ONLINE MATERIALS AND METHODS

Cigarette Smoke Initiates Oxidative Stress-Induced Cellular Phenotypic Modulation Leading to Cerebral Aneurysm Pathogenesis

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MATERIALS AND METHODS:

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by local Committees on the Ethics of Animal Experiments. All surgery was performed under general anesthesia with Ketamine and Xylaxine. All efforts were made to minimize suffering. Cerebral blood vessels (Circle of Willis) from rats were harvested for primary cell culture and for quantitative real-time polymerase chain reaction (RT-PCR), ROS production, and assessment following adenovirus promoter transfection. *In vitro* cigarette smoke extract (CSE) was carried out with cigarette smoke extract (Murty Pharmaceutical), and *in vivo* CSE was carried out within a smoking chamber (Kentucky Research cigarettes).

Rat Cerebral Vascular Smooth Muscle Cell Culture and CSE Exposure

Cerebral blood vessels (circle of Willis) from 7 week-old rats (Sprague-Dawley) were harvested for primary cell culture as previously described [1,2]. Cerebral blood vessels were placed in cold Hank's Balanced Salt Solution (HBSS). The vessels were washed, connective tissue and arachnoid were removed, and were placed in enzyme solution (Collagenase II 1mg/ml, Soybean Trypsin Inhibitor 1mg/ml, and Elastase 0.744 U/ml in HBSS) for 8-10 minutes. The adventitial layer was stripped. After an hour, the vessels were washed in media, plated in culture dishes in growth medium containing Dulbeco's Modified Eagle Medium/F12 media (20% FBS and 1% anti-anti) in a humidified atmosphere of 95% air and 5% CO_2. Cells were weaned to 10% FBS after passage 3-5. For experiments, unless otherwise noted, cells were grown to confluence and placed in serum free media containing DMEM/F12, L-ascorbic acid

(200 μM), apotransferrin (5 μg/ml) and selenium selenite (6.25 ng/ml), in addition to L-glutamine, and anti-anti (Invitrogen) for 72 hours prior to the start of the experiment.

Cells were treated with CSE dissolved in HEPES buffer for 24 hours for RT-PCR and 72 hours for Western blot. The CSE dose and timing was based on our preliminary studies and was selected to approximate levels of water-soluble components of cigarette smoke and nicotine present in plasma levels of human smokers [1].

Adenovirus Constructs and Infection

Adenoviruses encoding the flag-tagged NOX (Ad/NOX) gene, NOX1-antisense (Ad/AS-NOX1), and empty vector control were generated using standard methods.[3] VSMC were infected twenty-four hours after plating, at a multiplicity of infection (MOI) of 50 for 24-48 hours; resulting in greater than 95% infection .

Transient Transfection and Luciferase Assay

VSMC were transfected with a reporter plasmid (SM α-actin-luc (-2555/+2813 bp), SM MHC-luc (-4220/+11600bp), or pGL3 basic plasmid) using FuGENE (Roche Diagnostics Corp.) following the manufacturer's protocol. Luciferase activity was measured with luciferase assay substrate (Promega Corporation) and the data normalized to total protein content.

Quantitative Real-Time RT-PCR

Total RNA was prepared from VSMCs using a RNeasy Kit (Qiagen) following the manufacturer's instructions. Total RNA was isolated from CAs or cerebral blood vessels using an Ultra-Turrax (Sigma-Aldrich) isolation kit, and total RNA was prepared using RNeasy fibrous mini kit (Qiagen) according to manufacturer's instructions. cDNA was prepared using 0.5-1 μg of total RNA andRT-PCR conducted using iQ SYBR Green (Bio-Rad laboratories) Results were normalized to 18S rRNA gene expression. Primer sequences are listed in the online supplemental Table 1.

Dihydroethidium In Situ Superoxide Imaging

Cells were incubated with dihydroethidium (10 mmol/l; Sigma Aldrich) and imaged using fluorescence microscopy. *In vivo* specimens from mice were cut and mounted on slides and incubated with 20 mmol/l dihydroethidium and imaged using fluorescence microscopy.

Reactive Oxygen Species Assessment with Lucigenin-Enhanced Chemiluminescence

VSMC were lysed and the lysates added to lucigenin chemiluminescence (250 μM). Luminescence was measured every 15s for 3 minutes by a luminometer. The results are displayed as counts per mg of protein (percentage of control).

Cerebral Aneurysm Induction

CAs were induced in 8- to 10-week-old male NOX p47phox gene null (p47phox -/-) mice or wild type controls (Jackson Laboratory, Bar Harbor, ME) using previously described methods,[4-7] with alterations described below.

To induce hypertension, mice underwent a unilateral nephrectomy, followed by implantation of a deoxycorticosterone acetate (DOCA) pellet (Innovative Research of America) one week later.[7,8] On the same day as DOCA pellet implantation, animals were started on drinking water containing 1% NaCl [4-6,8] and 0.12% beta-aminoproprionitrile (BAPN) to reduce collagen cross-linking.[9] To induce aneurysm formation on the same day as DOCA pellet implantation, mice underwent a single stereotactic injection of elastase (Sigma Aldrich; 3.5 mU, 17.5 mU, and 35 mU) into the cerebrospinal fluid of the right basal cistern.[4-6] Sham control mice received a single stereotactic injection of vehicle (PBS). Animals were randomly assigned to the sham or CA groups.

Blinded daily neurological examination was performed as previously described.[5,7,10-12] All asymptomatic mice were euthanized 28 days after CA induction. To visualize the cerebral arteries as well as to assess for CA formation and SAH, mice were transcardial perfused with a gelatin - blue dye mixture. An aneurysm was defined as a localized outward bulging of the vascular wall whose diameter was greater than 1.5 times the parent artery diameter. [4,5]

Treatment with Apocynin

Mice were administered the antioxidant/NADPH oxidase inhibitor apocynin (acetovanillone, Sigma Aldrich), by dissolved in their drinking water (100 mg/kg/day). Controls received water only. Dosing was based on preliminary studies [1,2] and prior experiments.[13] Apocynin treatment was started three days prior to elastase injection and continued for 28 days.

Cigarette Smoke Exposure *In Vivo*

CS exposure was started 7 days prior to nephrectomy. The mice were repeatedly exposed to CS for a total of 200 minutes (Kentucky Research cigarettes (3r4f) each day. The CS exposure condition was evaluated weekly and CO2 concentration inside the chamber was measured using gas chromatography. Levels of CO2 were never above 1400 ppm; levels of O2 were never below 16%; and levels of carbon monoxide were never above 5%. Average CO2 concentration inside the chamber was 750 ± 50 ppm at the time point of burning for 15 min and 600 ± 50 ppm when the smoke was diffusing for another 25 min

Application of CSE to Rat Carotid Artery

In vivo experiments were carried out following application of pluronic gel (Sigma-Aldrich) containing CSE to the adventitial surface of rat carotid arteries, a validated model of carotid atherosclerosis and cerebrovascular disease [1,14-16]. Anesthesia was induced and maintained using Isofluorane. 100 µl of ice-cold F-127 Pluronic Gel (Sigma-Aldrich) containing CSE at 0.8 mg/ml (n=6) and vehicle (n=6) was applied to the adventitial surface of rat carotid arteries for 6–8 hrs for NOX and 12 hours for differentiation and inflammatory marker genes. Vessels were extracted, homogenized using Ultra-Turrax (Sigma-Aldrich), and total RNA was extracted using RNeasy fibrous mini kit (Qiagen) using manufacturer's protocol. Results were normalized to 18S rRNA gene expression and compared to non-treated vessels.

Statistical Analysis

With an alpha of 0.05 and a power of 80% and based on preliminary experimental studies on the incidence of CA formation according to various elastase doses, approximately 25 animals would be required in each cohort to detect differences in overall CA formation with an elastase dose of 35 mU and 30 animals with an elastase doses of 3.5 mU.

All experiments were performed with a minimum of triplicate samples and were conducted in 3 to 6 independent experiments unless otherwise indicated. Data are presented as mean and range for continuous variables, and as frequency for categorical variables. Analysis was carried out using Chi-square, Fisher's exact, Wilcoxon rank sum, and Kruskal-Wallis tests as appropriate. Secondary assessment of risk of rupture was carried out by Kaplan-Meier survival analysis with Cox regression analysis to assess hazard ratios (HR). Error bars represent standard error of the mean (SEM). Statistical significance was considered defined as $P<0.05$.

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