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Impaired mitochondrial respiration and protein nitration in the rat hippocampus after acute inhalation of combustion smoke

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ABSTRACT

Survivors of massive inhalation of combustion smoke endure critical injuries, including lasting neurological complications. We have previously reported that acute inhalation of combustion smoke disrupts the nitric oxide homeostasis in the rat brain. In this study, we extend our findings and report that a 30-minute exposure of awake rats to ambient wood combustion smoke induces protein nitration in the rat hippocampus and that mitochondrial proteins are a sensitive nitration target in this setting. Mitochondria are central to energy metabolism and cellular signaling and are critical to proper cell function. Here, analyses of the mitochondrial proteome showed elevated protein nitration in the course of a 24-hour recovery following exposure to smoke. Mass spectrometry identification of several significantly nitrated mitochondrial proteins revealed diverse functions and involvement in central aspects of mitochondrial physiology. The nitrated proteins include the ubiquitous mitochondrial creatine kinase, F1-ATP synthase α subunit, dihydrolipoamide dehydrogenase (E3), succinate dehydrogenase Fp subunit, and voltage-dependent anion channel (VDAC1) protein. Furthermore, acute exposure to combustion smoke significantly compromised the respiratory capacity of hippocampal mitochondria. Importantly, elevated protein nitration and reduced mitochondrial respiration in the hippocampus persisted beyond the time required for restoration of normal oxygen and carboxyhemoglobin blood levels after the cessation of exposure to smoke. Thus, the time frame for intensification of the various smoke-induced effects differs between blood and brain tissues. Taken together, our findings suggest that nitration of essential mitochondrial proteins may contribute to the reduction in mitochondrial respiratory capacity and underlie, in part, the brain pathophysiology after acute inhalation of combustion smoke.

Introduction

Massive smoke inhalation causes mortality and morbidity in victims of accidental fires, acts of terrorism and in combat, with severe immediate and delayed neurological impairments. The recognized neurotoxic factors in combustion smoke are carbon monoxide, hydrogen cyanide and toxicants, which in the brain tissue may combine and synergize with free radical-generating factors as well as hypoxia and acidosis, to perturb cellular homeostasis and precipitate brain injury (Hartzell, 1996; Rossi et al., 1996; Smith et al., 1996; Roohi et al., 2001; Alarie, 2002; Raub and Benignus, 2002; Stuhmiller et al., 2006).

To obtain insights into the progression of molecular events contributing to brain pathophysiology after acute exposure to combustion smoke, we have developed a rat model of smoke inhalation injury. Using this model, we show that blood parameters as well as cellular and molecular targets in the rat brain are significantly affected by acute inhalation of smoke (Lee et al., 2005). Importantly, the timing for onset and diminution of the various manifestations differs markedly, underscoring the complex nature of smoke inhalation pathophysiology. The major manifestations include an immediate elevation of blood carboxyhemoglobin and a reduction in oxygen saturation and blood pH, while in the brain the changes involve modulation of gene expression patterns, lipid peroxidation, DNA oxidation as well as significant modulations of the nitric oxide system (Lee et al., 2005; Chen et al., 2007).

It is noteworthy, that in humans after carbon monoxide (CO) insult, only limited predictions can be made based on blood carboxyhemoglobin (COHb) with respect to the potential for development of neuropathologies. This is likely due to a failure of blood COHb levels to reflect the tissue specific rates of CO clearance. In fact, it appears that during recovery tissue CO levels do not necessarily trail declining blood levels, and that tissue hypoxia slows down CO clearance during the later resolution stages, particularly in the brain and heart (Cronje et al., 2004). Since hypoxia is a major component in the setting of acute smoke inhalation, elevated brain tissue CO may persist, replacing oxygen bound to neuronal heme proteins and thus further impact oxygen availability and brain homeostasis. In our rat model, the very high COHb (72%) blood levels measured immediately after exposure, decline to near normal levels within the first 2 h of recovery.

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In contrast, even the early manifestations of smoke exposure in the brain tissue tend to peak at more delayed recovery times. For example, lipid peroxidation and DNA oxidation (Chen et al., 2007) as well as changes in gene expression patterns present throughout the 24-hour recovery post-smoke when the blood COHb, oxygen saturation and pH have returned to normal (Lee et al., 2005). These findings suggest that CO clearance from brain tissue lags behind its clearance from blood, and that severely reduced oxygenation, high CO, toxic gases and reduced pH persist in the brain after cessation of exposure.

Interestingly, the overall expression changes recorded after smoke are indicative of concomitant activation of genes associated with injurious as well as protective processes (Lee et al., 2005). Importantly, we observe this also in context of the nitric oxide system, as microarray analyses revealed that genes encoding the different components of the system including the endothelial nitric oxide synthase (eNOS) and capon, a NOS ligand/adaptor protein were upregulated after smoke (Lee et al., 2005). This finding is of particular interest in view of the previously reported upregulation of capon in a setting of cortical spreading depression-mediated neuroprotection from a subsequent ischemic insult (Wiggins et al., 2003). Thus, it is plausible that capon may be protective also in the setting of smoke inhalation injury. Notwithstanding, a significant elevation in 3-nitrotyrosine immunoreactivity was observed in our current and earlier work (Lee et al., 2005). This is consistent with earlier investigation of the nitric oxide (NO) system in the brain in the context of CO poisoning, which revealed elevation of NO, nitration of tyrosine residues (Ischiropoulos et al., 1996), increases in perivascular nitric oxide synthesis (Thom et al., 2003) with perivascular nitrotyrosine immunoreactivity in endothelial lining, and elevated synthesis of NO in the brain after CO poisoning (Thom et al., 2001). Interestingly, it was also found that the effects of CO-induced activation of neuronal NOS and resultant excitotoxicity could be reduced by pharmacological blockage of NMDA receptors (Thom et al., 2004). As CO is a major factor in a setting of smoke inhalation, it is plausible that NMDA mediated excitotoxicity contributes to the development of smoke inhalation induced brain injury.

Since modulations of the nitric oxide system have been strongly linked with reduced mitochondrial respiration (Brookes et al., 2003; Ischiropoulos and Beckman, 2003; Radi, 2004; Franco et al., 2006), in this study, we examine the effects of acute exposure to combustion smoke on mitochondrial protein nitration and respiratory capacity of rat hippocampal mitochondria. We show post-smoke increases in 3-nitrotyrosine immunoreactivity in the hippocampal region and demonstrate that mitochondrial proteins are a significant target for smoke-induced protein nitration. Identification of the post-smoke mitochondrial nitration targets revealed that the nitrated proteins are involved in a broad spectrum of mitochondrial functions. In addition, a substantial decline in respiratory capacity of hippocampal mitochondria was observed following exposure to smoke.

Methods

Smoke inhalation rat model. Awake Sprague Dawley male rats (250–300 g) were exposed to combustion smoke generated by burning wood shavings. Rats were kept in a smoke filled chamber for three successive 10-min periods separated by 30 s in room air, as we described previously (Lee et al., 2005). Sham controls were given similar treatment without smoke. Rats were recovered for 0, 2, 6 or 24 h and hemodynamic parameters were recorded. Compared to measurements taken immediately after exposure to smoke, a significant reoxygenation and reduction in blood carboxyhemoglobin were recorded already after a 2-hour recovery (Lee et al., 2005). Brains were harvested and fixed for immunohistochemistry or dissected for mitochondrial isolation and gradient purification. All experiments were conducted in accordance with mandated standards of humane care and were approved by the UTMB Institutional Animal Care and Use Committee.

Immunohistochemistry. Brains were immersion fixed in 4% paraformaldehyde and coronal, paraffin embedded sections at Bregma −3.14 (Paxinos and Watson, 1998) were prepared from control, and smoke exposed rats harvested at indicated times ($n=3$). Sections were dewaxed in xylene and rehydrated through graded ethanol series. Blocking was for 1 h with 1% BSA followed by 30 min with 3% goat serum. Sections were incubated with anti 3-nitrotyrosine antibody (#06-284, Upstate, Charlottesville, VA) at 1:150 for 1 h at room temperature, followed by three 5-minute washes and incubation with biotinylated goat anti-rabbit antibody. The avidin:biotinylated enzyme complex (ABC reagent) was used and sections were counterstained with hematoxylin. Sections processed identically with omission of the primary antibody served as a negative control. Sections were analyzed with a Nikon Eclipse 600 microscope using Plan Fluor and 40× Plan Apo objectives. Images were captured by Nikon DXM1200 digital camera. Biotinylated goat anti-rabbit IgG (BA-1000) and Vectastain ABC kit were from Vector Lab (Burlingame, CA). Hematoxylin Gill #2 (CS401-1D) and Permount (SP15-100) were from Fisher Scientific (Hampton, NH).

Subcellular fractionation. Dissected hippocampal tissues were homogenized in hypotonic buffer (10 mm Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml Pepstatin) supplemented with Complete Protease Inhibitor Cocktail tablet (#1836153 Roche). Homogenates were spun at 800 g for 5 min to remove nuclei. Supernatants were combined for separation of crude mitochondria by centrifugation at 12,500 *g* for 8 min. Prior to proteomic analyses the crude mitochondrial pellets were subjected to further purification through a Percoll density gradient adapted from Sims (1990) and Anderson and Sims (2000). Briefly, crude mitochondrial pellets were suspended in 14% Percoll in 1× MSHE (10 mM Hepes-KOH pH 7.6, 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.75 mM spermidine), layered over preformed Percoll gradient (19% on 40%) and spun at 30,700 *g* 10 min. Using a glass Pasteur pipette, the two upper layers were removed, while the opaque band formed at the interface of preformed 19% over 40% Percoll gradient was carefully collected and spun at 16,700 *g*. The resultant loose mitochondrial pellets were gently washed in isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA pH 7.5), centrifuged at 6900 *g* and the subsequently obtained firm mitochondrial pellets, were resuspended in isolation buffer for respiration assays or stored at −80 °C for lysis and protein extraction for 2D gel electrophoresis.

Mitochondrial lysis and Western blotting. Gradient purified mitochondrial pellets were incubated with lysis buffer recommended by Amersham (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 60 mM DTT, 1 mM PMSF), followed by 2× centrifugations at 20,000 *g* to remove remaining particulates. Equal amounts (30 μg/well) of extracted mitochondrial proteins were resolved in 10% SDS-PAGE, electrotransferred to a PVDF membrane, blocked overnight at 4 °C (20 mM Tris pH 7.5, 150 mM NaCl, 0.2% Tween-20, 2% BSA), and incubated with anti 3-nitrotyrosine monoclonal antibody (#05-233, Upstate, Lake Placid, NY) at 4 °C for 3 h followed by 5 washes and incubation with goat antimouse horseradish peroxidase conjugate for 3 h at RT and 5 vigorous washes prior to ECL mediated detection. Specificity of nitrotyrosine detection was confirmed by 'in membrane' reduction of nitrotyrosine to amino tyrosine, which does not react with the anti nitrotyrosine antibody. Reduction was accomplished by incubating the membrane with a solution of 100 mM sodium hydrosulfite (dithionite) in bicarbonate buffer (50 mM Na_2CO_3-Na HCO_3, pH 9) for 3 h at RT followed by washing with distilled water and equilibration with wash solution (20 mM Tris pH 7.5, 150 mM NaCl, 0.2% Tween 20) prior to overnight blocking as described above. Reduced and non-reduced PVDF membranes were processed and visualized simultaneously. Equal