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Ethanol suppresses phagosomal adhesion maturation, Rac activation, and subsequent actin polymerization during FcγR-mediated phagocytosis

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ABSTRACT

Clinical and laboratory investigations have provided evidence that ethanol suppresses normal lung immunity. Our initial studies revealed that acute ethanol exposure results in transient suppression of phagocytosis of *Pseudomonas aeruginosa* by macrophages as early as 3 h after initial exposure. Focusing on mechanisms by which ethanol decreases macrophage Fcγ-receptor (FcγR) phagocytosis we targeted the study on the focal adhesion and cytoskeletal elements that are necessary for phagosome progression. Ethanol inhibited macrophage phagocytosis of IgG-coated bead recruitment of actin to the site of the phagosome, dampened the phosphorylation of vinculin, but had no effect on paxillin phosphorylation suggesting a loss in "phagosomal adhesion" maturation. Moreover, our observations revealed that FcγR-phagocytosis induced Rac activation, which was increased by only 50% in ethanol exposed cells, compared to 175% in the absence of ethanol. This work is the first to show evidence of the cellular mechanisms involved in the ethanol-induced suppression of FcγR-mediated phagocytosis.

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1. Introduction

The consumption of alcohol (ethanol) is associated with increased morbidity and mortality, contributing to 3.8% of all death and 4.6% of disease globally [1–3]. Clinical observations and laboratory studies have linked acute ethanol exposure with suppressed immune function, specifically, decreased leukocyte activation in response to a pathogen or a pathogen-associated molecular pattern (PAMP) in many organs, including the lungs [4–8]. Previous work has provided evidence that acute exposure to ethanol can reduce monocyte/macrophage phagocytosis [9–14]. Specifically, Morland's group reported that acute ethanol exposure inhibited phagocytosis via the Fcγ-receptor (FcγR) [10]. Others have focused on the mechanisms involved in ethanol attenuation of cytokine production after ligand binding to Toll-like receptors (TLR) [4,15]. Decreased mitogen-activated protein kinase (MAPK) phosphorylation, nuclear factor-κB (NF-κB) dysregulation, and insufficient receptor clustering in lipid rafts have all been shown to contribute to this ethanol-mediated immunosuppression [4,9,15–17]. However, only limited progress has been made in understanding the underlying mechanisms involved in ethanol-mediated suppression of phagocytosis.

Phagocytosis can be initiated by binding of a PAMP with a phagocytic receptor or by opsonization of a pathogen by host molecules such as immunoglobulin G (IgG) or complement [18]. In parallel, pathogens can also activate numerous pattern recognition receptors (PRRs) including the Toll-like receptors, mannose receptors, and scavenger receptors, alerting the host to an inflammatory threat. Together, these immediate immune-mediate responses alert the host to an inflammatory threat, enhancing recognition and phagocytosis of the invading pathogen. The two most commonly studied modes of phagocytosis are via the FcγR and the complement receptor. Initiation of FcγR-mediated phagocytosis requires the binding of the receptor to the Fc portion of IgG. This interaction induces tyrosine phosphorylation of the gamma subunit of the receptor and subsequent downstream activation of the phagocytic pathway [19–20]. As with most types of phagocytosis, FcγR-mediated phagocytosis requires involvement of the focal adhesion molecules paxillin and vinculin, as well as the actin cytoskeleton [21–22]. In the context of phagocytosis, paxillin and vinculin are thought to link the extracellular pathogen bound to receptors with the cellular cytoskeleton. Once anchored to the pathogen, the "phagosomal adhesion" can induce pathogen/particle internalization through its interaction with actinomyosin contractions as well as many other molecules [23–24]. The inability to induce an adhesion or loss of cytoskeletal control, i.e. normal actin architecture, is known to inhibit phagocytosis [25–26]. Interestingly, ethanol has been shown to modulate

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paxillin, vinculin, and actin in non-professional phagocytes, such as astrocytes [27–28]. This study demonstrates that ethanol exposure decreases phosphorylation of these critical adhesion molecules and alters actin recruitment, implicating a potential role for ethanol mediated dysregulation of these molecules in phagosomal maturation.

Downstream of the FcR, small GTPases are integral to the activation of these focal adhesion molecules, and subsequent actin recruitment to the phagosome. Though there are conflicting data regarding the relationship between specific small GTPases and the type of receptor mediated phagocytosis, Rac and Cdc42 are typically considered the signaling molecules downstream of the FcRs, whereas Rho is generally associated with complement receptor-mediated phagocytosis [29–32]. In support of this, in RAW264.7 and J774.A1 macrophages, tumorigenic cell lines isolated from murine ascites, FcγR-mediated phagocytosis is inhibited following expression of the dominant negative Rac1 or Cdc42 plasmid Rac1N17 or Cdc42 N17, respectively [29–30]. This effect was also observed using IgE-opsonized particles and measuring RBL-2H3 mast cell phagocytosis after inhibition of Rac or Cdc42 [33]. Reports have also revealed that clustering of constitutively active Cdc42 or Rac1 leads to actin rearrangement, membrane ruffling, and particle engulfment [34–35], further highlighting the need for small GTPase activity in normal phagosomal development.

We sought to decipher the mechanisms involved in the ethanol-induced suppression of macrophage phagocytosis, specifically FcγR mediated phagocytosis. Initial work by our laboratory revealed that acute ethanol exposure suppressed bacterial phagocytosis [13]. In the present work, we broadened our understanding of the time dependent effects of ethanol on the phagocytosis of *Pseudomonas aeruginosa*. Additionally, we established that our *in vitro* ethanol experiments parallel our *in vivo* acute ethanol model, as demonstrated by decreased bacterial phagocytosis and diminished actin polymerization at the phagosome during IgG-induced FcγR-mediated phagocytosis. This suppression of actin polymerization was accompanied by reduced vinculin, but not paxillin, phosphorylation. Moreover, we expanded upon the differential role of two small GTPases, Rho and Rac, in FcγR-mediated phagocytosis, and revealed that ethanol primarily impairs Rac activation in the context of macrophage phagocytosis. Our study extends the current understanding of the suppressive effects of acute ethanol exposure during macrophage phagocytosis, and attributes the aforementioned observations to ethanol-induced decreases in Rac activity during FcγR-mediate phagocytosis.

2. Materials and methods

2.1. Animals, in vivo ethanol exposure, and alveolar macrophage isolation

Eight to 10 week old male C57BL/6 mice (Harlan, IN) were utilized to measure *in vivo* ethanol effects on alveolar macrophages. Prior to use, mice were acclimated for 1 week at the animal facility in Loyola University Medical Center. All animal studies described here were approved and performed with strict accordance to the rules and regulations set by the Loyola University Chicago Animal Care and Use Committee. Mice were subjected to a single intraperitoneal (i.p.) injection of 2.2 g/kg ethanol or saline control as previously described [4,13,15]. This dose of ethanol resulted in a transient elevation in blood alcohol concentration (BAC) which peaked at a level of 280 mg/dl at 30 min and returned to baseline levels by 3 h [13]. Following sacrifice, by CO_2 exposure and cervical dislocation, alveolar macrophages were harvested by bronchoalveolar lavage (BAL) at 0.5, 3, or 24 h after ethanol administration. Briefly, 8 sequential 800 µl saline lavages were performed per animal as previously described [13]. Roughly 600 µl of collected BAL fluid and cell suspension were isolated per lavage resulting in a total of ~5 ml of BAL fluid. In either ethanol or saline exposed mice, cellular characterization by flow cytometry revealed 85–95% of our BAL cells were alveolar macrophages as determined by F4/80$^+$ staining allowing studies to be performed on a purified primary macrophage without the use of receptor-mediated isolation (data not shown). Additionally, alveolar macrophages were utilized due to their frequent exposure to pathogens and their potential link to the increase in lung infection observed in people who abuse alcohol.

2.2. Cell culture and in vitro ethanol exposure

RAW264.7 cells, an immortalized macrophage cell line, were seeded (2.5×10^5) and incubated in 5% CO_2 in either 6 well culture plates or p35 MatTek glass bottom dishes for 48 h in complete media (RPMI with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Glutamine (PSG)) (Invitrogen; Eugene, OR). Cells were then cultured in complete media with or without 50 mM (~0.3%) ethanol for 0.5, 1, 1.5, 3, 6, or 24 h. Measurement of the ethanol concentration at these time points resulted in concentrations of 208.5, 217.5, 191, 179, 148, and 48 mg/dl, respectively. The RAW264.7 cell line was used as a model of primary culture macrophages due to the large number of cells needed for the molecular studies. This cell line has been used extensively in other studies examining the effects of alcohol on macrophage function [36]. Furthermore, to our knowledge, there is no published evidence suggesting the mechanisms involved in FcγR-mediated phagocytosis vary between different macrophage populations.

2.3. Phagocytosis and bead opsonization

Alveolar or RAW264.7 macrophages were cultured in media without antibiotics with 150 EGFP-*P. aeruginosa* per cell for 30 min in a 37 °C incubator under constant rotation or adhered to a plastic dish, respectively. The number of bacteria per cell were chosen after performing dose response analyses in which 5–600 bacteria per cell were tested and the midpoint of the linear range of fluorescence intensity per cell following phagocytosis was selected (data not shown). At specified times, phagocytosis was ceased using ice cold phosphate buffered saline (PBS), washed two additional times with PBS, and cultured with 5 µg/ml lysozyme for 30 min to eliminate any extracellular bacteria. RAW264.7 or alveolar macrophage phagocytosis of *P. aeruginosa* was then measured by flow cytometry (described below). The clearance of extracellular bacteria following lysozyme treatment was confirmed by fluorescence microscopy. FcγR-mediated phagocytosis consisted of opsonizing latex beads prior to phagocytosis. Three µm latex beads (Sigma LB30-1ML) were incubated in 10% bovine serum albumin (BSA) in PBS overnight at 4 °C. Beads were centrifuged and washed three times with PBS, then resuspended in 1 ml PBS. Ten microliters of mouse anti-BSA (Fisher MS-572-P1ABX) was added to the bead suspension and incubated at room temperature for 1 h under constant rotation. This resulted in >99% coating of the beads (data not shown). Beads were subsequently washed two times with PBS, once with RPMI, and finally resuspended in RPMI which was added to the distributing media. Warm RPMI medium with beads at a concentration of 20 (or 50 for western blot studies) beads per cell were added to each well. Wells were briefly centrifuged and immediately placed into a 37 °C incubator for specified times allowing phagocytosis to occur. Immediately following phagocytosis, cells were placed on ice and ice-cold PBS was added to the dish to stop phagocytosis. Cells were then either washed twice with ice-cold PBS and fixed with 4% paraformaldahyde for immunocytochemistry or lysed for molecular studies.

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