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Effects of acute ethanol exposure on cytokine production by primary airway smooth muscle cells

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ABSTRACT

Both chronic and binge alcohol abuse can be significant risk factors for inflammatory lung diseases such as acute respiratory distress syndrome and chronic obstructive pulmonary disease. However, metabolic basis of alcohol-related lung disease is not well defined, and may include key metabolites of ethanol [EtOH] in addition to EtOH itself. Therefore, we investigated the effects of EtOH, acetaldehyde [ACE], and fatty acid ethyl esters [FAEEs] on oxidative stress, endoplasmic reticulum (ER) stress, AMP-activated protein kinase (AMPK) signaling and nuclear translocation of phosphorylated (p)-NF-κB p65 in primary human airway smooth muscle (HASM) cells stimulated to produce cytokines using LPS exposure. Both FAEEs and ACE induced evidence of cellular oxidative stress and ER stress, and increased p-NF-κB in nuclear extracts. EtOH and its metabolites decreased p-AMPKα activation, and induced expression of fatty acid synthase, and decreased expression of sirtuin 1. In general, EtOH decreased secretion of IP-10, IL-6, eotaxin, GCSF, and MCP-1. However, FAEEs and ACE increased these cytokines, suggesting that both FAEEs and ACE as compared to EtOH itself are proinflammatory. A direct effect of EtOH could be consistent with blunted immune response. Collectively, these two features of EtOH exposure, coupled with the known inhibition of innate immune response in our model might explain some clinical manifestations of EtOH exposure in the lung.

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1. Introduction

Both acute and chronic EtOH consumption exert detrimental effect on lungs, and increase the risk for developing inflammatory lung diseases such as acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) (Moss and Burnham, 2003; Session, 2007; Kaphalia and Calhoun, 2013). Alcohol abuse has been identified as an independent risk factor for the development of ARDS, each year ~200,000 individuals develop ARDS in the US, and nearly 50% of these patients had a history of alcohol abuse (Boé et al., 2009). The incidence of ARDS is nearly doubled by alcohol abuse. Chronic alcohol abuse is shown to be associated with persistent fever, delayed resolution of symptoms, increased rate of bacteremia, and prolonged duration of hospital stay and increased use of intensive care (Moss, 2005). A U-shaped curve between alcohol consumption and 20 year chronic COPD mortality in middle-aged men was supported by cross sectional data on alcohol and pulmonary function (Tabak et al., 2001).

A significant amount of ingested alcohol reaches to the lungs (witness breath alcohol testing), and can be metabolized via both oxidative and non-oxidative pathways (Kaphalia and Calhoun, 2013). Accordingly, airways are exposed to EtOH and its multiple metabolites synthesized in the lungs. Acute and chronic alcohol ingestion impairs cellular components of innate immunity (Moss et al., 1996; Cook, 1998; Szabo et al., 2004). Much ingested alcohol is metabolized via hepatic alcohol dehydrogenase (ADH), in particular the ADH1 isoform, expression of which is generally reduced by chronic alcohol abuse and in alcoholic patients (Nuutinen et al., 1983; Panes et al., 1993, 1989). An alternative oxidative pathway from EtOH to ACE employs hepatic microsomal cytochrome P450 (CYP) 2E1 which itself is induced by chronic alcohol abuse (Lieber, 1997). However, this pathway generates additional reactive oxygen species (ROS) and amplifies oxidative stress (Cederbaum, 2010). Recently, we reported significant endoplasmic reticulum (ER) stress in the lungs of mice fed ethanol daily for 3 months (Kaphalia et al., 2014).

Abbreviations: ACE, acetaldehyde; ACC, acetyl CoA carboxylase; ADH, alcohol dehydrogenase; AMPK, AMP-activated protein kinase; ARDS, acute respiratory distress syndrome; CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β; COPD, chronic obstructive pulmonary disease; CYP2E1, cytochrome P-4502E1; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol-O-acyltransferase; EtOH, ethanol; FAEE, fatty acid ethyl ester; ER, endoplasmic reticulum; FAS, fatty acid synthase; GCSF, granulocyte-colony stimulating factor; GRP, glucose regulated protein; HASM, human airway smooth muscle; 4-HNE, 4-hydroxy-2-nonenal; HPLC, high performance liquid chromatography; IP-10, Interferon gamma-induced protein 10; LKB, liver kinase β; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species; SIRT, sirtuin; SREBP, sterol response element binding protein; TNF, tumor necrosis factor.

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In the setting of reduced ADH expression, non-oxidative metabolites of EtOH are produced, including fatty acid ethyl esters (FAEEs). Increased concentrations of these metabolites have been identified in the lungs of experimental animals in vivo after chronic EtOH feeding, in in vitro systems (Manautou and Carlson, 1991; Manautou et al., 1992), and in lungs of mice genetically deficient in hepatic ADH1 (Kaphalia et al., 2014). Therefore, we propose that understating the role of EtOH metabolites could dissect the metabolic basis of EtOH-associated diseases, and identify therapeutic targets.

One potential pathogenic mechanism is alteration in the secretion of cytokines, which mediate inter-cellular communications, and contribute to both disease pathogenesis and host defense (Kaphalia and Calhoun, 2013). Acute alcohol intoxication suppresses tumor necrosis factor (TNF)-α and interleukin-1β production by monocytes in response to challenge with a variety of stimuli, including both Gram-negative and Gram-positive bacterial products (Stoltz et al., 2000). Further, alcohol-induced cytokine suppression leads to decreased neutrophil recruitment and suppressed host defense (Taıeb et al., 2002).

EtOH exposure is known to alter cytokine levels in a variety of tissues including plasma, lung, liver, and brain. However, the precise mechanism whereby EtOH exerts its effects on cytokine release remains to be fully articulated. AMP-activated protein kinase (AMPK), a central regulator of cellular lipid metabolism, is linked to EtOH-induced target organ injury (You et al., 2004; Zhao et al., 2008), and could be an intermediary of EtOH-induced lung injury.

In contrast to an abundant literature in other tissues, the effect of EtOH and its oxidative and non-oxidative metabolites on human lungs and lung tissues are not well investigated. Based on existing literature, and our own preliminary data, we hypothesized that oxidative and non-oxidative metabolites of EtOH would influence cytokine secretion and cellular metabolism to a greater degree than would EtOH alone. We chose the human airway smooth muscle (HASM) cell model because of the important role HASM plays in modulating inflammatory responses in the airway (Lazaar and Panettieri, 2005; Guedes et al., 2015). In this study, we focused on the comparative effects of EtOH, and its primary oxidative metabolite (ACE), and key nonoxidative metabolites (FAEEs) on measures of inflammation and AMPK cell signaling using primary HASM cell culture model.

2. Materials and methods

Fatty acid ethyl esters (FAEEs), HPLC grade solvents, 200 proof ethanol (EtOH), and acetaldehyde (ACE) were obtained from Sigma-Aldrich (St. Louis, MO). Reagents for Western blot analysis were purchased from Invitrogen (Grand Island, NY). Antibodies against 4-hydroxynonenal (4-HNE), cytochrome P450 (CYP) 2E1, alcohol dehydrogenase (ADH)-1, and glucose-regulated protein (GRP) 78 were sourced from ABCAM (Cambridge, MA), and phosphorylated (p)-AMP-activated protein kinase (AMPK) α, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)β, liver kinase β (LKB) 1, acetyl CoA carboxylase (ACC)1, carnitine palmitoyltransferase (CPT)1α, sterol response element binding protein (SREBP) 1, diacylglycerol-O-acyltransferase (DGAT)1, sirtuin (SIRT)1 and p-NF-κB-p65 (proinflammatory transcription factor) were obtained from Cell Signaling (Cambridge, MA). Multiplex ELISA kit for cytokine and chemokine assays was from Bio-Rad (Hercules, CA).

2.1. Cell culture

We used primary human airway smooth muscle (HASM) cells, because of their prominent role in synthesis and release of a diverse repertoire of biologically active inflammatory mediators, including cytokines, chemokines, and growth factors and their contribution both to the inflammatory process and to airway remodeling within the bronchial wall (Johnson and Knox, 1997; Howarth et al., 2004; Chung, 2005).

About 3500 cells/cm^2 of primary HASM cells (cc-2576, Lonza, below 5 passage) were seeded into in culture flasks and grown at 37 °C in SmGM-2 Smooth Muscle Growth Medium-2. In the absence of stimulation, these cells are quiescent and produce little or no detectable cytokines. However, LPS exposure stimulates robust and broad production of inflammatory cytokines (Thorley et al., 2007). Therefore, for the cytokine and chemokine secretion studies, cells were cultured for 24 h and stimulated with 10 μM lipopolysaccharide (LPS), followed by incubation with EtOH (2, 4 or 8 mg/ml), FAEEs (10, 50 or 100 μg/ml) and ACE (0.45, 2.25 or 4.5 μg/ml) for 6 or 24 h based upon previous studies (Lindblad and Olsson, 1951; Christoloupos et al., 1973; Hammond et al., 1973; Berild and Hasselbalch, 1981; Nishiguchi et al., 2002; Kaphalia et al., 2004; Wu et al., 2006; Kaphalia et al., 2014). About 30% of initial EtOH added evaporates from cell culture during 6 h of incubation at 37 °C (Wu et al., 2006); hence the nominal concentrations we studied are the maximal concentration to which cells were exposed. FAEEs used in this study were used in the same ratio as they were produced in the lungs of mice after chronic EtOH feeding (Kaphalia et al., 2014). Cell viability was measured by MTT Assay Kit (Vybrant® MTT Cell Proliferation Assay Kit).

2.2. Immunoblotting and immunofluorescence

After culture, treated and untreated cells were harvested with lysate buffer containing protease and phosphatase inhibitors; protein concentration was measured by the Pierce BCA protein assay kit. An aliquot (45 μg) of protein from each sample, including nuclear fractions, were subjected to 10% SDS-PAGE. The proteins on gels were transferred to a nitrocellulose membrane for Western blot analysis using antibodies to ethanol metabolizing enzymes, AMPK signaling (up- and downstream)

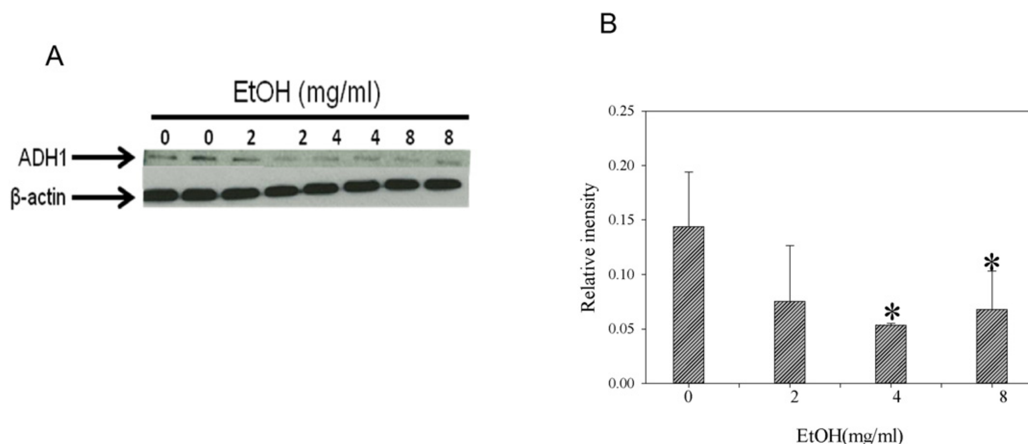

Fig. 1. Western blot analysis (A) and relative intensities (B) for ADH1 in HASM cells treated with EtOH for 24 h. Values are mean ± SD of four samples in each group. *p value ≤ 0.05.

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