Supplementary Material

Vitamin B12 Induces Hepatic Fatty Infiltration through Altered Fatty Acid Metabolism

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Supplementary material

Material and Methods

Cell culture

Hep G2 cell culture was done, following the protocol with slight modifications, as previously described elsewhere [26]. Briefly, using the custom-made B12 deficient Eagles' Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% streptomycin and/ penicillin, the cells were cultured in a T-75 flask under 37^{o}C incubation with 5% CO_2 saturation. Following attainment of 100% confluence, cells were trypsinized and seeded into six (6) well plates at 75,000 cells per well in four (4) different concentrations of B12 in EMEM media such as 500nM (Control), 1000pM, 100pM and 25pM. The respective B12-EMEM media for each condition was changed every 48 hours until 100% confluence was achieved where cells were (a) harvested for assays including; isolation of RNA, total intracellular triglyceride estimation and fatty acid profiling, (b) or maintained within plates for assays such as; oil red O (ORO) staining and radiochemical measurement of synthesized triglycerides.

Oil Red O staining and elution assay

Hep G2 cells, upon reaching 90-100% confluence, were gently washed twice with 1x phosphate buffered saline (PBS). Briefly, one-hour fixation of cells at room temperature using 10% formalin was performed, followed by washing off the formalin with PBS and allowing cells to completely dry. Equal volume of the ORO stain was dispensed into each well allowing staining of the cells for exactly 2-hours, followed by multiple washes with deionized water until no traces of the stain remained in the wells. Images of stained lipid droplets within Hep G2 cells were observed and captured using 40x objective of the light microscope. For the elution assay, after wells of plates were completely dry, 300µl absolute isopropanol was added to each well and placed on a shaker for 20-mins. 200µl of the isopropanol was collected from each well and absorbance measured spectrophotometrically at 520nm wavelength. Each absorbance was afterwards normalized with the amount of protein in Hep G2 cells in each well.

RNA isolation, cDNA synthesis and gene expression

Total RNA isolation was done using the Trizol method. The cDNA synthesis and gene expression assays were similar to the previously described protocol [27]. Also, qRT-PCR involved the use of 18s rRNA (Applied Biosystems, UK) for normalizing expression of RNA. All fatty acid, triglyceride, cholesterol biosynthesis and fatty acid oxidation genes were custom Taqman gene assays supplied by the Applied Biosystems, UK [14].

Total intracellular triglyceride estimation

Estimation of total intracellular triglyceride in Hep G2 was performed, following the manufacturer's protocol, using the commercial Triglyceride Quantification Kit (ab65336) from Abcam plc, Cambridge, UK. Briefly, frozen stock of 1mM triglyceride standard was thawed in water bath at 80^{o}C – 100^{o}C for 1 minute, gently vortexed for 30 seconds and then diluted to 0.2mM. The probe was warmed in water bath at 37^{o}C for 5-min to enable thawing of the dimethylsulfoxide solution, triglyceride enzyme mix and lipase were prepared by reconstituting each in 220µl of assay buffer and kept on ice throughout the test. Then the triglyceride standard was diluted with the assay buffer to a total volume of 50µl per well to generate standard concentrations of 0, 2, 4, 6, 8 and 10nmol/well. 2µl of lipase was added to each well and incubated for 20-minutes. A cocktail of reaction mix (50µl) comprising triglyceride assay buffer, probe and enzyme mix (46:2:2) was added to each well, gently mixed and the absorbance was measured at 570nm wavelength. The data was used to derive a standard curve. Then, the samples were prepared by homogenising cells in PBS and subsequently making up cell suspensions to 50µl of triglyceride assay buffer per well. 2µl of lipase was then added and incubated for 20-min at room temperature followed by addition 50µl of the reaction mix. The final content was gently mixed, and the absorbance was measured at 570nm. The concentration of triglycerides was calculated from the standard curve.

Radiochemical measurement of synthesized triglyceride

Upon reaching 100% confluence on 10th day of cell culture, the condition media in each well of Hep G2 cells were removed and replaced with 1ml of the ^{12}C-Oleate-labelled media and kept at 37^{o}C incubation for 2-hours, whereas background correction control cells were incubated for 5-mins. After the incubation, the labelled media was removed, and Hep G2 cells were harvested in 2ml of methanol. The extraction of total lipids from the Hep G2 cells was done using a mixture of chloroform and methanol (2:1 v/v) [28]. The resulting layer of chloroform containing the triglyceride fraction was then transferred into a glass tube and completely made dry under nitrogen gas. The dried content in the glass tube was then reconstituted in 500µl of chloroform and finally transferred gradually, 15µl at a time, onto a plate of silica gel 60-coated-TLC. The resultant radiolabelled triglyceride is separated by use of a mobile phase made of formic acid/diethyl ether/hexane (1:30:70, v/v/v). To accurately locate the band of the separated triglyceride from the total lipids, 10 nmol of glyceryltripalmitate (tripalmitin), a triglyceride standard was run alongside the test and included in each sample. Visualisation of the triglyceride bands was aided by use of iodine vapour. The bands were then scraped into scintillation vials and the radioactivity of triglyceride fraction was quantified by the scintillation counter (Beckman coulter LS6500 liquid scintillation counter, USA) [26]. Finally, the triglyceride concentration was calculated by normalizing with the protein

concentration in the chloroform extract using the Bradford method (Bio-Rad Laboratories, Hercules, USA) [29].

Analysis of fatty acid composition in total lipids of hepatic cell pellets

Fatty acid levels (µg), normalized per milligram protein of Hep G2 cells, were obtained following preparation of cell pellets of Hep G2 from cell culture in different B12 media. Confluent cells were harvested, following initial washes twice (2x) with PBS, by addition of 300µl of trypsin to cells in the plates and incubated at 37ºC for 5-minutes. 1000µl of EMEM media was then added to each well with the cell suspension transferred into Eppendorf tubes and centrifuged 2000rpm for 10-minutes at 4ºC.

The supernatant was carefully discarded whereas the pellets were frozen at -80ºC. Cell pellets were dissolved in 0.2 mL cell lysis buffer (Cell Signalling Technology, MA, EEUU) containing 1 mM phenylmethanesulfonyl fluoride [30]. Samples were sonicated for 10-mins and allowed to stand for 5-mins. Cell lysates were obtained from the supernatant after 15-min centrifugation at 10,000 g 4 ºC. The protein was quantified in the supernatants by Bradford assay [29]. Total lipids extraction into chloroform/ methanol (2:1 v/v) was carried out after adding 0.05mg pentadecanoic acid (internal standard) to cell pellets [28]. Drying of the lipid extract was achieved using a flow of nitrogen, followed by synthesis of fatty acid methyl esters (FAME) using 3mol/l methanolic HCl (Supelco, Bellafonte, PA, EEUU) for 1 hour at a temperature of 90ºC. Extraction of FAME was subsequently done into hexane which was kept at -20ºC until analysis. Finally, FAME were analyzed by gas-chromatography [31] on a Hewlett-Packard 6890 from Agilent Technologies, Inc. Palo Alto, CA, together with FID system for detection and SP-2560 capillary column (100 m x 0.25 mm x 20 µm) supplied by Supelco, Sigma Aldrich, St Louis, MO, EEUU. Peaks were identified by comparison of their retention times with appropriate FAME (Sigma Aldrich, St Louis, MO, CA, EEUU) and fatty acid concentrations determined in relation to peak area of internal standard.

Mitochondrial dysfunction assessment using seahorse extracellular flux assay

Cell culture:

Hep G2 cells were first cultured in T-75 flasks under four different B12 concentrations [500nM (Control), 1000pM, 100pM and 25pM] of EMEM media supplemented with 10% FBS, 1% L-Glutamine and 1% streptomycin and/ penicillin and placed under 37ºC incubation with 5% CO_2 saturation. The respective B12-media were changed every 48-hours until reaching almost 90-100% confluence on the 10^{th} day in the flasks. On the last day, following the last media change,

the cells were washed twice with 1x-PBS, followed by trypsinization, cell count and seeding into the XF-24 seahorse plates.

Cell density optimization and seeding for XF24 seahorse assay:

For the optimum number of Hep G2 cells in XF-24 seahorse plates for the experiment, we first optimized using different cells densities such as 10,000, 20,000, 30,000 and 50,000 cells / well. An optimum density of 50,000 cells/well, showing the highest oxygen consumption rate (OCR), was chosen for the experiment. The cells were first dispensed in 100µl EMEM-B12 media (supplemented with 10% FBS, 1% L-glutamine, 1% Penicillin and / streptomycin) into seahorse plates and securely incubated at 37^{o}C and 5%-CO_2 saturation for 1-hour to allow settling of cells on the plate. After this, 150µl of B12-EMEM media was further added to each well of seahorse plates and incubated at 37^{o}C and 5%-CO_2 saturation overnight (24-hours) and used for the seahorse assay.

Seahorse inhibitors:

Following optimisation for the optimum concentrations of respiratory inhibitors; oligomycin, carbonyl cyanide-p-tri-fluoro-methoxy-phenylhydrazone (FCCP) and rotenone/antimycin-A to be used for the seahorse assay, we chose optimum 10x concentrations such as 4.0 µM, 7.5 µM and 4.0 µM for oligomycin, FCCP and rotenone/antimycin-A respectively. The 10x concentration (stock) of the inhibitors were therefore prepared in KHB buffer to obtain final concentrations (1x) of oligomycin (0.4µM), FCCP (0.75µM) and rotenone/ antimycin (0.4µM) after injection into seahorse XF-24 plates. The inhibitors were loaded into the allocated ports of the seahorse cartridge which was initially hydrated in 1ml calibrant for 24-hours at 37^{o}C.

Sample preparation and seahorse assay.

Maximal respiratory capacity in Hep G2: After 24-hour incubation at 37^{o}C and 5% CO_2 saturation, Hep G2 cells cultured in different B12 conditions were washed in KHB buffer by removing 200µl EMEM media off the cells in seahorse plates, followed by addition of 1ml of KHB. 950 µl of the KHB in 24-well seahorse plates was then removed, followed by addition of another 675 µl of KHB to the wells of the plate. The plates were then incubated at 37^{o} C for 1-hour without CO_2 saturation. To run the seahorse assay, the cartridge was first loaded into the seahorse analyser for calibration and equilibration runs according to the manufacturers' protocol. Afterwards, the utility plate of the cartridge was replaced with the seahorse XF24 plate with Hep G2 cells for oxygen consumption rates (OCRs) measurement. Briefly, the OCR measurement was taken repeatedly at 8 mins intervals. First, measurement of a baseline cellular OCR was obtained, and this derived the basal respiration following the subtraction of

respiration of non-mitochondrial source. Then, the addition of the inhibitor of complex V or ATP synthase, Oligomycin, was done to produce an OCR which was useful to derive the ATP-linked respiration (Baseline OCR – Oligomycin rate) or respiration of proton leak (Oligomycin rate – non mitochondrial respiration). Next, the addition of FCCP was done to induce electron transport chain (ETC) to function to its maximal rate, following the FCCP-induced shutting off the inner membrane gradient. This resulted in the derivation of the maximal respiratory capacity (FCCP rate – non mitochondrial respiration). Finally, addition of both complex III inhibitor, antimycin A, and complex I inhibitor, rotenone, was done to shut down the action of the ETC resulting in the measurement of non-mitochondrial respiration.

Respiratory capacity in a limited-substrate (high-palmitate) supply

Then, to examine how the low B12 hepatocyte cell line function with the endogenous supply of high extracellular levels of palmitate and other limited substrate, we incubated the hepatocyte cell line in a limited-substrate KHB medium (containing only 0.5mM L-carnitine and 1.25mM glucose), which is poorly enriched with other supplements compared with the rich-substrate KHB medium, for one-hour. After the basal respiration (OCR) was measured in Hep G2, the cells were either exposed to 200μM palmitate (dissolved in 33.3 μM BSA) or 33.3μM BSA only (basal control) in the substrate medium to assess how Hep G2 cells efficiently uptake palmitate for ATP metabolism.

Statistical analysis

All quantitative measurements, where applicable, were obtained n=6 for standards, controls and cases in order to ensure precision of data. Data obtained from the oil red O staining, total intracellular triglyceride and scintillation assay of radio-labelled triglycerides were normalized with the total protein concentration (mg) in each sample (n) of Hep G2 cells to alleviate possible variations that might misrepresent the data. Differences between either parametric groups or non-parametric groups were observed respectively by performing Student's t-test or Mann-Whitney U test. P values of <0.05 were considered statistically significant.