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Implications of ER Stress, the Unfolded Protein Response and Pro-and Anti-apoptotic Protein Fingerprints in Human Monocyte Derived-Dendritic Cells treated with Alcohol

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Abstract

Background—Dendritic cells (DC) are responsible for the activation of T cells and B cells. There is accumulating evidence that psychoactive substances such as alcohol can affect immune responses. We hypothesize that this occurs by modulating changes in proteins triggering a process known as unfolded protein response (UPR). This process protects cells from the toxic effects of misfolded proteins responsible for causing endoplasmic reticulum (ER) stress. Although much is known about ER stress, little is understood about the consequences of ethanol use on DC's protein expression.

Methods—In the present study, we investigated alterations in proteins of human monocyte derived-dendritic cells (MDDC) treated with 0.1% of alcohol by two-dimensional (2D) gel electrophoresis followed by liquid chromatography-tandem mass spectrometry, protein identification, and confirmation at the gene expression level by qRT-PCR.

Results—Proteomes of related samples demonstrated 32 differentially expressed proteins that had a two-fold or greater change in expression (18 spots were up-regulated and 14 were down-regulated), compared to the control cultures (untreated cells). Alcohol significantly changed the expression of several components of the UPR stress-induced pathway that include chaperones, ER stress, antioxidant enzymes, proteases, alcohol dehydrogenase, cytoskeletal and apoptosis regulating proteins. qRT-PCR analyses highlighted enhanced expression of UPR and antioxidant genes that increased (18h) with alcohol treatment.

Conclusion—Results of these analyses provide insights into alcohol mechanisms of regulating DC, and suggest that alcohol induced specifically the UPR in DC. We speculate that activation of a UPR by alcohol may protect the DC from oxidant injury but may lead to the development of alcohol-related diseases.

Keywords

Dendritic cells; ethanol; Unfolded Protein Response; Mass Spectrometry; Proteomics

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Introduction

Alcohol is a potent immunosuppressive agent and has been recognized for many centuries as an important risk factor for the development of infections. Excessive alcohol use can be linked to direct immunomodulatory effects in several types of immune cells resulting in reduced T cell proliferative responses and impaired delayed-type hypersensitivity responses (Nelson and Kolls, 2002; Szabo and Mandrekar 2009). Both acute and chronic alcohol consumption have significant immunomodulatory effects of which alterations in innate immune functions contribute to impaired antimicrobial defense and inflammatory responses.

Among the immune cells, dendritic cells (DC) are bone marrow-derived leukocytes, that are responsible for the uptake, processing, and presentation of antigens (Ag) to T cells and serve as the first line of defense against infection. DCs express various co-stimulatory and/or adhesion molecules, produce regulatory cytokines, and activate naive T cells in a primary antigenic response. Thus, DCs play a central role in coordination of both innate and adaptive immune responses and are pivotal in activation of T lymphocytes in an antigen-specific manner.

One way that alcohol affects immune responses is by modulating Ag-specific T cell proliferation by affecting Ag-presenting cell (APC) function. Considering that monocytes are precursors of Ag-presenting dendritic cells, it is known that alcohol prevents monocyte-derived cell differentiation and maturation (Buttari et al., 2008). DCs are unique in their ability to provide Ag-specific activation of naïve T cells, and their functions are negatively impacted by acute and chronic alcohol exposure in humans (Laso et al., 2007). Impaired function of these key APCs may contribute to reduced adaptive immune responses and increased susceptibility to infections, particularly when acute alcohol intake coincides with exposure to pathogens. On the other hand, other reports have shown that DCs are less efficient than macrophages in Ag processing, suggesting the possibility of unique pathways and requirements for Ag processing and presentation by these two different cell types (Fonteneau et al., 2003).

Products of the alcohol oxidation metabolic pathway result in increased levels of reactive oxygen species (ROS) (Shiu et al., 2007). Recent studies (Sozio and Crabb, 2008) indicate that ROS interfere with protein folding in the ER and elicit a compensatory response termed the UPR. The importance of the UPR lies in its ability to alter the expression of a variety of genes involved in antioxidant defenses, inflammation, energy metabolism, protein synthesis, apoptosis, and cell cycle regulation.

In the present study, we assessed the effects of in vitro alcohol treatment on DCs by a comparative proteomic analysis and a gene expression analysis by qRT-PCR to test the hypothesis that alcohol treatment induces a UPR in DC. We found that *in vitro* alcohol treatment primarily affected key protein differential expression. This was mediated through modulation of ER and oxidative stress proteins, proteosomal proteins and other key proteins that may play a significant role in cell survival anti-apoptotic types of responses. These new findings may open new and significant avenue of research to detect potential biomarkers of therapeutic efficacy in alcohol-related diseases.

Materials and Methods

Isolation of monocytes

Human leukopacks were obtained from community blood centers of South Florida, Inc. Monocytes were isolated from the leukopack using the RosetteSep human monocyte enrichment cocktail (StemCell Technologies, NA, USA). The cocktail contains a

combination of mouse and rat monoclonal antibodies directed against CD2, CD3, CD8, CD19, CD56, CD66b cells and glycophorin A antigens on red blood cells (RBCs). It crosslinks unwanted cells in human whole blood to multiple RBCs, forming immunorosettes which pellet along with the free RBCs when centrifuged over Ficoll-Paque. Monocytes were collected at the interface between the plasma and Ficoll-Paque. All experiments were performed in biological and technical triplicates starting with the collection of monocytes from three different leukopacks.

Generation of monocyte-derived dendritic cells (MDDC)

Monocytes were cultured at a concentration of 1×10^6/mL in complete RPMI medium containing 20 ng/ml of recombinant human GM-CSF and 20 ng/ml of recombinant IL-4 (R&D systems, Inc., Minneapolis, USA) for 6 days. On alternate days, 1 mL medium was replaced with fresh medium containing GM-CSF and IL-4 at a concentration of 20 ng/mL. On day 6 the cells were positive for the expression of DC markers, CD40, CD80 and CD86.

Treatment of MDDC with alcohol

MDDC were cultured at a concentration of 5×10^5 cells/ml with ethanol (0.1% v/v) in 6 well plates. The source of ethanol used in the present experiments was Sigma Chemical Co (Cat# 7023). For proteomic analysis, MDDC were treated with ethanol (0.1%) and the protein profile was studied at the 24h time point, whereas gene expression studies were performed at the 18h time point.

Quantitative real time PCR

RNA from cell pellets was extracted using an RNAeasy mini kit (Qiagen, Valencia, CA) followed by cDNA synthesis using high capacity reverse transcriptase cDNA kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using the Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) for genes, ER 60 (Assay ID Hs00607126_m1), 78Kda Glucose Regulated Protein (Assay ID Hs99999174_m1) Cathepsin D (Assay ID Hs00157201_m1), Superoxide dismutase (Assay ID Hs00167309_m1) Vimentin (Assay ID Hs00185584_m1) and Pyruvate Kinase (Assay ID Hs00761782_s1). GAPDH (Assay ID Hs99999905_m1) served as an internal control. The relative abundance of each mRNA species was assessed using the SYBR green master mix from Stratagene (La Jolla, CA) to perform real-time semi-quantitative PCR using M×3000P instrument that detects and plots the increase in fluorescence vs. PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of the initial target in each PCR amplification, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each the amplification plot of each sample reaches the threshold (defined as the threshold cycle number (C_T). Differences in C_T are used to quantify the relative amount of PCR target contained within each tube. The mean fold change in expression of the target gene was calculated using the comparative C_T method (TAI=$2^{-\Delta\Delta CT}$). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control, untreated sample.

Two-dimensional gel electrophoresis

Two-hundred and fifty micrograms of protein from purified DCs were solubilized for 30 min with 2D rehydration/sample buffer (7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris) and 2% Bio-Rad IPG buffer, pH 3-10. Bio-Rad 11 cm ReadyStrip pH 3-10NL IPG strips were used to separate proteins according to charge. Solubilized DC proteins were adsorbed

on to the gel strip overnight and were then focused according to their isoelectric point with the Bio-Rad Protean IEF System. The program used was the following: 250 V rapid voltage ramping for 30 min, 10,000 V slow voltage ramping for 60 min, and 10,000 V rapid voltage ramping for 50 kV hours. The strips were incubated first in Equilibration buffer I with 6 M urea, 20% glycerol, 2% SDS, 2% DTT, and 0.375 M Tris, pH 8.8, for 10 min at room temperature, then in Equilibration buffer II with 6 M urea, 20% glycerol, 2% SDS, 2% iodoacetamide and 0.375 M Tris, pH 8.8. The strips were then loaded onto 12% SDS-PAGE gels and run at 50 V overnight to complete the second dimension of protein separation. ReadyPrep Overlay Agarose was added on top of the strip to secure it and included Bromophenol blue tracking dye. A molecular standard was used to estimate relative mass (M_r). Gels were pre-rinsed with water, and then stained overnight with Biosafe Coomassie blue stain, as stated in the manufacturer's protocol. Gels were distained in water and scanned with the Versadoc Model 1000 system (Bio-Rad, CA). Gel image analyses were performed with PD Quest software (Bio-Rad) version 7.4.0.

Protein identification by Peptide Mass Fingerprinting

Protein spots were excised and tryptic digests were analyzed at the University of Texas Medical Branch Proteomics Core Facility by LC-MS/MS as described previously (Gallaher et al, 2006). Briefly, protein spots from 2D gel were distained with 50% acetonitrile in 50 mM ammonium carbonate. In-gel tryptic digest was performed using reductively methylated trypsin (Promega, Madison, WI). Before digestion, samples were reduced with DTT (10 mM in 50 mM ammonium carbonate for 60 min at 56°C) and subsequently alkylated with iodoacetamide (55 mM in 50 mM ammonium carbonate for 45 min in the dark at room temperature). The digestion reaction was performed overnight at 37°C. Digestion products were extracted from the gel with a 5% formic acid/50% acetonitrile solution (2×) and one acetonitrile extraction followed by evaporation using an APD SpeedVac (Thermo Savant). The dried tryptic digest samples were cleaned with ZipTip (C_{18}; Millipore) before analysis by tandem mass spectrometry for protein identification. The digested sample was re-suspended in 10 μl of 60% acetic acid, injected via autosampler (Surveyor; ThermoFinnigan, San Jose, CA) and subjected to reverse phase liquid chromatography using a ThermoFinnigan Surveyor MS-Pump in conjunction with a BioBasic-18 100 × 0.18 mm reverse-phase capillary column (ThermoFinnigan). Mass analysis was done using a ThermoFinnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with a nanospray ion source using a 4.5 cm long metal needle (Hamilton; 950–00954) in a data-dependent acquisition mode. Electrical contact and voltage application to the probe tip took place via the nanoprobe assembly. Spray voltage of the mass spectrometer was set to 2.9 kV and heated capillary temperature at 190°C. The column was equilibrated for 5 min at 1.5 μl/min with 95% solution A and 5% solution B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) before sample injection. A linear gradient was initiated 5 min after sample injection ramping to 35% A and 65% B after 50 min and 20% A and 80% B after 60 min. Mass spectra were acquired in the mass-to-charge ratio 400–1800 range. Protein identification was performed with the MS/MS search software Mascot 1.9 (Matrix Science, Boston, MA) and confirmatory or complementary analyses were done with TurboSequest as implemented in the Bioworks Browser 3.2, build 41 (ThermoFinnigan) (Gallaher et al, 2006).

Results

Alcohol induced changes in MDDC proteome

To facilitate quantitative detection and to maximize identification of changes in the proteome induced by alcohol treatment, MDDC lysate were subjected to a 2D-gel electrophoresis LC-MS/MS proteomic analysis. Since it was not feasible to sequence all

resolved proteins, the selection criteria for sequencing was based on gel image quantitative analysis of the 2D analysis (Fig.1) with the goal of obtaining a broad scope of the proteins regulated and un-regulated by alcohol-treatment. Of 421 protein spots detected by gel image analyses, a total of 217 protein spots met the criterion for a two-fold or greater change in expression relative to control. Of these, 57 proteins were increased in expression after alcohol treatment, as measured via Biosafe Coomassie blue staining and imaging analyses. Conversely, 160 spots in the alcohol group were identified as having decreased expression relative to control. For identification by LC-MS/MS peptide sequencing, 32 differentially expressed spots that were significant at $p<0.05$ were selected from two categories: 1) alcohol-induced up-regulation (18 spots), 2) alcohol-induced down-regulation (14 spots), Figures 1 and 2 and Table 1. LC-MS/MS and Mascot database matching resulted in successful identification of 32 different proteins listed in Table 1. The proteome profile of alcohol-treated MDDC encompassed main functional categories such as ER and oxidative stress proteins, proteosomal proteins, cytoskeletal proteins, proteins associated with apoptosis and glycolytic enzymes (Fig. 2 and Table 1). Of the identified proteins up-regulated by alcohol should be noted ER stress markers such as GRP78 (glucose regulated protein 78kDa), PDI (protein disulfide-isomerase) and ER60; enzymes involved in antioxidant defense (peroxiredoxin, superoxide dismutase); proteolytic enzymes (cathepsin D and cathepsin B); anti-apoptotic protein (cystatin B); enzymes related to alcohol metabolism (alcohol dehydrogenase); cytoskeletal proteins (vimentin, tropomyosin, actin, cofilin); glycolytic enzymes (pyruvate kinase, phosphoglycerate kinase and alpha enolase) as located in the 2D gel and identified by peptide mass fingerprinting (Fig. 2 and Table 1). Two proteins, GRP-78 and PDI need special mention since they are involved in sensing and responding to the accumulation of unfolded or misfolded proteins in the ER. PDI and GRP78 were up-regulated by 2.6 and 2.2 fold, respectively, as determined by quantifying the spot densities over three gels per condition ($p < 0.05$, Fig.1 and Fig. 2). Other proteins are cathepsin-D and superoxide dismutase that were significantly up-regulated to respectively 2.49 and 3 fold relative expression in the alcohol-treatment group as compared to control ($*p < 0.05$; $n=15$) (Table 1). Likewise the expression of alpha-enolase was also significantly increased to 6.23-fold relative expression in the alcohol-treatment group as compared to control ($*p < 0.05$; $n = 17$). We also observed an increase in levels of expression in cystatin B, as well as aldehyde dehydrogenase, elongation factor 2 and in oxidative stress proteins (superoxide dismutase and peroxiredoxin) as well as some structural proteins such as vimentin and tropomyosin.

Furthermore, in the present analysis, we demonstrated that alcohol treatment induced a decreased DC expression of the ER stress protein ER-60, the protease cathepsin B, the chaperon Hsp 60, cytoskeletal proteins such as cofilin and actin, and proteins involved in inducing apoptosis (annexin A5, cyclophilin A and galectin-1). The average expression levels of these proteins ranged from 2.18 to 7.32 fold as compared to control ($p<0.05$). The remainder of the identified changes occurred in five proteins with differing functions. Alcohol increased antigen KI-67 and decreased serum albumin and macrophage capping protein (Table 1). The implications of changes in protein expression associated with ER stress, glycolytic metabolism, cytoskeletal changes, UPR and anti-apoptotic responses IN MDDC represent novel alcohol mechanistic avenues to pursue.

Alcohol induced changes in gene expression by MDDC

Consistent with the 2DE analysis, the expression changes identified by the 2DE LC-MS/MS analysis were validated by RT-PCR analysis to assess the effect of ethanol on mRNA levels of selected identified proteins. This was investigated by quantitative real time PCR. MDDC were cultured for 18h with ethanol (0.1% v/v), RNA was extracted, reverse transcribed and cDNA was amplified by Q-PCR using the gene specific primers. Data presented in Fig. 2

shows that gene expression for cathepsin D (fold change=1.58, $p<0.03$), superoxide dismutase (fold change=1.57, $p<0.04$), vimentin (fold change=1.43, $p=0.031$), GRP 78 (fold change = 1.29, $p<0.033$) and pyruvate kinase (fold change=1.53, $p=0.015$) were significantly up-regulated in ethanol treated dendritic cells compared to the untreated cells, where as expression of ER 60 (fold change=0.72, $p<0.03$) was significantly down-regulated in ethanol treated cultures compared to untreated control cultures. Thus, the gene expression data confirms our proteomic results. These data indicate that alcohol induces the gene expression of cytoskeletal, glycolytic, ER/oxidative stress, proteolytic, and UPR related proteins leading to an anti-apoptotic response in MDDC that is rapid in onset and concentration dependent (Figure. 2).

Discussion

We investigated the impact of alcohol on the MDDC proteome using 2D electrophoresis with subsequent protein identification by mass spectrometry. Identified changes in DC protein expression were then compared with RT-PCR analyses of DC gene expression. Based on our criteria of a two-fold or greater change in expression, the results indicate that alcohol significantly regulates key components of the UPR stress-inducing pathways that include chaperones, ER stress antioxidant enzymes, proteolytic enzymes, as well as enzymes related to alcohol metabolism, cytoskeletal proteins, and proteins involved in apoptosis.

Among the de-regulated proteins in MDDC induced by alcohol are noticeable ER stress markers such as GRP78, PDI and ER-60. These proteins are involved in sensing and responding to the accumulation of unfolded or misfolded proteins in the ER. Since these three proteins are part of the UPR, our data support our hypothesis that alcohol activates this compensatory cellular defense mechanism. Another important up-regulated protein as identified by peptide mass fingerprinting is EIF2a, which is a key protein involved in the initiation of the UPR (Wek and Cavener, 2007).

Upon the aggregation of unfolded proteins, GRP78 dissociates from the three ER stress receptors allowing them to dimerize, auto-phosphorylate, and thus become activated leading to repression of translation of other proteins relieving the accumulation of unfolded proteins (Boyce et al, 2005; Scheuner et al, 2001).

Among the proteins that play a crucial role in compensating for the stress of misfolded proteins, are chaperone proteins such as the heat shock protein, Hsp60. In the present study, this protein was up-regulated by alcohol treatment.

Several cytoskeletal proteins were found to be de-regulated by alcohol treatment in the MDDC proteome (actin, vimentin, tropomyosin and cofilin). Cytoskeletal changes after exposure to alcohol have been described in a number of cell types in adult rats and humans. Effects of alcohol on astrocytes induced a striking disorganization of the cytoskeleton. Since cytoskeletal integrity is a prerequisite for many cell functions, several studies have reported interactions between alcohol and cytoskeletal proteins (Tomás et al, 2003). It has been reported that alcohol-induced changes in the actin cytoskeleton is mainly mediated by acetaldehyde, the main metabolite of alcohol oxidation. In addition, actin dynamics can also affect the cellular and behavioral responses of flies and mice in response to alcohol (Offenhäuser et al., 2006). Our results demonstrated that cofilin, was found de-regulated in MDDC treated with alcohol.

We also observed glycolytic changes in the MDDC proteome following alcohol treatment. Pyruvate kinase, phosphoglycerate kinase, and alpha-enolase were up-regulated respectively 6.23, 2.66 and 2.37 fold. It has been reported that the targeting of Type II fibers by alcohol implicate glycolytic pathway lesions in myopathy and glycolytic insufficiency was

attributed to the fact that Type II fibers undergo specific atrophy (Fernandez-Sola et al, 2007). It was also noticed that in human muscle biopsies from chronic alcoholics there was a reduction in the pyruvate kinase activity before the first sign of myopathy appeared. Unlike the study mentioned above (investigating chronic ethanol effect) that lead to the down-regulation of pyruvate kinase, our data suggest that alcohol-induced up-regulation of pyruvate kinase and the glycolytic enzymes phosphoglycerate kinase and alpha-enolase might be the result of a protective cellular response triggered by the UPR mechanism to improve the glycolysis and energetic metabolism of DC status as a response to alcohol's deleterious effects.

Our results also showed a down-regulation of cyclophilin A expression following alcohol treatment in the MDDC proteome. Cyclophilin A also merits some discussion since this protein has a role in apoptosis (Bukrinsky 2002; Ivery 2000). Cyclophilin A is found in cell compartments where protein folding takes place, and complexes of cyclosporin A with cyclophilin A have been shown to inhibit calcineurin, a serine/threonine phosphatase inducing apoptosis (Ivery 2000). Cyclophilin A has also been shown to participate in the activation of the caspase cascade in neuronal cells (Capano et al, 2002).

Galectin 1 (Gal-1), a potent anti-inflammatory and immunoregulatory protein was triggered by alcohol treatment in our study as well. Evidence points to Gal-1 and its ligands as one of the master regulators of such immune responses as T cell homeostasis and survival, T cell immune disorders, inflammation and allergies as well as host–pathogen interactions (Camby et al, 2006). Several lines of evidence indicate that Gal-1 may function as a pro-apoptotic factor inducing cell death of immature thymocytes and activated, but not resting, mature T cells (Rabinovich et al, 2000) thus preserving homeostasis after the completion of an immune response. Gal-1 in its oxidized form plays a number of important roles in the regeneration of the CNS after injury (Horie et al, 2004).

Alpha enolase was up-regulated by the alcohol treatment of MDDC. Alpha enolase activity with both isoenzyme forms: non-neuronal enolase (NNE) and neuron specific enolase (NSE) have been shown to be modified in many injuries related to the glycolytic pathways. Increased activity of SOD (superoxide dismutase), also found up-regulated in our study, and NSE in blood cells may be related to liver injury mainly in alcoholism while increased NNE activity may also be a marker of alcohol abuse without liver injury. Significant increase of SOD activity in alcoholic patients with liver injury and mainly in cirrhotic patients with ascitis have been reported. While we speculate in our study that SOD activates ER stress as shown in several studies, alpha enolase product likely reflects increased glycolysis in alcohol-induced cells.

Cathepsin-D, active in intracellular protein breakdown, is another key protein that was up-regulated in MDDC treated with alcohol (Benes et al, 2008, Minarowska et al, 2008). Cathepsin-D is known to be involved in the antigen processing capacity of MDDC (Mohamadzadeh et al, 2004). As a protease, Cathepsin-D digests protein antigens that have been internalized by DC.

Cathepsin-B protease was up-regulated in MDDC induced with alcohol treatment. Macrophages and B cells have been reported to employ Cathepsins B, D, and/or E for digesting protein Ags.

We also observed that a protease known as cystatin B was up-regulated by alcohol treatment in DCs. Cystatin B (CSTB, also known as stefin B) may have an anti-apoptotic function in the cerebellum (Di Giaimo et al, 2002). While the molecular basis of such function is not clear, CSTB may possibly protect the cells against inappropriate cellular degradation by proteases. It was also demonstrated that oxidative stress induces the expression of CSTB in

cerebellar granule neurons and that the EPM1 (progressive myoclonus epilepsy type 1) patient-linked mutation of the cystatin B gene promoter impairs oxidative stress induction of CSTB transcription. CSTB knockout or knockdown sensitizes cerebellar granule neurons to oxidative stress-induced cell death. Interestingly, this CSTB deficiency-induced predisposition to oxidative stress in neurons is mediated by the lysosomal protease, cathepsin B (Lehtinen et al, 2009). Since our findings reveal an oxidative stress response, reflected by differential expression of antioxidants such as superoxide dismutase and peroredoxin, as well as cathepsin B, with an induced UPR response triggered by alcohol in DC, we speculate that CSTB could modulate this specific pathway as a protective mechanism for DCs against oxidant injury and protein misfolding.

To summarize our discussion, we propose the model highlighted in Figure 3. This model encompasses the alcohol-induced MDDC differentially expressed proteins detected in this study. After alcohol exposure, the MDDC becomes stressed due to the generation of high levels of reactive oxygen species (ROS), which interfere with proper protein folding in the ER, producing cellular stress and the synthesis of non-functional proteins. Alcohol oxidation, through aldehyde dehydrogenase (found up-regulated in our study), produces high levels of acetaldehyde, which is known to induce changes on the cytoskeleton. The stress generated by alcohol exposure promotes the increased expression of glycolytic enzymes (alpha enolase, pyruvate kinase, and phosphoglycerate kinase), proteolytic enzymes (cathepsin D and cathepsin B) and chaperones (Hsp60) to be able to generate energy and attenuate the accumulation of misfolded proteins under the UPR. One of the mechanisms activated by the cell to cope with the generated stress is to stop protein synthesis. The cell accomplishes this with the dissociation of GRP78 from stress receptors which results in a stoppage of protein synthesis and consequently the accumulation of misfolded proteins. We speculate that under a protective mechanism the cell will try to “eliminate” the misfolded proteins to evade the unfolded protein response (UPR)-activated apoptotic pathways induced by alcohol (Figure. 3). To cope with this apoptotic action, the MDDC then induces an anti-apoptotic machinery by down-regulating pro-apoptotic factors Gal-1 and cyclophilin A and by up-regulating anti-apoptotic proteins such as cystatin B to promote cell survival. In our study, interestingly, cyclophilin A another pro-apoptotic factor, was also found down-regulated in alcohol-induced DCs. Furthermore, this novel finding puts forward the evidence that alcohol induces in MDDC an unfolding response mediated by ER stress apoptotic as well as anti-apoptotic signals. For future studies, it will be interesting to further elucidate the Gal-1 apoptotic role and the putative anti-apoptotic proteins triggered by alcohol induction (such as cystatin B), as revealed in our study, to determine which specific mediated apoptosis response in alcohol-induced DCs (up- or down-regulation) would be triggered in non-constitutive receptors by Gal-1.

Overall, our findings revealed for the first time a UPR mechanism consistent with an ER-driven pathway activated through key ER stress and UPR proteins following alcohol treatment in DCs. It seems that activation of a UPR by alcohol may protect the DC from oxidant injury. Collectively, these data provide a plausible mechanistic rationale for alcohol action and suggest that the alcohol-inducible changes in the DC proteome could serve as potential biomarkers of therapeutic efficacy of reduced alcohol intake. Furthermore, alcohol-induced regulation of the UPR as well as pro- and anti-apoptotic protein signatures and the level of expression in DCs provide insight into targets and mechanistic strategies to prevent or alleviate alcohol abuse and dependence.

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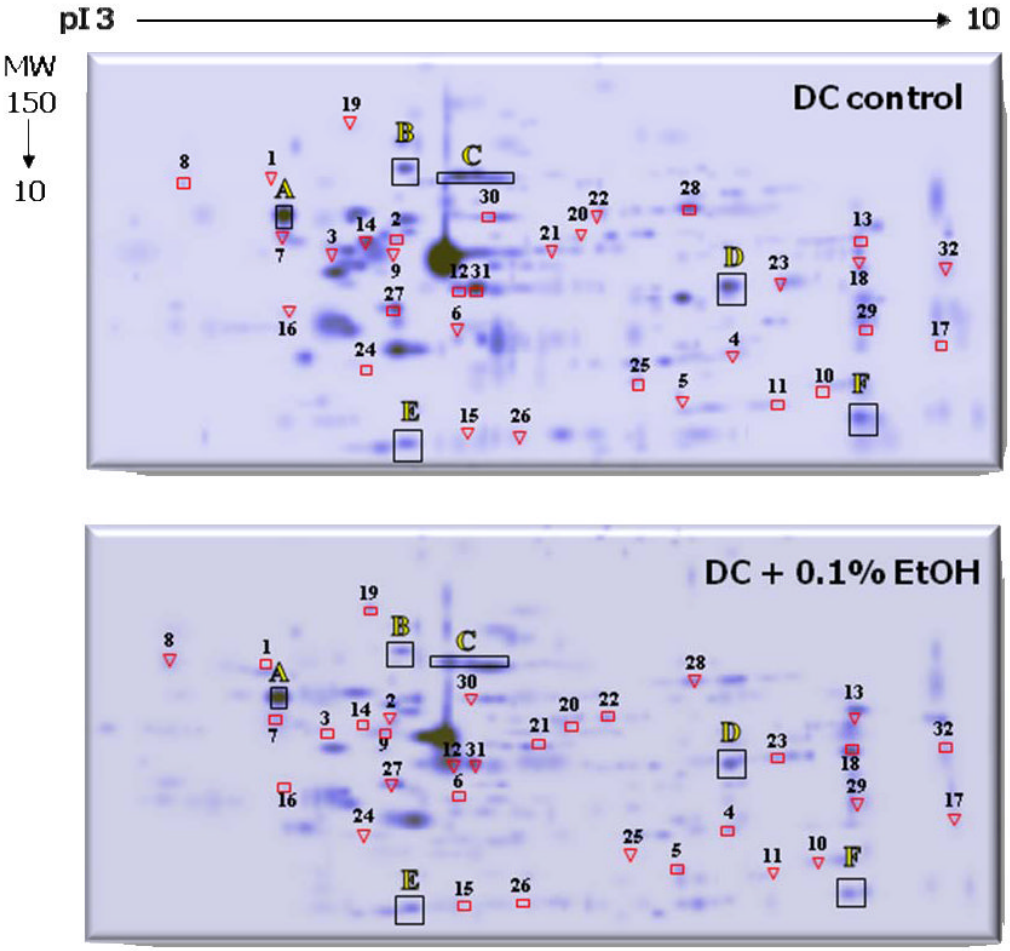

Figure 1.

Differentially expressed monocyte derived dendritic cell proteins identified by peptide mass fingerprinting. Down-regulated proteins are indicated with solid red squares in the DC control 2D gel image and up-regulated proteins are indicated with solid red squares in the DC+0.1% EtOH 2D gel image. The triangles represent less expression in the corresponding 2D gel. 2D spots are numbered as shown in table 1. The standard common proteins are indicated with black squares and letters.

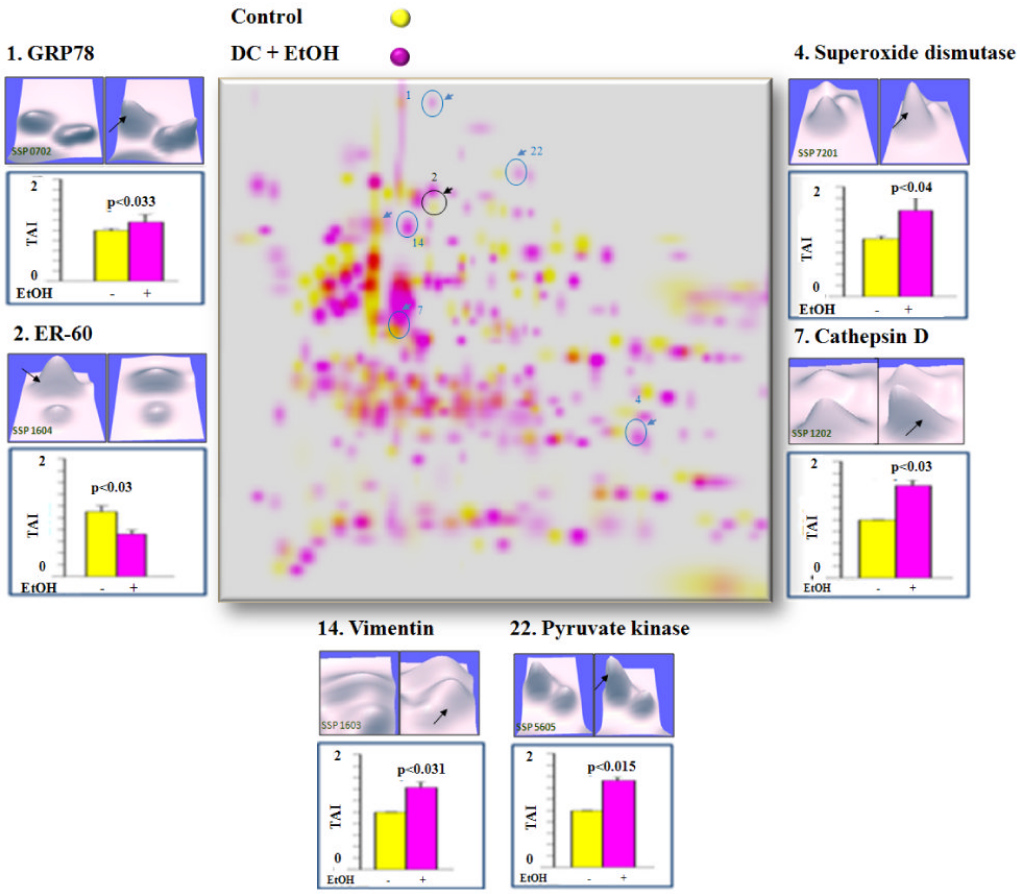

Figure 2.

Multichannel viewer of the overlapped images. Ethanol treatment (etOH) of monocytes derived dendritic cells treated with ethanol vs. control (non treated cells). Three-dimensional interpretation for six representative proteins identified by peptide mass finger printing and corresponding RT-PCR are shown around the 2D-GE images. Each pair of protein spots are 3D views of their relative peak volumes. The peak area comprising the entire volume represents the 2D-GE distributions of the protein spot in the gel, whereas the volume correlates to the protein concentration. The SSP # located in the respective 3D view images correspond to the protein volumes shown. Ethanol induced changes in gene expression. IDC (5×10^5 cells/ml) were treated with ethanol (0.1% v/v) for 18h. Total RNA was extracted, reverse transcribed and subjected to Q-PCR. Data are presented as mean fold change in gene expression + SE of 3 independent experiments. Statistical significance was calculated by students “t” test. The blue circles and arrows indicate downregulated proteins and the black circle and arrow indicate an upregulated protein.

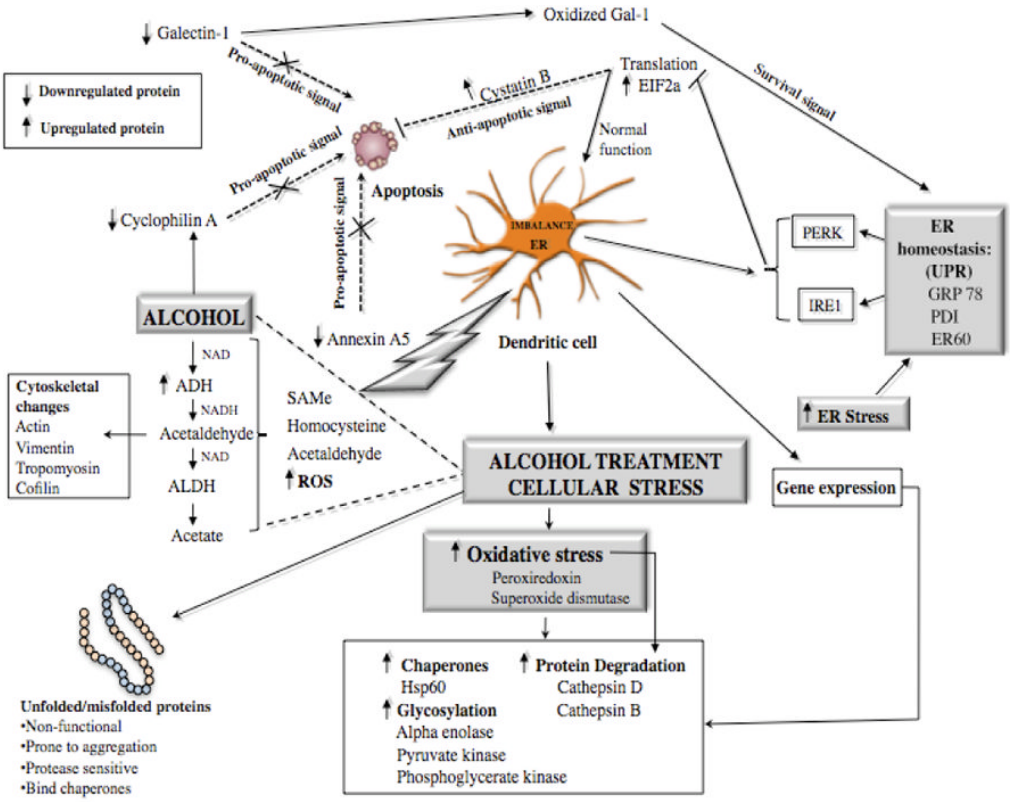

Figure 3.

Proposed model highlighting UPR (unfolded protein response) pathway with pro-apoptotic and anti-apoptotic protein signatures triggered by ER stress in alcohol treated monocyte derived dendritic cell.

Table 1

Proteins differentially expressed in monocyte derived dendritic cells treated with ethanol 0.1% and identified by peptide mass fingerprinting

Spot #	Protein ID	Access #	[1]MW [2]Theor./[3]Exp.	[4]Ip Theor./Exp.	[5]MS/MS score	Fold change	Peptides	Expression
UPR & Oxidative Stress								
1	78 kDa glucose-regulated protein	P11021	72.4/68.85	5.0/5.1	190	2.19	15	Up
2	ER-60 protein	AAC51518	57.14/52.55	5.8/5.4	694	2.18	25	Down
3	Protein disulfide-isomerase	P07237	57.47/26.34	4.8/4.0	899	2.04	29	Up
4	Superoxide dismutase	P04179	24.87/18.38	8.3/7.7	261	3.00	5	Up
5	Peroxiredoxin	CAI13097	10.67/65.69	9.5/4.2	78	2.79	2	Up
Protein Degradation								
6	Cathepsin B precursor	P07858	38.76/17.92	5.9/5.0	196	2.52	7	Up
7	Cathepsin D	1LYA_B	26.45/27.12	5.3/4.9	655	2.49	11	Up
8	Endoplasmic precursor	P14625	92.69/28	4.7/5.0	497	2.19	15	Down
Regulatory/Structural Proteins								
9	60 kDa heat shock protein	P10809	61.18/52.44	5.7/5.3	569	2.99	29	Up
10	Cofilin 1	NP_005498	17.38/18.71	8.2/8.2	452	2.24	10	Down
11	Cofilin 2	EAW74451	15.87/15.92	8.5/8.7	426	2.21	8	Down
12	Actin cytoplasmic 2	P63261	42.1/15.72	5.3/7.0	249	2.70	7	Down
13	Adenylyl cyclase-associated protein 1	Q01518	52.22/13	8.2/6.0	358	2.43	10	Down
14	Vimentin	AAA61279	53.73/52.12	5.0/5.0	609	1.83	34	Up
15	WD repeat protein 1	O75083	66.83/12.37	6.1/6.2	477	1.93	18	Up
16	Tropomyosin alpha-3 chain	P06753	32.85/22.36	4.6/5.0	465	1.60	12	Up
Enzymes/Transcription Factors								
17	Glyceraldehyde-3-phosphate dehydrogenase	ABD77190	24.6/17.92	9.4/5.0	135	2.20	2	Down
18	Elongation factor 2	P13639	95.33/77.09	6.4/8.9	435	2.25	19	Up
19	Vacuolar ATP synthase catalytic subunit A	P38606	68.65/77.66	5.3/5.5	626	2.06	24	Up
20	Aldehyde dehydrogenase	P05091	56.85/45.03	6.6/6.1	249	2.34	13	Up
Glycolytic Enzymes								
21	Alpha enolase	P06733	47.48/38.5	7.0/7.1	950	6.23	19	Up
22	Pyruvate kinase isozymes M1/M2	P14618	58.51/31.4	7.9/6.8	480	1.65	16	Up

Spot #	Protein ID	Access #	[1]MW [2]Theor./[3]Exp.	[4]Ip Theor./Exp.	[5]MS/MS score	Fold change	Peptides	Expression
23	Phosphoglycerate kinase 1	P00558	44.98/38.02	8.3/7.8	275	1.80	13	Up
Pro & Anti-Apoptotic Factors								
24	Galectin 1	P09382	15.04/10.31	5.3/5.7	656	2.53	9	Down
25	Cyclophilin A	5CYH_A	18.09/15.97	7.8/8.3	496	2.18	9	Down
26	Cystatin B	P04080	11.18/17.60	6.9/8.0	487	1.80	5	Up
27	Annexin A5	P08758	35.97/27.88	4.9/4.4	464	2.60	17	Down
Miscellaneous								
28	Hypothetical protein isoform 8	XP_001175084	59.40/46.52	7.8/7.9	564	2.66	20	Down
29	Unnamed protein	BAG62134	23.07/77.66	9.3/5.5	716	2.37	12	Down
30	Serum albumin precursor	P02768	71.31/79.69	5.9/6.4	190	7.32	7	Down
31	Macrophage capping protein	P40121	38.77/47.56	5.8/6.3	646	2.21	11	Down
32	Antigen KI-67	P46013	360.69/46.84	9.4/6.7	56	1.92	29	Up

[1]MW: Molecular weight (kDa),

[2]Theor: Theoretical,

[3]Exp: Experimental,

[4]Ip: Isoelectric point,

[5]MS/MS score: Tandem mass spectrometry.