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# The inflammatory footprints of alcohol-induced oxidative damage in neurovascular components

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ABSTRACT

Microvessels, the main components of the blood–brain barrier (BBB) are vulnerable to oxidative damage during alcohol-induced stress. Alcohol produces oxidative damage within the vessels and in the brain. Using our animal model of catheter implant into the common carotid artery (CCA), we trace the footprints of alcohol-induced oxidative damage and inflammatory process at the BBB and into the brain. The uniqueness of the finding is that ethanol causes oxidative damage in all neurovascular components by activating NADPH oxidase and inducible nitric oxide synthase in the brain. It is not the oxidants but the ethanol that traverses through the BBB because we found that the highly reactive peroxynitrite does not cross the BBB. Thus, oxidative damage is caused at the site of oxidant production in the microvessels and in the brain. Our data indicate that acetaldehyde (the primary metabolite of ethanol) is the inducer/activator of these enzymes that generate oxidants in brain neurovascular cells. Evidence for alcohol-induced BBB damage is indicated by the alterations of the tight junction protein occludin in intact microvessels. Importantly, we demonstrate that the site of BBB oxidative damage is also the site of immune cells aggregation in the microvessels, which paves the path for inflammatory footprints. These findings reveal the underlying mechanisms that ethanol-elicited BBB oxidative damage initiates the brain vascular inflammatory process, which ultimately leads to neuroinflammation.

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## 1. Introduction

Immediate inflammation is a defense mechanism of immune cells for elimination of foreign bodies from the site of tissue injury. In the brain microvessels (the main components of the blood–brain barrier, BBB), tissue injury is caused by reactive oxidative damage during stress. Brain cells are extremely susceptible to oxidative injury because exogenous and endogenous antioxidant levels are not abundant in the brain (Lakhan et al., 2009; Won et al., 2002).

Immune cells aggregate at the luminal site of vessel injury where they become highly activated and begin to infiltrate into the brain tissue. Chronic infiltration of immune cells into the brain as a result of pro-inflammatory mediators (cytokines and chemokines) secretion together with oxidative injury leads to the pathology of neuroinflammation. Leakiness of BBB and infiltration of immune cells was reported in acute autoimmune encephalomylitis (Floris et al., 2004). Neurovascular inflammation is integrated by a complex activation of brain endothelial cells, microglia, astrocytes and neuronal degeneration. In this chaotic inflammatory process, neurons are vulnerable to secreted toxic agents such as cytokines and oxidative products. Oxidative-mediated neuroinflammation is a common signature for many neurological diseases such as Alzheimer, Parkinson, multiple sclerosis and stroke (Lin and Beal, 2006; Maracchioni et al., 2007), which may be due to a consequence of BBB dysfunction (Daneman and Rescigno, 2009). Infact, development of inflammatory multiple sclerosis characterized by multi-focal perivascular infiltration of mononuclear cells in BBB breakdown has been reported recently (Sospedra and Martin, 2005).

Initiation of alcohol-induced neuroinflammation and neurodegeneration follow the similar pattern of tissue injury and immune adaptive response. There is growing evidence that alcohol mediates the production of cytokines in all major organs such as the livers, lungs, intestine and brain causing respective tissue damage (Crews et al., 2006; McClain et al., 2004). Interestingly, the intestinal microflora-derived lipopolysaccharide translocation seems to act as the axis of gut–liver–brain interactions in tissue damage and disease development in alcohol abuse (Wang et al., 2010). In deed, Fulton Crews lab demonstrated this interconnected pro-inflammatory path, in which secretion of pro-inflammatory mediators (TNFα, LPS and MCP-1) at systemic site and in glial cells

*Abbreviations:* Ach, acetaldehyde; BBB, blood–brain barrier; EtOH, ethanol; 3-NT, 3-nitrotyrosine; NOX1, NADPH oxidase 1; 4HNE, 4-hydroxynonenal; iNOS, inducible nitric oxide synthase; SIN-1, morpholinosydnonimine hydrochloride.

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leads to neuroinflammation and neuronal degeneration in chronic alcohol ingestion (Qin et al., 2008, 2007). Interestingly, neuroadaptations and neuroprotective response in human chronic alcoholics appear to correlate with dysregulation of NF-kB system in neuroinflammation and neurodegeneration (Okvist et al., 2007; Yakovleva et al., 2010). Potula et al. (2006) also showed that alcohol abuse enhances neuroinflammation and impairs immune responses in an animal model of human immunodeficiency virus-1 encephalitis (Potula et al., 2006).

Recently, we reported a novel finding that alcohol-induced oxidative production triggers the interactive phosphorylation of non-receptor protein tyrosine Src kinase and toll-like receptor-4 (TLR4) protein in primary human astrocytes (Floreani et al., 2010). It revealed that induction of phospholipase A2 and cyclooxygenase-2 by ethanol leads to secretion of inflammatory mediator through an interactive phosphorylation of Src kinase-TLR4 dependent manner. Similar to this finding, Alfonso-Loeches et al. (2010) showed the pivotal role of TLR4 in alcohol-induced neuroinflammation and neuronal degeneration in TLR4-deficient mice (Alfonso-Loeches et al., 2010). They alluded that chronic ethanol intake fails to activate astroglial cells for induction of inflammatory mediators in TLR4-deficient mice, suggesting that the presence of TLR4 response is essential in ethanol-induced neuroinflammation. In a review article, Gill et al. (2010) described the putative mechanism that stimulation of TLR connects the link between oxidative stress and inflammatory pathways (Gill et al., 2010).

It is now widely accepted that BBB (brain microvessels) is vulnerable to alcohol-induced oxidative damage and neuroinflammation via alcohol metabolism (Haorah et al., 2005a,b; 2007b). Ethanol metabolizing enzymes such as alcohol dehydrogenase, cytochrome P450 2E1 and catalase have been shown to localize in brain microvessel endothelium (Haorah et al., 2005b; Martinez et al., 2001; Zimatkin et al., 2006). Initially, metabolism of alcohol in brain endothelium generates oxidative and nitrosative products that cause oxidative damage to BBB and neuronal cells (Haorah et al., 2005a,b; 2008a). Oxidative disruption of BBB enhances permeability of toxic agents and migration of immune cells across the BBB and into the brain (Haorah et al., 2007a,b; 2008b). The uniqueness of alcohol-induced oxidative damage is that it occurs in all neurovascular cell types because each of cell type is capable of generating free radicals via the metabolism of ethanol (Floreani et al., 2010; Haorah et al., 2008a, 2007b). Interestingly, alcohol intake produces differential oxidative products and oxidative damage in these cell types, in which nitric oxide is detected in inducible nitric oxide synthase expressing neurons while ROS is detected predominantly in NADPH oxidase expressing astroglial cells (Rump et al., 2010).

The present studies demonstrated the underlying mechanisms of alcohol-induced oxidative damage in the microvessel (BBB) as the initial event for imprinting the inflammatory footprints. Using our animal model of catheter implantation into the common carotid artery (CCA), we are able to trace the oxidative damage in the microvessel caused by the infusion of 3-morpholinosydnonimine (SIN-1, spontaneous peroxynitrite donor), acetaldehyde into the CCA or in chronic alcohol intake animal. We also show the evidence that adhesion and migration of immune cells occur at these sites of oxidative damage in BBB vessels.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

We purchased the antibodies to 3-nitrotyrosine (3-NT), NADPH oxidase 1 (NOX1) and inducible nitric oxide synthase (iNOS) from Abcam (Cambridge, MA) and antibody to 4HNE (4-hydroxynononal) from Alpha Diagnostic, San Antonia, TX. The antibody to $\alpha$-actin was from Millipore (Billerica, MA). The primary antibody occludin and all secondary Alexa fluor antibodies and Fluo-3 were from Invitrogen (Carlsbad, CA, USA). Acetaldehyde (Ach) and 3-morpholinosydnonimine (SIN-1) were from Sigma–Aldrich (St. Louis, MO) and Lieber–DeCarli liquid-diets were from Dyets, Inc. (Bethlehem, PA).

### 2.2. Animals and treatments

Six weeks old male Sprague Dawley rats purchased from Jackson Laboratory (Bar Harbor, ME) were housed in UNMC animal facility following NIH Guide for Care and Use of Laboratory Animals. Rats weighing about $255 \pm 12.5$ g were acclimated to Lieber–DeCarli control and 29% calorie (5% vol/vol) ethanol (EtOH) liquid-diets from Dyets Inc. for 1 week prior to weight-match pair feeding regimens for 8–9 weeks. Pair feeding of control animals (12 rats, four rats for control, four rats for SIN-1 infusion and four rats for Ach infusion) was based on the amount of ethanol-liquid diets consumed by ethanol animals (six rats). The macronutrient composition of control-liquid diets as percent of total calories is 47% carbohydrate, 35% fat, and 18% protein; and that of ethanol-liquid diets is 35% fat, 18% protein, 19% carbohydrate and 29% ethanol caloric intake. Daily food intake and weekly body weights were recorded. After week 8, animals were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) ip injection as approved by AVMA Panel on Euthanasia. At the time of sacrifice, the average body weights were $357.6 \pm 7.5$ g for pair-fed control and $441.5 \pm 10.5$ g for ethanol–liquid diets, respectively. SIN1 (200 $\mu$M) or acetaldehyde (200 $\mu$M) in 100 $\mu$L volume was infused into the right common carotid artery was performed in control liquid-diets intake rats. Two hours after infusion of SIN1 or acetaldehyde (Ach), rats were euthanized and then microvessels were isolated and brain tissues were dissected.

### 2.3. Immunohistochemistry

Brain tissue sections (8 $\mu$m thickness) derived from chronic EtOH intake and pair-fed control rats or acute infusion of SIN1 or Ach in control rats were washed with PBS and fixed in acetone-methanol (1:1 v/v) fixative. Tissues antigen blocked with 3% bovine serum albumin at room temperature for 1 h in the presence of 0.4% Triton X-100 were incubated with respective primary antibodies such as mouse anti-3NT (Cat No.:ab61392; 1:250 dilution), rabbit anti-NOX1 (Cat No.:ab55831; 1:150 dilution), rabbit anti-4HNE (Cat No.:HNE-11S; 1:500 dilution), rabbit anti-iNOS (Cat No.:ab3523; 1:50 dilution) and rabbit anti-occludin (Cat No.: 71–1500; 1:60 dilution, without Triton X-100) for overnight at 4 °C. After washing with PBS, the tissues were incubated for 1 h with secondary antibody:anti-mouse-IgG Alexa fluor 594 for 3NT and anti-rabbit-IgG Alexa fluor 594 for 4HNE, NOX1 and iNOS. Then tissue slides were mounted with immunomount containing DAPI (Invitrogen). Fluorescence microphotographs were captured by fluorescent microscopy (Eclipse TE2000-U, Nikon microscope, Melville, NY) using NIS elements (Nikon, Melville, NY) software.

### 2.4. Western blotting

Brain tissue homogenates from respective experimental conditions were lysed with CellLytic-M buffer (Sigma) for 30 min at 4 °C, centrifuged at 14000 $\times$ g. Total lysates protein extracts were estimated by BCA for protein concentrations (Thermo Scientific, Rockford, IL). We loaded 20 $\mu$g protein/lane and resolved the various molecular weight proteins by SDS–PAGE on gradient gels (Thermo Scientific) and then transferred the protein onto nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies against mouse 3NT (Cat No.:ab52309; 1:1000), rabbit 4HNE (Cat No.:HNE-11S; 1:1000), rabbit NOX1

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