Aluminum-Induced Mitochondrial Dysfunction Leads to Lipid Accumulation in Human Hepatocytes: A Link to Obesity

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Key Words

Very low density lipoprotein • Apolipoprotein B-100 • Energy metabolism • Aluminum • Lipogenesis • Obesity

Abstract

Mitochondrial dysfunction is the cause of a variety of pathologies associated with high energy-requiring tissues like the brain and muscles. Here we show that aluminum (Al) perturbs oxidative-ATP production in human hepatocytes (HepG2 cells). This Al-induced mitochondrial dysfunction promotes enhanced lipogenesis and the accumulation of the very low density lipoprotein (VLDL). Al-stressed HepG2 cells secreted more cholesterol, lipids and proteins than control cells. The level of apolipoprotein B-100 (ApoB-100) was markedly increased in the culture medium of the cells exposed to Al. ¹³C-NMR and HPLC studies revealed a metabolic profile favouring lipid production and lowered ATP synthesis in Al-treated cells. Electrophoretic and immunoblot analyses pointed to increased activities and expression of lipogenic enzymes such as glycerol 3-phosphate dehydrogenase (G3PDH), acetyl CoA carboxylase (ACC) and ATP-citrate lyase (CL) in the hepatocytes exposed to Al, and a sharp diminution of enzymes

mediating oxidative phosphorylation. D-Fructose elicited the maximal secretion of VLDL in the Al-challenged cells. These results suggest that the Al-evoked metabolic shift favours the accumulation of lipids at the expense of oxidative energy production in hepatocytes.

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Introduction

Environmental pollution is a major cause of concern as numerous pollutants are known to be involved in a variety of diseases in humans [1]. Elevated levels of bioavailable metals have been implicated in cancers, neurological complications, pulmonary diseases and nephrological abnormalities [2-4]. In fact, millions of deaths are attributed annually to medical conditions arising from metal pollution [5]. Mercury (Hg) reacts with critical thiol moieties and severely impedes normal immunological responses and mental cognition [6]. On the other hand, a pollutant like lead (Pb) exhibits its toxic impact by dislocating the essential Zn localized in aminolevulinate

dehydratase (ALD), a key component of the heme-producing machinery [7]. This triggers medical complications associated with iron metabolism and energy production [8, 9].

Al, an important environmental toxin, has been implicated in Alzheimer's disease (AD), anemia and dialysis encephalopathy due to its ability to mimic iron (Fe). Al disturbs Fe homeostasis and consequently interferes with essential biochemical processes dependent on this redox-active metal [10]. This interaction leads to a further increase in the intracellular pool of labile Fe and a concomitant rise in reactive oxygen species (ROS) [11]. The Al-triggered oxidative environment subsequently contributes to the toxicity of the trivalent metal [12]. Various ion channels have been shown to be inactivated by micromolar quantities of Al as this trivalent metal disrupts biological membranes [12]. As the ionic radius of Al resembles Mg^{2+} , it can readily interact with Mg^{2+} requiring enzymes. Polymerization of tubulin has been shown to be promoted by Al^{3+} [13]. This trivalent metal also renders Ca^{2+} signaling networks dysfunctional due to its ability to bind to Ca-dependent proteins [14].

Although some molecular aspects of Al toxicity are becoming evident, its interaction with the various metabolic pathways that mediate the synthesis of ATP and the generation of a reducing environment in the cell has yet to be unravelled. Metabolism is the foundation of any biological system as it allows organisms to respond and to adapt to any intracellular and extracellular changes [15, 16]. It helps maintain a milieu conducive to the production and storage of energy and to cellular growth [17]. The glycolytic cycle, the tricarboxylic acid cycle (TCA), oxidative phosphorylation and the pentose phosphate pathway, due to their abilities to produce NADH, a pro-oxidant, NADPH, a reductant, ATP and essential anabolic precursors have to be intricately modulated if a cell is to survive [16]. We have recently shown that Al toxicity impedes the production of NADH via the TCA cycle and favours the synthesis of glycolytic ATP in hepatocytes [18, 19].

In this study, we have evaluated the impact of Al on hepatic metabolism in an effort to elucidate how this trivalent metal interacts with the ATP-producing machinery. Here, we show how Al limits mitochondrial ATP production and promotes the synthesis and accumulation of VLDL. This ineffective oxidative-energy production coupled with enhanced lipogenesis is characteristic of obesity. The relationship between Al toxicity, lipogenic enzymes, energy production, and obesity is discussed.

Materials and Methods

Cell Culture

HepG2 cells were a gift from Dr. D. Templeton, University of Toronto. The cells were seeded at 1×10^5 cells/ml and maintained in α -MEM containing 5% FBS and 1% antibiotics. Cells were passaged and cultured in 175cm² flasks with loosened caps and incubated with 5% CO₂ in a humidified atmosphere at 37°C.

Aluminum Treatment and determination of proteins and cholesterol

Upon reaching 70% confluency, cells were washed twice with PBS and re-supplemented with serum-free media containing 2.5mM citrate or 2.5mM citrate:10-500 μ M $AlCl_3 \cdot 6H_2O$ for a period of 24h (note: the amount of Al utilized ranged from 0.2 to 13.4 μ g/ml compared to 750 μ g/ml and > 0.4 μ g/ml in Alzheimer's and dialysis patient serum [20, 21]). The cultures were re-constituted with serum-free media containing 30mM D-glucose or 30mM D-fructose respectively and incubated for up to 24h. At various time intervals, the cells and the spent fluid was collected and stored at -86°C for further analyses. The cells were isolated by centrifugation at 250g for 10min at 4°C. The cellular pellet was washed and resuspended in cell storage buffer (CSB) (50mM Tris-HCl, 1mM phenylmethylsulphonylfluoride, 1mM dithiothreitol, 250mM sucrose, 2mM citrate, 0.5mM $MnCl_2$) and stored at -86°C until needed. The spent fluid collected from the D-glucose and D-fructose supplemented cultures were analyzed for their protein and cholesterol content without or with phosphotungstic acid precipitation. The latter treatment helped precipitate lipoproteins [22]. Protein levels were determined with the Bradford assay using BSA as the standard [23]. Total cholesterol was monitored using a kit based on cholesterol oxidation from Fisher Scientific.

¹³C -Nuclear Magnetic Resonance and HPLC Studies

NMR analyses were performed using a Varian Gemini 2000 spectrometer operating at 50.38 MHz for ¹³C. Experiments were executed with a 5mm dual probe (35° pulse, 1-s relaxation delay, 8 kilobytes of data, and 2000 scans). Chemical shifts were referenced to standard compounds under the same conditions. Following exposure to Al, HepG2 cells were ultrasonically disrupted and subjected to centrifugation at 450g for 30min at 4°C to afford a cell free extract (CFE) devoid of nucleus and intact cells. A 2mg/ml of HepG2 CFE was exposed to 10mM [2-¹³C] D-glucose, 1mM ADP, 1mM NAD⁺ for 24h at 37°C. Similar experiments were performed with control cells. The reaction was stopped by heating at 95°C for 5min. Whole cell experiments were performed in 24-welled collagen coated plates. Upon exposure to control and Al-stress conditions, cultures were re-constituted with serum-free media containing 30mM [2-¹³C] D-glucose and 10mM [¹³C] HCO₃⁻. The spent fluid was treated with phosphotungstate and the precipitate subject to spectral analysis. HPLC experiments were performed on mitochondria isolated from control and Al-exposed cells HepG2 cells. Following homogenization, mitochondria were isolated by centrifugation at 12000g at 4°C for 30min and re-suspended in

a phosphate reaction buffer. 2mg/ml of protein equivalent to mitochondria was incubated with 5mM citrate and 0.1mM NAD⁺ for 1h at 37°C. The reaction was stopped at 4°C with dilute acid to precipitate the protein and membranes [24]. The supernatant was analyzed using a Waters Alliance HPLC equipped a C₁₈-reverse phase column. A mobile phase consisting of 20mM KH₂PO₄ (pH 2.9) was used operating at a flow rate of 0.7ml/min. The eluants were measured by UV absorption at 254nm. The respective peaks were confirmed by running the appropriate standards and by spiking the desired samples.

Cell Fractionation and Electrophoretic Analyses

The cells were thawed and pelleted at 250g at 4°C for 10min and resuspended in CSB containing 1mg/ml of pepstatin A and 0.1mg/ml of leupeptin. The cell suspension was homogenized using a Brunswick sonicator for 20s in 2s bursts. Following homogenization, mitochondrial and soluble fractions were isolated by differential centrifugation [25, 26].

Native PAGE was performed according to a modified method by Schagger, et al. [27]. The BioRad MiniProtein™ 2 system was utilized and 4%-10% linear gradient gels were used for protein resolution. For proper ACC separation, 6% isocratic gels were favored. Soluble proteins were prepared in Native Protein buffer (NP buffer) (500mM 6-amino hexanoic acid, and 50mM BisTris (pH 7.0)). The mitochondria were prepared in NP buffer containing 10% (w/v) β-dodecyl-D-maltoside to disrupt the membranes. 30μg of protein/lane was loaded in each well. Gels were electrophoresed under native conditions at 80V. The voltage was increased to 200V once the proteins penetrated the resolving gel. The Ponceau S cathode buffer (50mM Tricine, 15mM BisTris, 0.013% Ponceau S (pH 7.0) at 4°C) was changed to colourless cathode buffer (50mM Tricine, 15mM BisTris, (pH 7.0) at 4°C) when the running front was half way through the separating gel [28]. 0.02% (w/v) Serva Blue was used in place of Ponceau S for the resolution of the mitochondrial proteins. The gel was then placed in equilibration buffer (25mM Tris, 5mM MgCl₂ (pH 7.4)) for 15min. Enzyme activity was visualized by the formazan precipitation assay in a reaction buffer consisting of equilibration buffer, the required substrate and cofactor(s), 0.4mg/ml iodonitrotetrazolium (INT), and 0.2mg/ml phenazine methosulfate (PMS). Cytoplasmic IDH-NADP⁺ was detected using equilibration buffer containing 5mM isocitrate, 0.5mM NADP⁺, INT, and PMS. Similarly G6PDH was visualized using 5mM glucose-6-phosphate, 0.5mM NADP⁺, INT, and PMS in equilibration buffer. G3PDH activity was ascertained using 10mM glycerol 3-phosphate and 0.75mM NAD⁺, PMS and INT. ATP-CL was detected by an enzyme-coupled reaction which required dichloroindophenol (DCIP) as an electron mediator [28]. This was accomplished using 20mM citrate, 0.75mM CoA, 0.37mM ATP, 1.5mM NADH, 5units/ml malate dehydrogenase (MDH), 16.7μg/ml DCIP, and INT (0.5mg/ml). The liberation of P_i as a result of ACC activity was monitored using the procedure described in [29]. Following electrophoresis, the gel was equilibrated and incubated in 10mM ATP, 10mM HCO₃⁻, 1mM acetyl-CoA for 1h. The gel was rinsed repeatedly with ddH₂O followed by a 10min incubation with phosphate precipitation reagent ((NH₄)₂MoO₄ (1.06g) in 1.37ml triethylamine and 9.2ml conc. HNO₃). The reactions were

stopped at the desired time with destaining solution (50% methanol and 10% glacial acetic acid). Cytochrome c oxidase (Cyt C Ox) was tested with equilibration buffer supplemented with 5mM KCN, diaminobenzidine (10mg/ml), cytochrome C (10mg/ml), and sucrose (562.5mg/ml). SDH was monitored with the aid of equilibration buffer containing succinate (60mM), 5mM KCN, INT, and PMS [19]. The activity bands were quantified using Scion Imaging for Windows (Scion Corp., Frederick, MD). All in-gel enzyme assays were performed in the absence of the required substrate(s) to ensure band specificity. Proper loading was maintained by Coomassie staining. Proteins levels for individual enzymes were determined by 2D Native gel electrophoresis. Activity bands were excised and electrophoresed as described above. Protein levels were ascertained by subjecting the gel slabs to silver staining.

To determine lipoprotein levels the spent fluid (from different experiments) was treated with phosphotungstic acid (1:1 v/v) to precipitate any lipoproteins. The resulting precipitate was isolated and resuspended in CSB and stored at 4°C until further use. The levels of the lipoproteins were determined by Blue Native (BN) PAGE analysis [30]. The gel slab was fixed and immediately treated with silver staining or Sudan Black IV treatment overnight. The gels were then destained to allow proper band resolution. The bands obtained from the Sudan Black IV treatment were cut out and subjected to 2D SDS PAGE analysis to confirm the presence of ApoB-100. Appropriate standards were utilized.

Immunoblot Assays

SDS-PAGE was performed on the cell extracts and spent fluid according to a modified method of Laemmli [31]. Samples were prepared in 62.5mM Tris-HCl (pH 6.8), 2% SDS, and 2% β-mercaptoethanol at 100°C for 5min. The proteins were electrophoresed and transferred to a Hybond™-P polyvinylidene difluoride membrane for immunoblotting. Nonspecific binding sites were blocked by incubating the membrane in 5% non fat skim milk in TTBS (20mM Tris-HCl, 0.8% NaCl, 1% Tween-20 (pH 7.6)) for 1h. ApoB-100, citrate synthase (CS), ATP-citrate lyase (CL) and VLDL were detected using anti-ApoB100 (Santa Cruz), monoclonal anti-VLDL (Abcam), polyclonal anti-ATP-citrate lyase (Abcam), and polyclonal anti-citrate synthase (Abcam). Native PAGE was used as described above for the VLDL immunoblot. The SDH antibody was a generous gift from Dr. Lemire, University of Alberta. The secondary antibody consisted of horseradish peroxidase-conjugated to goat anti-mouse or anti-rabbit (Santa Cruz). ACC was identified using horseradish peroxidase-avidin conjugate (Sigma), a compound specific for binding biotinylated proteins. Proper protein transfer and loading was established by detecting the levels of F-actin and voltage dependent anion channel (VDAC). The ChemiDoc XRS system (Biorad Imaging Systems) was utilized to document the bands. Band intensity was quantified using Alpha Innotech Software (Alpha Innotech Corporation, San Leandro, CA).

Fluorescent Microscopy

HepG2 cells were grown on glass cover slips to a minimal density and exposed to citrate or Al-citrate. Following washings

with PBS and 0.5mM EDTA, the cells were fixed with a methanol:acetic acid solution (3:1 v/v). The optical detection of intracellular lipid droplets was achieved using Oil Red-O (0.25% w/v in isopropyl alcohol). VLDL was detected using immunofluorescence. The cover slips were fixed and then incubated for 1h with 5% FBS dissolved in TTBS (0.1% Tween 20). The cover slips were washed thoroughly and incubated in primary antibody (anti-VLDL 1/200 dilution) for 2h and secondary antibody (anti-rabbit FITC conjugate 1/1000 dilution) for 1h, the cover slips were washed extensively with TBS. The nucleus was demarcated using propidium iodide (50ng/ml in PBS). Fluorescence was visualized using an inverted deconvoluting microscope (Zeiss) operating at the appropriate emission and excitation wavelengths.

Statistical Analysis

All experiments were performed in at least twice and in triplicate. Data were expressed as mean $\pm$ SD. Statistical correlations of data were checked for significance using the student t test.

Results

Aluminum triggers mitochondrial dysfunction

To assess the impact of Al on hepatic metabolism, ATP formation in the mitochondria was assessed. HPLC studies revealed that Al-stressed HepG2 mitochondria produced less ATP when exposed to 5mM citrate and 0.1mM NAD⁺ in comparison to control cells (Figure 1). This prompted us to monitor selected complexes involved in oxidative phosphorylation. In-gel activity analysis of SDH pointed to the diminished activity of this enzyme in Al-exposed hepatocytes (Figure 1). Immunoblot experiments revealed that there was a concomitant reduction in the expression of SDH. Cyt C Ox also exhibited lowered activity in the Al-stressed cells as opposed to control (Figure 1). Silver staining of the gel revealed a decrease in the expression of this complex in cells exposed to Al.

Determination of the lipoprotein levels in the spent fluid

The HepG2 cell line serves as an excellent model for studying the impact of environmental stress on human liver cells since they simulate the behaviour of normal hepatocytes [32]. The levels of protein and cholesterol in the spent fluid were markedly higher in the Al-stressed cultures in comparison to the control exposed to carbohydrate for 24h. D-Fructose elicited a more pronounced response compared to the D-glucose-treated cells (Table 1). These findings prompted us to examine

Fig. 1. Monitoring mitochondrial ATP production and enzymatic activities in A) mitochondria from control cells and B) mitochondria from Al-stressed cells. Panel 1: Detection of ATP levels in purified HepG2 mitochondria incubated for 1h with 5mM citrate and 0.1mM NAD⁺. Following the appropriate treatment of the mitochondria the nucleotides were detected at 254nm by HPLC on a C₁₈ column. Panel 2: In-gel detection of SDH activity and expression. I. Activity stain, II) Immunodetection of SDH. Panel 3: In-gel detection of Cyt C Ox activity and expression. I) Cyt C Ox activity assay. In-gel activity bands were quantified using Scion imaging software (n=3 mean $\pm$ S.D.) II) Analysis of Cyt C Ox protein levels. The activity bands were excised and resolved on a 2D-Native PAGE and then stained with silver staining solution. Note: in all experiments cells were stressed with 500 μ M Al-citrate except where indicated otherwise.

the lipoprotein contents in the spent fluid and the cells. HepG2 cells have the ability to secrete lipids complexed to ApoB-100 in the form VLDL, the major form of emulsified lipids found in serum [33]. The spent fluid from control and Al-stressed cultures was isolated following a 24h exposure to 30mM D-glucose and the pellet obtained from phosphotungstate precipitation was resolved by Native PAGE. As shown in figure 2(I), the spent fluid from the Al-stressed culture contained higher levels of VLDL in comparison to control. Native PAGE and silver staining confirmed the higher occurrence of this lipoprotein in the Al-stressed cultures. VLDL was significantly higher in Al-stressed cultures exposed to either 30mM D-glucose or D-fructose for 24h in contrast to control (Fig 2, II). Sudan black staining and immunoblot confirmed the presence of lipids and ApoB-100 in the phosphotungstate

Fig. 2. Detection of VLDL levels in A) control and B) Al-stressed HepG2 cells incubated with D-glucose or D-fructose. Cultures were supplemented with 30mM D-glucose or D-fructose and incubated for 24h as described previously. Spent fluid was collected and subject to phosphotungstate precipitation. The resulting pellet was electrophoresed and analyzed. I) The presence of VLDL in the spent fluid was confirmed by immunoblot experiment (Note: the LDL standard did not give any band). II) Detection of proteins using silver stain. III) Detection of lipids using Sudan Black IV. IV) To ascertain the levels of ApoB-100 expression, the bands from III were excised and electrophoresed further on a 2D-SDS PAGE and subject to immunoblotting. VLDL and LDL have similar mobilities on Native PAGE [30]. Due to its commercial availability LDL was utilized as the standard. V) Detection of VLDL in HepG2 cells exposed to A) control and B) Al-stressed conditions. Following the Al treatment the cells were exposed to 30mM D-glucose for 24h. VLDL levels were assessed using a secondary antibody conjugated to fluorescein isothiocyanate. Cells were visualized at 100x magnification. Scale bar: 50 μ m.

Table 1. Total protein and cholesterol in spent fluid of HepG2 culture exposed to 30mM D-glucose and 30mM D-fructose. S.D. in parenthesis n=3.

Experiment	Protein concentration (mg/ml)	Total cholesterol (mg/l)
Control (D-glucose)	0.194 $\pm$ (0.006)	0.149 $\pm$ (0.006)
Al-stress (D-glucose)	0.464 $\pm$ (0.012)	0.221 $\pm$ (0.019)
Control (D-fructose)	0.522 $\pm$ (0.022)	0.168 $\pm$ (0.015)
Al-stress (D-fructose)	1.568 $\pm$ (0.019)	0.310 $\pm$ (0.013)

pellet. Significantly higher amounts of lipids and ApoB-100 were evident in the Al-stressed cultures (Fig 2, III and IV). In addition cultures incubated with D-fructose produced higher levels of VLDL. The lipid content of the VLDL molecules was further analyzed by HPLC. Following hexane extraction VLDL pellets from Al-stressed cultures treated with either carbohydrate for 24h contained higher levels of lipids as opposed to control pellets (data not shown). This observation is consistent

with the ability of D-fructose to increase the levels of circulating VLDL. The identification of VLDL molecule was further verified using immunofluorescence (Fig 2, V).

As VLDL was a major constituent of the spent fluid of carbohydrate-treated Al-stressed cells, the intracellular levels of ApoB-100 were ascertained. ApoB-100 showed a slight increase in HepG2 cells isolated at 1h of incubation in Al-stressed cultures supplemented with 30mM D-

Fig. 3. Immunoblot detection of intracellular ApoB-100 levels in HepG2 cells exposed to A) control or B) Al-stressed conditions. I) Following a 24h exposure, HepG2 cultures were re-supplemented with α -MEM containing 30mM D-glucose. Cells were isolated at the desired time intervals and the soluble CFE was analyzed for ApoB-100 levels. II) HepG2 cultures exposed to 30mM D-fructose for 24h were isolated and analyzed for ApoB-100.

Fig. 4. Detection of VLDL levels and ApoB-100 levels in spent fluid isolated from cultures exposed to A) 100 μ M Al B) 10 μ M Al C) control (-FBS + citrate) and D) control (-FBS) cells. Cultures were supplemented with 30mM D-glucose and incubated for 24h as described previously. Spent fluid was collected and subject to phosphotungstate precipitation. The resulting pellet was analyzed by electrophoresis and immunoblotting. I) Detection of protein using silver stain. II) Detection of lipids using Sudan Black IV. III) Immunoblot detection of ApoB-100 levels.

glucose compared to the control. However, no significant variation was observed at subsequent time intervals (Fig 3. I). This is consistent with the ability of this protein to be complexed with lipids and excreted immediately into the extracellular environment within minutes of expression [34]. In addition there did not appear to be a variation in the the immunoreactivity towards ApoB-100 in HepG2 cells treated with D-fructose (Fig 3, II).

Dependence of Lipoprotein Secretion on Al Concentration

As Al appeared to promote the formation of a lipogenic environment, the sensitivity of the hepatocyte to Al concentration was discerned. HepG2 cultures were exposed to 100 μ M and 10 μ M Al for 24h followed by the addition of 30mM D-glucose. A noticeable change in VLDL, lipid, and ApoB-100 levels in the spent fluid of Al-stressed cultures was evident compared to the controls (Fig 4). In an effort to ascertain whether the metabolic shift favoring lipogenesis was specific to Al toxicity, the influence of other known inhibitors of the TCA cycle were examined. Exposure of the HepG2 cells to either Zn (50 μ M) or H₂O₂ (40 μ M) led to an increase in VLDL, lipids, and ApoB-100 secretion in comparison to control (Figure 5).

Fig. 5. Influence of Zn and H₂O₂ on lipoprotein secretion in HepG2 cells. A) control, B) Zn-stressed and C) H₂O₂-stressed HepG2 cells. Cultures were supplemented with 30mM D-glucose and incubated for 24h as described previously. Spent fluid was collected and treated with phosphotungstate. I) Protein detection by silver stain. II) Detection of lipids by Sudan black IV. III) Detection of ApoB-100 by immunoblot.

Fig. 6. Proton decoupled ^{13}C -NMR analysis of $[^{13}\text{C}] \text{HCO}_3^-$ metabolism and $[2-^{13}\text{C}] \text{D-glucose}$ in A) control and B) Al-stressed HepG2 cells. Following a 24h treatment, HepG2 spent fluid was isolated from control and Al-stressed cultures and treated with phosphotungstic acid. The resulting pellet was resuspended in a phosphate buffer and analyzed by NMR.

^{13}C -NMR Analysis of Phosphotungstate Pellet and Glucose Metabolism

As the Al-exposed cells secreted more lipoproteins, the metabolic enzymes favouring this process were monitored. Control and Al-stressed cells grown in 24 well collagen coated plates and incubated in 10mM $[^{13}\text{C}] \text{HCO}_3^-$ and 30mM $[2-^{13}\text{C}] \text{D-glucose}$ and the spent fluid was isolated. Following the precipitation of the lipoprotein, ^{13}C -NMR spectra were recorded. The phosphotungstate-precipitated pellet from Al-stressed cultures yielded signals at 41, 45, 85, 99, 111 and 123 ppm characteristic of the methylene and olefinic moieties found in lipids (Fig 6) [35]. The control pellet only yielded a signal at 168 ppm, indicating the near complete oxidative metabolism of these substrates.

These results prompted us to examine the role of the various cellular components involved in the biosynthesis of these lipoproteins. The CFE from control and Al-stressed cells (2mg/ml protein equivalent) were suspended in a phosphate reaction buffer containing 10mM $[2-^{13}\text{C}] \text{glucose}$ and incubated for 24h at 37°C (note: the purity of the CFE was confirmed with actin and histone 2A immunoblot assays). ^{13}C -NMR spectra obtained from Al-stressed cytoplasm reactions revealed two olefinic signals at 123 and 139 ppm (Fig 7, I). In control experiments two peaks at 56 ppm and 16 ppm attributable to malate and lactate respectively were evident (Fig 7, I). The nature of the peaks in the control experiment was confirmed by performing NMR analysis of lactate and malate standards (data not shown). The disparity in the metabolism of D-glucose in control and Al-stressed HepG2 cells was further analyzed using fluorescent microscopy. Al-stressed cells exposed to D-

glucose for 24h contained high amounts of oil droplets in the cytosol (Figure 7, II).

Lipogenic Enzymes in Al-exposed Hepatocytes

As Al toxicity seemed to promote enhanced lipogenesis, several lipogenic enzymes were monitored. The high levels of lipids and the disparate metabolic profile prompted us to analyze the activity and expression of ACC, the enzyme which catalyzes the initial rate limiting step of lipogenesis. ACC had at least a two fold higher activity in the Al-stressed cells exposed to D-glucose for 24h compared to the control (Fig 8, I). Biotin assays demonstrated the expression pattern of ACC in control and Al-stressed cells exposed to either D-glucose or D-fructose. The Al-stressed cells contained higher levels of ACC in comparison to control (Fig 8, II and III). The activity of G3PDH, which provides the glycerol backbone for triglyceride synthesis, was determined by BN PAGE. A more intense band was observed in Al-stressed cells in comparison to control (Fig 8, IV). 2D BN PAGE helped delineate an increased expression of this protein in Al-stressed culture. G6PDH and IDH-NADP⁺, which produces NADPH via the pentose phosphate pathway and decarboxylation of isocitrate to 2-oxoglutarate, also showed increased activity in Al-stressed cultures (Fig 8, V and VI). A marked increase in CS expression was observed in the Al-stressed cells treated with D-glucose for 24h compared to control cells (Fig 9, I). It is important to note that when citrate was the source of carbon, the expression of this enzyme was not prominent in the cells. ATP-CL, an enzyme that is known to trigger lipogenesis was increased in activity and expression in the cytoplasmic fractions of Al-stressed cells (Fig 9, II and III).

Fig. 7. I) Proton decoupled ^{13}C -NMR analysis of $[2-^{13}\text{C}]$ D-glucose metabolism in A) control and B) Al-stressed HepG2 cells. I) 2mg/ml equivalent of CFE from control and Al-stressed cultures was incubated at 37°C for 24h in a phosphate reaction buffer containing 10mM ^{13}C - labeled D-glucose. II) Fluorescent detection of intracellular lipid droplets. HepG2 cells were grown to a minimal density on glass coverslips and exposed to A) control, B) $10\mu\text{M}$ Al, and C) $500\mu\text{M}$ Al conditions. Cells were exposed to 30mM D-glucose as previously described. Visualization of the intracellular lipid droplets was achieved using Oil Red-O. Cells were visualized at 400x magnification. Scale bar: $10\mu\text{m}$.

Immunoblot experiments confirmed the upregulation of this lipogenic enzyme in Al-stressed cultures (Fig 9, IV). These data confirm that Al enhances the production of lipids through increased activity and expression of numerous lipogenic enzymes.

Discussion

The foregoing results point to an Al-evoked metabolic shift in hepatocytes resulting in enhanced production of VLDL. We have recently demonstrated the ability of this trivalent metal to severely impede mitochondrial functions in HepG2 cells. In this instance, various Fe-containing enzymes such as aconitase, and fumarase have been shown to be drastically perturbed [19]. The diminished TCA cycle in Al-stressed hepatocytes appears to limit ATP production via oxidative phosphorylation but increase lipid production. This phenomenon is common in obese individuals as they tend to experience a deficit in ATP and an accumulation in fat [36]. Al-triggered dyslipidemia may be due to the ability of Al to perturb iron metabolism and promote oxidative stress. Owing to its trivalent nature, Al is known to displace Fe and thus interferes with biological processes necessitating this essential redox

metal. TCA cycle and oxidative phosphorylation in particular cannot function efficiently under Fe-limited situations [19]. Hence, mitochondrial dysfunction will ensue. In this instance, a sharp reduction in the activities of SDH and Cyt c Ox, two critical enzymes in the electron transport chain, leads to a decrease in ATP production.

It is not unlikely that the Fe-deprived situation created by Al would favour a hypoxic environment with the concomitant stimulation of lipogenesis. The presence of lipoproteins in the spent fluid would argue for such a possibility. Indeed, the precipitate from the Al-stressed cultures contained markedly more protein and cholesterol compared to the control. ApoB-100 is a glycoprotein with a molecular mass of 100 kDa that plays a critical role in the formation of VLDL and LDL [37]. And as it forms insoluble aggregates in intracellular aqueous solutions, it is rapidly degraded co-translationally or post-translationally. The interaction of lipids with nascent ApoB-100 is mediated by the microsomal triglyceride transfer protein (MTP) and the mature VLDL is subsequently transported to different organs via a receptor-mediated process [37]. In this study, the enhanced lipogenesis triggered by Al would favor the stabilization and the subsequent secretion of VLDL. Indeed, the levels of ApoB-100 in the spent fluid coincided

Fig. 8. Activity and immunoblot detection of ACC and anaerobic enzymes in A) control and B) Al-stressed HepG2 cells. I) In-gel detection of ACC activity in HepG2 cells incubated with 30mM D-glucose. II) Immunoblot detection of ACC in HepG2 cells incubated in 30mM D-glucose. III) Immunoblot detection of ACC in HepG2 cells incubated with 30mM D-fructose. Assays were performed following a 24h exposure to the two monosaccharides. In-gel activity detection of anaerobic enzymes in HepG2 cells exposed to A) control and B) Al-stressed HepG2 cells. IV) Activity stain for G3PDH. Protein levels were ascertained using Native 2D PAGE. V) Activity stain for G6PDH. VI) activity stain for IDH-NADP⁺. CFE in I, IV, and V were used as loading controls.

with the concentration of Al in the culture media. This observation points to a direct connection between Al toxicity, lipogenesis, and VLDL secretion in hepatocytes. In addition, when D-fructose was the source of carbon, more lipids and ApoB-100 were detected in the spent fluid. The enhanced secretion of VLDL in the Al-stressed hepatocytes was further confirmed by incubating these

Fig. 9. Immunoblot detection and activity stain of CS and ATP-CL in A) control and B) Al-stressed HepG2 cells. I) CS levels were ascertained in cells exposed to control (A) and Al-stressed (B) conditions with or without supplementation with D-glucose for 24h. II) and III) Activity staining and protein expression of ATP-CL in control (A) and Al-stressed (B) cells (protein levels were ascertained by 2D-Native PAGE). IV) Immunodetection of ATP-CL in control (A) and Al-stressed (B) conditions with or without supplementation with D-glucose for 24h.

cells with ¹³C-labeled HCO₃⁻ and D-glucose. The VLDL isolated from the spent fluid of the Al-stressed cultures had signals at 123 ppm and 139 ppm attributable on olefinic carbon. In addition the signals at 41ppm and 45ppm suggest the presence of methylene groups. There was no discernable precipitate in the spent fluid from the control cultures.

Scheme 1. Al impedes ATP production and mitochondrial function thereby diverting incoming metabolites towards lipogenesis and VLDL production.

As there was no noticeable intracellular increase in ApoB-100 in the Al-stressed cells, it is quite likely that this apolipoprotein was secreted following its stabilization via the lipid moieties. Indeed, ApoB-100 is known to be constitutively synthesized and degraded; only in the presence of lipids that this glycoprotein is stabilized and packaged as VLDL [37]. Hence, its secretion in the Al-stressed cells had to be regulated and coordinated with lipid synthesis. It became apparent that the Al had drastically altered the metabolic network in the hepatocytes as revealed by NMR studies. Furthermore, more ATP production was evident in the control cells. We have recently shown that the mitochondria from the Al-exposed cells have markedly diminished membrane potential [19].

Lipogenesis involves the carboxylation and subsequent polymerization of acetyl CoA with the participation of NADPH, a critical anabolic nucleotide [38]. Three critical enzymes that control lipogenic activity had markedly increased activity and expression. ATP-CL, an enzyme responsible for generating acetyl CoA, was significantly higher in the citrate cultures compared to the cultures supplemented with the monosaccharides. ACC, the producer of malonyl CoA, an inhibitor of the transport of lipids into the mitochondria and an activator

of lipogenesis was markedly increased as evidenced by biotinylated probe [38]. CS also displayed higher expression in the Al-stressed cells. This may allow the routing of the hexose into citrate for its eventual processing into fatty acids. An ineffective aconitase and mitochondria would indeed favour such a metabolic shift [19, 39]. It is also worthy to note that the expression of CS was completely absent in cells treated with only citrate or Al-citrate.

G3PDH that diverts trioses from the glycolytic cycle into the lipid-producing machinery by producing glycerol, an essential component of triglycerides was also increased in the Al-stressed cells [40]. The supply of NADPH for the production of fatty acid was fulfilled by the enhanced activity of G6PDH and IDH-NADP⁺ as the increased activity of these enzymes was observed as a consequence of Al-toxicity. IDH-NADP⁺ has recently been shown to be the key producer of reducing equivalents for lipogenesis [41]. Thus, the increased synthesis of acetyl CoA and NADPH, two pivotal ingredients that drive lipogenesis, were favoured in the Al-challenged hepatocytes. The inability of the mitochondria to effectively utilize different carbon sources in ATP production may also be favouring this metabolic shift. We have recently demonstrated a sharp diminution in mitochondrial enzymes in Al-stressed hepatocytes [19].

In this study, although the presence of citrate and D-glucose did trigger the synthesis of lipids in Al-exposed HepG2 cells, maximal amount of lipid in the spent fluid was observed in the presence of D-fructose. The significance of this ketohexose in obesity has been well documented [42]. D-fructose is known to give rise to insulin resistance and hyperlipidemia [42]. In fact, an increase in triglycerides and VLDL has been observed in healthy individuals fed with D-fructose or sucrose but not with starch [42]. D-fructose is more lipogenic than D-glucose or starch. It is hypothesized that D-fructose encourages triglyceride production by evading fructose-2,6-biphosphatase/kinase, a regulatory node that controls D-glucose levels in the liver [42]. The inability to regulate the flux of D-fructose through glycolysis and glycogenesis may explain why the levels of VLDL and ApoB-100 were more pronounced in Al-stressed cultures fed with 30mM D-fructose.

¹³C-NMR data pointed to the conversion of D-glucose into peaks at 139 ppm and 123 ppm attributable to olefinic carbon. The inability of the CFE obtained from Al-challenged hepatocytes to completely utilize glucose was evident by a peak at 73 ppm indicative of the ¹³C₂-position of glucose. The control hepatocytes, on the other

hand, completely metabolized the glucose. It is also interesting to note that ATP-CL activity was most pronounced when citrate was the substrate. This situation may have been triggered by a metabolically ineffective aconitase in the cultures supplemented with Al. We have recently demonstrated the perturbation of the pivotal Fe-S cluster in aconitase in the presence of Al [15]. The diminished activity and/or inactivity of this citrate isomerizing enzyme may be an important mediator of lipogenesis. This enzyme may signal a lack of oxygen and iron, two key effectors that allow the mitochondria to produce ATP via oxidative phosphorylation. This metabolic dysfunction would result in increased cytoplasmic citrate, a potent activator of lipogenesis [44]. Indeed, Al has been shown to promote anaerobiosis via the stabilization of HIF-1 α , a harbinger of mitochondrial dysfunction [39].

Owing to its ability to interfere with iron-metabolism, Al severely affected normal mitochondrial metabolic function in this study. The inability of this organelle to produce energy via oxidative phosphorylation coupled by the diminished efficacy of the tricarboxylic acid (TCA) cycle due to ineffective enzymes like aconitase, would compel the hepatocyte to divert metabolites towards lipogenesis. This scenario is akin to what is observed in obesity whereby there is an imbalance in ATP production and nutrient intake. In this study, Al has compelled the cell to slow down its mitochondrial ATP formation and activate lipogenesis. This shift towards lipogenesis at the expense of ATP formation is a common phenomenon encountered in obese individuals. Incidentally, as has been demonstrated in numerous studies, D-fructose, a potent promoter of obesity, did evoke the maximal secretion of VLDL in Al-exposed hepatocytes. This Al-induced

dyslipidemia appeared to be pivotal in stabilizing ApoB-100 and the secretion of VLDL in the spent fluid.

Exposure to Zn and H₂O₂, known inhibitors of mitochondrial activity, did also induce the secretion of lipoproteins. Hence, the mitochondrial dysfunction appeared to be the trigger that provoked the hepatocyte to become lipogenic. In conclusion, our study demonstrates how Al toxicity may create a cellular environment conducive to the enhanced production of lipids and the diminished formation of mitochondrial ATP. Scheme I illustrates the ability of Al to disrupt mitochondrial function and promote lipogenesis and VLDL secretion. This situation is a common metabolic dysfunction that characterizes obese individuals. Hence, a link between mitochondrial dysfunction and obesity appears to be quite likely.

Abbreviations

VLDL (very low density lipoprotein); ApoB-100 (apolipoprotein B-100); ACC (acetyl CoA carboxylase); G3PDH (glycerol-3-phosphate dehydrogenase); ATP-CL (ATP-citrate lyase); CS (citrate synthase); IDH-NADP⁺ (isocitrate dehydrogenase NADP⁺-dependent); SDH (succinate dehydrogenase); FBS (fetal bovine serum); α -MEM (α -minimum Eagle medium); CSB (cell storage buffer).

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