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Research Paper

Role of Nrf2 and protective effects of Metformin against tobacco smoke-induced cerebrovascular toxicity

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ABSTRACT

Cigarette smoking (**CS**) is associated with vascular endothelial dysfunction in a causative way primarily related to the TS content of reactive oxygen species (**ROS**), nicotine, and inflammation. TS promotes glucose intolerance and increases the risk of developing type-2 diabetes mellitus (**2DM**) with which it shares other pathogenic traits including the high risk of cerebrovascular and neurological disorders like stroke *via* ROS generation, inflammation, and blood-brain barrier (**BBB**) impairment. Herein we provide evidence of the role played by nuclear factor erythroid 2-related factor (**Nrf2**) in CS-induced cerebrobvascular/BBB impairments and how these cerebrovascular harmful effects can be circumvented by the use of metformin (**MF**; a widely prescribed, firstline anti-diabetic drug) treatment. Our data in fact revealed that MF activates counteractive mechanisms primarily associated with the Nrf2 pathway which drastically reduce CS toxicity at the cerebrovascular level. These include the suppression of tight junction (**TJ**) protein downregulation and loss of BBB integrity induced by CS, reduction of inflammation and oxidative stress, renormalization of the expression levels of the major BBB glucose transporter Glut-1 and that of the anticoagulant factor thrombomodulin. Further, we provide additional insights on the controversial interplay between Nrf2 and AMPK.

1. Introduction

Globally, tobacco use causes approximately 6 million deaths per year, and predictions report that with current trends of tobacco use, more than 8 million deaths are expected annually by 2030. Cigarette smoking is accountable for more than 480,000 deaths each year in United States (US) and is the leading cause of preventable death in the US. What is further of concern is that 41,000 deaths out of 480,000 deaths results from secondhand smoke exposure. Nearly 50% of chronic smokers die prematurely as a result of the adverse effects of their tobacco addiction. For every individual who dies due to smoking, at least 30 people are affected and live with a severe smoking-related illness [1–3]. Smokers are 2–4 times more likely to suffer from coronary heart disease and stroke [4] and around 25 times more likely to suffer from lung cancer. The risk of developing diabetes is 30–40% higher for smokers in comparison to nonsmokers, which further increases with the number of cigarettes smoked [2,5]. Besides these major illnesses, smoking has also been associated with the onset of rheumatoid arthritis, pneumonia, asthma, blindness, hardening of the arteries, reduced fertility and enhanced risk of infections [2,3,5,6]. From a cerebrovascular and neurological perspective, tobacco smoking (**TS**) is associated with vascular endothelial dysfunction [7–9] in a causative and dose dependent way [10] primarily related to the TS content of reactive oxygen species (**ROS**) [8,11], nicotine [12–17], and smoking-induced inflammation [18]. As such TS is also a major prodromal factor for numerous central nervous system (CNS) disorders

Abbreviations: ARE, Anti-oxidant response element; BBB, Blood-brain barrier; COPD, Chronic obstructive pulmonary disease; CS, Cigarette smoke; CSE, Cigarette smoke extract; 2DM, Type 2 diabetes mellitus; FITC, Fluorescein isothiocyanate; FTC, Federal trade control; Glut–1, Glucose transporter; HG, Hyperglycemia; HO-1, Heme oxygenase 1; ICAM-1, Intercellular adhesion molecule-1; ISO, International organization for standardization; MF, Metformin; NQO-1, NAD(P)H: Quinone reductase I; Nrf2, Nuclear factor erythroid 2-related factor; PECAM-1, Platelet endothelial cell adhesion molecule-1; RITC, Rhodamine B isothiocyanate; ROS, Reactive oxygen species; SFN, Sulforaphane; TEER, Trans-endothelial electrical resistance; TJ, Tight junction; TS, Tobacco smoke; ZO-1, Zonulae occludentes-1

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including Alzheimer's, depression, cognitive impairment, stroke, and vascular dementia. TS promotes glucose intolerance and increases the risk of developing type-2 diabetes mellitus (**2DM**) [19,20] with which it shares other pathogenic traits *via* ROS generation, inflammation, and blood-brain barrier (**BBB**) impairment [21–23]. It also impairs cerebrovascular development in fetus during pregnancy [5].

Even though the Food and Nutrition Board of the National Academy of Sciences recommends a higher dietary allowance (**RDA**) of vitamin C for smokers (more than 200 mg/day as compared to 90 mg/day for non-smokers), the benefits of prophylactic antioxidant use in smokers remains controversial [24,25]. Considering this premise, it is evident that studies are needed to explore biologic mechanisms by which exposure to tobacco smoke compromises health, identify biomarkers of injury, and detect smokers at early stages of disease development and find clinical drugs with prophylactic/therapeutic benefits against smoke exposure effects.

From this perspective, recent findings from our group [26] support an additive release pattern of angiogenic and inflammatory factors (besides activation of common anti-oxidant mechanisms) by BBB endothelial cells in response to hyperglycemia (**HG**) with concomitant exposure to cigarette smoke extracts (**CSE**), thus suggesting the involvement of common pathogenic modulators of BBB impairment. To this end, metformin (**MF**; a widely prescribed, firstline anti-diabetic drug) treatment before and after stroke injury has been shown to reduces stress and inhibits inflammatory responses [27,28]. Herein we provide novel *in vitro* and *in vivo* data demonstrating how MF activates counteractive mechanisms which drastically reduce TS toxicity at the cerebrovascular level. These beneficial effects are mediated by MF's activation of nuclear factor erythroid 2-related factor (**Nrf2**) [27]. These novel findings support and build on previous research from our group demonstrating that Nrf2 plays a functional role in preserving BBB integrity [29] and that alterations of gene transcription/translation related to the Nrf2-ARE pathways were among the most predominant in human BBB microvascular endothelial cells exposed to TS [30].

2. Methods

2.1. Materials and reagents

Sterile culture wares were purchased from Fisher Scientific (Pittsburgh, PA, USA), reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Bio-rad laboratories (Hercules, CA, USA). Gel electrophoresis was carried out by using Mini-Protean® TGXTM gels 4–15% (#456–1084) from Bio-rad laboratories (Hercules, CA, USA). Dextran-Cascade Blue® (10,000 MW; #D-1976) was obtained from life technologies (Grand Island, NY, USA), while Fluorescein isothiocyanate (FITC)-dextran (3000–5000 MW; #FD4) and Rhodamine B isothiocyanate (RITC) - dextran (70,000 MW; #R9379) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used in this study were obtained from the following sources: Rabbit anti-ZO-1 (#402200) from Invitrogen; mouse anti-Occludin (#331500) from Life Technologies; mouse anti-Glut-1 (#12939), rabbit GFAP (#3670), p-AMPKα (#2535) and AMPKα (#5831) from Cell Signaling Technology; Rabbit anti-ZO-1 (# sc-10804), mouse anti-ICAM-1 (#sc-18853), mouse anti-VCAM-1 (#sc-13160), mouse anti-PECAM-1 (#sc-376764), rabbit anti-Nrf2 (#sc-722), mouse anti-NQO1 (#sc-376023), mouse anti-HO1 (#sc-390991) from Santa Cruz Biotechnology. Donkey anti-rabbit (#NA934) and sheep anti-mouse (#NA931) HRP-linked secondary antibodies were obtained from GE Healthcare (Piscataway, NJ, USA); goat anti-rabbit (#A11008, A21428) conjugated to Alexa Fluor® 488 and 555 respectively and anti-mouse (#A11001, A21422) conjugated to Alexa Fluor® 488 and 555 respectively from Invitrogen (Camarillo, CA, USA).

2.2. Experimental design (in vivo)

The animal protocol for this work was approved by the Institutional Animal Care and Use Committee, TTUHSC, Lubbock, Texas. Sixteen male C57BL/6 J mice, age range 8–10 weeks were purchased from Jackson Laboratories. They were divided into two groups either chronically exposed (via direct inhalation) to cigarette smoke (**CS**) mixed with oxygenated air or oxygenated air alone, 6 times/day; 2 cigarettes/hour, 6–8 h/day, 7 days/week for 2 or 4 weeks following International Organization for Standardization/ Federal Trade Commission (ISO/FTC) standard smoking protocol (35 ml draw, 2 s puff duration, 1 puff per 60 s). CS was generated using a Single Cigarette Smoking Machines (**SCSM**, CH Technologies Inc., Westwood, NJ, USA) following previously published methods [31]. Mice were sacrificed and samples were collected for further analysis. Based on the results, 4 weeks of CS exposure was selected as the time course for a subsequent study to evaluate the protective effects of MF treatment against CS exposure. MF (Sigma, St. Louis, MO, USA) was dissolved in sterile saline at a concentration of 30 mg/ml. MF was administered daily (via intraperitoneal Injections of doses of 100 or 200 mg/kg, 100 mg/kg - **MF100** and 200 mg/kg – **MF200** [28,32]) to mice either exposed to CS (mixed with oxygenated air) or oxygenated air alone (controls) for 4 weeks as earlier mentioned. An equal volume of saline (corresponding to 200 mg/kg MF dose) was used for the control group which received either oxygenated air or CS (mixed with oxygenated air). At the end of the study, mice were sacrificed and samples (plasma and brain) were collected for further analysis.

2.3. Tissue preparation

Mice were sacrificed within an hour of their last CS exposure cycle at the end of the day. Mice were decapitated under anesthesia to collect blood and brain for subsequent biochemical and molecular preparations.

2.4. Cotinine analysis

Plasma and brain homogenate cotinine levels were determined by using Cotinine EIA ELISA kit (1124EB) from OraSure Technologies, Incorporation (PA, USA). Analysis was carried out as per manufacturer's guidelines.

2.5. Glucose analysis

To determine glucose, a tail pin prick was performed on the anesthetized mice and the glucose level was determined using contour next blood glucose meter obtained from Bayer Healthcare (Indiana, USA).

2.6. Cell culture

C57BL/6 mouse primary brain endothelial cells (mBMEC, #C57-6023) were obtained from Cell Biologics (Illinois, USA). mBMEC (passages no. 4–7, obtained from vendor at P3) were seeded 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. Apart from primary cells, an immortalized mouse brain endothelial cell line, bEnd.3 (passage no. 22–25, ATCC® CRL-2299™) obtained from ATCC, VA, USA was also used in *in vitro* experiments. Immortalized bEnd.3 endothelial cells were seeded on uncoated cell culture flasks as per manufacturer's protocol and maintained at 37 °C with 5% CO_2 exposure. Cell culture medium for bEnd.3 consisted of ATCC- formulated Dulbecco's Modified Eagle's Medium (#30–2002 from ATCC, VA, USA), which was supplemented with 10% FBS (Atlanta Biologicals, GA, USA). The culture medium was changed every other day until the cells reached confluency. Phase contrast microscopy