International Journal of Biochemistry and Cell Biology 96 (2018) 51–62

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

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

GLA supplementation regulates PHD2 mediated hypoxia and mitochondrial apoptosis in DMBA induced mammary gland carcinoma

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ARTICLE INFO

Keywords:
Gamma linolenic acid
7, 12-Dimethylbenz (a) anthracene
Hypoxia
Mitochondria mediated death apoptosis
Breast cancer
alpha-7-nachr

ABSTRACT

The aim of the present study is to evaluate the effect of gamma linolenic acid (GLA) on mitochondrial mediated death apoptosis, hypoxic microenvironment and cholinergic anti-inflammatory pathway against 7, 12-dimethylbenz (a) anthracene (DMBA) induced mammary gland carcinoma. The effects of GLA were evaluated morphologically and biochemically against DMBA induced mammary gland carcinoma. The metabolic study was done for evaluation of biomarkers using ^{1}H NMR. The present study was also verified through immunoblotting and qRT-PCR studies for the evaluation of various pathways. GLA treatment has a delineate implementation upon morphology of the tissues when evaluated through carmine staining, hematoxyline and eosin staining and scanning electron microscopy. GLA also demarked a commendatory proclamation of the fifteen key serum metabolites analogous with amino acid metabolism and fatty acid metabolism when recognized through ^{1}H NMR studies. The immunoblotting and qRT-PCR studies accomplished that GLA mediated mitochondrial death apoptosis, curtail hypoxic microenvironment along with hindrance of *de novo* fatty acid synthesis and also mediate the cholinergic anti-inflammatory pathway to proclaim its anticancer effects.

1. Introduction

Polyunsaturated fatty acids (PUFAs) play a predominant role in the cell membrane formation and are also important for the functioning of membrane proteins and membrane fluidity. PUFAs synchronize several cellular processes, functions and gene expression (Kaur et al., 2014). GLA is a member of the ω-6 family of PUFAs and is transfigured into arachidonic acid (AA) by series of desaturation and elongation reactions. AA is further metabolized by cyclooxygenase enzyme into 2-series prostaglandins or through the 5-lipoxygenase enzymes into leukotrienes and 5-hydroxy-eicosatetranoic acid, which are the major determinants for cellular inflammation (Ricciotti and FitzGerald, 2011). GLA is found in animals and plants, oils like sunflower, soy bean and grape seed and is very much found in daily diet (Bederska-Łojewska et al., 2013). Considering the fact that GLA is metabolized to AA and majority of the product of AA metabolism are pro-inflammatory, the GLA has been also considered to be pro-inflammatory in nature. Consistent intake of GLA is expected to promulgate inflammatory cascade

Abbreviations: AA, arachidonic acid; AB, alveolar bud; AAA, aromatic amino acid; BCAA, branched chain amino acid; BSA, bovine serum albumin; BMRB, biological magnetic resonance databank; CEC, cuboidal epithelial cell; CPMG, carr–purcell–meiboom–gill; DMBA, 7, 12-dimethylbenz (a) anthracene; DCT, dense connective tissue; DTT, dithiothreitol; ECG, electrocardiograph; ECD, extracellular domain; EBSS, eagle's balanced salt solution; FASN, fatty acid synthase; FADD, fas associated death domain; GLA, gamma linolenic acid; GSH, glutathione; HR, heart rate; HRV, heart rate variability; HF, high frequency; HIF-1α, hypoxia inducible factor-1α; HBSS, hank's balanced salt solution; HMDB, human metabolome database; ICD, intracellular domain; LF, low frequency; LCT, loose connective tissue; LDL, low density lipoprotein; MEC, myoepithelial cell; MPTP, membrane potential transition pore; NAG, *n*-acetyl-glycoprotein; NO, nitric oxide; OAG, *o*-acetyl-glycoprotein; OPLS-DA, orthogonal projection to latent structure with discriminant analysis; PUFA, polyunsaturated fatty acid; PC, protein carbonyl; PCA, partial component analysis; PHD2, prolyl hydroxylase-2; RIP, receptor interacting protein; SEM, scanning electron microscopy; SOD, superoxide dismutase; TBARs, thiobarbituric acid reactive substances; TM, transmembrane; TMD, transmembrane domain; TRADD, tumor necrosis factor associated death domain; TMAO, trimethylamine; VLF, very low frequency; VLDL, very low density lipoprotein; VEGF, vascular endothelial growth factor; VLF, very low frequency; VLDL, very low density lipoprotein

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https://doi.org/10.1016/j.biocel.2018.01.011
Received 18 November 2017; Received in revised form 30 December 2017; Accepted 13 January 2018

and harmonize the risk of associated clinical conditions like cancer.

Several corroborations including *in vitro* and *in vivo* studies recommend the anticancer activity of GLA. It was previously reported that GLA hinders cell growth of several human neuroblastoma and several rat carcinosarcoma cell lines (Das and Madhavi, 2011; Xu and Qian, 2014). GLA also diminished the tumor growth in the implanted WBC256 rat model (Colquhoun, 2002). It was previously reported that supplementation with GLA rich diets suppressed the mammary gland carcinogenesis and transplanted tumor growth. GLA also inhibits the growth of various cultured human cancer cell lines like ZR-75-1, A549 and PC-3 cells (Xu and Qian, 2014). Thus, GLA seems to be an auspicious cancer therapeutic agent with prudent characteristics although the reason for its high selectivity in GLA induced antitumor effect is still unexplored. The mechanism for the same is elucidated herewith questioning several aspects of cancer progression.

2. Materials and methods

2.1. Chemicals

GLA (GLA-120) was acquired from Dr. Reddy's Laboratory (India). 7, 12-Dimethylbenz (a) anthracene (DMBA) (Sigma Aldrich, 57-97-6); eagle balanced salt solution (EBSS) (Gibco, 2018-11); RNase (SRL, 58895); ponceau S (Himedia, ML045); sodium cacodylate (Sigma Aldrich, C0250); collagenase type 4 (Himedia, TC-214); hyaluronidase (Himedia, TC331); hematoxylin (Himedia, S058); eosin (Himedia, S007); RIPA lysis buffer (Amresco, N653); protein assay kit (Amresco, M173); bovine serum albumin (BSA) (Genetix, PG-2330); transfer buffer (Genetix, GX-9411AR), trizol reagent (Sigma-T9424), cDNA synthesis kit (Genetix-K1612). Caspase 3 (SC-4263) and caspase 8 (SC-4267) assay kits were procured from Santacruz Biotechnology Inc., California, Delaware. All others chemicals were of molecular biology grade and purchased from Genetix Biotech Asia Pvt. Ltd., New Delhi.

2.2. Experimental protocol

The female albino wistar rats were housed in polypropylene cages and randomized into four groups. Each group contained eight animals per group and maintained with controlled condition of light and temperature (23 °C ± 2 °C, 12 h light: dark cycle). The animals were fed with a standard laboratory diet and water *ad libitum*. The animals were acclimatized for two weeks prior to the experimentation. The study was approved by the Animal Ethical Committee in accordance with approved guidelines for the treatment of laboratory animals (BBDNIIT/IAEC/021/2014). Animals randomized to group I received normal saline (0.9 ml/kg, p.o.); group II served as toxic control and received DMBA 8 mg/kg, i.v. through single tail vein injection; group III and IV served as treatment groups and received GLA (0.25 ml/kg, p.o) and GLA (0.5 ml/kg, p.o.) along with DMBA 8 mg/kg, i.v. respectively. Toxicity was induced by single tail vein injection of DMBA on day 1st. The GLA was administered from 7th to 110th day at the dose specified above. The study was terminated on 112th day and the animals were sacrificed by cervical dislocation under light ether anesthesia.

2.3. Hemodynamic changes

The changes in electrocardiogram (ECG) and heart rate variability (HRV) were recorded according to the method established in our laboratory (Mishra et al., 2016).

2.4. Morphological evaluation

2.4.1. Carmine staining

A detailed methodology for the carmine staining has been elaborated previously (Manral et al., 2016). The mammary gland whole mounts were examined under light microscope and evaluated for the presence of alveolar buds (ABs) (De Assis et al., 2010; Manral et al., 2016).

2.4.2. Histopathology of mammary gland tissue

The mammary gland tissues were promptly fixed in 10% formalin, embedded in paraffin wax. The sections were cut into 5 µm size by using microtome and then stained with hematoxyline and eosin (H&E). The histopathological sections were visualized and photographed using digital biological microscope (N120, BR-Biochem Life Sciences, New Delhi, India) (Feng et al., 2015; Líška et al., 2016).

2.4.3. Surface texture analysis of mammary gland tissue using scanning electron microscopy (SEM)

The mammary gland tissue was digested using collagenase (type 4) and fixed using glutaraldehyde. The tissue was processed further using the protocol elaborated by us previously and examined under SEM (JEOLJSM-6490LV) (Roy et al., 2017; Yasugi et al., 1989).

2.5. Antioxidant parameters

The mammary gland tissues (10% w/v) were homogenized in 0.15 M KCl and centrifuged at 10,000 rpm. The supernatants were evaluated for the presence of antioxidant parameters including thiobarbituric acid reactive substances (TBARs), superoxide dismutase (SOD), catalase, glutathione (GSH) and protein carbonyl (PC) using the methods established in our laboratory (Raj et al., 2014).

2.6. ^{1}H NMR spectroscopic analysis of serum samples

2.6.1. Serum sample preparation

The stored serum samples were thawed, vortexed and centrifuged at 10,000 rpm for 5 min at 40 °C to remove precipitates before the execution of NMR. 500 µl of the serum sample was taken from each group for NMR recording. The serum samples were prepared by mixing 250 µl of serum with 250 µl of saline sodium phosphate buffer (20 mM, pH 7.4) with 0.9% saline in D_2O (Kumar et al., 2016). The prepared serum samples were transferred into a 5 mm NMR tube, with a sealed coaxial insert containing the known concentration of 0.1 mM TSP (sodium salt of 3-trimethylsilyl-(2,2,3,3-d4)-propionic acid) prepared in D_2O, to provide lock for NMR experiments and as an external standard reference to aid chemical shift referencing for metabolite quantification and assignment.

2.6.2. ^{1}H NMR measurements

The ^{1}H NMR spectra was recorded at 300 K on a Bruker NMR spectrometer (Avance-III) operating at a ^{1}H frequency of 800.21 MHz and equipped with a Cryoprobe using a method described elsewhere (Roy et al., 2017).

2.6.3. Spectral assignment

The 1D ^{1}H Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra metabolite resonances were assigned using the Chenomx NMR Suite (Chenomx Inc., Edmonton, AB, Canada). The left over peaks in the CPMG ^{1}H NMR spectra was assigned as much as possible by connecting them with the chemical shifts of previously reported NMR assignments of metabolites (Guleria et al., 2014; Rawat et al., 2017) and compared against the data obtained from biological magnetic resonance data bank (BMRB) and human metabolome database (HMDB) (Ulrich et al., 2007; Wishart et al., 2007). The ^{1}H serum spectra consisted of resonances, mainly from lipoproteins, glycoproteins, glucose, amino acids, lactate, and choline-containing metabolites.

2.6.4. Multivariate statistical analysis

The NMR spectra was manually phased, baseline corrected and referenced internally to the methyl resonance of lactate at δ 1.3102 before the multivariate analysis. The CPMG δ (0.7–9.5) spectra were