Electronic Supplementary Material (ESI) for Toxicology Research.

Synergistic interaction between lipid-loading and doxorubicin exposure in Huh7 hepatoma cells results in enhanced cytotoxicity and cellular oxidative stress: Implications for acute and chronic care of obese cancer patients

S. AlGhamdi, V. Leoncikas, K. E. Plant, and N. J. Plant

Department of Biochemistry and Physiology, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, U.K.

Quantitation of Intracellular lipid by Flow cytometry

Methods

Huh7 cells were cultured in 75cm^2 tissue culture flasks at a density of 1x10^6 cells per flask in medium Dulbecco's Modified Eagle Medium with 2 mM L-glutamine, 4.5 g/L glucose, 100 units/mL each penicillin and streptomycin, and 10% foetal bovine serum (FBS), at 37˚C and 5% CO2. For lipid-loading of cells, palmitate and oleate were first dissolved in DMSO and conjugated to fatty acid free BSA at the required concentration as described by Wang et al[1]. Cells were exposed to the BSA-conjugated lipid mixture for 24h to allow lipid-loading to occur. At the end of the exposure period, cells were trypsinized, centrifuged at 800 x g for 5 minutes and the cell pellet washed twice with PBS. The cell pellet was resuspended at a concentration of 1x10^6 cells per ml in 1µM Nile Red staining solution. Following 15 minutes incubation, the cells were centrifuged at 800 x g for 5 minutes, washed once with PBS, and finally resuspended in 1 ml of PBS. For quantification of intracellular lipid, Nile Red fluorescence was determined by flow cytometry using a FACS Canto (BD Biosciences, CA, US) equipped with 488 nm argon laser source. BD FACS Diva software was used for data acquisition and analysis. For the measurements, Huh7 populations were gated using forward scatter channel versus side scatter channel plots. Data were collected for 10,000 events.

Results

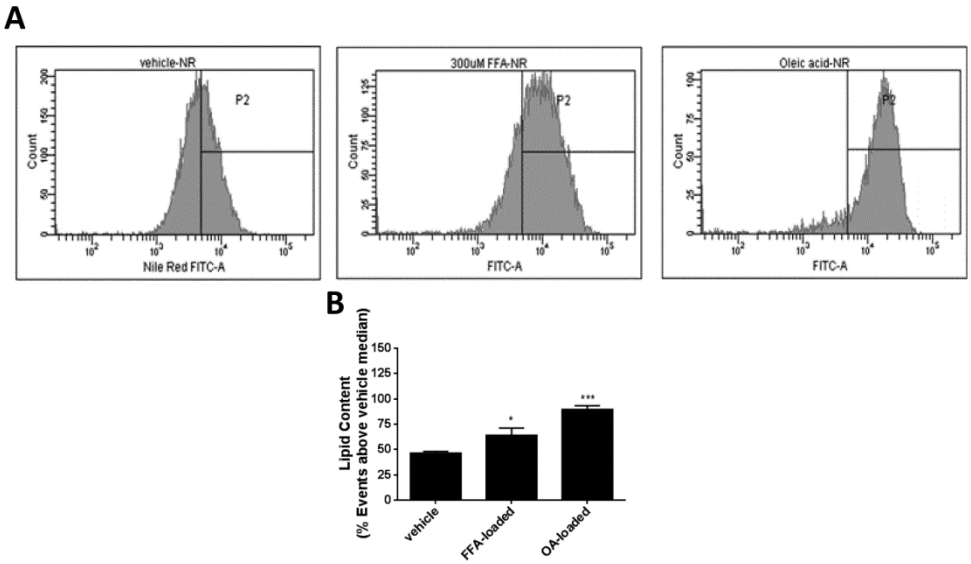

Figure S1: Additive lipid-accumulation between doxorubicin and lipid-loading in Huh7 cells. Huh7 cells were loaded with 300µM FFA mixture (2:1 v/v oleate:palimitate) or 1M oleate for 24h. Intracellular lipid accumulation was detected by Nile Red and quantified using flow cytometry (A) Representative flow cytometry histogram of Nile Red fluorescence, where P2 represents the number (percentage) of events above the median fluorescence value set for the vehicle control sample. (B) Percentage of cells expressing fluorescence above the vehicle median value from three independent experiments, with error bars representing the standard error of the mean (SEM). * = $p<0.05$, *** = $p<0.001$.

Quantitation of ROS by DCDF-DA assay

Methods

Huh7 cells were cultured in black 96-well plates at a density of $1x10^4$ cells per well in medium Dulbecco's Modified Eagle Medium with 2 mM L-glutamine, 4.5 g/L glucose, 100 units/mL each penicillin and streptomycin, and 10% foetal bovine serum (FBS), at 37˚C and 5% CO_2. For lipid-loading of cells, palmitate and oleate were first dissolved in DMSO and conjugated to fatty acid free BSA at the required concentration as described by Wang et al[1]. Cells were exposed to the BSA-conjugated lipid mixture for 24h prior to subsequent experiments to allow lipid-loading to occur. Naïve or lipid-loaded cells were exposed to various concentrations of doxorubicin for the indicated time. In the last three hours of incubation time, 400 µM H_2O_2 was added to separate wells as a positive control. One hour prior to the completion of the treatment, cells were exposed to 10µM DCF-DA dye prepared in phenol red free DMEM medium[2]. At the end of the incubation period, fluorescence was read with a Molecular Devices Spectramax Gemini XS fluorescence spectrophotometer (CA, US) at λex 485, λem 535 nm).

Results

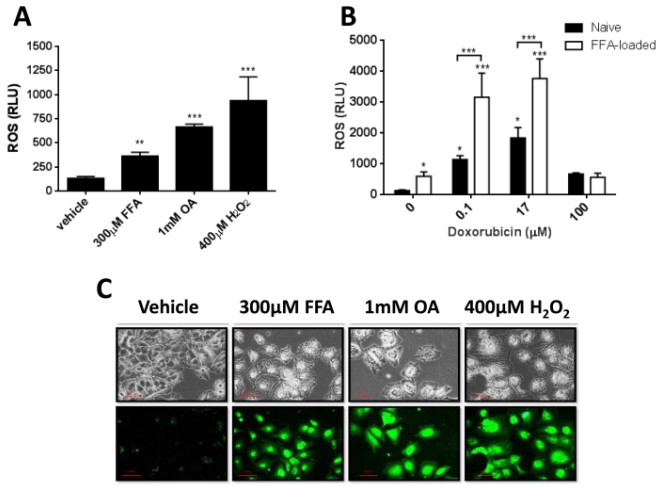

Figure S2: Synergistic oxidative stress between doxorubicin and lipid-loading in Huh7 cells. Huh7 cells were exposed to 300µM FFA mixture (2:1 v/v oleate:palimitate), 1mM oleate for 24h, or 400µM H_2O_2 for 3h then ROS production assessed by DCF assay. (B) Huh7 cells, either naïve or pre-loaded with 300µM FFA mixture (2:1 v/v oleate:palimitate) for 24h, were exposed to the indicated concentration of doxorubicin 24h and then ROS production assessed by DCF assay. (C) Visualised fluorescence microscopy for ROS in lipid-loaded Huh7 cells. For ROS quantitation, each data point represents the mean of three independent experiments, with error bars representing the standard error of the mean (SEM) * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Quantitation of Metallothionein protein expression

Methods

Huh7 cells were cultured in 6-well plates at a density of $1x10^6$ cells per well (Western blots) or in clear-bottom black 96-well plates at a density of $1x10^4$ cells per well (in-cell western and microscopy) in medium Dulbecco's Modified Eagle Medium with 2 mM L-glutamine, 4.5 g/L glucose, 100 units/mL each penicillin and streptomycin, and 10% foetal bovine serum (FBS), at 37˚C and 5% CO2. For lipid-loading of cells, palmitate and oleate were first dissolved in DMSO and conjugated to fatty acid free BSA at the required concentration as described by Wang et al[1]. Cells were exposed to the BSA-conjugated lipid mixture for 24h to allow lipid-loading to occur. Naïve or lipid-loaded cells were exposed to various concentrations of doxorubicin for the indicated time.

Western blot analysis was undertaken as previously described[3]. Briefly, total protein was extracted using RIPA buffer, and protein level quantified by the method of Lowry[4]. Thirty micrograms of total proteins was separated on precast 6-18% polyacrylamide gels, and then transferred to PVDF membrane. Metallothionein isoforms were detected using a pan-selective primary antibody (Santa Cruz Biotechnology sc11377; 1:1000) overnight at 4˚C, and an IRDye 800 CW secondary

(1:10,000) for one hour at room temperature. The membrane was then imaged using an Odyssey Family Imaging System (LI-COR Biosciences).

For in-cell protein detection, at the end of the treatment period cells were first imaged with using an Eclipse TS100 inverted microscope with DSVi1 digital camera and NIS-Elements imaging software (Nikon). Next, cells were fixed with 3.7% *para*-formaldehyde and permeabilised with 0.1% TX100 for 20 minutes. Following permeabilisation, non-specific binding was blocked with Odyssey® blocking buffer for 90 minutes. Metallothionein isoforms were detected using a pan-selective primary antibody (Santa Cruz Biotechnology sc11377; 1:100) overnight at 4˚C and IRDye 800 CW secondary (1:10,000) for one hour at room temperature. Cell Tag 700 Stain (0.2 µM) was also included in the incubation with secondary antibody, and then the membrane was then imaged using an Odyssey Family Imaging System (LI-COR Biosciences).

Results

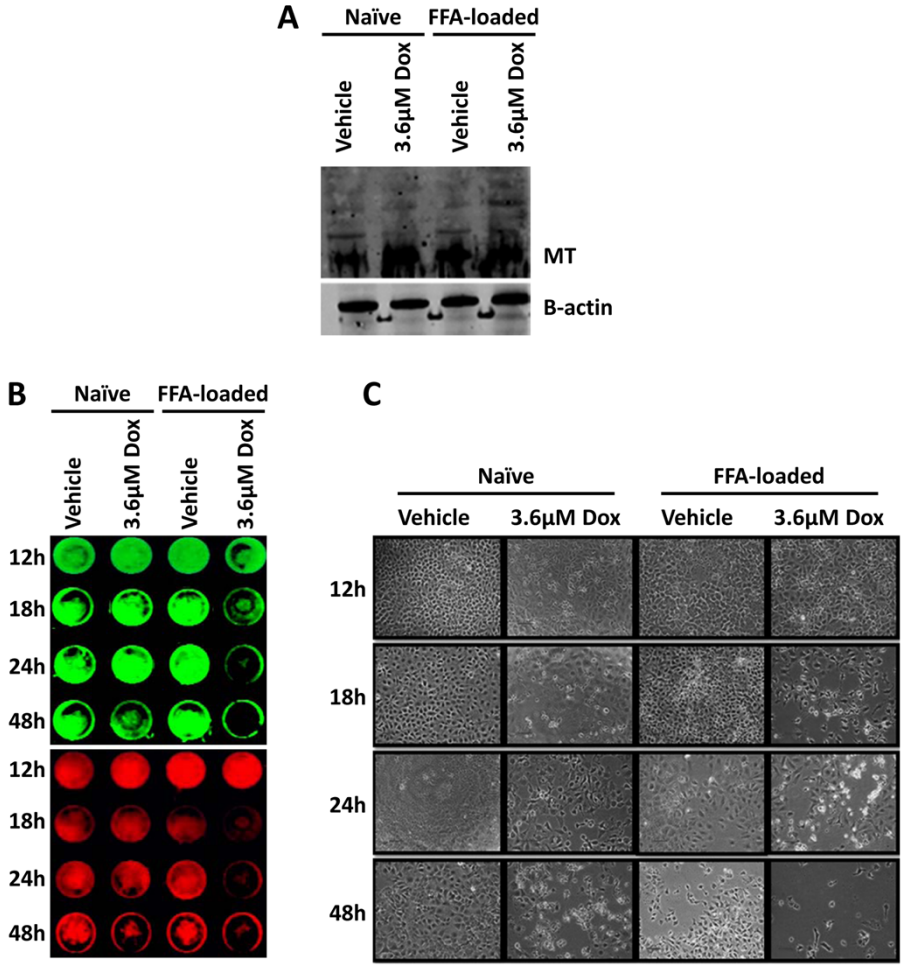

Figure S2: Doxorubicin exposure increases metallothionein expression in naïve Huh7 cells, but decreases expression in FFA-loaded Huh7 cells. Huh7 cells, either naïve or FFA-loaded, were exposed to 3.6µM DOX for the indicated time. (A) Following 12h of exposure total protein was extracted, separated by SDS-PAGE and total metallothionein detected by Western blot; B-actin protein levels were also detected to ensure even loading. (B) Following 12-48h or exposure, metallothionein expression was determined by in-cell Western (top, green stain), with cell number assessed using the CellTag 700 non-specific cellular stain (bottom, red stain). In addition, (C) light microscopy images were obtained for these samples using an original magnification of 100X. Scale bar = 200 µm.

References

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