Toxicology Letters 203 (2011) 219–226

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

The disruption of L-carnitine metabolism by aluminum toxicity and oxidative stress promotes dyslipidemia in human astrocytic and hepatic cells

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ARTICLE INFO

Article history:
Received 21 January 2011
Received in revised form 11 March 2011
Accepted 14 March 2011
Available online 23 March 2011

Keywords:
Aluminum toxicity
Dyslipidemia
Carnitine biosynthesis
Obesity
Neurological disorders

ABSTRACT

L-Carnitine is a critical metabolite indispensable for the metabolism of lipids as it facilitates fatty acid transport into the mitochondrion where β-oxidation occurs. Human astrocytes (CCF-STTG1 cells) and hepatocytes (HepG2 cells) exposed to aluminum (Al) and hydrogen peroxide (H_2O_2), were characterized with lower levels of L-carnitine, diminished β-oxidation, and increased lipid accumulation compared to the controls. γ-Butyrobetainealdehyde dehydrogenase (BADH) and butyrobetaine dioxygenase (BBDOX), two key enzymes mediating the biogenesis of L-carnitine, were sharply reduced during Al and H_2O_2 challenge. Exposure of the Al and H_2O_2-treated cells to α-ketoglutarate (KG), led to the recovery of L-carnitine production with the concomitant reduction in ROS levels. It appears that the channeling of KG to combat oxidative stress results in decreased L-carnitine synthesis, an event that contributes to the dyslipidemia observed during Al and H_2O_2 insults in these mammalian cells. Hence, KG may help alleviate pathological conditions induced by oxidative stress.

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1. Introduction

Industrialization, anthropogenic activities, natural phenomena and increased use of man-made chemicals, have triggered a sharp rise in pollutants in our daily life. This situation has led to a devastating impact on human health. Numerous diseases are known to be caused by these environmental toxins (Betarbet et al., 2000; Briggs, 2003; Sherer et al., 2002). While phthalates, chemicals widely utilized in consumer products, disrupt reproductive functions in males, pollutants like polychlorinated benzenes are responsible for various forms of cancers (Foster, 2006; Liu et al., 2010; Swan, 2008). Gaseous pollutants such as CO, NO_2, and SO_2 impair cardiac functions (Rosenlund et al., 2006). Environmental metal contaminants have also been documented to be responsible for a variety of medical conditions. Whereas manganese, a constituent of anti-knock agent induces Parkinson's disease-like disorders, the presence of arsenic in drinking water has been linked to cardiovascular diseases due to its ability to perturb nitric oxide (NO) homeostasis (Bhatnagar, 2006; Guilarte, 2010).

Aluminum (Al), the most widely occurring metal in the earth's crust has become a health threat due to its increased bioavailability in the environment and its presence in consumer goods (Kaizer et al., 2008). This trivalent metal has been associated with Alzheimer's disease, anemia, and osteomalacia (Kaizer et al., 2008). Its ability to mimic iron (Fe), interfere with calcium (Ca) signaling pathways, and bind to phosphate moieties appears to contribute to the toxicity of Al. We have recently demonstrated how Al triggers oxidative stress, disrupts oxidative phosphorylation, and compels HepG2 cells to adopt an anaerobic respiratory regime in an effort to generate ATP, a metabolic switch mediated by the stabilization of hypoxia inducible factor-1α (HIF-1α) (Mailloux and Appanna, 2007). Aconitase (ACN), the gate-keeper to the tricarboxylic acid (TCA) cycle is severely diminished in the presence of Al due to the perturbation of the Fe–S cluster (Mailloux et al., 2006; Middaugh et al., 2005; Zatta et al., 2000). These Al-induced metabolic changes, help to siphon carbohydrates towards lipogenesis turning hepatocytes into fat-producing engines (Mailloux et al., 2007a).

In this report, we have examined the molecular mechanisms responsible for the inability of two mammalian cell lines, namely HepG2 and astrocytoma (CCF-STTG1) to degrade lipids and their propensity to accumulate lipids when challenged by H_2O_2 and Al respectively. L-Carnitine is a non-essential amino acid involved in the transport of fatty acid derived acyl groups into the mitochondria (Muniyappa, 2010). Its synthesis is a multi-step enzymatic process that necessitates the participation of lysine, methionine, and KG. The homeostasis of this biomolecule plays a central role in the metabolism of lipids (Vaz and Wanders, 2002). Here, we demonstrate how Al and H_2O_2 interfere with L-carnitine biosynthesis by impeding two key enzymes mediating the synthesis of this metabolite. The significance of KG metabolism in anti-oxidative defense

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doi:10.1016/j.toxlet.2011.03.019

and L-carnitine biogenesis during Al and H_2O_2 toxicity is discussed. The resulting dyslipidemia and its implications in obesity and neurological disorders are also discussed.

2. Materials and methods

2.1. Culturing and isolating CCF-STTG1 and HepG2 cells

The human astrocytoma cell line (CCF-STTG1) was acquired from the ATCC, Manassas, VA, USA. The usefulness of this cell line is derived from its maintenance of normal astrocytic properties (Mentz et al., 1999). The astrocytic cell line was cultured as described in Lemire et al., 2008. HepG2 cells were a gift from Dr. Templeton (University of Toronto) and were grown as described in Mailloux et al. (2007a,b). It is a commonly utilized model system to study hepatic metabolism (Goya, 2009). Although these are cellular model systems, they do provide valuable information of the molecular workings of the respective organs (Donato et al., 2010; Mentz et al., 1999). When a confluency of 75% was reached, the cell monolayer was washed with Phosphate Buffered Saline [PBS (136 mM sodium chloride, 2.5 mM potassium chloride, 1.83 mM dibasic sodium phosphate, and 0.43 mM monobasic potassium phosphate) pH 7.4]. The astrocytic cells were re-supplemented with serum-free media containing 2.5 mM lactate chelated to varying amounts of Al (0.01–0.1 mM) or 2.5 mM lactate with 40 μM H_2O_2. Cells exposed to lactate (2.5 mM) alone served as the control. HepG2 cells were resupplemented with serum-free media containing 2.5 mM citrate complexed to varying amounts of Al (0.01–0.25 mM) or 2.5 mM citrate with 40 μM H_2O_2. Cells exposed to citrate (2.5 mM) alone served as the control (Mailloux et al., 2007a; Mailloux and Appanna, 2007). To perform recovery experiments, after the 24 h-stressing period, the cells were incubated with serum-free media resupplemented with 5 mM KG or 5 mM carnitine for 8 h. For lipid degradation experiments, HepG2 cells were given 2 mM palmitate complexed to BSA (defatted BSA served as a control) for 24 h following the stressing step. Purity of the fractions was deduced by detecting the levels of VDAC (mitochondria) and (F-actin) cytosol. Protein content was analyzed by the Bradford assay using Bovine Serum Albumin (BSA) as the standard (Bradford, 1976).

2.2. Metabolite analysis

To quantify the cellular levels of succinate, and α-ketoglutarate (KG), cells were treated with 1% perchloric acid and then analyzed by HPLC. Samples were injected into a C_{18}-reverse phase column (Phenomenex) working at a flow rate of 0.7 ml/min. The mobile phase consisted of 20 mM KH_2PO_4 (pH 2.9 with 6 N HCl). Total carnitine was measured by a modified method described in Minkler et al. (2008). Briefly, the soluble cell free extracts were subjected to a 1:4 digestion in 1 M KOH diluted in methanol for 60 min at 50 °C to remove the acyl groups from the carnitine (Minkler et al., 2008). The hydrolyzed carnitine was then injected into a in a C_{18}-reverse phase column (Phenomenex) working at a flow rate of 0.2 ml/min. The mobile phase consisted of 20 mM KH_2PO_4 (pH 7.0 with 6 N HCl) and 20% acetonitrile. Free L-carnitine levels were measured using tandem HPLC analysis. L-Carnitine and its derivatives were separated at a flow rate of 0.7 ml/min, at a retention time of 4 min. The samples were collected and then subsequently reinjected in a C_{18}-reverse phase column (Phenomenex) working at a flow rate of 0.2 ml/min. The mobile phase consisted of 20 mM KH_2PO_4 (pH 2.9 with 6 N HCl) and 20% isopropanol. Palmitate consumption was measured by isolating the cytoplasm by centrifugation at 180,000 × *g* for 2 h to remove any membranes. Following an extraction with hexane, the organic layer was analyzed for palmitate (Schotz et al., 1970). The mobile phase utilized was 95% hexane: 5% isopropanol. Fatty acids were detected at 210 nm. The separated metabolites were compared with known standards, and the metabolite mixtures were spiked with the given metabolites to confirm peak identities.

2.3. Fluorescence microscopy

To visualize ROS formation and lipid accumulation, immunofluorescence microscopy was utilized. CCF-STTG1 or HepG2 cells were grown to a minimal density on coverslips and treated with control, Al, and H_2O_2 conditions as described. The coverslips were washed with 0.5 mM EDTA and PBS and prepared for microscopic examination (Lemire et al., 2008). For the detection of ROS levels within the astrocytes, the cells were incubated with 20 μM of dicholorodihydrofluorescein diacetate (DCFH-DA) in α-MEM and 10% FBS for 1 h at 37 °C (Lemire et al., 2009). Lipids and triglycerides were visualized by incubating the cells in Oil Red O (0.5% in PBS) for 10 min at 37 °C (Mailloux et al., 2007a). The nucleus was identified using Hoechst 33528 (2.5 μg/ml in PBS). The cells were subsequently examined using an inverted deconvolution microscope (Zeiss, Peabody, MA, USA).

2.4. Blue Native PAGE (BN PAGE) and In-gel activity assays

BN PAGE was performed as described in Mailloux et al. (2007b) and Schagger and von Jagow (1991). Gradient gels (4–16%) were preferentially used for these assays. Briefly, 2 μg of protein equivalent/μl was prepared in blue native buffer [500 mM 6-amino hexanoic acid, 50 mM BisTris (pH7.0), and 1% β-dodecyl-D-maltoside]. For soluble proteins, β-dodecyl-D-maltoside was omitted. Each well of the native gel was loaded with 30 μg of prepared protein samples. Once the electrophoresis was completed, the native gel was incubated in equilibration buffer [25 mM Tris–HCl, 5 mM $MgCl_2$ (pH 7.4)] for 15 min. In-gel activity for ATP-citrate lyase (CL) was monitored by adding 20 mM citrate, 0.75 mM CoA, 0.37 mM ATP, 1.5 mM NADH, 5units/ml malate dehydrogenase (MDH), 16.7 μg/ml dichlorophenolindophenol (DCIP), and iodonitrotetrazolium chloride (INT) (0.5 mg/ml) (Mailloux et al., 2007a). Acetyl-CoA carboxylase (ACC) activity was ascertained by the release of Pi as described in (Simonovic et al., 2004). Briefly, the gel was equilibrated and incubated in 10 mM ATP, 10 mM HCO_3^-, and 1 mM acetyl-CoA for 1 h. The gel was rinsed thrice with ddH_2O followed by 10 min incubation with the phosphate precipitation reagent [$(NH_4)_2MoO_4$ (1.06 g) in 1.37 ml triethylamine and 9.2 ml conc. HNO_3] (Mailloux et al., 2007a). BADH activity was monitored by adding 5 mM γ-butyrobetaine (Sigma), 0.5 mM NADH, 16.7 μg/ml DCIP, and 0.4 mg/ml INT in equilibration buffer. BBDOX activity was ascertained by the addition of 5 mM γ-butyrobetaine, 5 mM α-ketoglutarate, 2.5 mM sodium ascorbate, 0.15 mM ferrous sulfate, and 0.4 mg/ml INT in equilibration buffer. Activity bands were achieved by coupling of INT by ascorbate, a method modified from (May et al., 1995). The reactions were halted using a destaining solution (50% methanol and 10% glacial acetic acid). Negative controls were run routinely with activity stains omitting either substrate or cofactor to ensure band specificity. Bands were quantified using the densitometry suite in the Scion Imaging™ software (Scion Corp. Frederick, MD, USA) (Liu et al., 2009).

2.5. Expression analysis

SDS PAGE and 2D SDS PAGE were performed using the modified method catalogued in (Laemmli, 1970; Lemire et al., 2008). Briefly, cells were sonicated and CFE obtained after centrifugation at 400 × *g* were solubilized in 62.5 mM Tris–HCl (pH 6.8), 2% SDS, and 2% β-mercaptoethanol at 100 °C for 5 min and electrophoresed on a 10% isocratic denaturing gel. For 2D analysis of protein levels, activity bands from native gels were precision cut from the gel and incubated in denaturing buffer (1% β-mercaptoethanol, 5% SDS) for 30 min and then loaded vertically into the SDS gel. Electrophoresis was carried out as described above. Proteins were detected using a silver staining kit purchased from Bio-Rad (Chenier et al., 2008). Following electrophoresis, the proteins were blotted on to a nitrocellulose membrane (Li-COR) for immunoblotting. Non-specific binding sites on the membrane were blocked by treatment with 5% non-fat skim milk dissolved in TTBS [20 mM Tris–HCl, 0.8% NaCl, 1% Tween-20 (pH7.6)] for 1 h. Primary antibodies were goat polyclonal to carnitine palmitoyl transferase 1 (CPT1) (Abcam), mouse monoclonal to voltage dependent anion channel (VDAC) (Abcam), mouse monoclonal to BBDOX (Abcam), and a mouse monoclonal to actin (Abcam) and were utilized as recommended by the manufacturers. Secondary antibodies consisted of rabbit anti-goat horseradish peroxidase (HRP) conjugated (Sigma), goat anti-mouse horseradish peroxidase (HRP) conjugated (Sigma) or goat anti-mouse Infrared (IR) 700 (LI-COR) conjugated, donkey anti-mouse IR 680 conjugated (LI-COR), respectively. Detection of the immunoblots was documented via a ChemiDoc XRS system (Bio-Rad Imaging Systems) or using an Odyssey Infrared Imager and accompanying software (LI-COR. Lincoln, NE, USA).

2.6. In-cell immunoblots

In-cell western assays were modified from the Odyssey® Infrared Imaging System protocol document (Li-COR doc# 988-08599). Briefly, CCF-STTG1 cells were seeded in 96-well plates at 1.0×10^6 cells/ml. Following seeding (48 h), the cells were grown to 75% confluency and then stressed as described above. After treatment, the media was removed and the cells were washed thrice with PBS. The cells were then fixed with 37% formaldehyde for 20 min at room temperature. The fixing solution was then removed and the cells were rinsed with 0.1% tween-20 in PBS. Blocking ensued using Odyssey® blocking buffer for 2 h. Primary antibody incubations occurred over a 1 h period with gentle shaking. Mouse monoclonal BBDOX (Abcam), mouse monoclonal to actin (Abcam) were both diluted to a concentration of 1:200 in blocking buffer. Secondary antibodies consisted of donkey anti-mouse IR 680 (LI-COR) diluted to 1:1000. Actin served as a relative control. The infrared signal was detected using an Odyssey® Infrared Imager (LI-COR. Lincoln, NE, USA).

2.7. Statistical analysis

All experiments were performed at least three times and in duplicate. Where appropriate the Student *T* test was utilized to assess significance.

3. Results

3.1. Al and H_2O_2 insult lead to the accumulation of the antioxidant, KG

Following a 24 h treatment with H_2O_2 or Al (a pro-oxidant), the CFE from both the astrocyte and HepG2 cells were found to contain increased levels of KG and succinate when subjected to HPLC analyses (Fig. 1). The latter moiety is known to accumulate due to the non-enzymatic decarboxylation of KG by ROS (Fedotcheva

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