, NJ, USA) according to previously published methods [17,18]. Extracts were prepared fresh for each cycle and used in culture at a 5% dilution [18,19].

2.4. In vivo experimental design and tissue preparation

The animal protocol for this work was approved by the Institutional Animal Care and Use Committee, TTUHSC, Lubbock, Texas. C57BL/6J male mice (age range 8–10 weeks old) were purchased from Jackson Laboratories. Mice of both gender (50:50) were divided into 2 groups including control and TS exposed. Mice were chronically exposed (via direct inhalation) to TS mixed with oxygenated air or oxygenated air alone, 6 times/day; 2 cigarettes/hour, 7 days/week for 4 weeks following previously established exposure method simulating realistic human smoking pattern[20]. Mice were sacrificed within an hour of their last TS exposure cycle at the end of the day. Mice were then decapitated under anesthesia to collect blood and brain for subsequent biochemical and molecular preparations.

2.5. Mitochondrial membrane potential

Following treatment, mitochondrial membrane potential was assessed by using the cationic dye, JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), and a mitochondrial membrane potential disrupter, CCCP (carbonyl cyanide 3-chlorophenylhydrazone), as a positive control. Cells were washed with PBS and incubated with 50 μM (final concentration) of CCCP for minutes. 2 μM JC-1 dye (in PBS) was added to both control and treated groups along with the CCCP exposed group and incubated for 20 min. Afterwards, images were captured with EVOS inverted fluorescence microscope at 40X or fluorescence was measured in plate reader (excitation wavelength 488 nm, emission wavelength green (~529 nm) and red (~590 nm).

2.6. Immunofluorescence and western blotting

Following treatment, cells were rinsed with PBS and fixed with ice-cold 4% buffered formalin for 10–15 min. Later, cells were permeabilized and subsequently blocked with 10% goat serum in PBS followed by overnight cold incubation with primary antibodies in blocking buffer (1:100–150) reactive against human or mouse proteins (Glut1, Nrf2, Nqo1 and Slc40a1). Next day, cells were washed with blocking buffer and incubated for 1 h with Alexa Fluor 488 or 555-conjugated anti-rabbit or anti-mouse antibodies (1:1000). Cells were washed and cover-slipped with DAPI in Prolonged Gold Anti-fade reagent (Invitrogen, Carlsbad, CA). Images were captured with EVOS inverted fluorescence microscope at 40X.

Total protein was collected by cell lysis in RIPA buffer with protease inhibitors and quantitated by BCA assay [21]. Equal amounts of protein across samples were subjected to SDS-PAGE separation (4–20% graded gels) as described earlier [21,22]. Following gel to PVDF membrane electrotransfer of the protein bands, membranes were blocked with blocking buffer (0.1% tween-20 in TBS) containing 5% non-fat dry milk and 1% BSA. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies in blocking buffer (in range of 1:200-1:500), washed repeatedly and probed with HRP-conjugated secondary antibodies (1:5000). Bands were visualized by chemiluminescence reagent using X-ray film-based autoradiography and densities were analyzed by Li-Cor Image Studio software with β-actin as a loading control.

2.7. Quantitative RT-PCR

Total RNA (1 μg) extracted as mentioned above was reverse transcribed to cDNA by Superscript III first strand synthesis kit (Life Technologies, Carlsbad, CA). The cDNA strands were mixed with gene-specific forward and reverse primer pairs (Integrative DNA technologies, Coralville, IA; Table 1) and SYBR select master mix (Life Technologies). Template-free and RT-negative samples served as internal controls [21]. Amplification was performed in triplicates/sample on Bio-Rad CFX96 Touch Real-Time PCR system (95 °C for 5 min followed by 40 cycles of 95 °C–30 s, 58 °C–1 min and 72 °C–1 min and a terminal reaction at 72 °C–2 min). The threshold values (counts, Ct) for target genes and reference genes (Rpl21) were determined for each sample and relative expression of each target gene was calculated by $^{\Delta\Delta}$Ct method [21].