RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2016, **6**, 114969

Bilobalide safeguards 3T3-L1 adipocytes from hypoxia through protecting mitochondrial bioenergetics, biogenesis and dynamics

Priyanka A., Anupama Nair, Anusree S. S., Nisha V. M. and Raghu K. G.*

Natural products are the cornerstone of modern therapeutics. Bilobalide was found to be effective against hypoxia induced alterations in innate antioxidant status in our earlier study. Adipose tissue hypoxia in obesity contributes to insulin resistance *via* mitochondrial dysfunctions. Mitochondria are a central control point of many metabolic pathways and various pathophysiological conditions. In the present investigation, we evaluated the effect of hypoxia on crucial mitochondrial functions in 3T3-L1 adipocytes and possible protection with bilobalide. Hypoxia for 24 hours substantially increased ($P \leq 0.05$) HIF-1α expression (5.3 fold) as well as PDK-1 expression (2.3 fold) at the protein level in 3T3-L1 adipocytes. The aconitase enzyme activity was significantly ($P \leq 0.05$) reduced (4.5 fold) in the hypoxic group indicating an elevated level of mitochondria-generated ROS production. It also affected mitochondrial bioenergetics like oxygen consumption (2.23 fold), ATP synthesis (4.32 fold), and the activities of respiratory chain complexes such as complexes I, III and IV (2.05, 2.35 & 2.9 fold) in hypoxic adipocytes. Hypoxia also impaired ($P \leq 0.05$) mitochondrial dynamics such as mitochondrial biogenesis and fusion/fission balance in 3T3-L1 adipocytes. Bilobalide protected the 3T3-L1 adipocytes from adverse effects of hypoxia by safeguarding mitochondrial bioenergetics and dynamics, *via* downregulating HIF-1α expression. These findings suggest that bilobalide could be used as a therapeutic agent for adipocyte hypoxia-mediated mitochondrial dysfunctions in obesity.

Received 14th September 2016
Accepted 1st December 2016

DOI: 10.1039/c6ra22298f

www.rsc.org/advances

1. Introduction

Natural products exhibit an extensive effect on mitochondria. Data concerning differential effects on cells from different disease samples are particularly interesting for possible, therapeutic application of these compounds as bioactives. Furthermore, most of the lifestyle related diseases is connected with mitochondrial oxidative stress in virtually every tissue.[1] The use of these compounds, particularly as prophylactic agents, is very promising. However, detailed pharmacological evaluations are needed before considering a wide, systematic use of these compounds in clinical practice. Quercetin, resveratrol, and curcumin have been evaluated in detail for their effects on mitochondria to elucidate the mechanisms of their medicinal properties.[2] Bilobalide from *Gingko biloba* was found to be effective against hypoxia induced alteration in mitochondrial integrity and associated generation of oxidative stress and inflammation[3] from previous data. We also found significant up-regulation of HIF-1α expression.[3] It also caused depletion of innate antioxidant content. Mitochondrial dysfunctions are closely associated with a large variety of disorders.[4] This is because of central role of mitochondria in energy metabolism and associated cellular physiology. These processes are very conspicuous during various stress conditions like hypoxia and ER stress.[5] Cells under hypoxia adjust with surrounding physiology *via* regulation of various metabolic pathways. If low oxygen levels are lengthened, cells undergo adapting process, through hypoxia-inducible factor 1 (HIF-1). Function of this factor is strictly related to the mitochondrial function, which in turn is dependent with the oxygen level.

Mitochondrial abnormalities have been reported in obesity.[6] The mitochondria are dynamic organelles that consistently fuse and divide. Equilibrium between fusion and fission is required for proper functioning of mitochondria.[7] Characterization of the participants of the fission and fusion process has progressed considerably, and we are now anxious to know the exact role of mitochondrial dynamics in mitochondrial and cellular functions. Its importance has been highlighted by the discovery of human diseases caused by alterations in dynamics.[4] Likewise mitochondrial biogenesis and OXPHOS have been reported to play significant role in genesis as well as amplification of various disorders.[6]

There is substantial evidence that hypoxia develops in adipose tissue as the tissue mass expands, and the secretion of a number of inflammation-related adipokines is upregulated by

Agroprocessing and Natural Products Division, CSIR – National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, Kerala, 695019, India. E-mail: raghukgopal@niist.res.in; Fax: +91-471-2491712; +91-471-249158; Tel: +91-9495902522

hypoxia.[3] There is a switch from oxidative metabolism to anaerobic glycolysis.[5] Overall, hypoxia has pervasive effects on the function of adipocytes and appears to be a key factor in adipose tissue dysfunction in obesity.

On the basis of our preliminary study, a detailed investigation is planned with bilobalide exclusively on mitochondrial bioenergetics, biogenesis and dynamics to check whether mitochondrial dysfunction can be taken as druggable target for development of lead for hypoxia mediated disorders especially obesity.

2. Materials and methods

2.1. Chemicals and cell culture reagents

Bilobalide, acriflavine, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, dimethyl sulfoxide (DMSO), and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), fetal calf serum (FCS) and penicillin–streptomycin antibiotics were from Himedia (Himedia Pvt. Ltd, India). Antibodies were from Santacruz (Santa Cruz, CA, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

3T3-L1 preadipocytes (ATCC) were maintained in DMEM (4.5 g L^{-1} high glucose) supplemented with 10% FBS, antibiotic (100 U penicillin per ml, and 100 μg streptomycin per ml) and incubated at 5% CO_2 and 37 °C. To induce differentiation, 2 days post confluent 3T3-L1 preadipocytes were stimulated for 48 h with 0.5 mM isobutyl methyl xanthine, 0.25 mM dexamethasone, and 10 μg ml^{-1} insulin in DMEM. Then differentiated adipocytes were maintained in and refed every 2 days with DMEM containing 10 μg ml^{-1} insulin.

2.3. Hypoxia induction and treatment

In order to induce hypoxia, differentiated 3T3-L1 adipocytes at 9^{th} day were incubated in a hypoxic chamber (Galaxy 48R, New Brunswick, Eppendorf, Germany) at an atmosphere of 1% O_2, 94% N_2, 5% CO_2, and at 37 °C for 24 h. The control cells were incubated in an atmosphere of 21% O_2 and 5% CO_2 at 37 °C. The cells were treated with different concentrations (10, 20 & 50 μM) of bilobalide or acriflavine (5 μM) during hypoxic period (24 h). For mRNA and protein expression studies, only higher doses of test materials were used. Acriflavine was used as positive control.

2.4. Determination of aconitase activity

Aconitase activity was assayed using kit purchased from Cayman chemicals (USA) as per manufacturer's instructions. After respective treatments, cells were washed with cold PBS (pH 7.4). Then fresh PBS was added to cover the cells and centrifuged the cells at 800 × *g* for 10 minutes (min) at 4 °C. Then supernatant was discarded and resuspended the cell pellet in 1 ml of homogenization buffer. The cell suspension was sonicated for 5 seconds and centrifuged the cell suspension at 20 000 × *g* for 10 min at 4 °C. This supernatant was used for the assay of aconitase. 50 μl of the sample was added to 5 μl of assay buffer, 50 μl of $NADP^+$ reagent, 50 μl of isocitric dehydrogenase and 50 μl of aconitase substrate solution and incubated for 15 min at 37 °C. The absorbance was taken once in every min at 340 nm for 10 min.

2.5. Determination of the activity of mitochondrial respiratory complexes

For determining the activity of mitochondrial respiratory complexes after normoxic and hypoxic treatments, mitochondria were isolated using mitochondria isolation kit (Sigma-Aldrich, USA). The isolated mitochondria were then dissolved in CelLytic M, cell lysis reagent with protease inhibitor cocktail [1 : 100 (v/v)] for further analysis.

The effect of hypoxia on complex I mediated electron transfer (NADH dehydrogenase) was studied using NADH as the substrate and menadione as electron acceptor as described previously by Paul *et al.* (2008). The reaction mixture containing 200 μM menadione and 150 μM NADH was prepared in phosphate buffer (0.1 M, pH 8.0). To this, mitochondria (100 μg) was added, mixed immediately and observed quickly for change in the absorbance at 340 nm for 8 min (UV-2450 PC; Shimadzu, Kyoto, Japan). Rotenone (10 μM) was used to inhibit the complex I.

Complex II mediated activity (succinate dehydrogenase) was measured spectrophotometrically at 600 nm using dichlorophenolindophenol (DCPIP) as an artificial electron acceptor and succinate as substrate. The extent of decrease of absorbance (ΔOD) was considered as the measure of the electron transfer activity of complex II as described previously by Paul *et al.* (2008). The reaction mixture was prepared in 0.1 M phosphate buffer (pH 7.4) containing 10 mM EDTA, 50 μM DCPIP, 20 mM succinate and mitochondria (50 μg). The change in absorbance was observed immediately for 8 min at 30 °C. Malonate (25 μM) was used to inhibit the complex II.

Complex III (ubiquinol–cytochrome c reductase) activity was determined as previously described by Sudheesh *et al.* (2009). In brief, mitochondrial protein (50 μg) was mixed with 100 μM of EDTA, 2 mg of BSA, 3 mM of sodium azide, 60 μM of ferricytochrome c, decylubiquinol (1.3 mM) and phosphate buffer (50 mM, pH 8.0) in a final volume of 1 ml. The reaction was started by the addition of decylubiquinol and monitored for 2 min at 550 nm and again after the addition of 1 μmol l^{-1} of antimycin A. The activity was calculated from the linear part of absorption–time curve, which was not less than 30 seconds. Activity of complex III was expressed as μmoles of ferricytochrome c reduced per min per mg protein. Antimycin A (10 μM) was used as standard inhibitor of complex III.

Complex IV activity of mitochondria was assayed using kit from Sigma Aldrich chemicals (USA) as per manufacturer's instructions. Briefly, 950 μl of 1× assay buffer was added to a cuvette and then 10 μg of mitochondrial suspension was added and brought the reaction volume to 1.05 ml with 1× enzyme dilution buffer. The reaction was initiated by the addition of 50 μl of ferrocytochrome c substrate solution. Absorbance was read at 550 min^{-1}.

2.6. Oxygen consumption rate assay

The rate of oxygen consumption in the cells was determined using oxygen consumption rate (OCR) assay kit from Cayman in

accordance with the manufacturer's protocol. The phosphorescence of MitoXpress-Xtra, a phosphorescent oxygen probe, is quenched by oxygen and the phosphorescent signal is inversely proportional to the amount of oxygen present. The OCR was calculated from the change in MitoXpress probe signal over time (excitation: 380 nm; emission: 650 nm).

2.7. ATP determination assay

The ATP content was measured using a luciferase-based bioluminescence assay kit (ATP Determination Kit, Molecular Probes, Invitrogen). The cells, after treatment were homogenized in an ice-cold ATP releasing buffer (100 mM potassium phosphate pH-7.8, 2 mM EDTA, 1 mM DDT, 1% Triton X 100). Then 90 μl of standard reaction mixture (8.9 ml distilled water, 0.5 ml 20× reaction buffer, 0.1 ml 0.1 M DTT, 0.5 ml of 10 mM D-luciferin, 2.5 μl of 5 mg ml^{-1} firefly luciferase) and 10 μl of samples were gently mixed and the luminescence was read at 560 nm in a multiplate reader (Tecan Infinite 200PRO, Austria). Using ATP standard provided with kit, ATP concentrations were determined and then normalized to protein concentrations.

2.8. Determination of mitochondrial biogenesis

Mitochondrial biogenesis was analysed by using Abcam MitoBiogenesis In-CellElisa kit according to the manufacturer's protocol. Briefly, 3T3-L1 preadipocytes were seeded in 96 well plate and were allowed to differentiate. After respective treatments, cells were fixed in 4% paraformaldehyde. The levels of two mitochondrial proteins were measured in each well, one protein being subunit I of complex IV (COX-I), which is mitochondrial DNA-encoded, and the other being the 70 kDa subunit of complex II (SDH-A), which is nuclear DNA encoded. The expression level of COX-I is an indication of mitochondrial biogenesis.

2.9. Quantitative real-time PCR

Total RNA from 3T3-L1 adipocytes was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). 2 μg of total RNA was reverse transcribed using SuperScript III reverse transcriptase and random hexamers (Life technologies, Invitrogen, USA). The gene expression levels were analysed by quantitative real-time RT-PCR, conducted using the CFX96 Real Time PCR system (Bio-Rad, USA) using the following conditions: an initial denaturation for 10 min at 95 °C, followed by 39 cycles of 15 s denaturation at 95 °C, 30 s annealing at the optimal primer temperature and 10 s extension at 72 °C. Each sample was assayed in triplicate in a 20 μl reaction volume containing 1 μl of cDNA, 10 μl of SYBR Green master mix (Power SYBR® Green PCR Master Mix, Life Technologies, Invitrogen, USA), 5.81 μl of DEPC water and 1.6 μl of appropriate primer. Negative controls (no template) were run as well to ensure the absence of contamination. Analysis was performed according to the $\Delta\Delta C_t$ method using β-actin as the housekeeping gene. The nucleotide sequence of primers is given in Table 1.

We selected Tfam, Pgc1a (master regulator of biogenesis), Nrf1, MtDNA, Cyt b genes due to their relevance in the functional features of mitochondria like mitochondrial genome replication, mitochondrial biogenesis, mitochondrial DNA transcription, respiration and replication, mitochondrial DNA and electron transport chain respectively.

Table 1 Nucleotide sequences of qRT primers

mRNA		Primer sequence
Tfam	Forward	5′-GGAATGTGGAGCGTCCTAAAA-3′
	Reverse	5′-TGCTGGAAAAACACTTCGGAATA-3′
Pgc1a	Forward	5′-CGGAAATCATATCCAACCAG-3′
	Reverse	5′-TGAGGACCGCTAGCAAGTTTG-3′
Nrf1	Forward	5′-TGGTCCAGAGAGTGCTTGTG-3′
	Reverse	5′-TTCCTGGGAAGGGAGAAGAT-3′
MtDNA	Forward	5′-CCACTTCATCTTACCATTTA-3′
	Reverse	5′-ATCTGCATCTGAGTTTAATC-3′
Cyt b	Forward	5′-TTTTATCTGCATCTGAGTTTAATCCTG-3′
	Reverse	5′-CCACTTCATCTTACCATTTATTATCGC-3′
β-Actin	Forward	5′-AGTACCCCATTGAACGC-3′
	Reverse	5′-TGTCAGCAATGCCTGGGTAC-3′

2.10. Western blot analysis

Treated cells were washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitors. The cell suspensions were centrifuged at 12 000 rpm for 15 min at 4 °C, and the supernatants was collected. Proteins were quantified using the bicinchoninic acid protein assay kit (BCA kit; Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. Equal amount of proteins (50 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes using turbo transblot apparatus (BD Bioscience). The membranes were blocked with 5% BSA in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20) for 1 h at room temperature. The membrane was washed 3 times with TBST for 10 min each. The membrane was incubated at 4 °C overnight in 5% BSA in TBST containing primary antibodies to one of the following: HIF-1α (biomarker of hypoxia), PDK-1 (biomarker of hypoxia from TCA cycle), OPA1 (mitochondrial fusion protein) 1 : 500, MFN2 (mitochondrial fusion protein) 1 : 500, DRP (mitochondrial fission protein) 1 : 500, FIS (mitochondrial fission protein) 1 : 500 or β-actin 1 : 1000. After washing with TBST, the membrane was incubated with peroxidase-conjugated corresponding secondary antibodies for 1 h at room temperature. After washing, membranes were developed using 3,3′-diaminobenzidine tablets (DAB; Sigma Aldrich, St Louis, MO, USA) and H_2O_2. The immunoblot results were analysed using quantity one software in Gel doc (BD Bioscience, USA).

2.11. Statistical analysis

All the experiments were repeated thrice with duplicate ($n = 6$). Results are expressed as means ± standard deviations. Data were subjected to one-way ANOVA and the significance of differences between means were calculated by Duncan's multiple range tests using SPSS for Windows, standard version 7.5.1 (SPSS), and significance was accepted at $P \leq 0.05$.

3. Results

3.1. HIF-1α expression in normoxic and hypoxic adipocytes

The expression of HIF-1α was determined in normoxic and hypoxic groups of 3T3-LI adipocytes at mRNA and protein level. The expression was significantly increased in hypoxia-treated cells compared with normal cells (Fig. 1A and B). This confirmed the induction of hypoxia in cells. The mRNA and protein level expression of HIF-1α were checked in normoxic and hypoxic groups. The HIF-1α mRNA level was significantly ($P \leq 0.05$) increased after 24 h of hypoxic treatment compared to normoxia. The treatment with bilobalide (50 μM), significantly ($P \leq 0.05$) inhibited hypoxia induced upregulation of HIF-1α mRNA level (Fig. 1A). In this study, acriflavine, which is an HIF-1α inhibitor and prevents transcriptional activities of HIF-1, is used as positive control. This compound also prevented hypoxia induced

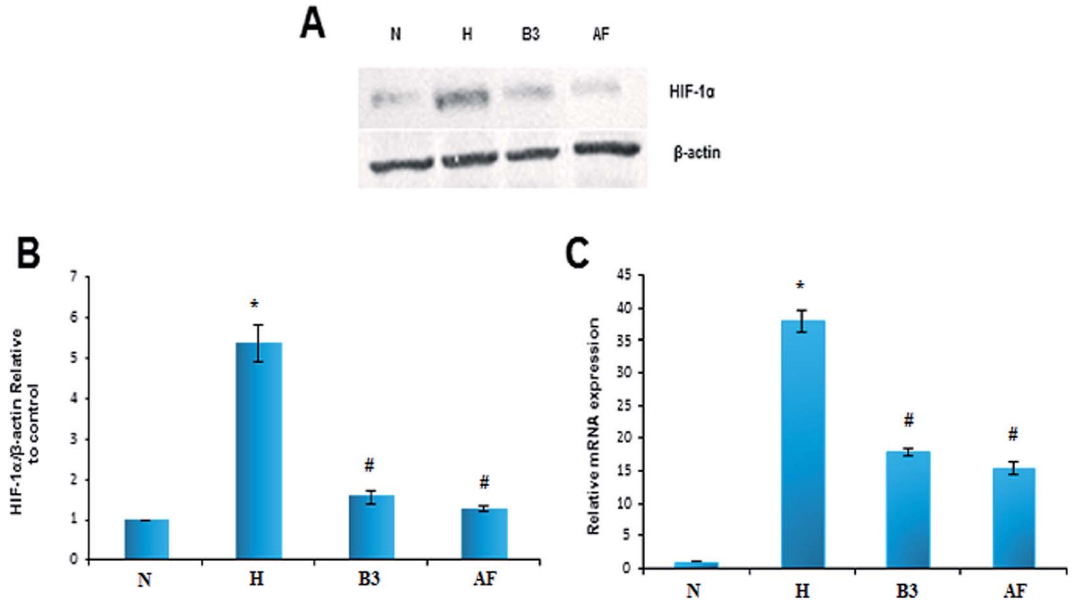

Fig. 1 Expression of HIF-1α transcription factor in normoxic and hypoxic groups: (A) immunoblot analysis of HIF-1α. (B) Quantification of protein level normalized to β-actin. (C) mRNA expression of HIF-1α normalised to β-actin. N-normoxia, H-hypoxia, B3-50 μM of bilobalide, and AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 3$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

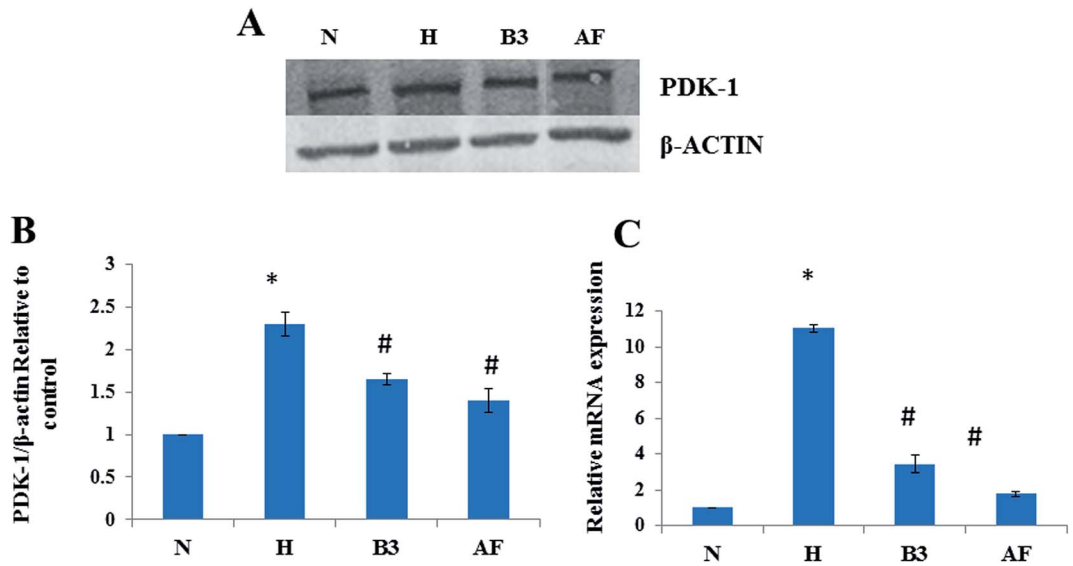

Fig. 2 Expression of pyruvate dehydrogenase kinase 1 in normoxic and hypoxic adipocytes: (A) immunoblot analysis of PDK-1. (B) Quantification of protein level normalized to β-actin. (C) mRNA expression of Pdk-1 normalised to β-actin. N-normoxia, H-hypoxia, B3-50 μM of bilobalide, and AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 3$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

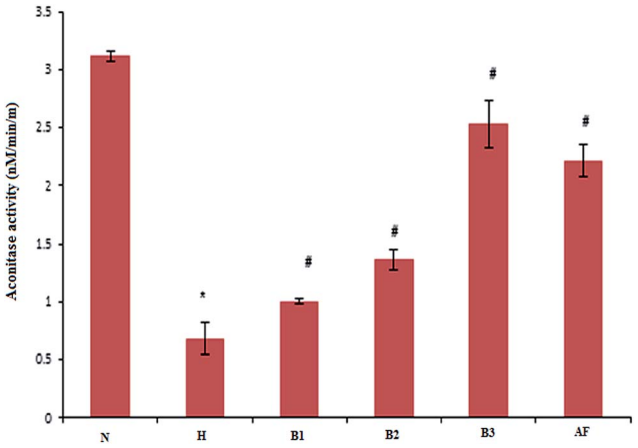

Fig. 3 Aconitase activity in normoxic and hypoxic groups: hypoxia treatment significantly reduced the aconitase activity. Co-treatment of bilobalide restored aconitase activity in a dose dependent manner. N-normoxia, H-hypoxia, B1-10 μM, B2-20 μM, B3-50 μM of bilobalide, AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 6$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

upregulation HIF-1α mRNA level in 3T3-L1 adipocytes. Similar results were obtained with western blot analysis. Hypoxic group showed a significant ($P \leq 0.05$) increase in protein level of HIF-1α (5.34 fold) compared to normoxia. Bilobalide or acriflavine significantly reduced the protein level of HIF-1α (Fig. 1B and C).

3.2. Pyruvate dehydrogenase kinase 1 expression and aconitase activity in normoxic and hypoxic adipocytes

We analysed the expression of pyruvate dehydrogenase kinase 1 (PDK1) by qRT-PCR and immunoblot analysis. PDK1 is the protein that maintains mitochondrial function in hypoxic condition. Results showed an increased expression of PDK1 at protein level as well as mRNA level in hypoxia treated cells compared with normoxic cells. The treatment with bilobalide (50 μM) or acriflavine (5 μM) significantly ($P \leq 0.05$) reduced PDK 1 expression in hypoxic 3T3-L1 adipocytes (Fig. 2A and B).

Aconitase activity was significantly ($P \leq 0.05$) reduced in hypoxic group (4.54 fold) when compared with control cells. Bilobalide (10, 20, 50 μM) treatment dose dependently improved (1.47, 1.99, 3.69 fold) the activity significantly ($P \leq 0.05$) and brought back the activity near to normal. Acriflavine also improved aconitase activity (3.24 fold; Fig. 3). Thus, inhibition of aconitase activity in hypoxia indicates elevated level of mitochondria generated ROS.

3.3. Activities of mitochondrial respiratory chain complexes in normoxic and hypoxic adipocytes

Table 2 shows the activities of mitochondrial respiratory complexes in normoxic and hypoxic groups. The activities of respiratory chain complexes such as complexes I, III and IV were significantly decreased (2.05, 2.35 & 2.9 fold; Table 2) in hypoxic adipocytes ($P \leq 0.05$) compared to normal cells. Bilobalide (50 μM) or acriflavine (10 μM) treatment prevented the reduction (1.31, 1.36, 1.28 fold; 1.4, 1.6, 1.3 folds respectively; Table 2) of respiratory chain complexes activities in hypoxic 3T3-L1 adipocytes ($P \leq 0.05$). Typical inhibitors of the various steps of respiratory chain complexes like rotenone, inhibited complex I activity by 3.87 fold, malonate inhibited complex II activity by 3.40 fold, antimycin A inhibited complex III activity by 2.44 fold and KCN inhibited complex IV activity by 7.45 fold. There were no significant changes in complex II activity in normoxic and hypoxic groups.

3.4. Oxygen consumption rate and ATP content in normoxic and hypoxic adipocytes

The oxygen consumption rate (OCR) was analysed using Cayman's O_2 consumption assay kit. The kit utilizes a phosphorescent probe MitoXpress®, signal of which is quenched by oxygen and resulting in a signal that is inversely proportional to the amount of oxygen present. Adipocytes under hypoxia showed a significant ($P \leq 0.05$) decrease in OCR (2.23 fold) compared with the normoxic cells (Fig. 4). There was also a significant decrease in oxygen consumption rate in antimycin A treated

Table 2 Activities of mitochondrial respiratory chain complexes[a]

	NADH:ubiquinone oxidoreductase (complex I) (ΔOD 340 nm)	Succinate-CoQ reductase (complex II) (ΔOD 600 nm)	Cytochrome c reductase (complex III) (μM of ferricytochrome c reduced per min per mg protein)	Cytochrome c oxidase (complex IV) (μM of ferrocytochrome c oxidized per min per mg protein)
N	0.391 ± 0.006	0.245 ± 0.008	5.14 ± 1.015	6.11 ± 0.18
H	0.191 ± 0.003*	0.239 ± 0.01	2.19 ± 0.16*	2.90 ± 0.25*
B3	0.251 ± 0.015#	0.237 ± 0.016	3.95 ± 0.41#	4.11 ± 0.29#
AF	0.245 ± 0.011#	0.249 ± 0.012	3.18 ± 0.52#	3.88 ± 0.41#
Rotenone	0.101 ± 0.008*			
Malonate		0.072 ± 0.021*		
Antimycin A			2.11 ± 0.24*	
KCN				0.82 ± 0.52*

[a] N – normoxia, H – hypoxia, B3 – 50 μM of bilobalide and AF – 5 μM of acriflavine treated hypoxic groups. Values are means, with standard deviations ($n = 6$).* Mean values are significantly different from the control cells ($P < 0.05$). # Mean values are significantly different from hypoxia treated cells ($P < 0.05$).

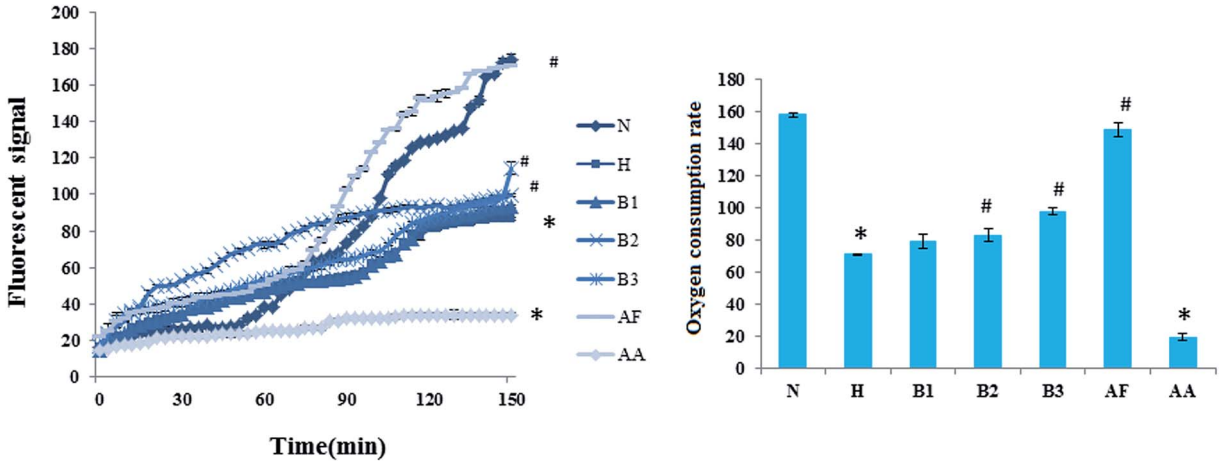

Fig. 4 Changes in oxygen consumption in normoxic and hypoxic groups: hypoxia significantly reduced the oxygen consumption rate compared with the control group. Bilobalide and acriflavine co-treatment partly restored the oxygen consumption rate. N-normoxia, H-hypoxia, B1-10 μM, B2-20 μM, B3-50 μM of bilobalide, AF-5 μM of acriflavine treated hypoxic groups, and AA-antimycin A treated group. Values are means with standard deviations represented by vertical bars ($n = 6$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

cells. Antimycin A is a typical electron transport chain inhibitor. It inhibits mitochondrial electron transport between cytochromes b and c, and causes an inhibitory response on cellular oxygen consumption. However, treatment with bilobalide or acriflavine dose dependently promoted oxygen consumption rate. 10, 20, 50 μM of bilobalide significantly accelerated 1.11, 1.37, & 1.67 fold oxygen consumption rate respectively in hypoxic adipocytes ($P \leq 0.05$; Fig. 4A and B). Acriflavine (5 μM) also increased ($P \leq 0.05$) oxygen consumption rate (2.09 fold) in hypoxic adipocytes, indicating protection against the defect in oxygen consumption.

ATP content in hypoxic group was significantly ($P \leq 0.05$) reduced (4.32 fold) when compared to normoxia (Fig. 5). Treatment with bilobalide or acriflavine restored (2, 2.34, & 3.08; 3.55 fold respectively; $P \leq 0.05$; Fig. 5) ATP content, further confirming the protective effect of these compounds against alterations in mitochondrial function.

3.5. Mitochondrial biogenesis in normoxic and hypoxic adipocytes

The mitobiogenesis in cell ELISA assay measures the specific activities of two mitochondrial proteins, the subunit I of complex IV (cytochrome c oxidase-1, COX-1), which is mtDNA encoded, and a subunit of complex II (succinate dehydrogenase-A, SDH-A), which is nuclear DNA-encoded. The specific activity of mtDNA encoded COX-1 was significantly ($P \leq 0.05$) depleted (1.45 fold) in hypoxic group compared with normoxia indicating, loss of mitochondrial biogenesis. The treatment with bilobalide (10, 20, 50 μM), or acriflavine (5 μM) significantly ($P \leq 0.05$) restored COX-1 activity (1.07, 1.10, 1.26 fold; 1.22, 1.38, 1.41 fold; 1.23 fold; Fig. 6A) in a dose dependent manner, showing protection from loss of mitochondrial biogenesis. But there was no significant changes in the activity of SDH-A, the nuclear DNA encoded protein in normoxic and hypoxic groups. These results strongly support hypoxia induced mitochondrial dysfunctions in adipocytes.

3.6. Mitochondrial biogenesis marker expression, mtDNA copy number and alterations in proteins involved in mitochondrial structural dynamics in normoxic and hypoxic adipocytes

Next, we examined the mRNA level expression of the mitochondrial biogenesis related factors, Pgc1a, Nrf1, mtTFA and Cyt b in normoxic and hypoxic adipocytes. The results showed

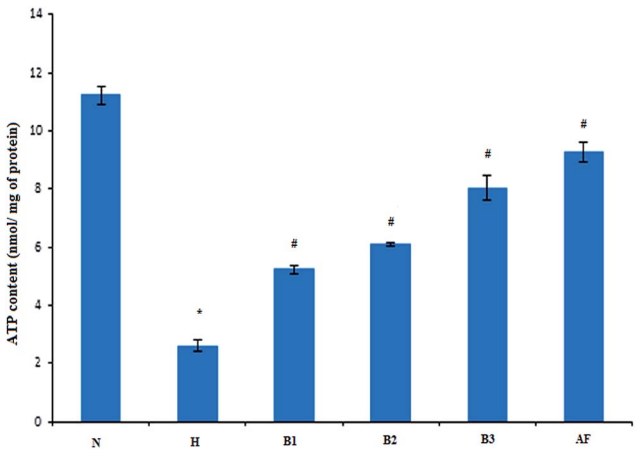

Fig. 5 Determination of ATP content in normoxic and hypoxic group: hypoxia significantly reduced ATP content compared with the normoxic group. Bilobalide and acriflavine co-treatment partly restored ATP content in hypoxic groups. N-normoxia, H-hypoxia, B1-10 μM, B2-20 μM, B3-50 μM of bilobalide, and AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 6$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

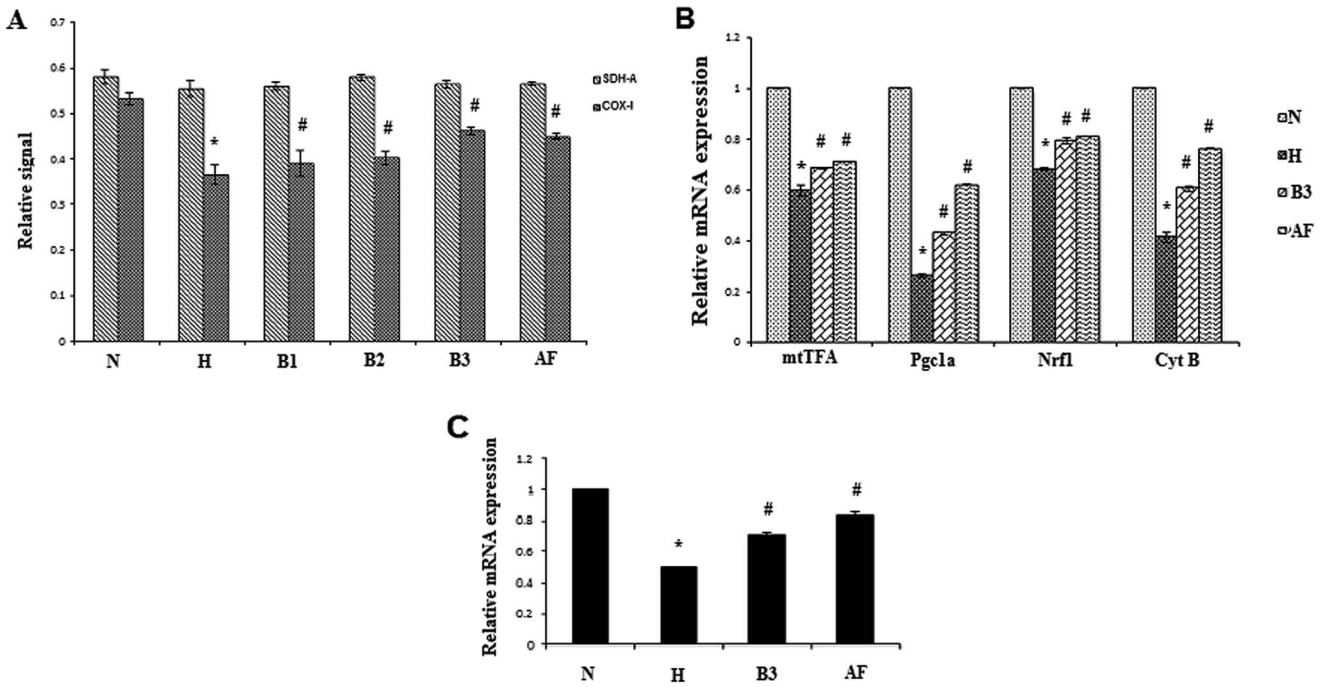

Fig. 6 Studies on mitochondrial biogenesis in normoxic and hypoxic groups: (A) mitochondrial biogenesis in normoxic and hypoxic group based on the specific activities of subunit I of complex IV (COX-1), which is mtDNA encoded, and subunit of complex II (SDH-A), which is encoded in nuclear DNA. (B) The relative expression of genes involved in mitochondrial biogenesis in normoxic and hypoxic groups. (C) Mitochondrial DNA copy number in normoxic and hypoxic groups determined by qRT PCR. N-normoxia, H-hypoxia, B1-10 μM, B2-20 μM, B3-50 μM of bilobalide, and AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 6$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

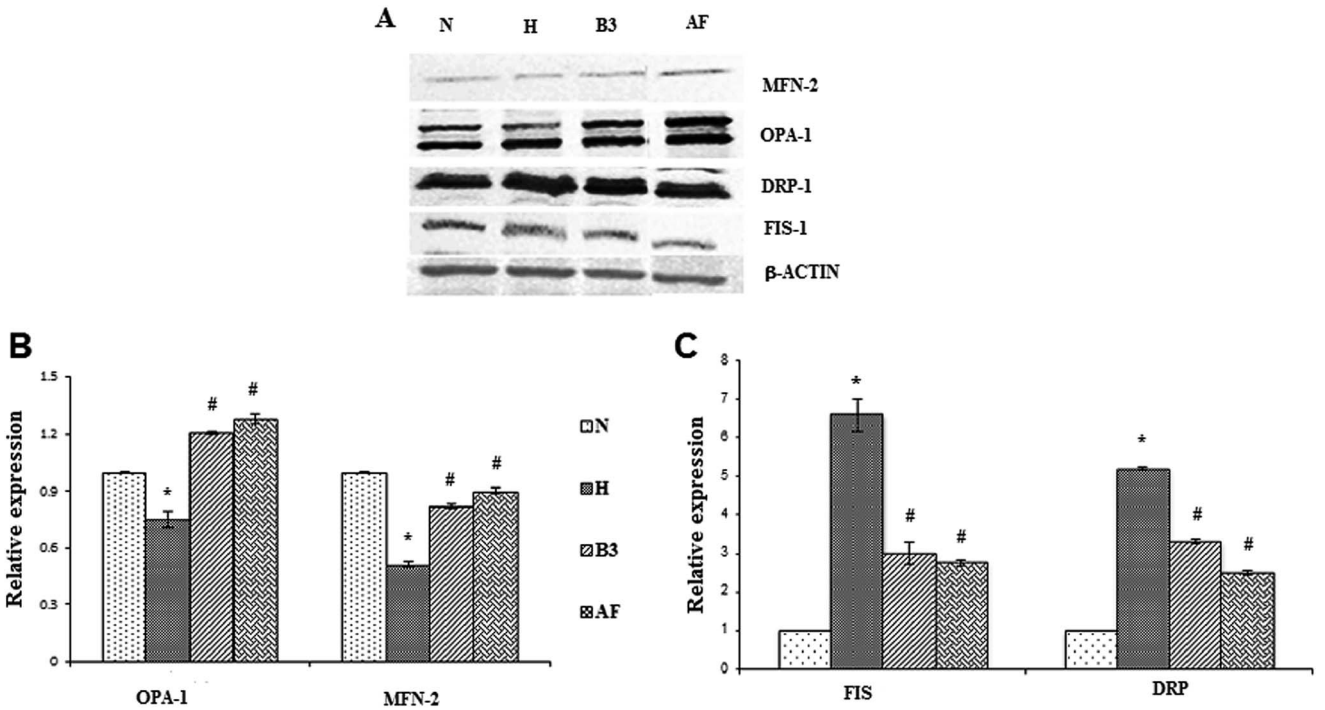

Fig. 7 Studies on mitochondrial structural dynamics in normoxic and hypoxic groups: (A) immunoblot analysis of proteins involved in mitochondrial fusion (MFN2 & OPA-1) and fission (DRP1 & FIS1). (B) Quantification of protein level of OPA-1 and MFN-2 normalized to β-actin. (C) Quantification of protein level of FIS-1 and DRP-1 normalized to β actin. N-normoxia, H-hypoxia, B3-50 μM of bilobalide and AF-5 μM of acriflavine treated hypoxic groups. Values are means with standard deviations represented by vertical bars ($n = 3$).* Mean values are significantly different from the control cells ($P \leq 0.05$). # Mean values are significantly different from hypoxia treated cells ($P \leq 0.05$).

that the gene level expression of Pgc1a, Nrf1, mtTFA and Cyt b were significantly ($P \leq 0.05$) decreased after hypoxia treatment with respect to normoxia (Fig. 6B). Similarly qRT PCR analysis of mtDNA copy number was also found to be significantly decreased in hypoxic group (Fig. 6C). The treatment with bilobalide (50 μM), or acriflavine (5 μM) significantly ($P \leq 0.05$) restored mitochondrial biogenesis related proteins and mtDNA copy number almost to normal level in hypoxia treated groups (Fig. 6B and C). The results showed a significant ($P \leq 0.05$) decrease in MFN2 & OPA1 (fusion proteins) expression and significant increase in DRP1 & FIS1 (fission proteins) in hypoxic adipocytes indicating impaired mitochondrial function. The treatment with bilobalide (50 μM), or acriflavine (5 μM) restored the fusion and fission proteins in normal range (Fig. 7A–C).

4. Discussion

The investigations on the effects of biologically active natural compounds of vegetal origin on cell physiology is now centre of attraction for having better prospects in the development of nutraceuticals and phytoceuticals. Many of these compounds are found to exert their functions *via* mitochondrial functions.[8] Mitochondria are "powerhouse" of the cell, where tricarboxylic acid cycle (TCA) and β-oxidation take place and chemical energy is converted into ATP.[9] Its dysfunction is a causing factor to obesity.[10] It is involved in many pathways in the adipocyte, including differentiation and maturation.[11] So any defect in mitochondrial biology may lead to alterations in adipocytes. HIF-1α is a major biochemical agent of the hypoxia in the inhibition of mitochondrial function.[12,13] Mitochondria, which are crucial oxygen sensors are getting affected with adipocyte hypoxia.[3] Therefore, in present study, detailed investigations on the effect of bilobalide on mitochondrial dynamics, biogenesis and OXPHOS functions during hypoxia were conducted.

The induction of hypoxia was confirmed by evaluating the expression of HIF-1α. The treatment with bilobalide inhibited the expression of HIF-1α in 3T3-L1 adipocytes showing protection from hypoxia. Studies by various authors have already established PDK-1 as a hypoxia responsive protein that regulates the function of the mitochondria under hypoxia, by reducing pyruvate conversion to acetyl-CoA, resulting in a drop in mitochondrial oxygen consumption.[14,15] In another study by Wigfield *et al.*, (2008),[16] it was confirmed that hypoxic upregulation of PDK-1 is under the control of the HIF-1. In our study also, the PDK-1 expression is substantially increased showing impairment in mitochondrial functions with hypoxia. Bilobalide restored its expression to normal level *via* downregulating HIF-1α.

The mitochondrial ETC is a major site of ROS production. ROS has been described in WAT of many mouse models of obesity, such as the KKAy, diet-induced obesity, and db/db mice.[17–19] In our previous study, we reported, an increased level of ROS along with surplus generation of mitochondrial superoxide in hypoxic adipocytes.[3] Mitochondrial ROS generated at complex III, causes stabilization of HIF-1α during hypoxia.[20,21] Mitochondrial aconitase is an enzyme that plays a central role in carbohydrate and energy metabolism and is responsible for the interconversion of citrate to isocitrate.[22] The activity of this enzyme is sensitive to oxidative stress and superoxide radicals.[23,24] Thus, the assay for aconitase activity is a marker of mitochondrial-generated ROS level.[25] In agreement with several other reports,[26,27] the present study confirmed the paradoxical phenomenon of hypoxia-induced oxidative stress and damage in 3T3-L1 adipocytes. This leads to a reduction in aconitase activity. Bilobalide protected mitochondria from ROS as well as superoxide production in hypoxic cells which is evident from increased aconitase enzyme activity. Bilobalide could attenuate ROS in different cells and act as a free radical scavenger.[28]

Hypoxia is associated with disturbances of ATP synthesis resulting from depressed functions of ETC and oxidative phosphorylation in the respiratory chain.[29] The mitochondrial membrane potential provides the driving force for ATP synthesis. We have already reported a dissipation of $\Delta\psi_m$ in hypoxia[30] which ultimately leads to disruption of ETC, ATP synthesis, and oxygen consumption. Mitochondrial oxygen consumption and ATP synthesis can also be regulated by PDK1 expression.[15] It is a direct target of HIF-1, which phosphorylates and inactivates pyruvate dehydrogenase enzyme that converts pyruvate to acetyl-coenzyme A. Thereby it inhibits pyruvate metabolism *via* TCA cycle.[30] Since TCA cycle is coupled to electron transport, regulation of the PDH complex by PDK-1 is critical for mitochondrial respiration and ROS production.[14] We found an increased PDK-1 expression in hypoxia and treatment with bilobalide restored its expression to normal. So by regulating HIF-1α and PDK-1 expression, bilobalide maintained ATP synthesis and oxygen consumption rates to normal during hypoxia. Many factors including age, obesity, and T2DM could reduce the mitochondrial content in white adipocytes.[31,32] Recent studies also reported that transgenic, as well as high-fat diet obese mice, have less mitochondrial density compared to lean control. This is an indication of less mitochondrial biogenesis[33] in obese condition. Obesity induced alterations in mitochondrial biogenesis substantially impair white adipocyte metabolism. Herein we analysed, how hypoxia affects the major factors involved in mitochondrial biogenesis. Of several regulatory factors involved in biogenesis, peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α) and nuclear respiratory factors (NRF1 and NRF2) are master regulators.[34,35] We found a reduction in the expression of PGC-1α in hypoxia-treated 3T3-L1 adipocytes. NRF1 and mtTFA are essential transcription factors for mitochondrial biogenesis and PGC-1α. NRF1 can stimulate the transcription of many nuclear-encoded mitochondrial genes, such as OXPHOS genes and respiratory complexes. There were significant reductions in NRF1, mtTFA expression and mtDNA copy number after hypoxia, which was consistent with the decreased expression of PGC-1α. Our study also showed that bilobalide prevented downregulation of mitochondrial biogenesis influencing factors under hypoxic condition. Bilobalide improved the expression of PGC-1α and all other PGC-1α regulated downstream factors of biogenesis. The previous study by Krishnan *et al.*, (2012),[36] reported that HIF-1α inactivation promotes mitochondrial biogenesis specifically in white adipocytes. We have already found bilobalide inhibits HIF-

1α at mRNA and proteins level and thus protects hypoxic cells from mitochondrial biogenesis impairment.

Mitochondria are dynamic organelles, continuously undergoing fission/fusion and biogenesis to form a network that spans the entire area of cells to meet the demands of cellular function.[7] These processes are essential for proper maintenance of the structure as well as every aspect of mitochondrial function of cells including the formation of reactive oxygen species (ROS), ATP production, and respiration.[7] It is also associated with cell death mechanisms.[37] The dysfunction of mitochondrial fission/fusion and biogenesis is associated with many diseases, in particular, metabolic syndromes. No detailed informations are available on the effect of hypoxia in adipocyte on mitochondrial fission/fusion. Herein we explored whether adipocyte hypoxia was associated with imbalance in mitochondrial fission/fusion and beneficial properties of bilobalide on mitochondrial dynamics during hypoxia.[38] We, therefore, investigated the expression of the fusion proteins, MFN2 and OPA1, and fission proteins, DRP1 and FIS1 in hypoxic and normoxic cells by immunoblot analysis. Although opposing, the fusion and fission processes work in concert to maintain mitochondrial morphology, size, and number.[39] Key mediators of mitochondrial fission/fusion include the DRP1 and FIS1 which are essential for fission, and OPA1, MFN1 and MFN2 which mediate fusion. A recent study by Liu *et al.*, (2014)[40] found that proteins controlling mitochondrial fusion MFN1 and MFN2 but not OPA1 were decreased and proteins governing mitochondrial fission FIS1 and DRP1 were increased in skeletal muscle of HFD-fed mice when compared to normal. Our study has exhibited significantly lower expression of MFN2 and OPA1 in hypoxic condition when compared with the normoxic group. The previous report described PGC-1α acts as a mediator of mitochondrial biogenesis and transcriptional co-activator of MFN2, a protein involved in fusion.[41] Hence this lower expression of MFN2 could be attributed to reduced expression of PGC-1α in hypoxic condition. We also observed an increased expression of fission proteins DRP1 and FIS1 in hypoxia. It is already reported in cancer studies that HIF-1α promotes mitochondrial fission by upregulation of DRP1 and FIS proteins.[42] From this, we conclude that obesity-related hypoxia in adipocytes promotes mitochondrial fission by overexpression of proteins DRP1 and FIS which downregulate mitochondrial biogenesis, oxygen consumption, OXPHOS, and ATP synthesis. Bilobalide restored the expression of proteins DRP1 and FIS1 to a normal level and protected the cells from enhanced mitochondrial fission by inhibiting HIF-1 activation as well as by improving PGC-1α expression.

Bilobalide, a sesquiterpene trilactone from *Ginkgo biloba* has been proposed to have protective effects on mitochondrial function. Most of the effect of EGb761 containing high content of bilobalide on energy metabolism and protection against hypoxia are explained by a stabilization of mitochondrial function.[43] The beneficial effects on complex I, IV and V of the mitochondrial respiratory chain against NO-induced damage was observed after treating young and old mice with EGF761.[44] Direct evidence for an improvement of mitochondrial function by bilobalide was provided by Janssens *et al.*, 1995.[45] It protects endothelial cells against hypoxia induced ATP decrease. In addition, it increases the respiratory control ratio of mitochondria isolated from liver of orally treated rats.[45] The protection of ATP content and the delay in glycolysis activation are explained by a protection of mitochondrial respiratory activity. It appears to improve the ability to form ATP and reduced the requirement for cellular glycolysis by preservation of ATP generation in mitochondria.[45] Further investigations provided evidence that bilobalide allows mitochondria to maintain their respiratory activity in ischemic condition by protecting complex 1 and probably complex 3 activities.[46] EGb761 was found to prevent mitochondrial dysfunction at low concentrations as determined by measurement of ATP levels and mitochondrial membrane potential.[47]

In conclusion, overall results of this study provide a new insight into hypoxia induced impairment of mitochondrial function in 3T3-L1 adipocytes and possible recovery with bilobalide.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

A. P. designed and conducted the experiments, analysed the results and prepared the manuscript. N. A. has involved in preparation of manuscript and contributed in cell culture and investigations related to mitochondria. S. S. A. and V. M. N. also conducted the experiments and analysed the data. K. G. R. contributed to the conception and design of the study, the acquisition and interpretation of the data, performed the analysis and drafted the manuscript for important intellectual content. All the authors participated sufficiently in the work.

Acknowledgements

Priyanka A. thanks University Grants Commission, Govt. of India, for financial assistance in the form of research fellowship. We are also thankful to the CSIR 12th 5-year plan project 'NaPAHA CSC 0130' for financial support. We thank the Director, CSIR-NIIST for providing necessary facilities.

References

1 L. Vitetta and B. Anton, Lifestyle and nutrition, caloric restriction, mitochondrial health and hormones: scientific interventions for anti-aging, *Clin. Interventions Aging*, 2007, **2**(4), 537–543.

2 L. Gibellini, E. Bianchini, S. De Biasi, M. Nasi, A. Cossarizza and M. Pinti, Natural Compounds Modulating Mitochondrial Functions, *J. Evidence-Based Complementary Altern. Med.*, 2015, **2015**, 527209.

3 A. Priyanka, V. M. Nisha, S. S. Anusree and K. G. Raghu, Bilobalide attenuates hypoxia induced oxidative stress, inflammation, and mitochondrial dysfunctions in 3T3-L1

adipocytes *via* its antioxidant potential, *Free Radical Res.*, 2014, **48**, 1206–1217, DOI: 10.3109/10715762.2014.945442.

4 K. H. Lee, Y. M. Cho, S. H. Kwak, S. Lim, K. S. Park and E. B. Shim, Mitochondrial dysfunction and metabolic syndrome—looking for environmental factors, *Biochim. Biophys. Acta*, 2010, **1800**(3), 282–289.

5 M. J. Schönenberger and W. J. Kovacs, Hypoxia signaling pathways: modulators of oxygen-related organelles, *Front. Cell Dev. Biol.*, 2015, **21**(3), 42, DOI: 10.3389/fcell.2015.00042.

6 J. C. Bournat and C. W. Brown, Mitochondrial dysfunction in obesity, *Curr. Opin. Endocrinol., Diabetes Obes.*, 2010, **17**(5), 446–452.

7 C. A. Galloway and Y. Yoon, Mitochondrial morphology in metabolic diseases, *Antioxid. Redox Signaling*, 2013, **19**(4), 415–430.

8 B. C. Cavalcanti, P. M. da Costa, A. A. Carvalho, F. A. Rodrigues, R. C. Amorim, E. C. Silva, *et al.*, Involvement of intrinsic mitochondrial pathway in neosergeolide-induced apoptosis of human HL-60 leukemia cells: the role of mitochondrial permeability transition pore and DNA damage, *Pharm. Biol.*, 2012, **50**(8), 980–993.

9 M. J. Goldenthal and J. Marín-García, Mitochondrial signaling pathways: a receiver/integrator organelle, *Mol. Cell. Biochem.*, 2004, **262**, 1–16, DOI: 10.1023/B:MCBI.0000038228.85494.3b.

10 J. Ye, Emerging role of adipose tissue hypoxia in obesity and insulin resistance, *Int. J. Obes.*, 2009, **33**, 54–66, DOI: 10.1038/ijo.2008.229.

11 A. De Pauw, S. Tejerina, M. Raes, J. Keijer and T. Arnould, Mitochondrial (Dys)function in adipocyte (De)differentiation and systemic metabolic alterations, *Am. J. Pathol.*, 2009, **175**, 927–939, DOI: 10.2353/ajpath.2009.081155.

12 X. Zhang, K. S. Lam, H. Ye, S. K. Chung, M. Zhou, Y. Wang, *et al.*, Adipose tissue-specific inhibition of hypoxia-inducible factor 1α induces obesity and glucose intolerance by impeding energy expenditure in mice, *J. Biol. Chem.*, 2010, **285**, 32869–32877, DOI: 10.1074/jbc.M110.135509.

13 M. K. Jang, Y. Son and M. H. Jung, ATF3 plays a role in adipocyte hypoxia-mediated mitochondria dysfunction in obesity, *Biochem. Biophys. Res. Commun.*, 2013, **431**, 421–427, DOI: 10.1016/j.bbrc.2012.12.154.

14 J. W. Kim, I. Tchernyshyov, G. L. Semenza and C. V. Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia, *Cell Metab.*, 2006, **3**, 177–185, DOI: 10.1016/j.cmet.2006.02.002.

15 I. Papandreou, R. A. Cairns, L. Fontana, A. L. Lim and N. C. Denko, HIF-1 mediates adaptation to hypoxia by actively down-regulating mitochondrial oxygen consumption, *Cell Metab.*, 2006, **3**, 187–197, DOI: 10.1016/j.cmet.2006.01.012.

16 S. M. Wigfield, S. C. Winter, A. Giatromanolaki, J. Taylor, M. L. Koukourakis and A. L. Harris, PDK-1 regulates lactate production in hypoxia and is associated with poor prognosis in head and neck squamous cancer, *Br. J. Cancer*, 2008, **98**, 1975–1984, DOI: 10.1038/sj.bjc.6604356.

17 J. M. Curtis, P. A. Grimsrud, W. S. Wright, X. Xu, R. E. Foncea, D. W. Graham, *et al.*, Downregulation of adipose glutathione s-transferase a4 leads to increased protein carbonylation, oxidative stress, and mitochondrial dysfunction, *Diabetes*, 2010, **59**, 1132–1142, DOI: 10.2337/db09-1105.

18 N. Houstis, E. D. Rosen and E. S. Lander, Reactive oxygen species have a causal role in multiple forms of insulin resistance, *Nature*, 2006, **440**, 944–948, DOI: 10.1038/nature04634.

19 S. Furukawa, T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, *et al.*, Increased oxidative stress in obesity and its impact on metabolic syndrome, *J. Clin. Invest.*, 2004, **114**, 1752–1761, DOI: 10.1172/JCI200421625.

20 N. S. Chandel, D. S. McClintock, C. E. Feliciano, T. M. Wood, J. A. Melendez, A. M. Rodriguez, *et al.*, Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O_2 sensing, *J. Biol. Chem.*, 2000, **275**, 25130–25138, DOI: 10.1074/jbc.M001914200.

21 B. Chhunchha, N. Fatma, E. Kubo, P. Rai, S. P. Singh and D. P. Singh, Curcumin abates hypoxia-induced oxidative stress based-ER stress-mediated cell death in mouse hippocampal cells (HT22) by controlling Prdx6 and NF-κB regulation, *Am. J. Physiol.: Cell Physiol.*, 2013, **304**, C636–C655, DOI: 10.1152/ajpcell.00345.2012.

22 K. H. Tsui, L. C. Chung, S. W. Wang, T. H. Feng, P. L. Chang and H. H. Juang, Hypoxia upregulates the gene expression of mitochondrial aconitase in prostate carcinoma cells, *J. Mol. Endocrinol.*, 2013, **51**, 131–141, DOI: 10.1530/JME-13-0090.

23 M. K. Paul, R. Kumar and A. K. Mukhopadhyay, Dithiothreitol abrogates the effect of arsenic trioxide on normal rat liver mitochondria and human hepatocellular carcinoma cells, *Toxicol. Appl. Pharmacol.*, 2008, **226**, 140–152, DOI: 10.1016/j.taap.2007.09.020.

24 N. P. Sudheesh, T. Ajith and K. K. Janardhanan, *Ganoderma lucidum* (Fr.) P. Karst enhances activities of heart mitochondrial enzymes and respiratory chain complexes in the aged rat, *Biogerontology*, 2009, **10**, 627–636, DOI: 10.1016/j.taap.2007.09.020.

25 P. R. Gardner, Aconitase: sensitive target and measure of superoxide, *Methods Enzymol.*, 2002, **349**, 9–23, DOI: 10.1016/S0076-6879(02)49317-2.

26 J. Magalhaes, A. Ascensao, J. M. Soares, M. J. Neuparth, R. Ferreira, J. Oliveira, *et al.*, Acute and severe hypobaric hypoxia-induced muscle oxidative stress in mice: the role of glutathione against oxidative damage, *Eur. J. Appl. Physiol.*, 2004, **91**, 185–191.

27 C. Regazzetti, P. Peraldi, T. Grémeaux, R. Najem-Lendom, I. Ben-Sahra, M. Cormont, F. Bost, *et al.*, Hypoxia decreases insulin signaling pathways in adipocytes, *Diabetes*, 2009, **58**, 95–103, DOI: 10.2337/db08-0457.

28 L. J. Zhou and X. Z. Zhu, Reactive oxygen species-induced apoptosis in PC12 cells and protective effect of bilobalide, *J. Pharmacol. Exp. Ther.*, 2000, **293**, 982–988.

29 L. D. Lukyanova, Mitochondrial Signaling in Hypoxia, *Open J. Endocr. Metab. Dis.*, 2013, **3**, 20–32, DOI: 10.4236/ojemd.2013.32A004.

30 M. C. Sugden and M. J. Holness, Therapeutic potential of the mammalian pyruvate dehydrogenase kinases in the prevention of hyperglycaemia, *Curr. Drug Targets: Immune, Endocr. Metab. Disord.*, 2002, **2**, 151–165.

31 H. J. Choo, J. H. Kim, O. B. Kwon, C. S. Lee, J. Y. Mun, S. S. Han, *et al.*, Mitochondria are impaired in the adipocytes of type 2 diabetic mice, *Diabetologia*, 2006, **49**, 784–791, DOI: 10.1007/s00125-006-0170-2.

32 T. Schöttl and M. Klingenspor, Boosting mitochondrial biogenesis in white adipocytes: a route towards improved insulin sensitivity?, *Mol. Metab.*, 2013, **2**, 128–129, DOI: 10.1016/j.molmet.2013.07.002.

33 J. X. Rong, Y. Qiu, M. K. Hansen, L. Zhu, V. Zhang, M. Xie, *et al.*, Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone, *Diabetes*, 2007, **56**, 1751–1760, DOI: 10.2337/db06-1135.

34 J. Liu, W. Shen, B. Zhao, Y. Wang, K. Wertz, P. Weber and P. Zhang, Targeting mitochondrial biogenesis for preventing and treating insulin resistance in diabetes and obesity: hope from natural mitochondrial nutrients, *Adv. Drug Delivery Rev.*, 2009, **61**, 1343–1352, DOI: 10.1016/j.addr.2009.06.007.

35 R. C. Scarpulla, R. B. Vega and D. P. Kelly, Transcriptional integration of mitochondrial biogenesis, *Trends Endocrinol. Metab.*, 2012, **23**, 459–466, DOI: 10.1016/j.tem.2012.06.006.

36 J. Krishnan, C. Danzer, T. Simka, J. Ukropec, K. M. Walter, S. Kumpf, *et al.*, Dietary obesity-associated HIF-1α activation in adipocytes restricts fatty acid oxidation and energy expenditure *via* suppression of the Sirt2–NAD^+ system, *Genes Dev.*, 2012, **26**, 259–270, DOI: 10.1101/gad.180406.111.

37 D. F. Suen, K. L. Norris and R. J. Youle, Mitochondrial dynamics and apoptosis, *Genes Dev.*, 2008, **22**(12), 1577–1590.

38 M. Liesa, M. Palacín and A. Zorzano, Mitochondrial dynamics in mammalian health and disease, *Physiol. Rev.*, 2009, **89**, 799–845, DOI: 10.1152/physrev.00030.2008.

39 D. C. Chan, Fusion and fission: interlinked processes critical for mitochondrial health, *Annu. Rev. Genet.*, 2012, **46**, 265–287, DOI: 10.1146/annurev-genet-110410-132529.

40 R. Liu, P. Jin, L. Yu, Y. Wang, L. Han, T. Shi, *et al.*, Impaired Mitochondrial Dynamics and Bioenergetics in Diabetic Skeletal Muscle, *PLoS One*, 2014, **9**, e92810, DOI: 10.1371/journal.pone.0092810.

41 Y. Y. Chiang, S. L. Chen, Y. T. Hsiao, C. H. Huang, T. Y. Lin, I. P. Chiang, *et al.*, Nuclear expression of dynamin-related protein 1 in lung adenocarcinomas, *Mod. Pathol.*, 2009, **22**, 1139–1150, DOI: 10.1038/modpathol.2009.83.

42 A. Zorzano, Regulation of mitofusin-2 expression in skeletal muscle, *Appl. Physiol., Nutr., Metab.*, 2009, **34**, 433–439, DOI: 10.1139/H09-049.

43 W. E. Müller, A. Eckert, C. Kurz, G. P. Eckert and K. Leuner, Mitochondrial dysfunction: common final pathway in brain aging and Alzheimer's disease–therapeutic aspects, *Mol. Neurobiol.*, 2010, **41**, 159–171, DOI: 10.1007/s12035-010-8141-5.

44 S. Augustin, G. Rimbach, K. Augustin, R. Cermak and S. Wolffram, Gene regulatory effects of *Ginkgo biloba* extract and its flavonol and terpenelactone fractions in mouse brain, *J. Clin. Biochem. Nutr.*, 2009, **45**, 315–321, DOI: 10.3164/jcbn.08-248.

45 D. Janssens, C. Michiels, E. Delaive, F. Eliaers, K. Drieu and J. Remacle, Protection of hypoxia-induced ATP decrease in endothelial cells by *Ginkgo biloba* extract and bilobalide, *Biochem. Pharmacol.*, 1995, **50**, 991–999, DOI: 10.1016/0006-2952(95)00227-Q.

46 D. Janssens, J. Remacle, K. Drieu and C. Michiels, Protection of mitochondrial respiration activity by bilobalide, *Biochem. Pharmacol.*, 1999, **58**, 109–119, DOI: 10.1016/S0006-2952(99)00061-1.

47 R. Abdel-Kader, S. Hauptmann, U. Keil, I. Scherping, K. Leuner, A. Eckert, *et al.*, Stabilization of mitochondrial function by *Ginkgo biloba* extract (EGb761), *Pharmacol. Res.*, 2007, **56**, 493–502, DOI: 10.1016/j.phrs.2007.09.011.