Online Data Supplement

Role of Nerve Growth Factor in Ozone-Induced Neural Responses in Early Postnatal Airway Development

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METHODS

Ozone Exposure

Technical details of our ozone exposure apparatus have been published (37). Fisher-344 rat pups were exposed for 3 hrs to either O_3 (2ppm) or FA at various postnatal ages in a stainless steel-and-glass exposure chamber. O_3 was produced by passing hospital-grade air through a drying and high-efficiency particle filter and then through an ultraviolet light source. The O_3 concentration in the chamber was measured by chemiluminescence with a calibrated O_3 analyzer (model OA 350-2R, Forney, Carrollton, TX). In the FA exposed animals, procedures were identical to those described above except O_3 was not delivered to the exposure chamber.

NGF or Rhodamine-Labeled Latex Microspheres Instillation

PD6 rat pups were anesthetized via hypothermia by submergence in ice for approximately 3-5 minutes and were placed inside a latex sleeve to protect their skin. PD21 rat pups were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After sufficient anesthesia, NGF-ß, (5 uls, 10 ug/ml, Sigma), saline or rhodamine labeled latex microspheres (10 uls, LumaFluor Inc., Naples, FL) were instilled into the tracheal lumen using a Hamilton syringe. Incisions were closed using sterile surgical tape. Animals were revived using gentle warming and artificially ventilated if needed. When rat pups regained consciousness and were breathing normally, they were returned to their mother.

Bronchoalveolar Lavage

A cannula was inserted into the trachea and phosphate buffered saline (PBS) was instilled into the lungs in situ. A standard volume of PBS, adjusted for age and lung size, was instilled for each age group, collected and centrifuged at 10,000 rpm. The supernatant was removedand utilized for ELISA analysis. Resulting cell pellet was resuspended in 1 ml cold PBS, plated on glass slides at a density of $1.5X10^5$ cells/ml using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) and stained with Wright-Giemsa. A total of 100 cells were classified as neutrophils, eosinophils, basophils, lymphocytes, macrophages or epithelial cells using light microscope (Olympus AX70, Center Valley, PA) with a 40x magnification objective. The percentage and total numbers of inflammatory cells were recorded for each slide.

NGF Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of NGF (7.8-250 pg/ml) in each lavage supernatant sample was assayed using the NGF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. The concentration of NGF in each lavage sample was extracted from an NGF standard curve and then multiplied by the standard instilled volume for the respective age group to determine the amount of NGF in the entire lung. NGF concentrations were then normalized to the dry lung weight (g) for the respective postnatal age. All samples were run in duplicate or triplicate. As a negative control, triplicate PBS samples were run with each assay.

Tracheal Epithelial Cell Isolation and RNA Extraction

Tracheas were excised and immersed in ice cold Hank's Buffered Salt Solution (HBSS) while excess tissue was removed. Next, the tracheas were placed in pre-warmed (37˚C)

dissociation medium (10mls, 0.5% protease in Dulbecco"s Modified Eagle Medium (DMEM)-F12/5%Pen-Strep) and incubated at 37˚C with agitation for 1hr. Fetal bovine serum (1ml, FBS) was added to the dissociation medium containing the tracheal tissue to stop the protease enzymatic reaction and then removed and rinsed in a second vial containing culture medium (10mls, 5% FBS and 4% DNase I in DMEM-F12/5%Pen-Strep). The tracheal pieces were discarded and the culture medium and dissociation medium cell suspensions were pooled. The combined cell suspension was incubated on anti-IgG antibody coated plates at 37˚C for 1hr to remove immune cells. The cell suspension was collected, centrifuged (1,000rpm/10min) and supernatant was discarded. Total RNA was extracted from the pellet according to the manufacturer's instructions (RNeasy Plus Micro Kit, Qiagen, Valencia, CA).

Real-Time PCR

Total RNA was transcribed into cDNA using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions**.** The cDNA was then amplified in the 7500 RT-PCR System (Applied Biosystems) using their NGF Taq Man gene expression assays. The Relative Quantification Method ($2^{-\Delta\Delta Ct}$) was used to analyze the NGF mRNA expression data. NGF expression levels were normalized using β-actin expression as an internal control.

SP Immunocytochemistry and Nerve Fiber Density (NFD) Measurement

All procedures for tissue preparation, immunocytochemistry and analysis have been described in our previous publications (38). Briefly, lungs were fixed by intra-tracheal inflation with picric acid-formaldehyde for three hours. After three hours, the lungs were rinsed twice with a 0.1 M phosphate-buffered saline containing 0.15% Triton X-100 (PBS-Tx, pH 7.8) and remained in PBS-Tx overnight at 4°C. The next day, extraneous lung tissue was removed leaving only the airways, which were further dissected into extrapulmonary and intrapulmonary regions. The airways were placed on corks, covered with Tissue Tek O.C.T. compound (Sakura, Torrance, CA) and frozen in isopentane cooled with liquid nitrogen.

Cryostat sections(12 μm thick) of airway tissue were incubated with rabbit anti-SP primary antiserum(1:200; Peninsula, Belmont, CA) diluted in PBS-Tx + 1% bovine serum albumin (BSA) (PBS-Tx-BSA, pH 7.8) in a humid chamber at 37°C for 30 minutes. Sections were rinsed 3X for 5 minutes each with PBS-Tx-BSA, covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobin IgG (1:100; ICN Pharmaceuticals, Inc, Costa Mesa, CA), incubated at 37°C for 30 min, rinsed 3X for 5 min in PBS-Tx-BSA and mounted on glass slides using Fluoromount (Southern Biotechnology, Birmingham, AL). Sections were observed using an Olympus AX70 fluorescence microscope equipped with a fluorescein filter. Controls consisted of testing the specificity of primary antiserum by absorption with 1μg/ml of the specific antigen. Nonspecific background labeling was determined by omission of primary antiserum.

SP-immunoreactivity (IR) on airway sections was observed on a Zeiss LSM 510 confocal microscope (40X) equipped with an argon laser (Zeiss, Oberkochen, Germany). Serial images of SP-containing nerve fibers located in airway smooth muscle were collected, saved to adatabase and exported as a black and white electronic TIF images. SP-NFD was measured for each TIF image using Optimus software (Bioscan, Edmonds, WA). The entire perimeter of smooth muscle was traced on each airway image. Athreshold was optimized so that only SP- IR nerve fibers were visible. The SP-NFD was calculated by dividing the SP-IR nerve fiber area by the total smooth muscle outlined. This represents the proportional cross-sectional area occupied by SP-IR nerve fibers.

Evaluation of SP-immunoreactivity in Airway Labeled Neurons

SP immunocytochemistrywas described in the above section and used to evaluate SP-IR in airway neurons. SP-IR was evaluated only in cell bodiescontaining rhodamine-labeled latex microspheres.Localization of the rhodamine microspheres identified sensory neurons that projected to the airway epithelium. Rhodamine-labeled neurons were identified using the Olympus AX70 fluorescence microscope equipped with a rhodamine filter and a black and white image was captured using a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). The perimeters of cell bodies containing microspheres were traced using Optimus Image Analysis Software (Media Cybernetics, L.P., Silver Springs, MD). Using the same field, an identical black and white image was captured with the fluorescein filter. The cell body outline obtained using the rhodamine filter was superimposed on the fluorescein image. The intensity of SP-IR labeling was determined by calculating the mean gray value (MGV) for each neuron using Optimus software. Neurons with an MGV< 50 were considered negative and neurons with a MGV$\geq$50 were classified immunoreactive for the protein SP. The cut-off range from positive to negative is based on an initial survey of several neurons in the ganglia directly observed to be positive or negative with the naked eye and then digitally analyzed. The percentage of SP-IR neurons innervating the airways was determined by dividing the total number of SP positive microsphere-containing neurons (MGV$\geq$50) by the total number of microsphere-labeled neurons.

Data Analysis

The results are expressed as means (±SE). Statistical analysis was evaluated by using a one- or two-way (age and O_3/Air) ANOVA. When an effect was considered significant, a pair-wise comparison was made with a *post hoc* analysis. A value of P less than 0.05 was considered significant for each endpoint and *n* represents the number of animals studied per experimental group.