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Differential Expression and Functional Role of Cannabinoid Genes in Alcohol Users*

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Abstract

Background—Genetic factors account for about fifty percent of the risk for alcoholism and alcohol dependence (AD) has been reported to be influenced by cannabinoid receptors (CBRs) and the endocannabinoid system (ECS). Previous studies have focused on cannabinoids and alcohol-related effects in the CNS; however, the role CBRs play on alcohol effects in the immune system has not been elucidated yet. Since alcohol can affect immune responses and have detrimental effects on immune cells such as dendritic cells (DCs), we hypothesize that alcohol can exert its effects on DCs by modulating changes in CBRs, which in turn can regulate important DC functions such as cytokine production.

Methods—Therefore, we studied the expression of CNR1 and CNR2, and the novel cannabinoid G protein-coupled receptor (GPCR) 55 (GPR55) in human monocyte-derived dendritic cells (MDDCs) from alcohol users. CNR1, CNR2, and GPR55 genes were measured by qRT-PCR and protein by flow cytometry. MDDCs from alcohol users show significantly higher levels of CNR2 and GPR55 compared to MDDCs from non-users. These findings were further confirmed using MDDCs treated with alcohol. Inflammatory cytokines were measured in EtOH-treated and non-treated cells by antibody array.

Results—Functional effects of CBRs on MDDCs were shown by CB2 and GPR55 siRNA transfection. Transfected EtOH-treated cells showed significantly higher levels of proinflammatory cytokine production as measured by IL-1 expression. Our results provide insights into alcohol mechanisms of DC regulation and show, for the first time, that alcohol is inducing CNR2 and GPR55 in human DCs.

Keywords

alcohol; cannabinoids; cannabinoid receptors; human dendritic cells

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1. INTRODUCTION

According to NIAAA, 18 million Americans suffer from alcohol use disorders (AUDs; NIAAA, 2007), and genetic factors account for fifty percent of the risk for alcoholism (Foroud et al., 2010; Schuckit, 2009). Molecular and behavioral studies confirm a critical role of the innate immune system in mediating the effects of alcohol and support a neuroimmune hypothesis of alcohol addiction (Mayfield et al., 2013). Although it has been known that alcohol interferes with immune functions, and as a result, alcoholics may be more susceptible to infections (Szabo, 1997); to date, one of the least appreciated medical consequences of AUDs is their effects on the immune system leading to a growing demand for research to elucidate the molecular mechanisms of alcohol-induced immunomodulation. Therefore, our study aims to elucidate the effects of alcohol on human dendritic cells (DCs) which are pivotal antigen presenting cells of the innate immune system.

Alcoholism is a complex system influenced by CBRs (Pava and Woodward, 2012), and the ECS has been shown to be involved in mechanisms underlying AD (Erdozain and Callado, 2011; Marcos et al., 2012). The neurological effects of cannabinoids are mainly reported to be associated with CB₁ (Gaffuri et al., 2012) while the peripheral actions to be mediated by CB₂ (Miller and Stella, 2008). However, both receptors are known to play major roles in CNS (Mechoulam and Parker, 2013) and periphery (Kaplan, 2012). CB₂ activation, in particular, has a potential role in neuroinflammation and immunomodulation as recently reviewed (Rom and Persidsky, 2013). Overall, CBRs are known to have immunomodulatory properties; for instance, murine studies have reported a role of CBRs in DC regulation during inflammation (Lu et al., 2006a, 2006b; Newton et al., 2004). In terms of CB₂, for instance, agonists have been reported as potent anti-inflammatory and neuroprotective agents with beneficial effects of CB₂ activation in the case of concomitant HIV infection and alcohol abuse (Persidsky et al., 2011). Other reports from human brain tissues and microvascular endothelial cells have demonstrated that CB₂ is highly up-regulated during inflammation (Ramirez et al., 2012). However, most of the studies correlating CBRs to AUDs, have been done in the context of other inflammatory disorders in the CNS. Therefore, the alcohol-induced alterations of CBRs and their functional role in immunomodulatory mechanisms are still unclear. Moreover, there is evidence that additional CBRs may exist such as the novel GPR55 (Ryberg et al., 2007); which has been suggested to be involved in metabolism and glucose homeostasis (Romero-Zerbo et al., 2011); and in inflammatory responses mediated by neutrophils (Balenga et al., 2011).

Since alcohol is known to impair innate immune cell function (Szabo, 1997; Szabo et al., 2012), CBRs have been reported to regulate DCs (Kaplan, 2012), and the ECS is involved in AUDs (Erdozain and Callado, 2011), we hypothesize that alcohol can exert its effects on DCs by modulating CBRs, and subsequently CBRs may alter DC functions. We are the first ones to report the effects of alcohol drinking on CBRs produced by MDDCs and to show a functional role of CB₂ and GPR55 on cytokine production using a siRNA transfection approach. Analyzing the functional role of CBRs in alcohol-induced modulation of MDDCs will contribute to the current literature that steers towards a major role of the ECS in AUDs (Pava and Woodward, 2012).

2. MATERIALS AND METHODS

2.1 Participants

Blood donors were recruited from the Borinquen Health Care Center, Inc., Miami. Consents were obtained consistent with Florida International University (FIU) and the National Institutes of Health (NIH) policies. The protocol was approved by the IRB of FIU. Exclusion criteria were polydrug use, Hepatitis, HIV, other medical conditions, age <18 and

>52 years, and pregnancy. Prior to enrollment in the study, alcohol users (n=7) and non-users (n=7) completed a questionnaire. Alcohol users reported drinking an average of 5 days/week and an average of 7 drinks/day. Race/ethnicity of the participants includes African American, White, and Hispanics. Participants' average age is 35.

2.2 Differentiation of MDDCs

Blood samples from alcohol users and non-users were drawn. MDDCs were prepared from peripheral blood mononuclear cells (PBMCs) as previously described by us (Nair et al., 2005, 2009).

2.3 Gene Expression by Quantitative real-time PCR (qRT-PCR)

Gene expression was assessed as previously described by us (Agudelo et al., 2011; Agudelo et al., 2012) using Taqman assays (Applied Biosystems) for the expression of CB₁ (Hs00275634_m1), CB₂ (Hs00275635_m1), GPR55 (Hs00995276_m1), and IL-1 (Hs01555410_m1). GAPDH (Hs99999905_m1) and 18S rRNA (catalog # 4333760F) were used as endogenous controls.

2.4 Analysis of intracellular CB₁, CB₂ and GPR55 by flow cytometry

After MDDC differentiation, the cells were treated with 10–40 mM EtOH for 24 hours, harvested, blocked, fixed, and permeabilized with cytofix/cytoperm (BD Bioscience). CB₁ (catalog # sc-20754), CB₂ (catalog # sc-25494), and GPR55 (catalog # 10224) proteins were detected with primary rabbit-polyclonal, (Santa Cruz Biotechnology, Santa Cruz, CA; and Cayman Chemicals, Ann Arbor, MI respectively) and secondary goat anti-rabbit IgG fluorescein (FITC)-conjugated (Millipore) antibodies. Cells were acquired on an Accuri C6 flow cytometer (BD Accuri; Ann Arbor, MI) and analyzed with FlowJo software (Tree Star, Inc.; Ashland, OR).

2.5 Cytokine array

Non-treated and treated cells were harvested and whole cell lysates were prepared in protein extraction reagent (Pierce Biotechnology, Rockford, IL). 50 ug of protein were added to the array membranes (catalog # AAH-INF-3-8, RayBiotech, Norcross, GA). The intensities of signals were quantified by densitometry using Image J software. Data were analyzed using the RayBio Analysis Tool.

2.6 siRNA transfection against CNR2 and GPR55

CNR2 and *GPR55* silencing was performed using siRNA transfection kit following manufacture's protocol. (Santa Cruz Biotechnology, catalog #sc-45064) along with control (catalog #sc-37007), CB₂ (catalog # sc-39912) or GPR55 siRNA (catalog #sc-75183). Gene silencing was confirmed by qRT-PCR.

3. RESULTS

3.1 MDDCs from alcohol users and treated with EtOH have higher levels of CNR2 and GPR55

MDDCs from alcohol users (figure 1a) show significantly higher *CNR2* and *GPR55* compared to MDDCs from controls; and there were no significant effects on *CNR1*. And when MDDCs were treated with alcohol (0.05, 0.1 and 0.2%), there was an increased and bell curved pattern in *CNR2* and *GPR55*, with a more significant effect at the 0.1% alcohol concentration (figure 1b).

3.2 MDDCs treated with EtOH have higher levels of CB₂ and GPR55 protein

MDDCs were treated with alcohol (0.1 and 0.2%) for 24 hours and analyzed by flow cytometry. Accordingly, *in vitro* protein results (figure 1c) correlate with the gene data showing an increased in CB₂ and GPR55.

3.3 EtOH induces up-regulation of inflammatory cytokines

Since one of the main functions of DCs is the production of cytokines and there is evidence that alcohol-induced inflammation is mediated by proinflammatory cytokines (Lippai et al., 2013), we measured and compared the levels of cytokines in untreated and EtOH-treated MDDCs (Figure 2a) using antibody array (RayBiotech). EtOH significantly up-regulated 19 cytokines compared to the untreated control (Supplementary Table 1¹). Cytokine levels in the EtOH-treated cells ranges from 2.3–18.6 folds higher than untreated cells.

3.4 CB₂ and GPR55 siRNA transfection

In order to establish potential mechanisms of receptor up-regulation in the context of alcohol exposure, we used siRNA against CB₂ and GPR55 to analyze the effect of gene silencing on MDDCs function. As confirmed by qRT-PCR, CB₂ and GPR55 were significantly knockdown compared to the untransfected controls and EtOH-treated samples, and siRNA transfected controls (figure 2b).

3.5 Functional effects of CB₂ and GPR55 siRNA transfection on cytokine production

After confirming the CB₂ and GPR55 transfections were successful, cytokine levels were measured in untransfected and transfected controls and EtOH-treated MDDCs. Surprisingly, CB₂ and GPR55 transfected EtOH-treated cells show significantly higher levels of IL-1 (>20 fold difference) compared to the untransfected EtOH-treated cells (figure 2c).

3.6 Statistics

Data were analyzed using GraphPad Prism software. Comparisons between groups were performed using paired t-test and two-way ANOVA. Differences were considered significant at $p < 0.05$. Data are expressed as mean $\pm$ SEM. Experiments were performed at least three times in triplicates. Arrays were performed once; each array can detect 40 cytokines in duplicates.

4. DISCUSSION

There are several reports in humans and animals that associate *CBRs* with alcohol and drug dependence and emphasize the relevance of the ECS system in AUDs (Colombo et al., 2002; Erdozain and Callado, 2011; Freedland et al., 2001; Gessa et al., 2005; Marcos et al., 2012; Persidsky et al., 2011; Ramirez et al., 2012). However, most of the reports on the effects of alcohol on CBRs have focused on the neurological aspects, and little is known about the effects of alcohol use on CB₂ and GPR55 in the context of the immune system, in particular alcohol regulation of CBRs in MDDCs. Therefore, we are the first ones to provide evidence of upregulation of CB₂ and GPR55 by MDDCs from alcohol users (figure 1a), and to measure the subsequent functional role of CBRs in the context of alcohol- induced DC modulation and cytokine production. Our findings are novel, in particular the effects of alcohol on *GPR55*, since *GPR55* has been recently proposed as a new CBR (Ryberg et al., 2007), and its physiological role is mostly unexplored. Moreover, in our study, MDDCs from alcohol users have lower *CNR1* levels compared to non-users; and alcohol treatment

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has no significant effects on *CNR1*. It is relevant to point out that CB₁ expression and role in the immune system are unclear.

Our study provides insights into alcohol mechanisms of DC regulation and is the first one to show that alcohol induces *CNR2* and *GPR55* by MDDCs from alcohol users and *in vitro* alcohol treatment (figure 1a–c). Furthermore, EtOH is significantly modulating MDDCs to produce higher levels of pro-inflammatory cytokines (figure 2a). 19 out of 40 cytokines (Supplementary Table 1²) were increased after EtOH treatment. IL-1 showed the highest upregulation (18.6 folds). To test whether the alcohol-induced inflammatory response in MDDCs was regulated by CBRs, siRNA transfection was performed to silence CB₂ and GPR55 prior to measuring IL-1 levels. Surprisingly, the siRNA transfected MDDCs treated with alcohol have shown a significantly higher production of IL-1 (>20 fold) leading us to conclude that these genes provide a protective and anti-inflammatory effect that is lost when they are silenced (figures 2b–c).

Although the molecular mechanisms still need to be elucidated, we can speculate that CBRs are working together to modulate the immune system as shown by functional GPR55 and a rewarding GPR55-CB₂ partnership in leukocytes (Balenga et al., 2011). This and other reports have concluded that the crosstalk between these GPCRs may be crucial to abolish tissue injury (Balenga et al., 2011; Irving, 2011). Also, a recent review has highlighted the role of signaling through CB₂ as part of a protective machinery where inflammation and tissue injury may trigger elevations in the ECS, which in turn regulate signaling responses in immune cells modulating their critical functions (Pacher and Mechoulam, 2011).

In the context of alcohol research, CBRs may be playing a similar immunoprotective role during immune dysfunction caused by alcohol; hence, the observed increased in GPR55 and CB₂ (figures 1a–c). And, since alcohol-induced activation contributes to increased IL-1 during inflammation ((Lippai et al., 2013); and the lack of CB₂ and GPR55 caused uncontrollably elevated proinflammatory IL-1 levels by transfected EtOH-treated MDDCs (figure 2c), we speculate that CBRs may be playing a protective role in MDDCs by regulating cytokine production.

Further studies will be required to clarify the mechanisms that mediate the functional role of CBRs in alcohol-induced inflammation. Perhaps, innovative system-based approaches as recently described (Palmer et al., 2012) will identify mechanisms that may provide new targets for the treatment of AUDs.

One of the main limitations of our study is the sample size. This project is in its initial stages, the total five-year enrollment plan is 60 subjects. Another limitation is the alcohol consumption classification, which was assessed by self-report; therefore, it is subject to self-report biases. However, self-report is considered a reliable approach for measuring use of alcohol and other substances since it can provide useful estimates of consumption (Del Boca and Noll, 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Differential gene (1a, 1b) and protein (1c) expression of CNR1, CNR2 and GPR55 by MDDCs from alcohol users (1a) and *in vitro* alcohol treatment (1b). Primary human monocytes were isolated from PBMCs of blood samples from alcohol users (n=7) and non-users (n=7) (1a) and allowed to differentiate into MDDCs. RNA was extracted and reverse transcribed followed by qRT-PCR for CB₁, CB₂, GPR55 and housekeeping GAPDH and 18S rRNA gene expression. Data are expressed as mean $\pm$ SEM of TAI values performed in triplicates. Comparisons between alcohol users and non-users were performed using paired t-test and two-way ANOVA analyses with GraphPad Prism Software. For the *in vitro* alcohol treatments (1b), MDDCs from non-users (n=3) were treated with EtOH (0.05, 0.1 and 0.2%). Data are expressed as mean $\pm$ SEM of TAI values. Statistical significance was calculated by paired t-test with GraphPad Prism software. Fig. 1c, shows differential protein expression of CB₂ and GPR55 by MDDCs after alcohol treatment. MDDCs from non-users were treated with alcohol (0.1 and 0.2%) and stained with specific antibodies against human CB₂ and GPR55. Stained cells were acquired in an Accuri flow cytometer and analyzed with FlowJo software. Red histogram represents unlabeled controls, blue represents untreated controls, yellow represents treatment with EtOH 0.1%, and green represents treatment with EtOH 0.2%.

Figure 2.

Cytokine Modulation and siRNA knockdown for CB₂ and GPR55. Fig. 2a shows two arrays of inflammatory cytokines. RayBio Inflammation arrays were incubated with protein lysates from MDDCs untreated and treated with EtOH (0.1%) for 24 hrs. Chemiluminescence signals were detected by a film developer and analyzed by densitometry. Fig. 2b shows siRNA transfection studies against CB₂ and GPR55 in untreated and EtOH (0.1%) treated MDDCs (n=2). mRNA was extracted and reversed transcribed for quantitation by qRT-PCR. Fig. 2c shows IL-1 expression by MDDCs after siRNA transfection to knockdown CB₂ and GPR55. Data are expressed as mean $\pm$ SEM of TAI values. qRT-PCR experiments were performed at least three times in triplicates. Comparisons between groups were performed using paired t-test and two-way ANOVA analyses with GraphPad Prism Software.