Ethanol Suppresses PGC-1α Expression by Interfering with the cAMP-CREB Pathway in Neuronal Cells

Zilong Liu1,4, Yongping Liu1, Rui Gao1, Haixia Li2, Tiffany Dunn3, Ping Wu3, Robert G. Smith1, Partha S. Sarkar1,3, Xiang Fang1*

1 Department of Neurology, University of Texas Medical Branch, Galveston, Texas, United States of America, 2 Department of Internal Medicine/Gastroenterology, University of Texas Medical Branch, Galveston, Texas, United States of America, 3 Department of Neuroscience & Cell Biology, University of Texas Medical Branch, Galveston, Texas, United States of America, 4 Department of Forensic Medicine, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

Abstract
Alcohol intoxication results in neuronal apoptosis, neurodegeneration and manifest with impaired balance, loss of muscle coordination and behavioral changes. One of the early events of alcohol intoxication is mitochondrial (Mt) dysfunction and disruption of intracellular redox homeostasis. The mechanisms by which alcohol causes Mt dysfunction, disrupts cellular redox homeostasis and triggers neurodegeneration remains to be further investigated. Proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) plays critical roles in regulating Mt biogenesis and respiration, cellular antioxidant defense mechanism, and maintenance of neuronal integrity and function. In this study, we sought to investigate whether alcohol causes Mt dysfunction and triggers neurodegeneration by suppressing PGC-1α expression. We report that ethanol suppresses PGC-1α expression, and impairs mitochondrial function and enhances cellular toxicity in cultured neuronal cell line and also in human fetal brain neural stem cell-derived primary neurons. Moreover, we report that cells over-expressing exogenous PGC-1α or treated with Rolipram, a selective phosphodiesterase-4 inhibitor, ameliorate alcohol-induced cellular toxicity. Further analysis show that ethanol decreases steady-state intracellular cAMP levels, and thus depletes phosphorylation of cAMP-response element binding protein (p-CREB), the key transcription factor that regulates transcription of PGC-1α gene. Accordingly, we found PGC-1α promoter activity and transcription was dramatically repressed in neuronal cells when exposed to ethanol, suggesting that ethanol blunts cAMP→CREB signaling pathway to interfere with the transcription of PGC-1α. Ethanol-mediated decrease in PGC-1α activity results in the disruption of Mt respiration and function and higher cellular toxicity. This study might lead to potential therapeutic intervention to ameliorate alcohol-induced apoptosis and/or neurodegeneration by targeting PGC-1α.

Introduction
Alcohol (ethanol) is a common psychoactive substance, which is causally correlated with more than 60 different medical conditions, particularly the central nervous system disorders and contributes to 4% of the global burden of disease [1]. It has been well-demonstrated that acute and chronic alcohol intoxication results in significant alterations of brain structure and function, and induces neuronal apoptosis and neurodegeneration [2–4]. Alcohol intoxication manifests clinically as impaired balance, loss of muscle coordination and behavioral changes [5,6].

One of the most important underlying mechanisms involved in ethanol-induced neuronal apoptosis and/or neurodegeneration is the disruption of intracellular redox homeostasis [7]. Alcohol increases the productions of intracellular reactive oxygen species (ROS) [8], which has been implicated in the etiology of many alcohol-induced pathological conditions. Mitochondria (Mt) are known to be an important metabolic source of ROS in most cells. Dysfunction of mitochondrial energy metabolism have been described following acute and chronic ethanol exposure, leading to reduced ATP production and exacerbated ROS generation, contributing to increased susceptibility of cellular apoptosis and/or death to oxidative stressors [8,9].

Peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) is a member of transcription co-activator family. It plays important roles in regulating cellular energy metabolism, Mt biogenesis/respiration, cellular antioxidant surveillance mechanism, and maintenance of neuronal integrity and function [10–12]. PGC-1α interacts with a variety of transcription factors that regulate Mt biogenesis, Mt respiration, glucose transport and metabolism, fatty acid oxidation and cellular anti-oxidant properties. For example, PGC-1α protects neuron from H₂O₂-induced injury by up-regulating transcription of several antioxidant genes [11]. Decreased activity of PGC-1α has been implicated in the pathogenesis of numerous neurodegenerative diseases such as Huntington disease, Alzheimer disease, and Parkinson diseases [11,13–15]. Furthermore, PGC-1α is also involved in many
metabolic and traumatic brain diseases [11,16–18]. Acute alcohol exposure has been shown to suppress PGC-1α expression resulting in reduced expression of PGC-1α target genes regulating ROS metabolism contributing to liver injury in vivo animal model [19]. Taken together, we hypothesized that PGC-1α might play an important role in alcohol-induced Mt dysfunction and neurodegeneration.

The purpose of the present study was to investigate whether alcohol inflicts cellular toxicity via suppressing PGC-1α expression and to delineate the mechanism by which alcohol suppress PGC-1α expression. We have used human neuroblastoma SH-SY5Y cells as well as human fetal brain neural stem cell-derived primary neurons to test our hypothesis.

Materials and Methods

Cell culture and alcohol treatment

Two types of human neural cells were used in the study: 1) Human neuroblastoma SH-SY5Y cells, and SH-SY5Y cells stably over-expressing PGC-1α. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro, Manassas, VA, USA) supplemented with 1% nonessential amino acids, 10% fetal bovine serum (FBS, Cellgro), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in 5% CO₂. 2) Human fetal brain neural stem cells (hNSC, line K048) originally provided by Dr. Clive Svendsen were cultured according to our previous published protocol [20]. Cells were expanded as neurospheres in growth medium containing a basic medium supplemented with 20 ng/ml recombinant human epidermal growth factor (EGF), 5 µg/ml heparin, 10 ng/ml recombinant human leukemia inhibitory factor (LIF), and 20 ng/ml recombinant human basic fibroblast growth factor (bFGF) at 37°C with 8.5% CO₂. The basic medium is serum-free and contained DMEM (high glucose, L-lactamine)/Ham’s F12 (3:1), 1.5% D-glucose, 15 mM HEPEs, 25 µg/ml bovine insulin, 67 µl/ml/67 µg/ml penicillin/streptomycin, 100 µM putrescine, 100 µg/ml human transferrin, 30 nM sodium selenite, 20 nM progesterone and 2 mM L-glutamine. The cells were passaged every 10 to 11 days as previously described [21].

Half of the medium was changed to fresh medium after 2 days. For cell differentiation, 5 days after cultured in priming medium, 80% of the medium was changed to a B27 medium containing DMEM/Ham’s F12 (3:1), 1.5% D-glucose, 15 mM HEPEs, 67 µl/ml/67 µg/ml penicillin/streptomycin, and B27 (Invitrogen/GIBCO). Cells in B27 medium were cultured at 37°C with 5% CO₂.

Following an overnight culture in a serum free medium, the cells were incubated in medium containing different concentrations of alcohol at 37°C for 24 h. According to previous studies, 100, 300, 500, and 700 mM of ethanol (Aaper Alcohol and Chemical Co, Shelbyville, KY, USA) were used for our experiments [22–25]. To maintain and stabilize the ethanol concentration in the medium, all cultures were maintained in sealed containers [26].

Cell counting

Cell counting was performed to determine total amount of cells and to ensure that cells were not detached from the surface of the plates due to ethanol treatment [27]. Briefly, the culture medium was carefully aspirated from the well without disturbing the attached cells, and the aspirated medium was examined under microscope to ensure that no cell was floating in the medium or accidentally drawn off. Then 0.30 ml solution containing 0.125% trypsin and 0.05% EDTA and 0.20% Trypan blue in 0.01 M PBS was added to the well after medium removed to detach the cells. The number of both viable (Trypan blue-negative) and dead/dying (Trypan blue-positive) cells was counted using a hemocytometer.

MTS assay

CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA), containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) [28], was used to determine the cell viability and mitochondrial function. In brief, SH-SY5Y cells (1x10⁴ cells/ml) were seeded in 96-well plates. Following overnight growth, at 37°C in humidified chamber containing 5% CO₂, the medium was removed and replaced with 100 µL medium containing 0, 100, 300, 500 or 700 mM ethanol. After 24 hours exposure to ethanol, 20 µL MTS solution (Sigma, St Louis, MO, USA) was added into each well and incubated for 2 to 4 hours. The absorbance was measured at 490 nm using a microplate reader (FluoStar OPTIMA, BMG, USA).

Cell Toxicity (LDH) assay

The release of the cytosolic enzyme LDH into the medium was measured to determine the cellular toxicity due to ethanol treatment according to the instruction provided by the manufacturer (Sigma-Aldrich) [29]. Briefly, the cells were exposed to 0, 100, 300, 500 or 700 mM ethanol with or without 30 µM Rolipram, (Sigma-Aldrich Co., St Louis, MO, USA) [a selective phosphodiesterase-4 inhibitor] in medium for 24 h or 300 mM ethanol for various times. After incubation, the medium was collected for the LDH measurement. The reaction mixtures were kept in dark incubation chamber for 30 min, and then the reactions was terminated with the addition of 1/10 volume of 1 N HCl into each well. Absorbance was measured at 490 nm with a multi plate reader (FluoStar OPTIMA).

Analysis of intracellular ATP levels

The cultured SH-SY5Y cells were treated with alcohol in 24-well plates, intracellular ATP was extracted from 100 µL of cell suspension by adding 1.5% trichloroacetic acid, according to the manufacturer’s recommended protocol (ENLITEN ATP assay system bioluminescence detection kit, Promega, Madison, WI). The extracted ATP solution was neutralized by 0.75 M Tris acetate buffer (pH 7.75) and centrifuged for 5 min at 4,500 x g. The resulting supernatant was used in ATP determination assays. The reaction was initiated by adding 15 µL of the extract to 100 µL of the luciferin-luciferase luminous reagent (Promega) in 96-well white plate. Light output from the reaction was measured with Fluostar OPTIMA well plate reader (BMG, Germany) in luminescence mode. The concentration of the intracellular ATP levels was normalized by total cell counts.

Transient transfections and luciferase assay

The SH-SY5Y cells were transfected with 0.8 µg/well of PGC-1α-delta-CRE-promoter-luciferase reporter plasmid (the CREB-binding sequences were deleted), PGC-1α-promoter-luciferase plasmid (encoding wild type PGC-1α promoter sequences upstream of luciferase reporter gene), PGL negative control plasmid or CMV-luciferase positive control plasmid respectively using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). Cells transfected with the plasmid DNA mixtures were cultured for 48 h. After washing with PBS, the cells were lysed with the lysis buffer (Promega, USA). The cell lysates were mixed with Luciferase Assay Reagent (Promega, USA) in 96-well plate, and the light was measured using a 96-well microplate luminometer (Veritas, Promega, USA) [30].
Figure 1. Time- and dose-dependent effects of ethanol on cellular toxicity in SH-SY5Y cells. The confluent SH-SY5Y cells were treated with 300 mM ethanol for various times (Panel A), or various concentrations of ethanol for 24 hours (Panel B). The cell culture medium was collected, and the LDH release was measured to assess ethanol-induced cellular toxicity. Mean ± SE, n = 4; **P < 0.01 vs. control. doi:10.1371/journal.pone.0104247.g001

Western blot analysis

Total proteins from cells were extracted and quantified with a BCA Protein Quantitative Analysis Kit (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated on 8%–12% SDS-PAGE Gels (Life Technologies, Carlsbad, CA) and subsequently transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with blocking buffer (0.1% Tween 20) for 5 min at room temperature for 30 min and then incubated with antibodies against PGC-1α (1/1000, Santa Cruz Biotechnology, San Diego, CA, USA), CREB (1/1000, Cell Signaling Technology), anti-phospho CREB (p-CREB, 1/1000, Cell Signaling Technology), and β-actin (1/5000, Abcam, Cambridge, MA, USA) in blocking buffer at 4°C overnight. After washing with PBST (PBS and 0.1% Tween 20) for 5 min for 3 times, the membrane was incubated with HRP-conjugated secondary antibody (1/5000) at room temperature for 2 h. The immunoreactive proteins were visualized by chemiluminescent reagent ECL (Pierce Biotechnology). Anti-β-actin antibody was used as loading control.

Effect of Ethanol on PGC-1α Expression

Total RNA from SH-SY5Y cells before and after ethanol treatment was isolated using TRIzol reagent (Invitrogen, USA). Equal amount of total RNA (2 μg) were reversely transcribed using the SuperScript-II RT preamplification system (Invitrogen). Quantitative RT-PCR amplification (qRT-PCR) was carried out using specific primer pairs designed with Oligo Calculator and synthesized by IDT (MI, Brazil). Quantitative PCRs were carried out in an Applied-Biosystem StepOne Plus real-time cycyler and done in quadruplicate. The PCR program was used as follows: 5 min at 95°C; 30 cycles of 5 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C; and a final extension step of 10 min at 72°C. The following primer pairs were designed using the primer 5.0 software: PGC-1α forward 5'-CAAGTCTCCAGGCAGTA-3', reverse 5'-CACAGGTGGTAGGT-3', and β-actin forward 5’-CAACTGGGACGATATGGAGAAG-3', reverse 5’-TCTCCTTCTGCA TCCCTCAG-3’. β-actin served as an internal control and for normalization. Expression of Mitochondrial uncoupling protein genes (UCP2 and UCP3), the PGC-1α target genes, were also measured by qRT-PCR. The UCP2 and UCP-3 primers were purchased from Qiagen (CA, USA).

Construction of plasmid DNA and transfection

The full length PGC-1α cDNA was PCR-amplified from plasmid pcDNA4-myc-PGC-1α-alpha (kindly provided by Dr. Toren Finkel, Addgene plasmid 10974) [31] using appropriate primers and the full-length PGC-1α cDNA was sub-cloned in plasmid pCDA3.1-hygro (Invitrogen, USA) to construct pcDNA-hygro-PCG-1α. The sequence integrity of PGC-1α cDNA in the expression plasmid was verified by sequencing. Plasmid DNA expressing PGC-1α was transfected into SH-SY5Y cells using Lipofectamine 2000 reagent (Invitrogen, USA) and the positive clones were selected with hygromycin (Invitrogen, USA). The effective expression of PGC-1α in the stable clones was verified by Western blot analysis using anti-PCG-1α antibody (Santa Cruz Biotechnology, USA). Plasmid DNA encoding 2 kb of the wild type PGC-1α promoter sequences (PGC-1α promoter 2 kb luciferase) and plasmid DNA wherein the CRE sequences in the PGC-1α promoter were deleted (PGC-1α promoter luciferase delta CRE) were kind gifts from Dr. Bruce Spiegelman (Addgene plasmids 8888 and 8887) [32].

Statistical analysis

All data are expressed as mean ± SE from at least three independent experiments. Comparisons of quantitative data were performed using one-way analysis of variance (ANOVA) followed by Tukey-test for multiple treated group. The difference between two treated groups was analyzed by student t-test. A P-value less than 0.05 was considered as significant.

Results

Ethanol-induced cellular toxicity in human neuroblastoma SH-SY5Y cells

To assess the extent to which alcohol causes cellular toxicity in neuronal cells, we treated human neuroblastoma SH-SY5Y cells with ethanol and assayed the alcohol-induced cellular toxicity using the Cell Toxicity Assay Kit (Sigma, USA). Previous studies showed that the high concentrations of alcohol are needed to produce cellular toxicities [22–25]. To assess cellular toxicity, the SH-SY5Y cells were incubated with cell culture medium containing 100, 300, 500 and 700 mM of ethanol for 24 hrs, or 300 mM ethanol for various times, and cell toxicity as well as cell viability was measured. The cellular viability was significantly...
decreased in all ethanol treatment groups compared to control group following 24 hrs of ethanol exposure (data not shown). Treatment of the SH-SY5Y cells with 300 mM ethanol produced a time-dependent and robust cytotoxicity as measured by LDH release by the ethanol-treated cells. Our data show that the amount of LDH release was increased by 10-folds when treated with ethanol for 72 hrs (Figure 1A). We also found a dose-dependent increase in cell toxicity (LDH release) when cells were exposed to increasing alcohol concentrations. Compared to untreated control group, the LDH release from the ethanol-treated cells was increased by 86% with 100 mM ($P = 0.009$ vs. control), 110% when treated with 300 mM ($P = 0.002$ vs. control), 254% when treated with 500 mM ($P = 0.0004$ vs. control) and 362.0% when treated with 700 mM ($P = 0.0002$ vs. control) ethanol (Figure 1B). Consistent with the LDH release data, the cell viability was also decreased by 86% with 100 mM ($P = 0.02$ vs. control), 75% with 300 mM ($P = 0.0002$ vs. control), 63% with 500 mM ($P < 0.0001$) and 59% with 700 mM ($P < 0.0001$) ethanol treatment compared with the control groups.

**Ethanol suppresses PGC-1α expression and blunts mitochondrial function**

We next investigated the possible mechanism by which ethanol causes cellular toxicity and impairs Mt function. First, we sought to test whether alcohol impairs Mt respiration and suppresses PGC-1α expression. To test this idea, we treated SH-SY5Y cells with varying concentrations of ethanol and assessed Mt function using MTS assay, a versatile method to measure Mt function [2]. MTS assay measures the activity of Mt NAD(P)H-dependent cellular oxidoreductase enzyme succinic dehydrogenase. As shown in Figure 2A, ethanol treatment of the SH-SY5Y cells significantly decreased the MTS absorbance in a concentration-dependent manner. The MTS absorbance was decreased at least one-third in presence of 500 mM ethanol (Figure 2A). Since one of the major Mt functions is ATP production, we measured intracellular ATP production in SH-SY5Y cells treated with various concentration of ethanol for 24 hrs. Similar to MTS measurement, ethanol also produced a dose-dependent inhibition in intracellular ATP production (Figure 2B). The ATP production by SH-SY5Y cells was significantly suppressed at least ~70% in the presence of 500 mM ethanol as compared with control group (Figure 2B).

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**Figure 2. Concentration-dependent effect of ethanol on mitochondrial function, and expression of the PGC-1α in SH-SY5Y cells.** The SH-SY5Y cells were treated with various concentration of ethanol for 24 hours, and MTS activity and intracellular ATP level were measured to assess mitochondrial function (Panel A & B). Total protein was isolated from the ethanol-treated and control cells and expression of PGC-1α was measured by Western blot analysis, and a representative Western blot is shown in Panel C. The protein density in each Western blot was quantitatively analyzed, and is shown in Panel D. Mean ± SE, n = 3, *$P < 0.05$ vs. control, **$P < 0.01$.
doi:10.1371/journal.pone.0104247.g002
Since PGC-1α is a key regulator of the Mt biogenesis and function [10–12], we tested whether ethanol suppresses expression of PGC-1α. To test this idea, the SH-SY5Y cells were incubated with various concentrations of ethanol for 24 hours, and the steady-state levels of PGC-1α were measured by Western blot using a specific antibody against human PGC-1α. The Western blot data showed that ethanol decreased levels of PGC-1α protein in a dose-dependent manner (Figure 2C). The protein density analysis indicated that 500 mM and 300 mM ethanol decreased expression of PGC-1α protein by 66% \( (P = 0.006) \) and 26% \( (P = 0.03) \), respectively when the SH-SY5Y cells were treated with ethanol for 24 hrs (Figure 2D). Furthermore, we also tested whether ethanol affects expression of PGC-1α target genes. Expression of the UCP-2 and UCP-3 mRNA in the SH-SY5Y cells treated with or without ethanol was analyzed by qRT-PCR. We found that ethanol decreased expression of the UCP-2 and UCP-3 by \( \sim 70\% \) when the cells were treated with 500 mM ethanol for 24 hrs.

**Ethanol reduces PGC-1α levels by repressing transcription of PGC-1α**

To determine whether ethanol suppresses expression of PGC-1α at the transcriptional level, we first determined the effects of ethanol on PGC-1α promoter activity in the ethanol-treated cells. The PGC-1α promoter reporter plasmid was constructed as described in the methods and materials section. Our results showed that treatment of cells with 500 mM ethanol inhibited
PGC-1α promoter activity by 83% when cells were incubated with ethanol for 24 hours (Figure 3A). Consistent with this data, we also found ethanol suppressed PGC-1α mRNA levels by ~80% when the cells were exposed to 500 mM ethanol (Figure 3B).

These results suggested that the ethanol suppress PGC-1α expression by inhibiting its transcription.

Ethanol suppresses PGC-1α transcription by reducing phosphorylation of CREB

Several transcriptional factors have been identified that regulate PGC-1α transcription. For example, phosphorylated cAMP-responsive element binding protein (p-CREB) has been shown to bind the CRE consensus sequences proximal to the PGC-1α promoter and regulate PGC-1α transcription [33,34]. Therefore, we tested whether ethanol suppresses transcription of PGC-1α by repressing phosphorylation of CREB. To test this idea, the SH-SY5Y cells were incubated with cell culture medium containing various concentrations of ethanol for 24 hrs. After incubating the cells with ethanol, cells were harvested and the cell extracts were analyzed to assess the expression levels of CREB and p-CREB by Western blot analysis using specific antibodies against CREB and p-CREB. As shown in Figure 4A, ethanol treatment significantly decreased the steady-state levels of p-CREB, while levels of total CREB remained unchanged after ethanol treatment for 24 hrs. The protein density analysis indicated that level of p-CREB was significantly suppressed by ethanol in a dose-dependent manner (Figure 4B).

To further investigate the possible role of CREB pathway in the regulation of ethanol-mediated suppression of PGC1-α, we tested whether ethanol inhibits the promoter activity of PGC-1α. We used two plasmids for the promoter activity assay; plasmid PGC-1α-promoter-2 kb-luciferase [26], where the wild type PGC-1α promoter sequences (~2 kb) was cloned upstream of reporter luciferase; and plasmid PGC-1α-promoter-luciferase-delta CRE, wherein the CREB-binding CRE sequences were deleted...
The delta-CRE-PGC-1α promoter plasmid and PGC-1α-promoter-2 kb-luciferase control plasmid, was transfected into alcohol-treated and untreated SH-SY5Y cells and luciferase activity was measured. We found that PGC1-α promoter-luciferase activity was, 70% decreased in cells transfected with wild type PGC-1α promoter plasmid; and about 40% decreased compared with the cells transfected with delta-CRE-PGC-1α promoter plasmid when the cells were treated with 500 mM ethanol for 24 hrs. This data suggests that CREB-binding sequences are important in alcohol-induced suppression of PGC-1α transcription, and indicates that cAMP → CREB signaling pathway might be affected by alcohol. To test this hypothesis, we investigated the possible mechanism by which ethanol decreases CREB phosphorylation. Since cAMP levels play an important role in regulating phosphorylation and activity of CREB, we tested whether ethanol decreases intracellular cAMP levels. As shown in Figure 5, our data showed that ethanol produced a dose-dependent decrease in the steady-state intracellular cAMP levels. We observed a ~70% reduction of intracellular cAMP when cells were treated with 500 mM ethanol compared with untreated control. These results suggest that alcohol suppresses CREB phosphorylation and expression of the PGC-1α, at least in part, by...

Figure 6. Over-expression of PGC-1α protect SH-SY5Y cells from ethanol-induced cellular toxicity. The SH-SYSY cell line constitutively over-expressing PGC-1α was treated with various concentrations of alcohol and cellular toxicity (relative cell viability: panel A; LDH release: panel B) was measured as described in Figure 1. Mean ± SE, n = 4, *P<0.05, **P<0.01 vs. control.
doi:10.1371/journal.pone.0104247.g006
decreasing intracellular cAMP levels and thus by interfering with the cAMP-CREB-dependent signaling pathway.

Moreover, recent studies have shown that the Zn-finger transcriptional repressor protein ZNF746, also known as PARIS plays an important role in regulating PGC-1α transcription [15]. This study shows that PARIS acts as transcriptional repressor which binds with the insulin response sequences (IRS) present in the PGC-1α promoter sequence and suppresses PGC-1α transcription. Since alcohol dramatically decreased PGC-1α expression, we therefore tested whether ethanol alters expression of PARIS in neuronal cells and contributes to the suppression of PGC-1α expression. The Western blot analysis of the alcohol-treated cells showed that ethanol indeed increased the expression of PARIS by 2 fold when cells were exposed to higher concentration of ethanol (300 mM) (Figure 4A & C). These data suggest that alcohol not only decreases cAMP levels and CREB activity but also stimulates PARIS expression to suppress PGC-1α expression.

**Over-expression of PGC-1α ameliorated ethanol-induced cellular toxicity in SH-SY5Y cells**

Since ethanol suppressed expression of PGC-1α, and PGC-1α has protective effects against oxidative stress-induced cellular injury [11], we next tested whether over-expression of PGC-1α protects against alcohol-mediated cellular toxicity. To test this idea, we treated a SH-SY5Y stable cell line ectopically expressing PGC-1α and control SH-SY5Y cells with various concentrations of ethanol for 24 hrs, and assessed the cell viability and cell toxicity (LDH activity) in the treated and control untreated cells. Our data show that the stable cells over-expressing PGC-1α showed significantly higher resistance to alcohol-induced toxicity (Figure 6). The cell viability was also significantly improved when cells over-expressing PGC-1α were treated with 500 mM and 700 mM ethanol (P = 0.003 and 0.02, respectively) (Figure 6A). The protective effect of PGC-1α against ethanol-induced cytotoxicity was further demonstrated as the LDH release in the culture medium was significantly decreased in the group expressing PGC-1α. The cell toxicity (as assessed by LDH release) was decreased by about 65% when the cells expressing PGC-1α were treated with 500 mM and 700 mM ethanol as compared with the cells expressing control plasmid (P = 0.0003 and 0.02, respectively) (Figure 6B).

**Protective effect of Rolipram on ethanol-induced cellular toxicity and suppression of PGC-1α expression in SH-SY5Y cells**

Since ethanol suppresses expression of PGC-1α through a cAMP-pCREB dependent mechanism, then we tested whether increased intracellular cAMP can protect the cells from alcohol-induced cellular toxicity, and reverse alcohol-mediated suppres-
sion of PGC-1α expression using a pharmacological approach. Rolipram, a selective phosphodiesterase (PDE)-4 inhibitor, has been shown to increase intracellular cAMP level in SH-SY5Y neuronal cells [35]. The cells were treated with or without 30 μM Rolipram in the presence of 300 mM ethanol for different times, or various concentrations of ethanol for 12 hrs. The amount of LDH in the medium and expression of PGC-1α protein in the cells were determined as described in the section of Methods. As shown in Figure 7, Rolipram provided significant protective effects against alcohol-induced cellular toxicity in a time- and dose-dependent manner as compared with ethanol alone group. 30 μM Rolipram decreased the LDH release by ~25 to 30% when the cells were exposed to 100 to 500 mM ethanol (Figure 7A & B). Furthermore, Rolipram also reversed ethanol-induced suppression of PGC-1α protein (Figure 7C). The protein density analysis indicated that expression of the PGC-1α was almost back to the baseline when the cells were treated with 30 μM Rolipram in the presence of 300 mM ethanol (Figure 7D).

**Ethanol suppressed PGC-1α and decreased CREB phosphorylation in human fetal brain neural stem cell (hNSC)-derived primary neurons**

Since alcohol inhibits neurogenesis in a CREB dependent mechanism [2], we tested the effect of ethanol on PGC-1α expression in hNSC-derived primary neurons. Similar to the data obtained using the SH-SY5Y cells, the ethanol treatment also produced a concentration-dependent toxicity in the hNSC-derived primary neurons (data not shown). We next tested whether ethanol suppressed expression of PGC-1α in the primary neurons. As shown in Figure 8, ethanol significantly inhibited the expression of PGC-1α in a dose-dependent manner (Figure 8A & B). Similarly, ethanol also suppressed phosphorylation of CREB, whereas total CREB levels remained unaltered upon ethanol treatment (Figure 8A & C). These data strongly support our hypothesis that alcohol can suppress PGC-1α transcription via interfering with the cAMP-CREB signaling pathway.

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**Figure 8.** Dose-dependent effect of ethanol on the expression levels of PGC-1α, CREB, and p-CREB in human fetal brain neural stem cell-derived primary neurons. The primary neuronal cells were incubated with various concentrations of ethanol for 24 hrs, and after incubation with ethanol, the cells lysates were analyzed by Western blot to determine expression levels of PGC-1α, CREB, and p-CREB (Panel A). β-actin was used as loading control. The protein density in each Western blot was quantitatively analyzed, and is shown in Panels B and C. Mean ± SE, n = 3, *P<0.05, **P<0.01 vs. control. doi:10.1371/journal.pone.0104247.g008
Discussion

In the present study, we report that ethanol 1) produces a dose- and time-dependent cellular toxicity as well as blunts Mitochondrial (Mt) respiration; 2) interferes with the PGC-1α promoter activity and thus suppresses expression of PGC-1α; 3) suppresses expression of the PGC-1α target genes UCP2 and UCP3; 4) diminishes CREB phosphorylation via reducing intracellular cAMP levels; 5) increases expression of Zn-finger protein Paris in SH-SY5Y cells; 6) decreases expression of PGC-1α and phosphorylation of CREB in hNSC-derived primary neurons. We also find that 1) overexpression of PGC-1α protects neuronal cells from alcohol-induced cell damage; 2) inhibition of PDE-4 by Rolipram protects ethanol-induced cellular toxicity and reverses ethanol-induced suppression of PGC-1α expression.

Mt dysfunction and disruption of intracellular redox homeostasis play important roles in alcohol-induced neurodegeneration [2]. Previous studies have shown that alcohol increases intracellular ROS level, produces mitochondrial damage which provokes mitochondrial-mediated apoptosis, and neuron death [36,37]. It is well-demonstrated that disruption of intracellular redox status causes impairment of function of proteins, lipids, nucleic acids, and triggers cascades of ROS-dependent signaling that activates programmed cell death [38]. Various antioxidants/ROS scavengers have been found to provide protective effects against alcohol-induced cell damage in in vivo and in vitro models [7,37,39–41]. Our unpublished data also showed that ethanol increased intracellular ROS levels, and NADPH oxidase-dependent superoxide production in SH-SY5Y cells (unpublished observation). Our present findings show that alcohol dramatically impairs Mt function (Figure 2) in SH-SY5Y cells. Considering a crucial role of PGC-1α in the regulation and maintenance of intracellular redox status and mitochondrial function, we tested the effects of ethanol on the expression of PGC-1α. Our data clearly show that ethanol decreases the expression of PGC-1α in a dose-dependent manner via suppressing PGC-1α promoter activity. These results suggest that ethanol regulates expression of PGC-1α at the transcriptional level. Consistent with our observation, alcohol has been shown to suppress expression of PGC-1α in H4-IIE cells and hepatic tissues [16]. Many PGC-1α target genes that are involved in regulation of mitochondrial biogenesis, muscle integrity, ROS detoxification, fatty acid metabolism, and insulin sensitivity have been identified. UCPs are those PGC-1α target genes that regulate mitochondrial transmembrane proton leakage, and ATP synthesis. We also found that the expression of UCP-2, and UCP-3 genes was downregulated when the SH-SY5Y cells were treated with ethanol.

Several signaling pathways have been implicated in the regulation of PGC-1α transcription, e.g., CaMK, calcineurin A, PKC, and cAMP-CREB pathways [42–45]. cAMP is a second messenger derived from adenosine triphosphate (ATP) by a reaction catalyzed by adenylyl cyclase. Although our data clearly show that alcohol decreases steady-state cAMP levels, and we are yet to establish whether alcohol decreases cAMP levels by mitigating activity of adenylyl cyclase. It is also possible that alcohol might stimulate activity of phosphodiesterase to catalyze degradation of cAMP and thus reduces steady-state cAMP levels. Clearly additional experiments are necessary to further establish how alcohol decreases cAMP levels. Activation of the cAMP-CREB pathway results in the phosphorylation of CREB (p-CREB), and p-CREB binds the CREB-response element (CRE), located proximal to the PGC-1α promoter, and activates its transcription [46]. It has been reported that p-CREB stimulates the expression of PGC-1α by interacting with the consensus CRE sequences in the PGC-1α promoter region in neuronal cells [13]. Recent study revealed a suppressive effect of alcohol on CREB.
activity, which might be involved in ethanol-induced cell damage [47]. In the present study, we also found that ethanol produced a dose-dependent suppression of phosphorylation of CREB in SH-SY5Y neuronal cells; whereas total CREB levels remained unchanged upon alcohol treatment. Furthermore, using mutated ΔCRE binding site at PGC-1α promoter, inhibitory effect of ethanol on PGC-1α promoter activity was significantly decreased suggesting that suppression of PGC-1α expression by alcohol is at least partly mediated via a CREB-dependent pathway.

The fact that deletion of CRE binding sequences proximal to the PGC-1α promoter sequences did not completely restore the inhibitory effect of ethanol on PGC-1α promoter activity suggests that additional transcriptional regulatory factors might also play an important role in alcohol-mediated suppression of PGC-1α transcription. Recently, the Zn-finger protein PARIS, also known as Znf146 has been identified which suppresses transcription of PGC-1α by binding to the insulin response sequence (IRS) present in the proximal region of the PGC-1α promoter sequences [15,48]. The activity of both human and mouse PGC-1α promoters is markedly decreased by PARIS, suggesting that the transcriptional repressor PARIS might play an important role in neurodegenerative disorders [15,49]. Shin et al [15] reported that PARIS is differentially expressed in the brain and is localized to neurons, the over-expression of PARIS leads to the loss of neurons mediated via repression of PGC-1α. Thus, we tested whether increased PARIS expression might also contribute to the ethanol-mediated expression of PGC-1α. When SH-SY5Y cells were treated with ethanol, expression of PARIS was significantly increased, concurrently with the decrease in PGC-1α expression. However, it is not clear how ethanol stimulates PARIS expression, and we are currently investigating the possible mechanisms by which ethanol increases PARIS expression. Our present findings thus suggest that ethanol suppresses transcription of PGC-1α by interfering with multiple but independent signaling pathways.

Previous studies have suggested that PGC-1α has neuroprotective effects against oxidative stress [11,19]. Since ethanol suppressed PGC-1α expression, we determined whether over-expression of PGC-1α can protect the neuronal cells from ethanol-induced damage. Our findings showed that over-expression of PGC-1α significantly prevented ethanol-mediated cell cytotoxicity as measured by cell viability and LDH release by the ethanol-treated SH-SY5Y cells. Consistent with the previous findings [11,19], we found that over-expression of PGC-1α also decreased the level of intracellular ROS when the cells were treated with ethanol (unpublished observations). It is likely that the antioxidant properties of PGC-1α might contribute to its anti-apoptotic and neuroprotective actions. However, additional effects such as the prevention of alcohol-induced dysfunction of mitochondrial energy metabolism [10–12] that can potentially contribute to the neuroprotective actions of PGC-1α still need to be further investigated.

Since PGC-1α is regulated by a cAMP-CREB dependent signaling pathway in ethanol-treated neuronal cells, an alternative approach is to determine whether inhibition of cAMP decomposition can protect the neuronal cells from ethanol-induced cellular damage, and reverse ethanol-mediated suppression of PGC-1α. Intracellular cAMP is further metabolized by a process catalyzed by PDE in most mammalian cells. Rolipram, a selective PDE-4 inhibitor, has been shown to increase intracellular cAMP in SH-SY5Y cells, and improve survival of spiral ganglion neurons [35,50]. Furthermore, PDE-4 inhibitors also increase the survival of dopaminergic neurons in primary cell culture [51]. We found that elevated intracellular cAMP by inhibiting PDE-4 can diminish ethanol-mediated suppression of PGC-1α expression, and therefore, protect the neuronal cells from ethanol-induced cell damage. These results further support our hypothesis that PGC-1α plays an important role in alcohol-induced pathogenesis in neuronal cells.

Furthermore, we found that ethanol suppressed expression of PGC-1α as well as reduced phosphorylation of CREB in hNSC-derived primary neuronal cells. Altered levels of CREB phosphorylation is implicated in neurodegeneration and neurogenesis during alcohol intoxication and abstinence. For example, regeneration of brain is related to increased CREB transcription, increased neurogenesis and cell genesis during alcohol abstinence [2]. Since mitochondrial dysfunction is involved in many neurodegenerative diseases and aging [52], and PGC-1α is a key regulator of mitochondrial function, our findings suggest that CREB target PGC-1α might play an important role in alcohol-induced process of neurodegeneration.

In conclusion, our study clearly shows that alcohol suppresses transcription of PGC-1α in both SH-SY5Y cells as well as in hNSC-derived primary neurons. Alcohol-induced suppression of PGC-1α might contribute to alcohol-medicated cell damage, and neurodegeneration that are commonly associated with alcohol overuse and alcoholism. Suppression of PGC-1α transcription by ethanol is mediated by interfering with the CREB and PARIS signaling pathways in these neurons. The proposed mechanisms for ethanol-mediated regulation of PGC-1α expression and neurodegeneration are illustrated in Figure 9. The fact that over-expression of PGC-1α has protective effects against alcohol-induced neuronal damage suggests that modulation of PGC-1α expression can be a novel therapeutic approach for the treatment of alcohol-induced apoptosis and/or neurodegeneration.

Acknowledgments

Authors thank Raymond Fang and Hongxia Pei for their technical assistances.

Author Contributions

Conceived and designed the experiments: XF ZLL PSS. Performed the experiments: ZLL YPL RG TD. Analyzed the data: XF PSS ZLL HXL. Contributed reagents/materials/analysis tools: PW RGS. Wrote the paper: XF ZLL PSS.

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Effect of Ethanol on PGC-1α Expression