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COMBUSTION SMOKE EXPOSURE INDUCES UP-REGULATED EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR, AQUAPORIN 4, NITRIC OXIDE SYNTHASES AND VASCULAR PERMEABILITY IN THE RETINA OF ADULT RATS

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Abstract—Retinal cells respond to various experimental stimuli including hypoxia, yet it remains to be investigated whether they react to smoke inhalation. We show here that retinal cells in rats, notably the ganglion cells, Müller cells, astrocytes and blood vessels responded vigorously to a smoke challenge. The major changes included up-regulated expression of vascular endothelial growth factor (VEGF), aquaporin 4 (AQP4) and nitric oxide synthase (NOS). VEGF expression was localized in the ganglion cells, Müller cells, astrocytes and associated blood vessels. AQP4 was markedly enhanced in both astrocytes and Müller cells. Increase in vascular permeability after smoke exposure was evidenced by extravasation of serum derived rhodamine isothiocyanate which was internalized by Müller cells and ganglion cells. The tracer leakage was attenuated by aminoguanidine and N^G-nitro-L-arginine methyl ester (L-NAME) treatment which suppressed retinal tissue NOS and nitric oxide (NO) levels concomitantly. It is suggested that VEGF, AQP4 and NO are involved in increased vascular permeability following acute smoke exposure in which hypoxia was ultimately implicated as shown by blood gases analysis. NOS inhibitors effectively reduced the vascular leakage and hence may ameliorate possible retinal edema in smoke inhalation.

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Abbreviations: AG, aminoguanidine; AQP4, aquaporin 4; BE_{ecf}, base excess in extracellular fluid; BRB, blood–retinal barrier; COHb, carboxyhemoglobin; eNOS, endothelial nitric oxide synthase; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GS, glutamine synthase; iBRB, inner blood–retinal barrier; INL, inner nuclear layer; iNOS, inducible nitric-oxide synthase; IPL, inner plexiform layer; L-NAME, NG-nitro-L-arginine methyl ester; NeuN, neuronal nuclear antigen; NFL, nerve fiber layer; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; OLM, outer limiting membrane; ONL, outer nuclear layer; O_2Hb, oxyhemoglobin; PCO_2, carbon dioxide partial pressure; pO_2, oxygen partial pressure; RhIC, rhodamine isothiocyanate; RPL, retinal pigment epithelium layer; sO_2, oxygen saturation; TBS, Tris-buffered saline; VEGF, vascular endothelial growth factor.

Key words: smoke exposure, vascular endothelial growth factor, aquaporin 4, nitric oxide synthases, vascular permeability, retinal glia and neurons.

Smoke inhalation frequently occurs during all kinds of fires, leakage of noxious chemicals, explosion and even in wars. Among the various fires, urban terrain fire has been the biggest factor contributing to smoke inhalation or exposure. It has been estimated that about 6000–7000 fires break out each year in the world, the number of times and the death toll being the highest in major cities in Europe and North America. Combustion smoke inhalation injury is generally caused by direct thermal burns, hypoxia and inhalation of gases and particulate matter, acting alone or in combination. Absorption of toxic gases, by means of inhalation of carbon monoxide, or cyanide, causes methemoglobinemia (Fitzpatrick et al., 1996; Leopoldo et al., 2003; Whitehead et al., 2003). Smoke inhalation may be life-threatening as it can result in a series of organ and tissue damages. There is a great deal of research about acute or chronic hypoxic injuries, but combustion smoke inhalation correlational research has remained unexplored. In the latter, the main focus has been on the respiratory system (Whitehead et al., 2003), with very few studies focusing on the CNS (Lee et al., 2005). Indeed, as far as can be ascertained reports on retinal changes following combustion smoke inhalation appear to be lacking. Some earlier studies have reported that chemical and thermal injuries of the eyes destroy surface epithelia and cause ischemic necrosis of conjunctiva, cornea, sclera, iris, ciliary body and eyelids (Reim, 1992; Reim et al., 1993; Pfister et al., 1993; Fischern et al., 1998). This was followed by an inflammatory response with leukocytic infiltration and release of inflammatory mediators (Pfister et al., 1993; Reim et al., 1993). However, these studies were mainly aimed at the eye that suffered serious injuries by some direct factors; studies on the retina have remained elusive. Some studies have reported that a large number of firefighters and patients suffered from acute symptoms of eye irritation following fires, such as conjunctival vessels engorgement, lacrimation and pain (Duclos et al., 1990; Gallanter et al., 2002). These studies only described the symptoms of the eye without mentioning the retinal changes. The mechanism of secondary injury of combustion smoke inhalation affecting the eye has not been fully explored, notably the retina. In view of this, the present study was aimed to determine whether the retinal struc-

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tural features would be affected by combustion smoke inhalation. In this connection, it was hypothesized that vascular endothelial growth factor (VEGF), aquaporin 4 (AQP4) and nitric oxide synthase (NOS) associated with the retina in adult rats may be altered following combustion smoke inhalation. This is because these factors are altered in the retina exposed to hypoxia (Stone et al., 1995; Neroev et al., 2003; Kashiwagi et al., 2003; Werdich et al., 2004; Kaur et al., 2007). Furthermore, VEGF (Simonetti et al., 2006; Clavel et al., 2007), AQP4 (Lu and Sun, 2003; Kleindienst et al., 2006) and NOS (Lidington et al., 2007) are known to associate with vasodilation, tissue edema and inflammatory reaction. We therefore also sought to determine if these factors would be involved in changes in vascular permeability in combustion smoke inhalation rats.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (280–320 g) were used for smoke inhalation model. Prior to surgery, the rats were deprived of food for 24 h but allowed free access to water. They were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally for the insertion of cannula and were maintained under anesthesia for the duration of the surgical procedure. The femoral artery was cannulated for withdrawal of blood samples for blood gases analysis. After the procedure, the rats were allowed free access to food and water. Twenty-four hours after the surgical procedures, they were placed in the smoke challenge chamber and after the baseline parameters were stabilized, the rats were subjected to smoke injury challenge with a setup mentioned below. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The project was approved by the Institutional Animal Care and Use Committee (IACUC), National University of Singapore (Protocol 088/07). All efforts were made to reduce the number of rats used and their suffering.

Smoke challenge

A system inhalation model was designed and built to achieve a constant smoke challenge of smoke toxicants from cotton burning. The entire smoke-inhalation testing setup was designed with reference to Lee et al. (2005) and Whitehead et al. (2003). Briefly, the setup consisted of three main parts: a furnace, an equilibrium chamber to contain the volume of smoke being generated by the furnace and, the animal exposure chambers. Smoke was generated by burning of 33 g of cotton towel in the 290 °C furnace for 5 min and then allowed to collect and cool in the equilibrium chamber. At the same time, un-anesthetized animals were placed in the exposure chamber individually and allowed to acclimatize for 15 min before release of the smoke from the equilibrium chamber. Gas concentration was monitored by a carbon monoxide (CO) and oxygen (O_2) combustion meter (Testo AG, Lenzkirch, Germany). In this experimental paradigm, we have established that the following smoke challenge conditions would result in an approximate mortality rate of 10%: CO level 2200–2500 ppm; O_2 level >19%; smoke exposure of 60 min. Fresh air was allowed to recirculate into the test chambers if either the CO reaches over 2500 ppm or O_2 drops below 19% in order to prevent outright hypoxia death and CO poisoning death.

Blood gases analysis

A blood sample of 0.3 ml was collected with a heparinized syringe from the femoral artery of rats before exposure to smoke and immediately after (0.5 h) and at 3, 24 and 72 h following exposure to smoke for blood gases analysis using the Rapidlab® 1265 System (Siemens Medical Solutions Diagnostics, New York, USA). Blood parameters including pH, oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), base excess in extra-cellular fluids (BE_{ecf}), oxygen saturation (sO_2), lactate, oxyhemoglobin (O_2Hb) and carboxyhemoglobin (COHb) were measured. Withdrawn blood was replaced by equivalent volume of 0.9% saline to prevent disruption to hemodynamic parameters.

Drug administration

To determine the roles of NOS in the retina of rats subjected to combustion smoke inhalation, the experimental rats were given treatment of aminoguanidine (AG, Sigma, MO, USA), a selective inducible nitric-oxide synthase (iNOS) inhibitor and N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), a nonspecific NOS blocker. The retinal tissue was then removed and prepared for Western blotting analysis and measurement of nitric oxide (NO) production. Along with this, some rats were examined for vascular permeability in the retina following smoke inhalation. The rats were given i.p. injections of AG (100 mg/kg body weight) (Jiang et al., 2004) or L-NAME (300 mg/kg body weight) (Jones et al., 1998), with the first injection given immediately after the smoke exposure, followed by an injection every 24 h till the respective harvest time points (four rats each at 3, 24 and 72 h of every group after smoke exposure). The matching group received an injection of same amount of saline intraperitoneally after exposure to smoke.

Double immunofluorescence

Normal control rats ($n=4$) and smoke exposure rats (four rats each sacrificed at 0.5, 3, 24 and 72 h after smoke inhalation) were used for immunofluorescence studies. At designated time points after smoke exposure, the rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally and then perfused transcardially with saline, followed by fixation with 2% paraformaldehyde in 0.1 M phosphate buffer. The eyes were removed, post-fixed for 2 h in the same fixative, then cryoprotected in 15% sucrose for 24 h. Frozen sections at 20 μm of the eye were cut coronally through the optic nerve with a cryostat (Leica CM 3050) and mounted onto gelatin-coated microscopic slides and stored at −20 °C until use. Tissue sections at different time points were incubated in primary antibodies (see Table 1). After incubation, FITC-conjugated and Cy3-conjugated secondary antibodies were added. Images representing at least one eye each from four rats at different time points were captured under an Olympus Fluoview TM1000 confocal microscope. Immunofluorescence labeling for the respective antibodies directed against the various cell types was consistent and reproducible across different rats.

Table 1. Primary antibodies used in immunofluorescence studies

Name	Dilution	Source
AQP4	1:250	Santa Cruz Biotechnology, Inc., CA, USA
VEGF	1:100	Santa Cruz Biotechnology, Inc.
iNOS	1:800	BD Biosciences, CA, USA
nNOS	1:100	BD Biosciences
eNOS	1:100	BD Biosciences
NeuN	1:100	Chemicon, CA, USA
GS	1:500	Chemicon
GFAP	1:500	Chemicon