Trichostatin A Shows Transient Protection from Chronic Alcohol-Induced Reactive Oxygen Species (ROS) Production in Human Monocyte-Derived Dendritic Cells

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Abstract

Objective: The objective of this study was to understand whether histone deacetylase (HDACs) inhibitor Trichostatin A or TSA can block and/or reverse chronic alcohol exposure-induced ROS in human monocyte-derived dendritic cells (MDDCs). Additionally, since nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a known regulator of antioxidant responses, we studied the effects of alcohol and TSA on ROS production and modulation of Nrf2 by MDDCs.

Methods: Intra-cellular, extra-cellular, and total ROS levels were measured in MDDCs treated chronically with alcohol (0.1 and 0.2 % ETOH) using 2',7'-dichlorofluorescin diacetate (DCF-DA) followed by detection of ROS in microplate reader and imaging flow cytometer. Nrf2 expression was analyzed by qRT-PCR and western blot. In addition, NFE2L2 (Nrf2), class I HDAC genes HDAC1, HDAC2, and histone acetyltransferase genes KAT5 were analyzed in silico using the GeneMania prediction server.

Results: Our results confirmed alcohol’s ability to increase intracellular ROS levels in MDDCs within minutes of treatment. Our findings have also demonstrated, for the first time, that TSA has a transient protective effect on MDDCs treated chronically with alcohol since the ability of TSA to reduce intracellular ROS levels is only detected up to 15 minutes post-chronic alcohol treatment with no significant protective effects by 10 hours. In addition, chronic alcohol treatment was able to increase the expression of the antioxidant regulator Nrf2 in a dose dependent manner, and the effect of the higher amount of alcohol (0.2%) on Nrf2 gene expression was significantly enhanced by TSA.

Conclusion: This study demonstrates that TSA has a transient protective effect against ROS induced by chronic alcohol exposure of human MDDCs and chronic long-term exposure of MDDCs with alcohol and TSA induces cellular toxicity. It also highlights imaging flow cytometry as a novel tool to detect intracellular ROS levels. Overall, the effect of TSA might be mediated through Nrf2; however, further studies are needed to fully understand the molecular mechanisms.

Keywords: Human dendritic cells; Imaging flow cytometry; Oxidative stress; Reactive oxygen species; Trichostatin A

Introduction

Alcohol has been known to upregulate reactive oxygen species (ROS) production thereby causing increased oxidative stress leading to the development of diseases [1]. Recently, it has been discussed that alcoholics have a heightened pro-inflammatory response due to the alteration of the activity of innate immune cells [2]. This deviation results in an increase in pro-inflammatory cytokine levels; as well as, a decrease resistance against colonization leading to alterations in organ microbiomes [2-4]. Therefore, approaches to reduce the inflammatory effects of alcohol are of interest to the alcohol research field. Some of the compounds gaining a lot of attention are the histone deacetylase inhibitors (HDACi) since they are currently being pursued to modulate a variety of human disorders involving chronic inflammatory diseases.

For instance, HDACi (vorinostat and BML281) were shown to exhibit anti-inflammatory activity in macrophages [5] and trichostatin A [6] is known to suppress cytokine production and gene expression associated with inflammation and innate immune responses in microglia and astrocytes [7]. Besides reducing the levels of pro-inflammatory cytokines and innate immune responses, TSA has also been shown to ameliorate endotoxin-induced neuronal inflammation and cognitive dysfunction in mice and microglial cells [8].

Under additional inflammatory effects induced by alcohol and other substances of abuse and toxins, HDACs and HDACi such as TSA have been implicated in the regulation of neuro-inflammation [9]. Further research findings have also demonstrated that treatment with HDACi such as sodium butyrate blocks both the development and the expression of ethanol-induced behavioral sensitization in mice [10]. In parallel, we have previously demonstrated the role of alcohol towards modulation of HDACs in human central nervous system (CNS) and peripheral cells [9,11,12]. For instance, we have demonstrated the ability of TSA to exert its neuroprotective effects by reducing alcohol-induced reactive oxygen species (ROS) production in the human...
neuroblastoma cell line (SK-N-MC) [9] and to modulate oxidative stress-related genes in human monocye-derived dendritic (MDDCs) cells [12]. For the past five years, the main focus of our lab has been the study of alcohol abuse on MDDCs, one of the primary antigen presenting cells of the immune system [13] that have been shown to get functionally altered due to alcohol exposure [12,14-20]. However, most of the studies were performed under acute alcohol conditions, which do not depict the effects of chronic alcohol dependence. Furthermore, the ability of TSA to block and/or reverse ROS production induced by chronic alcohol exposure of human innate immune system cells remains to be elucidated.

Therefore, in the current study, we analyzed the ability of TSA to ameliorate the effects of chronic alcohol exposure in MDDCs and demonstrated, for the first time, the implementation of single cell imaging flow cytometry as a novel tool to detect intracellular and extracellular ROS levels. In addition, when it comes to studying mechanisms behind the protective action of anti-oxidants, nuclear factor erythroid 2-related factor 2 (Nrf2), has been repeatedly shown to play a role as an important anti-oxidant gene transcription regulator [21-23]; therefore, we also analyzed the effects of alcohol and TSA on the regulation of Nrf2 by MDDCs.

Methods

MDDC isolation

Human buffy coats from healthy anonymous blood donors were purchased from the community blood bank (One Blood, Miami, FL, USA). Human blood studies in Dr. Agudelo’s lab were reviewed and approved by the Institutional Review Board of FIU. Total peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coats. Monocytes isolated from the PBMCs were differentiated into monocyte-derived dendritic cells (MDDCs) in CRPMI medium for 5 to 7 days with cytokines IL-4 and GM-CSF as previously described by us [16,24,25]. Cells obtained from each buffy coat are considered independent biological replicates.

Treatments

MDDCs were treated with 0.1% (~50 mM) or 0.2% (~100 mM) of alcohol (Ethanol or EtOH) (catalog #E7023, Sigma-Aldrich, St. Louis, MO, USA) for 5 days. These in vitro chronic alcohol treatments are equivalent to the physiologically blood alcohol concentrations (BAC) of 100 mg/dl and 200 mg/dl, respectively, and are close to the legal limit for driving under intoxication of 0.08% (80 mg/dl) and corresponds to BAC in sober alcohol users (200 mg/dl) seen in an emergency room [26]. Control MDDCs were not treated with alcohol but received media change at the same time as the rest. Alcohol-treated MDDCs were kept in a separate incubator humidified with alcohol. Additionally, to address evaporation issues, alcohol treatments were replenished in full every 24 h. MDDCs receiving TSA were pre-treated for 2 h with 50 nM TSA (catalog #1406, Tocris, Bio-Technne Corporation, Minneapolis, MN) as previously reported by us [9,12]. TSA treatment was replenished during media change every 48 h. Both ethanol and TSA concentrations were re-added to cells after 5 day post chronic effect and prior to initiation of ROS measurement, as further explained.

ROS assay

All cells were harvested at day five post chronic alcohol exposure (0.1%-0.2%, EtOH). MDDCs were harvested and plated in 96 well plates at 100,000 cells per well for microplate fluorescence detection or aliquoted into 1 million cells per mL of medium for single cell imaging flow cytometry. MDDCs receiving TSA were pre-treated with TSA for two hours followed by addition of 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA) (catalog #D6883, Sigma, St. Louis, MO). Cells that were used for ROS kinetic analysis were kept under chronic alcohol conditions for an additional 24 hours post DCF-DA treatment. For positive control, hydrogen peroxide (50 μM H₂O₂) was added, and the untreated control cells received just media. Co-treatment with TSA and H₂O₂ was used as an additional control to show that variations in the readings as a result of the addition of DCF-DA are not due to direct interference of TSA with H₂O₂ but rather through cellular signaling mechanisms. Following alcohol treatments, ROS measurement was carried out by two separate techniques. The first technique involved measuring fluorescence (excitation 495/ emission 530) in a Biotek Synergy HT plate reader using the kinetic setting, which measures total (intracellular and extracellular) ROS at different time points up to 24 h. Each sample was read at least in quadruplets and data were analyzed by combining relative fluorescent units (RFU) from different experiments. The second technique used for the detection of intracellular ROS was single cell imaging flow cytometry, where post TSA, alcohol, or H₂O₂ treatment, viability dye DAPI was added to the cells, and 10,000 live single cell images were acquired per sample using Aminis FlowSight. Data were analyzed using Ideas software. To measure extracellular ROS, the supernatants from the cells were plated in quadruplets and fluorescence was measured using a plate reader as mentioned above. A schematic diagram for the ROS assay is depicted in Supplementary Figure 1.

Nrf2 gene expression

Post differentiation, MDDCs were pre-treated with 50 nM TSA and then chronically treated with alcohol (0.1% and 0.2%). TSA was replenished with every media change. After 5 days of treatment, total RNA was isolated from control and treated MDDCs. Extracted RNA was reverse transcribed, followed by qRT- PCR using Taqman assays (Applied Biosystems, Thermo Fisher Scientific) for Nrf2 (assay id Hs00975961_g1). 18s RNA (catalog # 4333760F) was used as internal control.

Nrf2 Protein expression

Post differentiation, MDDCs were pre-treated with 50 nM TSA and then chronically treated with alcohol (0.1% and 0.2%). TSA was replenished with every media change. After 5 days of treatment, total protein was isolated from control and treated MDDCs. 30 μg of protein was ran on a SDS-PAGE gel and immunoblotted with mouse monoclonal anti-Nrf2 primary antibody (catalog # sc-365949, Santa Cruz) and secondary anti-mouse IgG peroxidase antibody (catalog # A9044, Sigma).

In silico analysis

NFE2L2, HDAC1, HDAC2, and KAT5 were further analyzed in silico using the GeneMania prediction server (University of Toronto) for collating gene and pathway interactions.
Figure 1: Alcohol increases intracellular ROS levels within minutes and this effect is transiently blocked by TSA: After five days of chronic alcohol exposure, cells were retreated with TSA for two hours, DCF-DA was added followed by EtOH, then intracellular ROS levels were analyzed in MDDCs by single cell imaging flow cytometry. Panel a shows representative single cell images where column 1 is BF or Bright Field, column 2 is ROS or DCF-DA, column 3 is SSC or Side Scatter, column 4 is DAPI or viability dye and column 5 is BF/ROS or overlay of Bright Field and ROS or DCF-DA image. Panel b shows percentage of ROS positive cells for each treatment. Significant effect was observed [F (6,50)=30.05, p<0.0001]. Post hoc comparisons using the Tukey’s multiple comparisons test indicated that the mean score for EtOH 0.1% (M=75.46, SEM=3.371, p=0.0005) and EtOH 0.2% (M=57.47, SEM=2.418, p=0.0001) was significantly different than EtOH 0.1%+TSA and EtOH 0.2%+TSA condition (M=31.17, SEM=5.419; M=23.09, SEM=5.586). Panel c show representative histogram overlays of intensity of ROS for all treatments. Panel g shows percentage of ROS positive cells at 10 h post DCF-DA and EtOH treatment [F (6, 32)=0.675, p=0.6706]. Panel h shows a representative histogram overlay of intensity of ROS after 10 h. post DCF-DA and EtOH treatment. The experiment was carried out from 5 different buffy coats. 1-way ANOVA was carried out to test for significance. Data represented as Mean ± SEM with * representing p ≤ 0.05.

Statistics

All data have been represented as mean ± SEM. Statistical analysis was carried out using GraphPad Prism software (La Jolla, CA). Single cell imaging flow cytometry data were analyzed using Ideas Software. Mean differences across the treatment groups were assessed using analysis of variance (ANOVA) with appropriate multiple comparison tests. 2-way ANOVA and paired t-test were used where appropriate for comparing statistical differences among experimental groups. Differences among experimental groups were considered significant at p ≤ 0.05. Each experiment was repeated at least thrice as specified in the Figure legends.

Results

Alcohol increases intracellular ROS levels within minutes and this effect is transiently blocked by TSA

After five days of chronic alcohol exposure, cells were retreated with TSA for two hours, DCF-DA was added followed by EtOH, then intracellular ROS levels were analyzed in MDDCs by single cell imaging flow cytometry. TSA exerted a transient protective effect by blocking chronic alcohol-induced intracellular ROS levels within 15 minutes of retreating the cells with alcohol. Figure 1, Panel c-f show representative histogram overlays of the intensity of ROS for all treatments. Panel c shows TSA was able to reduce ROS production when compared to control while panel d shows rightward shift or increased intensity of 0.1 and 0.2% EtOH treated MDDCs compared to control.
control. Panel e and f show the leftward shift of intensity of 0.1% EtOH + TSA and 0.2% EtOH+TSA compared to 0.1% and 0.2% EtOH; respectively, indicating the protective effect of TSA. Panel a shows representative single cell images. Panel b shows the percentage of ROS positive cells for each treatment. Positive control or H2O2 treated MDDCs shows 99.2% ± 0.5 cells positive for ROS. TSA was able to significantly reduce the percentage of cells (39.2% ± 4.3, p=0.03) expressing intracellular ROS compared to untreated MDDCs (49.5% ± 2.4). 0.1% EtOH (75.4% ± 3.01, p=0.005) and 0.2% EtOH (57.4% ± 2.3, p=0.01) treated MDDCs significantly increased the percentage of ROS producing cells compared to untreated MDDCs. 0.1%+TSA (38.5% ± 3.3, p=0.0005) and 0.2%+TSA (29.6% ± 5.4, p=0.0001) were able to significantly decrease the percentage of cells expressing intracellular levels of ROS compared to 0.1% EtOH or 0.2% EtOH respectively. TSA significantly blocked the effect of EtOH; however, this effect is transient since after 10 hours of alcohol treatment, there were no differences in intracellular ROS levels among treatments and the protective effect of TSA is lost as measured by single cell imaging flow cytometry (Figure 1, panel g). Panel h shows a representative histogram overlay of the intensity of ROS in which only the MDDCs treated with H2O2 show a high intensity (rightward shift) compared with all other treatments, which show the same levels of ROS as untreated control (no shift).

**Alcohol differentially induces extracellular ROS production and this effect is transiently blocked by TSA**

To corroborate the transient protective effect of TSA, extracellular ROS levels were also measured at different time points after adding DCF-DA and re-treating the cells with alcohol (Figure 2). In panel a, TSA still shows a protective effect by 15 minutes as indicated by significantly lower (1013.2 ± 49.9 RFU, p=0.03) extracellular ROS levels in supernatants from TSA-treated MDDCs compared to untreated MDDCs (1195.9 RFU ± 59.2). Although the extracellular levels of ROS are lower in the supernatants from TSA-treated cells compared to the supernatants from EtOH-treated cells, the effects are non-significant. Additionally, at 15 minutes, there is also a significant increase in extracellular ROS levels in supernatants from 0.2% EtOH (1513.8 RFU ± 135, p=0.04) treated MDDCs compared to untreated MDDCs. In panel b, there are higher levels of extracellular ROS detected by 10 hours compared to the ROS levels detected at 15 minutes; additionally, 0.2% EtOH-treated MDDCs have a significantly higher amount of extracellular ROS (7241 RFU ± 452, p=0.03) compared to untreated MDDCs (5644.6 RFU ± 479.9). Finally, after 24 hours, as shown in panel c, both EtOH concentrations, 0.1% (30910 RFU ± 8492, p=0.02) and 0.2% (15149 RFU ± 1804, p=0.02) have caused the MDDCs to release significantly higher amounts of ROS into the extracellular environment compared to untreated MDDCs (10055 RFU ± 1063.9). However, based on the extracellular ROS measurements, TSA is having no protective effect on the EtOH-induced release of ROS.

**Alcohol increases total ROS production over time and this effect plateaus by 12 h**

Since the effects of alcohol and TSA on the intracellular and extracellular ROS production were diverse, we proceeded to elucidate the effects on total ROS production (intracellular and extracellular) by MDDCs. In Figure 3, panel a, MDDCs chronically treated with 0.1 or 0.2% alcohol show upregulated ROS production compared to control MDDCs as measured by RFU of total ROS levels; however, the effects of alcohol on total ROS were not significant. In panel b, total ROS levels measured at different time points for MDDCs treated with 50 nM TSA are plotted along with untreated control, positive control H2O2 treated MDDCs, and for blank or no cells. At 9, 10, and 12 h Post-chronic alcohol treatment, the MDDCs pre-treated with 50 nm TSA, show significantly reduced ROS levels (3507.8 RFU ± 129.9, p=0.05, 3873.6 RFU ± 151.4, p=0.007 and 4604.9 RFU ± 195.9, p=0.001) compared to untreated control MDDCs (4109.1 RFU ±257.7, 4611.6 RFU ± 307.2 and 5657.3 RFU ± 415.6). In panel c, 12 h post-chronic alcohol treatment, the MDDCs treated with 0.1% alcohol and pre-treated with TSA (5263.1 RFU ± 348, p=0.03) show significantly reduced ROS levels compared to MDDCs treated with 0.1% alcohol only (6333.9 RFU ± 496.9). In panel d, MDDCs treated with 0.2% alcohol and pre-treated with TSA show reduced ROS levels compared to MDDCs treated with 0.2% alcohol only; however, this reduction in ROS was observed at earlier time points for up to 12 h. At later time points for up to 24 h, there is an opposite trend which may be related to increased cytotoxicity. From 20-24 h, there is significant increase in ROS levels in MDDCs treated with EtOH 0.2%+TSA (5370 RFU ± 59.6, p=0.02-0.0322 RFU ± 63.8, p=0.0009) when compared to MDDCs treated with EtOH 0.2% (4029 RFU ± 83.1-4495 RFU ± 119). In summary, TSA’s reduction in alcohol-induced ROS is only transient and the ROS levels get exacerbated over time as demonstrated with a significant increase in ROS production by MDDCs exposed to both EtOH 0.2%+TSA.

**Chronic alcohol and TSA exposure exacerbates ROS levels ultimately affecting cellular viability**

After chronic EtOH exposure (5 days) and after adding DCF-DA and re-treating the cells with alcohol for additional 10, 12, and 24 h, cell viability was measured by single cell imaging flow cytometry using DAPI. Supplementary Figure 2 shows that above 80% of the cells are still viable for up to 12 h post-chronic EtOH and TSA exposure and post-DCF-DA treatments; however, by 24 h, MDDCs viability drops drastically indicating that the cells begin to die due to the toxic environment created not only by the release of ROS induced by the presence of TSA, alcohol, and DCF-DA, but it might also be due to other mechanisms including apoptosis induced by chronic TSA and/or alcohol exposure. Thus, the observed effects on ROS levels post-chronic EtOH exposure may be due to ROS exacerbation and cytotoxicity.

**Alcohol and TSA modulate the antioxidant regulator Nrf2**

To understand the underlying mechanism of TSA’s transient anti-oxidative effect, we analyzed Nrf2 gene expression levels. Nrf2 is a transcription factor that is associated with antioxidant gene regulation [27]. We studied gene expression of Nrf2 in MDDCs chronically treated with 0.1% and 0.2% EtOH and in presence or absence of TSA. Gene expression studies showed (Figure 4, panel a) both 0.1% (2.04 ± 0.4, p=0.02) and 0.2% EtOH (9.93 ± 1.2, p=0.0007) upregulated transcription of Nrf2 significantly compared to untreated MDDCs. While 0.1% EtOH+TSA treatments did not significantly modulate transcription of Nrf2 compared to 0.1% EtOH treatment alone, 0.2% EtOH + TSA (18.01 ± 2.3, p=0.006) treatments significantly increased Nrf2 gene expression compared to 0.2% EtOH treatment alone. Nrf2 protein expression was also measured by western blotting (Figure 4, panel b) showing a similar trend as gene expression results. Overall, 0.2% EtOH+TSA treatments showed the highest expression of Nrf2; however, there was no significant difference between treatments.
In silico analysis reveal gene pathway interactions among HDACs and Nrf2

To further understand the association between Nrf2 and TSA's protective activity, in silico analysis was performed using gene MANIA, online software for understanding gene pathway interactions. Since TSA is an established non-specific HDAC inhibitor and our previous studies (Agudelo et al.) have demonstrated alcohol effects on class I HDACs, HDAC1 and HDAC2 were selected to understand the association between TSA and Nrf2. Additionally, since Nrf2 regulates antioxidant responses [28] and the major source of ROS are membrane-associated NAD(P)H-oxidases, also known as Nox enzymes and Nox4 subunit expression is strongly correlated with an increase of NAD(P)H-oxidase activity [29], we wanted to analyze the effects of alcohol and TSA on ROS production and modulation of markers of oxidative stress such as Nrf2 in the periphery, particularly in human MDDCs treated with alcohol. Besides the main interest on analyzing the effects of alcohol on deacetylation and HDACs, we selected histone acetyltransferase TIP60 for the in silico analysis since we have recently demonstrated that chronic alcohol significantly induces acetylation and histone acetyltransferases may play a role under chronic alcohol conditions [17]. Therefore, the in silico analysis suggests the interaction of HDAC1, HDAC2, and histone acetyltransferase TIP60 gene (KAT5) with Nrf2. Moreover, Figure 4 panel c indicates, genes for Nrf2 (NFE2L2), HDAC1, HDAC2, and KAT5 interact and are co-expressed suggesting that the inhibition of HDACs by TSA might be somehow resulting in the overexpression of Nrf2 gene corresponding to an indirect increase in acetylation.

Discussion

In the current study, we have demonstrated the ability of TSA to diminish chronic EtOH-induced ROS production by human MDDCs using a novel imaging flow cytometry method to measure intracellular ROS levels; however, the effect of TSA is only transient. HDAC inhibitors have been extensively studied for their anti-inflammatory properties as demonstrated by their anti-inflammatory activity in human macrophages in a rat model of arthritis [5] and modulation of leukocyte differentiation and inflammation [30]. TSA has been extensively studied for its pleiotropic effects especially in its role to reduce inflammation as a therapeutic tool. For instance, TSA was shown to protect against cisplatin-induced cell damage via regulation of IL-4 and STAT 6 signaling pathway [31]. TSA was also shown to protect against reperfusion-induced lung damage in perfused rat lung model by rescinding inflammation and apoptosis-related signaling pathways [32]. TSA was also found to protect liver cells against sepsis by inhibiting toll like receptor signaling in an in vitro cell culture study [33].

Apart from studies that show TSA's anti-inflammatory activity in different organs, studies have shown its protective effects in immune cells like dendritic cells. TSA prevented the onset of arthritis in a mice model by making the dendritic cells more tolerogenic in phenotype [34]. TSA was also shown to reduce type 1 interferon production by plasmacytoid dendritic cells making it an effective therapeutic target towards the treatment of autoimmune diseases as type 1 interferon plays a major role in auto-immune diseases [35]. In addition, TSA has been shown to improve differentiation of dendritic cells in cases of leukemia and highlighted as a potential therapeutic target towards leukemia [36,37]. Through literature, we know that inflammation and ROS causing oxidative stress are deeply associated [38]. Therefore, TSA's anti-inflammatory properties have led researchers to study its potential to block ROS production. We have also shown previously that TSA is able to protect neuronal cells from alcohol-induced oxidative stress by reducing ROS production [9]. In the current study, however, our focus was to study chronic alcohol-induced ROS in the periphery using human MDDCs and to elucidate the protective kinetics of TSA in these innate immune system cells. Since Nrf2 is a known regulator of antioxidant responses [28] and the major source of ROS are membrane-associated NAD(P)H-oxidases, also known as Nox enzymes and Nox4 subunit expression is strongly correlated with an increase of NAD(P)H-oxidase activity [29], we proceeded to analyze the effects of alcohol and TSA on ROS production and modulation of markers of oxidative stress such as Nrf2 in the periphery, particularly in human MDDCs treated with alcohol.

As shown in Figure 1, by using single cell imaging flow cytometry, we were able to demonstrate that TSA has a transient protective effect on MDDCs. We call this effect transient since when we measured intracellular ROS levels, the protective effect of TSA was only detected up to 15 minutes post-treatment with no significant effects by 10 hours post-treatment (Figure 1). This observed transient effect of TSA on intracellular ROS levels might also be due to the release of ROS out of cells under oxidative stress. Extracellular ROS has previously been studied and shown to cause altered ROS production, lipid peroxidation, energy efficiency, lipid handling, and differentiation in human adipocytes that were treated with lactate and pyruvate to generate extracellular ROS [39]. This signaling due to extracellular ROS can also lead to the localization of immune cells at the site of infection, leading to clearance of the infection but also to an increase in injury through inflammation. To take extracellular ROS levels into consideration, in parallel to measuring intracellular levels through imaging flow cytometry, extracellular ROS levels were also measured in the cell culture supernatants. Our results demonstrate that there is a significant increase in extracellular ROS in alcohol treated cells compared to control for up to 24 hours (Figure 2). Moreover, TSA reduces ROS production compared to untreated control and EtOH-treated cells (Figure 2); however, these effects were not significant. A possible explanation for the differential and transient protective effects of TSA might be that TSA has the ability to regulate the intracellular levels of ROS at early time points following alcohol exposure; however, when chronic alcohol exposure starts exacerbating the release of ROS from the cells, TSA is no longer effective and fails to block alcohol-effects.

Besides measuring intracellular and extracellular levels of ROS, total ROS was also measured in chronically treated MDDCs. When total ROS levels (intracellular and extracellular) were measured, the protective effect of TSA was detected for up to 12 hours (Figure 3) and then plateaus. To understand this plateauing of ROS levels after 12 hours and further decreased in ROS levels beyond that time point, we measured MDDCs viability. The viability of MDDCs was 80% and above for up to 12 hours after 5 days of chronic alcohol and TSA exposure and post-DCF-DA assay (supplementary Figure 2); however, due to the accumulation of intracellular and extracellular ROS production, the viability of cells dropped drastically by 24 h post-DCF-DA as ROS levels got exacerbated.
Figure 2: TSA has a temporal effect on alcohol-mediated extracellular ROS production. To corroborate the protective effect of TSA, extracellular ROS levels were measured by plate reader at different time points after adding DCF-DA and re-treating the cells with alcohol. Panel a, b and c: is a graphical representation of extracellular ROS levels as measured in supernatants of cells after adding DCF-DA and re-treatment with EtOH after 15 minutes (panel a), 10 h. (panel b) and 24 h. (panel c). The experiment was done from 3 different buffy coats and each treatment plated in quadruplets. For panel a, at 15 minutes, 2-way ANOVA showed significant row factor (F (12, 60)=14.81, p<0.0001) and significant column factor (F (5, 60)=1.15, p<0.0001). Post hoc analysis by Tukey's multiple comparisons test showed mean for EtOH 0.2% (M = 1513.85, SEM=135, p=0.006) was significantly different compared to control (M=1195.9, SEM=59.2). When analyzed by paired T-Test, TSA (M=1013.2, SEM=49.9, p=0.03) showed significant difference compared to control, however, Tukey's multiple comparisons test was not significant for the same. For panel b at 10 h, 2-way ANOVA showed significant row factor (F (11, 55)=150.8, p<0.0001) and significant column factor (F (5, 55)=11.92, p=0.0001). Post hoc analysis by Tukey's multiple comparisons test showed mean for EtOH 0.2% (M=7241, SEM=452, p<0.0001) was significantly different compared to control (M=5644.6, SEM=479). For panel c at 24 h, 2-way ANOVA showed significant row factor (F (11, 55)=2.532, p=0.0116) and significant column factor (F (5, 55)=5.128, p=0.0006). Post hoc analysis by Tukey's multiple comparisons test showed mean for EtOH 0.2% (M=30910, SEM = 8492, p=0.0058) was significantly different compared to control (M=10055.6, SEM=1063.9). When analyzed by paired T-Test, EtOH 0.2% (M=15149, SEM=1804, p=0.02 showed significant difference compared to control, however, Tukey's multiple comparisons test was not significant for the same. 2-way ANOVA with post hoc analysis of Tukey's multiple comparisons test and paired T-test were carried out to test for significance. Data represented as Mean ± SEM with * representing p ≤ 0.05.

Even though the cell viability is compromised overtime after 12 hours of DCF-DA and additional treatment of alcohol, the kinetic studies using the microplate reader to measure total ROS levels was able to show the partial protective properties of TSA to transiently block chronic alcohol-induced oxidative stress. Additionally, it is relevant to point out that histone deacetylase inhibitors are known for their anti-tumor properties; in particular TSA has been shown to inhibit breast cancer cell viability and proliferation while inducing cell apoptosis due to mitochondrial ROS [40]. TSA in combination with nanoparticles has been shown to enhance apoptosis in human cancer cells [41,42] and to promote apoptosis of osteosarcoma cells through p53 signaling pathway activation [43]. Moreover, chronic alcohol exposure for more than five days in culture can also be contributing to the cytotoxic effects observed since in vivo experiments using binge ethanol in an animal model of chronic ethanol exposure resulted in augmented levels of necrosis and steatosis in the liver [44]. In summary, chronic ethanol exposure and the interaction with TSA and DCF-DA might be also inducing the accumulation of ethanol metabolites limiting the protection from TSA and inducing cytotoxic effects in MDDCs.
Figure 3: Alcohol increases total ROS production over time and this effect plateaus by 12 h. After chronic treatment of MDDCs, cells were harvested, plated and treated with TSA followed by DCF-DA and retreated with EtOH and total ROS production (intra-cellular and extra-cellular) by MDDCs was measured. Panel a, MDDCs chronically treated with 0.1 or 0.2% EtOH show upregulated ROS production compared to control MDDCs as measured by relative fluorescence units (RFU) of total ROS levels. 2-way ANOVA showed significant row factor (F (10, 670)=91.92, p<0.0001) and post hoc analysis with Tukey’s multiple comparisons test did not find any significant difference between control, EtOH 0.1% and EtOH 0.2%. In panel b, total ROS levels measured at different time points for MDDCs chronically treated with 50 nM TSA are plotted along with untreated control, positive control H$_2$O$_2$ treated MDDCs, and for blank or no cells. 2-way ANOVA showed significant row factor (F (10,373)=138, p<0.0001) and significant column factor (F (1, 373)=30.65, p<0.0001). Post hoc analysis with Sidak’s multiple comparisons test shows at 9th h, mean for TSA (M=3507.8, SEM=129.9, p=0.059) showed significant difference compared to control (M=4109.1, SEM=257.7). At 10th h, mean for TSA (M=3873.6, SEM=51.4, p=0.007) showed significant difference compared to control. Finally, at 12th h, mean for TSA (M=4604.9, SEM=195.9, p=0.0001) showed significant difference compared to control. Panel c, MDDCs treated with EtOH 0.1% and TSA show reduced ROS levels compared to MDDCs treated with EtOH 0.1% only. 2-way ANOVA showed significant row factor (F (10, 578)=75.68, p<0.0001) and significant column factor (F (1, 578)=6.625, p=0.0103). Post hoc analysis with Sidak’s multiple comparisons test shows at 12 h, mean for EtOH 0.1%+TSA (M=5263.1, SEM=348, p=0.003) showed significant difference compared to EtOH 0.1% (M = 6333.9, SEM = 496.9). In Panel d, MDDCs treated with EtOH 0.2% and TSA show reduced ROS levels compared to MDDCs treated with EtOH 0.2% only. 2-way ANOVA showed significant interaction (F (10, 562)=7.345, p<0.0001), significant row factor (F (10, 562)=111.7, p<0.0001) and significant column factor (F (1, 562)=4.966, p=0.0262). However, post hoc analysis with Sidak’s multiple comparisons test did not find any significant difference between EtOH 0.2% and EtOH 0.2% + TSA up to 12 h. At 20th, 22nd and 24th h, there is significant difference between EtOH 0.2% + TSA (20th h: M=5370, SEM=59.6, p=0.02, 22nd h: M=5801, SEM=63.1, p=0.005, 24th h: M=6232, SEM=63.8, p=0.0009) and EtOH 0.2% (20th h: M=4029, SEM=83.1, 22nd h: M=4260, SEM=101.2, 24th h: M=4495, SEM=119). The experiment was carried out from 3 buffy coats and each treatment was plated at least in quadruplets. 2-way ANOVA was used to test for significance. Sidak’s multiple comparisons test was used when comparing two treatments while Tukey’s multiple comparisons test was used when comparing more than two treatments. Data represented as Mean RFU ± SEM with * representing p ≤ 0.05.
Figure 4: Alcohol and TSA modulate the antioxidant regulator Nrf2 possibly by interactions between HDACs, HATs and Nrf2. Post chronic treatments, total RNA and protein were isolated. Nrf2 gene expression was studied through reverse transcription qPCR and represented as Transcript Accumulation Index (TAI) [50] in panel a. 2-way ANOVA showed significant column factor \(F(5,65)=38.33, p<0.0001\). Post hoc comparisons using the Tukey’s multiple comparisons test indicated that the mean score for EtOH 0.2% (M=9.937, SEM=1.308, \(p=0.0007\)) was significantly different than control. EtOH 0.1% (M=2.04, SEM=0.4, \(p=0.02\)) was significantly different than control. EtOH 0.2% + TSA condition (M=18.01, SEM=2.3, \(p=0.006\)) was significantly different than EtOH 0.2%. Western blotting was used to visualize protein expression as shown in panel b. The qPCR and western blot experiments were carried out from 5 different buffy coats. Representative western blot is depicted in Figure 4, panel b. Optical density accounts for 33.11\% of the total variance \(F(4, 20) = 3.996, p=0.0153\). Post hoc Tukey's multiple comparisons tests showed no significance between treatments, only a variance between optical densities of Nrf2. Statistical test 2 way ANOVA was carried out to test for significance. Data are represented as mean ± SEM with \(*\) representing \(p \leq 0.05\). In silico analysis in panel c show, genes for Nrf2 (NFE2L2), HDAC1 and HDAC2 and histone acetyl transferase (HAT) TIP60 gene KAT5 interact primarily through physical interactions and co-expression.

Interestingly, there was overall higher extra-cellular and total ROS production in MDDCs exposed to both 0.2% EtOH+TSA compared to 0.2% EtOH treated MDDCs at 24 hours post DCF-DA and EtOH retreatment (Figure 2c and Figure 3d). This ROS provoking effect needs further clarification. Since we do not observe this effect intracellularly, we can conclude it is primarily due to extra-cellular ROS exacerbation. However the difference between seeing TSA/protective effect with 0.1% EtOH treatment while a ROS provoking effect with 0.2% EtOH treatment may stem from differences on how the cells process different concentrations of alcohol. According to the literature, studies done in zebrafish, which have comparable ethanol metabolism to that of mammals, showed that, treatment with differential levels of alcohol alters the activity of the enzyme alcohol dehydrogenase in a bell shaped curve [45]. Higher concentration of alcohol lowered the activity of alcohol dehydrogenase in zebrafish liver [45]. Similar curves were also studied for acute and chronic alcohol exposure in zebrafish [46]. In human brain endothelial cells treated with ethanol, superoxide dismutase activity was measured over 240 hours, which also exhibited a bell shaped curve for activity demonstrating a differential effect on ROS metabolizing enzymes based on the duration of ethanol exposure [47]. These studies show a functional difference exists between how cells use different mechanisms to cope with a lower and a higher concentration of alcohol. Hence, further studies are needed to understand these effects in-depth.

From our previous studies, there is evidence that acute alcohol or binge drinking increases histone deacetylases [12], TSA exert its neuroprotective effects by reducing alcohol-induced ROS production by human CNS cells [9], and TSA modulates oxidative stress related...
genes in human immune cells [12]. Moreover, the current study demonstrates that the protective effect of TSA on EtOH-treated cells is transient. In order to further elucidate the molecular mechanisms behind the protective action of TSA, expression of the nuclear factor Nrf2 was analyzed after in vitro alcohol and/or TSA treatment in MDDCs. Nrf2 is a key transcription factor that has been consistently associated with anti-oxidative properties and has been shown to regulate anti-oxidant genes in the human body [48]. Nrf2 has also been shown to protect the liver from alcohol induced oxidative stress [49]. Therefore, we wanted to analyze if Nrf2 was playing a role in the alcohol-induced oxidative stress and TSA's transient effect in MDDCs. Nrf2 gene expression was analyzed in untreated and MDDCs treated with alcohol and/or TSA and the results demonstrated that chronic alcohol by itself upregulates Nrf2 expression and this effect is enhanced when alcohol is combined with TSA. This upregulation could be a stress response mechanism for the anti-oxidant regulator to control ROS in MDDCs. Other reports also support that HDAC inhibition increases ROS in MDDCs. TSA inhibition of HDACs since there are studies that show acetylation is an important factor for activating Nrf2 transcription [51]. Therefore, by decreasing HDACs with TSA, there is an increase in acetylation and hence activation of Nrf2 bringing about the transient anti-oxidant properties of TSA. We further carried out in silico analysis of Nrf2 gene (NFE2L2), HDAC 1, HDAC 2, and histone acetyl transferase TIP60 gene KAT5. There were physical interactions and co-expression, suggesting that, the inhibition of HDACs by TSA might be somehow resulting in the overexpression of Nrf2 gene through the increase of acetylation. This can be deducted since KAT5 is associated with NFE2L2 indirectly through KEAP1, which codes for Keap1, a substrate adaptor protein for the Cullin 3 (Cul3)-dependent E3 ubiquitin ligase complex. Keap1 suppresses Nrf2 expression by helping its ubiquitination followed by proteasomal degradation [52]. Literature also shows an extensive interaction between histone acetylation and deacetylation regulating expression of Nrf2. For instance, one such study shows histone acetyltransferase hMOF acetylates Nrf2 and locates it to the nucleus and increases transcription of its downstream genes in lung cancer cells [53]. Another in vitro study demonstrated that CREB binding protein acetylates Nrf2 to retain it in the nucleus while heterologous sirtuin (SIRT1) deacetylates Nrf2 concluding that acetylation and deacetylation of Nrf2 regulates its transcriptional activity and nucleocyttoplasmic localization. These studies further suggest an intricate relationship between histone acetylation or deacetylation and expression of Nrf2. However, further studies can be carried out to understand the exact nature of this relationship in MDDCs under chronic alcohol stress. Previous studies on alcohol-induced ROS have pointed towards dysfunction of NADPH oxidases [54] and the role of xanthine oxidoreductase (in alcohol induced oxidative stress [55]). Additionally Nox 1, Nox 2, and Nox 4 protein levels were increased in alveolar macrophages from alcoholic patients compared to controls [54]. Other studies have also shown that the role of NADPH oxidase dependent ROS production in murine macrophages under the effect of alcohol is mediated by matrix metalloproteinase-12 expression [56]. Therefore, the same remains of interest in the case of TSA's transient anti-oxidant protective effects and exacerbation of ROS when combined with in vitro chronic alcohol exposure. These molecular pathways of oxidation may be pursued in future studies to elucidate the mechanism of TSA's and alcohol interactive effects.

In summary, chronic alcohol treatments increased intracellular, extracellular, and total ROS in human MDDCs. Although TSA was able to transiently protect MDDCs from oxidative stress, it is evident that chronic long-term exposure of MDDCs with alcohol and TSA induces cellular toxicity. These effects may be mediated through transcription of Nrf2 promoted by acetylation; however, further studies are needed to fully understand the molecular mechanisms and the therapeutic capacity of TSA.

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Conflict of Interest

The authors declare no competing interests.

References
