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RESEARCH ARTICLE

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Essential role for albumin in preserving liver cells from TNFα-induced mitochondrial injury

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Abstract

Cytokine-induced inflammation and mitochondrial oxidative stress are key drivers of liver tissue injury. Here, we describe experiments modeling hepatic inflammatory conditions in which plasma leakage leads to large amounts of albumin to reach the interstitium and parenchymal surfaces to explore whether this protein plays a role in preserving hepatocyte mitochondria against the damaging actions of the cytotoxic cytokine tumor necrosis factor alpha (TNFα). Hepatocytes and precision-cut liver slices were cultured in the absence or presence of albumin in the cell media and then exposed to mitochondrial injury with the cytokine TNFα. The homeostatic role of albumin was also investigated in a mouse model of TNFα-mediated liver injury induced by lipopolysaccharide and D-galactosamine (LPS/D-gal). Mitochondrial ultrastructure, oxygen consumption, ATP and reactive oxygen species (ROS) generation, fatty acid β-oxidation (FAO), and metabolic fluxes were assessed by transmission electron microscopy (TEM), high-resolution respirometry, luminescence-fluorimetric-colorimetric assays and NADH/$FADH_2$ production from various substrates, respectively. TEM analysis revealed that in the absence of albumin, hepatocytes were more susceptible to the damaging actions of TNFα and showed more round-shaped mitochondria with less intact cristae than hepatocytes cultured with albumin. In the presence of albumin in the cell media, hepatocytes also showed reduced mitochondrial ROS generation and FAO. The mitochondria protective actions of albumin against TNFα damage

Abbreviations: ATF3, activating transcription factor 3; ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; Ccl2, monocyte chemotactic protein 1; D-gal, D-galactosamine; ETC, electron transport chain; FADH, flavin adenine dinucleotide; FAO, fatty acid β-oxidation; GSH, glutathione; HO-1, heme oxygenase-1; HSA, human serum albumin; IRG1, immunoresponsive gene 1; LPS, lipopolysaccharide; NADH, nicotinamide adenine dinucleotide; NRF2, nuclear factor erythroid-2-related factor 2; OI, 4-octyl itaconate; OXPHOS, oxidative phosphorylation; PCLS, precision-cut liver slices; ROS, radical oxygen species; ROX, residual oxygen consumption; SDH, succinate dehydrogenase complex; TAG, triacylglycerol; TCA, tricarboxylic acid; TEM, transmission electron microscopy; TNFα, tumor necrosis factor alpha.

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were associated with the restoration of a breakpoint between isocitrate and α-ketoglutarate in the tricarboxylic acid cycle and the upregulation of the antioxidant activating transcription factor 3 (ATF3). The involvement of ATF3 and its downstream targets was confirmed in vivo in mice with LPS/D-gal-induced liver injury, which showed increased hepatic glutathione levels, indicating a reduction in oxidative stress after albumin administration. These findings reveal that the albumin molecule is required for the effective protection of liver cells from mitochondrial oxidative stress induced by TNFα. These findings emphasize the importance of maintaining the albumin levels in the interstitial fluid within the normal range to protect the tissues against inflammatory injury in patients with recurrent hypoalbuminemia.

KEYWORDS

hepatocytes, liver injury, mitochondrial dysfunction, mitochondrial oxidative stress, mitochondrial respiration, tricarboxylic acid cycle

1 | INTRODUCTION

The generation of reactive oxygen species (ROS) is inherent to living cells and characteristic ROS, such as superoxide anions, are byproducts of the normal functioning of the mitochondrial respiratory chain.[1] If this process is excessive and not properly controlled, it can give rise to oxidative stress, which is a common pathological factor in many complex diseases including liver disease, that leads to cell and tissue injury and organ dysfunction.[2] Oxidative stress is frequently associated with inflammation and both processes are mutually synergizing. For example, the production of inflammatory cytokines by cells of the innate immune system can directly trigger oxidative stress and tissue injury.[3] Specifically, exposure to the cytokine tumor necrosis factor (TNF)α, predominantly produced by monocytes/macrophages, is known to damage the mitochondria and to increase mitochondrial ROS production.[4,5]

Albumin is the most abundant protein in the bloodstream and is the major contributor to the maintenance of the plasma oncotic pressure.[6] In the intravascular compartment and besides its oncotic power, albumin exerts pleiotropic effects such as detoxification, and endothelial stabilization, with these effects being related to the ability of this protein to bind endogenous and exogenous molecules.[6–9] Apart from these scavenging properties, albumin is internalized in the endosomal compartment of peripheral leukocytes where it regulates toll-like receptor signaling and immune response to pathogens.[10] Likewise, in the extravascular compartment and interstitium, albumin exhibits non-oncotic properties in addition to maintaining the osmotic gradient.[11,12] For instance, in the liver parenchyma, albumin protects hepatocytes from TNFα-induced cell-death under conditions of actinomycin D transcriptional arrest.[13] The antiapoptotic actions of albumin on liver cells were demonstrated to be unrelated to the scavenging properties of this molecule,[13] placing albumin as a key element in liver tissue homeostasis.

In the current study, we investigated in vitro, ex vivo and in vivo how important is albumin in the maintenance of mitochondrial homeostasis when liver cells are exposed to an inflammatory microenvironment. Specifically, we cultured hepatocytes and precision-cut liver slices (PCLS) in the absence or presence of albumin and then induced mitochondrial injury with the cytokine TNFα. In our experiments, we employed albumin concentrations close to those encountered in the interstitial fluid.[12] In addition, we performed experiments with and without actinomycin D-induced transcriptional arrest to compare the mitochondrial damaging actions of this cytokine when hepatocyte survival is not compromised. We also investigated the homeostatic role of albumin in a mouse model of TNFα-mediated liver injury. Our data indicate that when hepatocytes are growing in an inflammatory milieu, the albumin molecule is required to preserve the ultrastructure and mitochondrial function in these cells from the damaging actions of TNFα. Importantly, the presence of albumin in the hepatocyte media is required for protecting these cells from mitochondrial oxidative stress induced by TNFα. These findings uncover the importance of maintaining the albumin levels within the normal range to have protection against inflammatory tissue injury in conditions characterized by recurrent hypoalbuminemia, such is the case of advanced liver disease.

2 | MATERIALS AND METHODS

2.1 | Animal models

Wild-type C57BL/6J male mice (Charles River Laboratories) were housed in cages with woodchip bedding at 50%–60% humidity and a 12-h light/dark cycle with free access to food and water. For in vitro and ex vivo experiments, mice ($n = 45$) were anesthetized with ketamine and xylazine before collection of hepatocytes or PCLS, as described below. For in vivo experiments, mice ($n = 15$) received an intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 20 μg/kg body weight [b.w.]) and D-galactosamine (D-gal, 600 mg/kg b.w.) as a model of TNFα-mediated liver injury. To test the effects of albumin, two doses of human serum albumin (Albutein, Grifols) of 1.5 g/kg b.w. or its control stabilizer were administered via i.p. 72 and 24 h prior to injection of LPS+D-gal. Six hours later, mice were euthanized by an overdose of anesthetic (a mixture of 0.1 mg ketamine/g b.w. and 0.01 mg xylazine/g b.w.), and peripheral blood was collected and serum obtained by centrifugation at 1200 ***g*** for 10 min. Liver tissue was excised and rinsed in Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$) and either fixed in 10% formalin and embedded in paraffin or kept at −80°C or snap-frozen in N_2 for further analysis. Animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the University of Barcelona following EU laws.

2.2 | Hepatocyte isolation

Hepatocytes were isolated from mice by a three-step in situ perfusion procedure using 0.04% of collagenase IV (Sigma) through the vena cava and posterior digestion with 0.001% of DNAse (Sigma)[14,15] (Figure S1). The cell suspension was filtered through a 100-μm cell strainer (Becton Dickinson) and subsequently washed by centrifugation at 600 ***g*** for 10 min with cold Hank's balanced salt solution (HBSS) followed by a centrifugation at 70 ***g*** for 2 min at 4°C. The pelleted cells were resuspended in cold William's E medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (200 mM), penicillin (50 U/mL), streptomycin (50 μg/mL), insulin (1 μM), Hepes (15 mM), and β-mercaptoethanol (50 μM). The viability of hepatocytes was determined by trypan blue exclusion and the cell number was determined in a Countess II cell counter (Life Technologies). Isolated hepatocytes were seeded on collagen I-coated 12-well (5×10^5 cells/well), 6-well (9×10^5 cells/well), or 96-well (4×10^4 cells/well) plates with William's E medium. Four hours later, hepatocytes were washed three times with $DPBS^{++}$ and visualized by phase contrast microscopy. Thereafter, hepatocytes were grown overnight in a humidified 5% CO_2 incubator at 37°C in 1% FBS William's E medium before incubation with TNFα and/or albumin. To test the effects of albumin on TNFα-induced injury, experiments were carried out in pretreatment (hepatocytes were incubated first with albumin [15 mg/mL] 30 min before TNFα [20 ng/mL] addition) and treatment (hepatocytes were incubated with albumin [15 mg/mL] 1 h after the addition of TNFα [20 ng/mL]) modes. Some experiments were performed with mannitol (15 mg/mL, M1902, Sigma-Aldrich). In other experiments, hepatocytes were pre-treated with 4-octyl itaconate (OI, 125 μM) for 2 h or tempol (3 mM, Sigma-Aldrich) for 30 min. When indicated, experiments were performed in the presence of actinomycin D (250 ng/mL) to induce transcriptional arrest. At the end of the incubation period (5 h), supernatants and hepatocytes were collected for further assessments.

2.3 | PCLS and transmission electron microscopy (TEM)

For PCLS preparation, the inferior vena cava of anesthetized mice was cut off and blood was allowed to drain for 1 min. The liver was excised and placed into ice-cold DPBS without calcium and magnesium ($DPBS^{--}$). Blocks (0.5 cm^3) were cut from the main lobe with a scalpel and embedded in 4% UltraPure low-melting agarose (Invitrogen) diluted in HBSS. An agarose cube with the tissue inside was cut into 250-μm-thick slices using a vibrating blade VT1000S microtome (Leica Microsystems) as described elsewhere.[14,15] PCLS were individually transferred to P-35 Petri dishes containing Millicell cell culture inserts (EMD Millipore) and prebalanced for 20 min with warmed William's E medium containing GlutaMAX-I, D-glucose (25 mM), and gentamicin (50 μg/mL). PCLS were maintained at 37°C in a 5% CO_2 incubator for 120 min and then incubated with albumin (15 mg/mL) for 30 min before the addition of vehicle or TNFα (10 ng/mL) for 22 h. At the end of the incubation period, PCLS were collected and fixed for 30 min at room temperature with 2% paraformaldehyde and 2.5% glutaraldehyde in sodium phosphate buffer (0.1 M, ph 7.4) and kept at 4°C. The samples were post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide, dehydrated in acetone, and embedded in Spurr's epoxy resin. Four ultrathin sections were obtained from each sample, which were then post-stained with uranyl acetate and lead citrate and examined under a JEOL J1010 TEM.

2.4 | Measurement of mitochondrial oxygen consumption

High-resolution respirometry was performed using Oroboros Oxygraph-2k system. Hepatocytes were seeded in 6-well plates under the conditions described above. A minimum of 300 000 hepatocytes were trypsinized and resuspended in mitochondrial respiration medium (MiR05) and added into the oxygraph chamber at a final volume of 2 mL. Oxygen consumption was measured at 37°C under basal conditions and following the sequential addition of oligomycin (2 μM), repeated additions of uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP), rotenone, and antimycin A, all at 1 μM. Oligomycin inhibits adenosine triphosphate (ATP) synthase allowing the assessment of the proton leak across the inner mitochondrial membrane. CCCP renders the mitochondrial inner membrane permeable to protons, leading to rapid oxygen consumption without ATP generation, allowing the assessment of uncoupled maximal respiration. Rotenone and antimycin A are inhibitors of mitochondrial I and III complexes and allow the assessment of residual oxygen consumption (ROX) or non-mitochondrial respiration.

2.5 | Measurement of ATP levels

ATP levels in hepatocytes incubated under the conditions described above were determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, hepatocytes were seeded in 96-well opaque plates at a concentration of 40 000 cells/well and equilibrated to room temperature for 30 min before the addition of CellTiter-Glo® Reagent for 2 min to induce cell lysis. Luminescence was recorded in a plate reading luminometer (FLUOstar Optima, BMG Labtech) and background signal from control wells was subtracted.

2.6 | Assessment of fatty acid oxidation (FAO)

A non-radioactive FAO kit from Biomedical Research Service Center based on the oxidation of octanoyl-CoA and generation of nicotinamide adenine dinucleotide (NADH), which is coupled to the reduction of iodonitrotetrazolium to formazan, was used. Briefly, hepatocytes (500 000 cells/well in 12-well plates) incubated under the conditions described above were washed with $DPBS^{--}$, lysed with 100 μL of Cell Lysis Solution, and incubated on ice for 5 min. Lysates were centrifuged at 16 000 ***g*** for 5 min, supernatants were collected and protein concentrations were determined by the micro-BCA method. Fifty microliters of FAO Assay solution or control solution was added to 10 μL of the protein sample and the plate was kept in a non-CO_2 incubator for 60 min at 37°C. Optical density was read at 492 nm with a FLUOstar Optima spectrophotometer. Blank readings were subtracted and enzyme activities were corrected by the amount of protein.

2.7 | Assessment of mitochondrial oxidative stress

Hepatocytes were seeded in 96-well black-walled plates at a concentration of 40 000 cells/well and mitochondrial oxidative stress was determined using MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes) according to the manufacturer's protocol. MitoSOX is a fluorogenic dye and superoxide indicator specifically targeted to mitochondria in live cells. Oxidation of the MitoSOX reagent by mitochondrial superoxide is widely used to detect mitochondrial ROS, especially superoxide. Briefly, the MitoSOX reagent was dissolved in 13 μL of dimethyl sulfoxide to prepare a 5 mM stock solution and subsequently a 5 μM working solution by dilution with HBSS. Then, the MitoSOX reagent was added to the cells for 10 min at 37°C and 5% CO_2 protected from light. Cells were then washed once with $DPBS^{++}$ to remove background fluorescence and the plate was then read with a monochromator fluorescence reader (Infinite 200 PRO, TECAN Life Sciences) at 510 and 580 nm as excitation and emission wavelengths.

2.8 | Assessment of mitochondrial membrane potential

Hepatocytes were seeded in 96-well black-walled plates at a concentration of 50 000 cells/well, and following the incubations described above, the supernatants were removed and cells were exposed to the JC-1 dye (2 μM) (MitoProbe™, Molecular Probes) for 30 min at 37°C. After washing with warmed PBS, fluorescence was measured in the FLUOstar Optima microplate reader, first at 550/600 nm and later at 485/535 of excitation/emission wavelengths to calculate the ratio between the red and green signals. CCCP at 50 μM was used as a positive control.

2.9 | Assessment of mitochondrial metabolic function

Metabolic flux analyses were performed using MitoPlate S-1 (Biolog) based on the production of NADH and flavin

adenine dinucleotide ($FADH_2$) from various substrates, which feed electrons to the electron transport chain (ETC).[16] A tetrazolium-based redox dye acts as an electron acceptor at the distal end of the ETC and the color formation, which indicates the utilization of the substrate present in each well, is read kinetically. For the preparation of the substrates, the MitoPlate S-1 plates were solubilized with 30 μL per well of a solution containing 2× Biolog Mitochondrial Assay Solution, 6× tetrazolium redox dye MC reagent and saponin (30 μg/mL) for 1 h at 37°C. Thereafter, 30 μL of each of the solubilized substrates was added to a 96-well half area plate with hepatocytes seeded at a concentration of 40 000 cells/well, and the optical density at 590 nm was monitored at 37°C for 12 h with a kinetic reading of 5 min intervals in the FLUOstar Optima microplate reader. The value of the area under the curve was used as the measurement unit.

2.10 | Measurement of glutathione (GSH) levels

GSH levels in liver tissue were determined by the GSH-Glo™ Glutathione Assay (Promega) according to the manufacturer's instructions. Liver tissue (10 mg) was homogenized in 1 mL of PBS containing 2 mM EDTA, centrifuged at 16 000 ***g*** for 20 min at 4°C and 50 μL of the supernatant was incubated with 50 μL of prepared GSH-Glo™ Reagent 2× at room temperature in a 96-well plate. After 30 min, 100 μL of reconstituted Luciferin Detection Reagent wasadded and the plate was incubated for 15 min at room temperature. Luminescence was recorded in a plate reading luminometer (FLUOstar Optima) and readings were corrected by the tissue weight.

2.11 | Measurement of triacylglycerol (TAG) levels

Liver tissue was homogenized in NP40 Substitute Assay Reagent (Cayman Chemicals) containing protease inhibitors (Complete Mini; Roche Diagnostics) whereas hepatocytes were resuspended in 500 μL of cold diluted Standard Diluent and sonicated 20 times at 1 s bursts. Tissue and cell homogenates were centrifuged 10 min at 10 000 ***g*** at 4°C and supernatants assayed using a TAG colorimetric assay (Cayman Chemicals), according to the manufacturer's instructions.

2.12 | Measurement of succinate levels

Hepatocytes were seeded onto 96-well plates at a concentration of 40 000 cells/well. Extracellular levels of succinate were determined using the EnzyChrom™ Succinate Assay Kit (Bioassay Systems) according to the manufacturer's instructions. Briefly, 20 μL of cell supernatants was assayed in duplicate, one with the internal standard (1 mM succinate standard), and the other without. Then, 80 μL of working reagent of the proprietary kit was added to the cells for 30 min at room temperature and optical density was read at 570 nm in the microplate reader FLUOstar Optima. Intracellular levels of succinate in hepatocytes were measured with the Succinate Colorimetric Assay kit (Sigma) after homogenizing the cells on ice-cold Succinate Assay Buffer, centrifugation at 10 000 ***g*** for 5 min, and collection of supernatant. Thereafter, 50 μL of the diluted samples was added onto 96-well plates containing 50 μL of the Reaction Mix of the proprietary kit for 30 min at 37°C. Optical density was read at 450 nm in the microplate reader FLUOstar Optima.

2.13 | Analysis of gene expression by real-time polymerase chain reaction (PCR)

Isolation of total RNA from tissue and hepatocytes was performed using the TRIzol reagent and RNA concentration was assessed in a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis from 0.5 to 1 μg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR analysis for immunoresponsive gene 1 (*Irg1*, Mm01224532_m1), succinate dehydrogenase complex subunits (A, *Sdha*, Mm01352360_m1; B, *Sdhb* Mm00458272_m1; C, *Sdhc* Mm00481172_m1; and D, *Sdhd* Mm00546511_m1), activating transcription factor 3 (ATF3) (*Atf3*, Mm00476032_m1), nuclear factor erythroid-2-related factor 2 (NRF2) (*Nrf2*, Mm00477784_m1), isocitrate dehydrogenases (NADP+ *Idh1*, Mm00516030_m1; *Idh2* Mm00612429_m1; *Idh3* alpha [*Idh3a*], Mm00499674_m1; and *Idh3* beta, *Idh3b*, Mm00504589_m1), TNFα (*Tnf*, Mm00443258_m1), monocyte chemoattractant protein-1 (MCP-1) (*Ccl2*, Mm00441242_m1), interleukin (IL) 6 (*Il6*, Mm00446190_m1), and heme oxygenase-1 (HO-1) (*Hmox1*, Mm00516005_m1) was performed using validated and predesigned *TaqMan* Gene Expression Assays purchased from Applied Biosystems in a 7900HT Fast Real-Time PCR System using β-actin (*Actb*, Mm00607939_s1) as endogenous control. The PCR results were analyzed with Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative C_t method. The amount of target gene normalized to β-actin and relative to a calibrator was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative C_t method.

2.14 | Analysis of protein expression

Total protein from tissue was extracted with RIPA buffer (Sigma) supplemented with a mixture of protease (Complet Mini) and phosphatase (PhosStop) inhibitors (Roche Diagnostics). Total protein from hepatocytes was extracted by scraping the cells into 150 μL of RIPA buffer (Sigma) supplemented with the same mixture of inhibitors. Homogenates were incubated on ice for 10–15 min and centrifuged at 16 000 ***g*** for 20 min at 4°C. The supernatant was dissolved in 2× Laemmli buffer, heated for 5 min at 95°C, and separated by 10% (vol/vol) SDS-PAGE for 90 min at 120 V. Transfer was performed using the iBlot Dry Blotting System (Invitrogen) on PVDF membranes at 20 V over 7 min. The membranes were then soaked for 1 h at room temperature in Tris-buffered saline containing 0.1% of Tween 20 (T-TBS) and 5% (wt/vol) nonfat dry milk. Blots were washed three times for 5 min each with T-TBS and incubated overnight at 4°C with primary monoclonal rabbit anti-mouse NRF2 antibody (12721, Cell Signaling Technology (CST), 1:1000 dilution), ATF3 (33593 CST, 1:1000 dilution), and β-actin (5125 CST, 1:1000 dilution) in T-TBS containing 5% of BSA. Thereafter, the blots were washed three times for 5 min each with 0.1% of T-TBS and incubated for 1 h at room temperature with a horseradish-peroxidase-linked donkey anti-rabbit antibody (406401, BioLegend, 1:2000 dilution) in 0.1% of T-TBS containing 5% of nonfat dry milk. For the assessment of IκB and JNK phosphorylation, blots were incubated overnight at 4°C with primary polyclonal rabbit anti-mouse phospho-SAPK/JNK (Thr183/Tyr185) antibody (9251 CST, 1:250 dilution) and mouse monoclonal anti-mouse phospho-IκB-α (9246 CST, 1:500 dilution). To assess total JNK and total IκB-α protein expression, membranes were stripped at 37°C for 15 min in Restore Western blot stripping buffer (Thermo Fisher Scientific) and reblotted overnight at 4°C with rabbit monoclonal SAPK/JNK (56G8) (9258 CST, 1:250 dilution) and polyclonal IκB-α (9242 CST, 1:1000). The bands were visualized with the EZ-ECL chemiluminescence detection kit (Biological Industries) in ImageQuant LAS 4000 equipment (GE Healthcare Life Sciences).

2.15 | Statistics

The statistical analysis was performed with GraphPad Prism software version 9.0.1. Statistical differences of the endpoints were evaluated with the unpaired *t* test with Welch's correction or one-way ANOVA for multiple comparisons corrected with Tukey posttest. Results were expressed as mean ± SEM and differences were considered significant at $p < .05$.

3 | RESULTS

3.1 | Albumin is required to preserve the mitochondrial ultrastructure of liver cells in front of TNFα-induced damage

Structural mitochondrial damage accompanies the cytotoxic effects of the cytokine TNFα on liver cells.[5] Figure 1 shows TEM images of the hepatocyte mitochondria ultrastructure at two different magnifications in PCLS incubated for 22 h with vehicle (left panels), TNFα in the absence of albumin (middle panels), and TNFα in the presence of albumin (right panels). Unless indicated, all experiments described in the following paragraphs were performed in the absence of transcriptional arrest (i.e., PCLS or hepatocytes were not exposed to actinomycin D), avoiding the induction of cell death and merely focusing on the mitochondrial damaging actions of TNFα. As compared to vehicle control, in the absence of albumin, more mitochondria per cell were present in hepatocytes treated with TNFα and these mitochondria were of smaller size and abnormally shaped with cristae rarefication (Figure 1). These TNFα-induced mitochondrial disturbances were less evident in PCLS incubated in the presence of albumin (Figure 1).

3.2 | Albumin is required to prevent mitochondrial oxidative stress in hepatocytes challenged with TNFα

We next determined changes in hepatocyte mitochondrial membrane potential, which is essential for energy production during oxidative phosphorylation.[17] Whereas the uncoupling agent CCCP, used as positive control, induced a severe alteration in mitochondrial membrane potential monitored by the JC-1 fluorescence assay (Figure S2A), no changes in this parameter were observed with TNFα in the presence or absence of albumin (Figure 2A). We also determined changes in mitochondrial oxidative stress using the Mitosox dye assay. As shown in Figure 2B, in the absence of albumin, TNFα-induced mitochondrial oxidative stress in hepatocytes, an effect that was not seen when these cells were pre-treated with albumin. To exclude the possibility that albumin could interfere with the binding of TNFα to hepatocytes, the experiments were repeated adding albumin 1 h after the stimulation of hepatocytes with TNFα (a condition in which this cytokine has already interacted and activated its membrane receptors). As shown in Figure 2B, under these conditions, albumin also reduced TNFα-induced mitochondrial oxidative stress. Of interest, albumin reduced TNFα-induced mitochondrial oxidative stress to a similar extent to tempol,

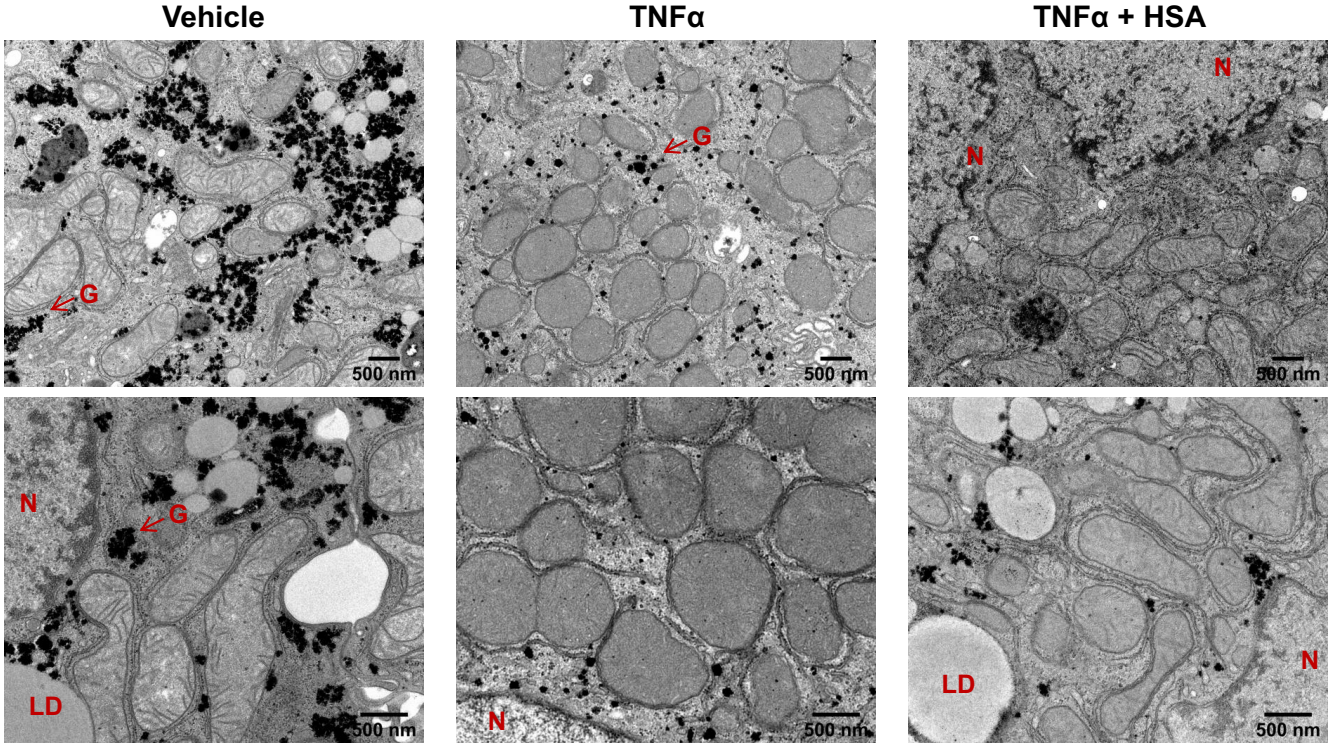

FIGURE 1 Influence of albumin in preserving hepatocyte mitochondrial ultrastructure. Representative electron microscopy images of PCLS incubated for 22 h with vehicle (left panels), TNFα (20 ng/mL) (middle panels), and albumin (HSA, 15 mg/mL) for 30 min and then challenged with TNFα (right panels). Images were taken at ×20 000 and ×30 000. G, glycogen; N, nucleus; LD, lipid droplet.

an intracellular antioxidant that preserves mitochondria against oxidative damage (Figure 2B). The antioxidant effects of albumin were not mirrored by mannitol, another oncotic agent, and were not related to the stabilizer contained in the albumin solution, which did not affect TNFα-induced mitochondrial oxidative stress (Figure S2B). We also observed that albumin disrupted the hepatocyte intracellular signaling of TNFα and significantly reduced the induction of IκB-α phosphorylation produced by this cytokine (Figure 2C). In these cells, JNK1 and JNK2 phosphorylation remained unchanged in response to both TNFα and albumin (Figure 2D).

3.3 | Albumin is not required to preserve mitochondrial respiration in hepatocytes challenged with TNFα

To investigate the mechanisms by which hepatocytes growing in the presence of albumin had reduced TNFα-induced mitochondrial oxidative stress, we monitored changes in mitochondrial ETC, one of the major sources of ROS.[18] In particular, we measured oxygen (O_2) consumption by high-resolution respirometry in the presence and absence of protein uncouplers and inhibitors of the ETC, as schematized in Figure S3. We first established that incubation for 5 h with TNFα was enough to induce significant changes in basal hepatocyte O_2 consumption (Figure 2E) and this time-period was selected for subsequent experiments. As compared to vehicle and in the absence of albumin, hepatocytes exposed to TNFα showed significantly increased basal and uncoupled O_2 consumption (Figure 2F,G) as well as maximal and residual (ROX) respiration (Figure S4A). Increased mitochondrial respiration induced by TNFα did not translate into higher ATP production (Figure 2H). Albumin did not modify the increased respiratory response to TNFα (Figure 2F,G and Figure S4A). Also, albumin per se did not induce changes in resting hepatocyte respiration or ATP levels (Figure S4B,C). Similarly, ATP levels were not affected by shorter incubation time periods (2 h) (Figure S4D). In contrast, albumin significantly reduced the induction in mitochondrial FAO produced by TNFα, as assessed by a non-radioactive assay based on the oxidation of octanoyl-CoA (Figure 2I).

3.4 | Albumin is required for the homeostatic control of the hepatocyte TCA cycle in front of TNFα

We next performed functional flux analysis with MitoPlates S-1 to monitor changes in mitochondrial metabolism that can lead to the generation of the reducing agents NADH and $FADH_2$. The results were graphed in lollipop charts

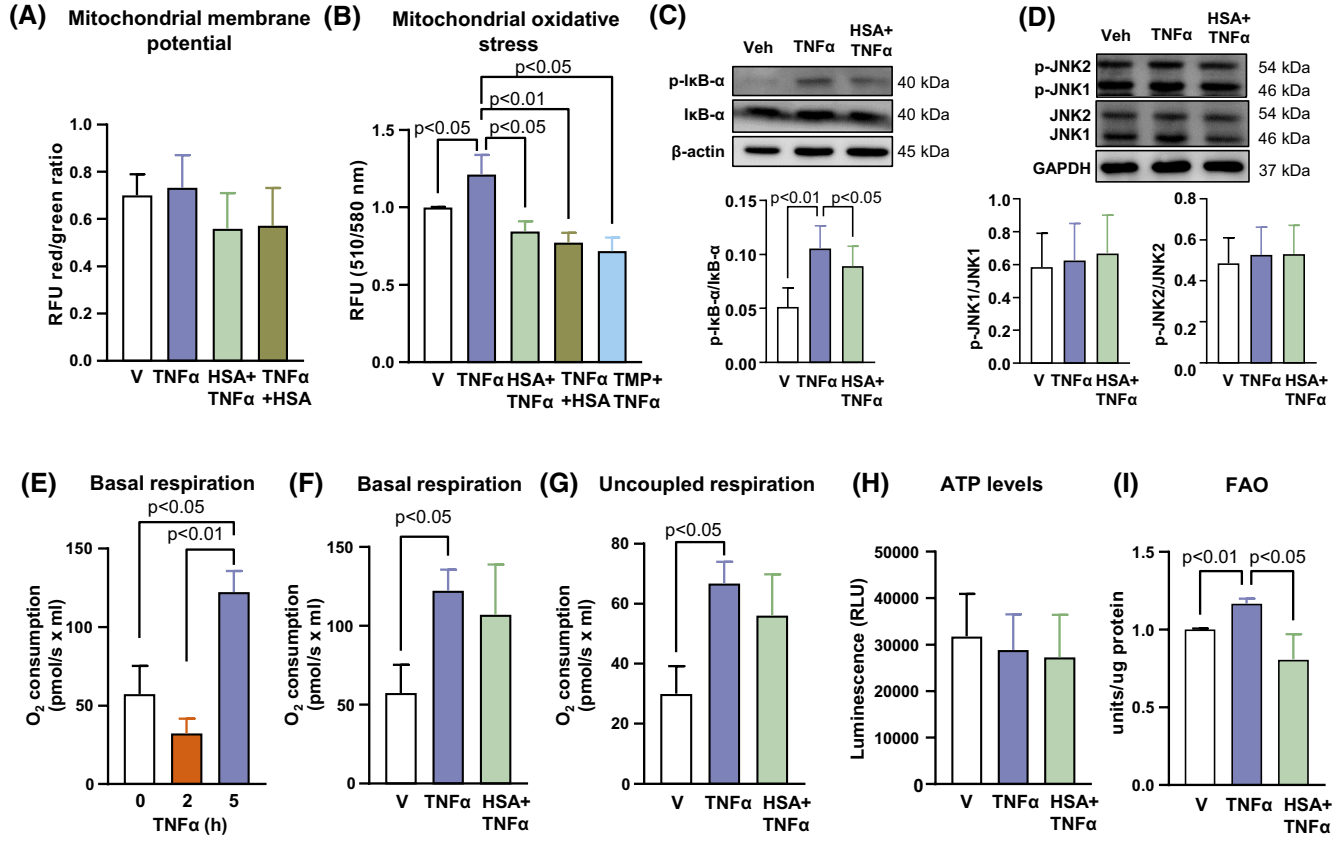

FIGURE 2 Albumin reduces mitochondrial oxidative stress and modulates fatty acid β-oxidation (FAO). (A) Mitochondrial membrane potential in hepatocytes incubated with vehicle (V) or albumin (HSA) for 30 min and then challenged with TNFα for 5 h or treated with HSA 1 h after TNFα addition. (B) Mitochondrial oxidative stress in hepatocytes incubated with V, has, or tempol (TMP) for 30 min and then challenged with TNFα for 5 h or treated with HSA 1 h after TNFα addition. (C) Representative Western blot of p-IκB-α and IκB-α protein expression in hepatocytes incubated with V or HSA for 30 min and then challenged with TNFα for 5 h. (D) Representative Western blot of p-JNK1, p-JNK2, JNK1, and JNK2 protein expression in hepatocytes incubated with V or HSA for 30 min and then challenged with TNFα for 5 h. (E) Basal respiration in hepatocytes challenged with TNFα for 2 or 5 h. (F) Basal respiration in hepatocytes incubated with or without HSA and challenged with TNFα for 5 h. (G) Uncoupled respiration from hepatocytes incubated as described in (F) in the presence of the ATP synthase inhibitor oligomycin (2 μM). (H) ATP levels in hepatocytes incubated as described in (F). (I) FAO in hepatocytes incubated as described in (F). Results from three or more independent experiments assayed in duplicate and expressed as mean ± SEM. RLU, relative luminescence units; RFU, relative fluorescence units.

using three pairwise comparisons: TNFα versus vehicle (Figure 3A), HSA+TNFα versus vehicle (Figure S5A), and HSA+TNFα versus TNFα (Figure 3D). Acetylcarnitine was the most favored mitochondrial substrate in hepatocytes exposed to TNFα, whereas isocitrate was the mitochondrial substrate less utilized by hepatocytes stimulated by this cytokine (Figure 3A). The utilization of these substrates in hepatocytes incubated with TNFα with respect to that of resting hepatocytes (incubated with vehicle) is shown in Figure 3B. These findings are consistent with the view that TNFα modulates mitochondrial FAO and induces a breakpoint in the TCA cycle between isocitrate and α-ketoglutarate leading to less utilization of isocitrate (Figure 2I and Figure 3C). The TNFα-induced mitochondrial overutilization of acetylcarnitine and the impairment in mitochondrial utilization of isocitrate were restored in hepatocytes in which albumin was present in the cell culture medium (Figure 3D). The utilization of these substrates in hepatocytes incubated with TNFα and albumin with respect to that of hepatocytes incubated with TNFα alone is shown in Figure 3E. The utilization of these substrates in hepatocytes incubated with TNFα and albumin with respect to that of hepatocytes incubated with vehicle alone is shown in Figure S5B,C. The presence of the isocitrate breakpoint in hepatocytes incubated with TNFα alone suggests that anaplerotic reactions might replenish the TCA cycle under inflammatory injury conditions. Indeed, the utilization of the amino acid glutamine, which enters the TCA cycle at the α-ketoglutarate level (Figure 3C), was higher in hepatocytes exposed to TNFα alone than in hepatocytes exposed to TNFα in the presence of albumin (Figure S6A). The restoration by albumin of the breakpoint

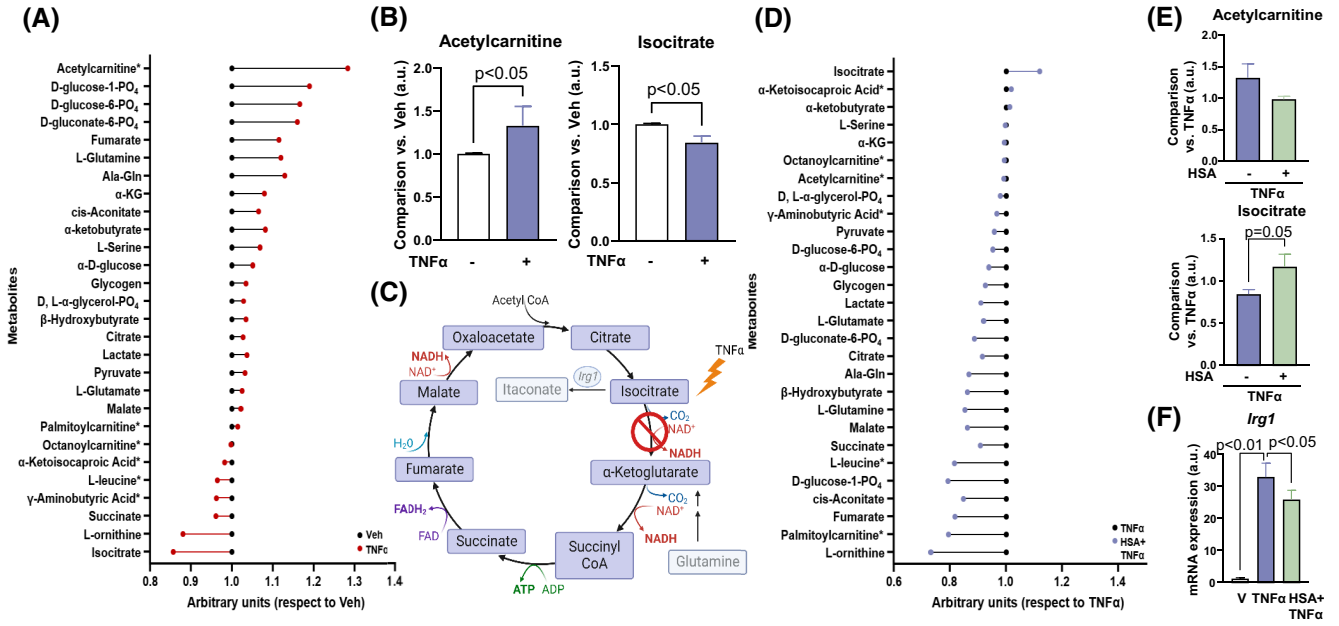

FIGURE 3 Albumin modulates the tricarboxylic acid (TCA) cycle. (A) Utilization of different energy substrates comparing hepatocytes incubated with TNFα for 5 h from hepatocytes incubated with vehicle (Veh). (B) Acetylcarnitine and isocitrate utilization in hepatocytes incubated with TNFα for 5 h in comparison to vehicle. (C) Schematic diagram of the damaging actions of TNFα on the TCA cycle. (D) Utilization of different energy substrates comparing hepatocytes incubated with TNFα and albumin (HSA) for 5 h from hepatocytes incubated with TNFα but without HSA. (E) Acetylcarnitine and isocitrate utilization in hepatocytes incubated with or without HSA for 30 min and then challenged with TNFα for 5 h. (F) *Irg1* expression in hepatocytes incubated as described in (E). Results from three independent experiments and expressed as mean ± SEM.

at the isocitrate level induced by TNFα was consistent with a decrease in the expression of the *Irg1* gene, a proxy of the diversion of the isocitrate metabolic intermediate cis-aconitate into itaconate[19] (Figure 3F). The reduced utilization of isocitrate in hepatocytes challenged with TNFα in the absence of albumin was neither related to changes in the expression of isocitrate dehydrogenase enzymes, which convert isocitrate to α-ketoglutarate (Figure S6B) nor in the expression of genes coding for the dehydrogenation of the biologically active TCA metabolite succinate (Figure S6C). No changes in the utilization of glycogen or the ketone body β-hydroxybutyrate were observed after TNFα stimulation, although the latter was significantly reduced by albumin treatment (Figure S7A,B). Finally, no lipid (i.e., TAG) changes were observed in hepatocytes exposed to TNFα in the absence or presence of albumin (Figure S7C).

3.5 | Albumin is also essential for the homeostatic control of mitochondrial TCA cycle when hepatocytes stimulated with TNFα are submitted to transcriptional arrest

When hepatocytes are under transcriptional arrest, TNFα-induced mitochondrial injury translates into programmed cell death (apoptosis).[13,20] To investigate how important is the albumin molecule in this situation, we repeated the mitochondrial flux analysis in the presence of actinomycin D. Figure 4A shows a lollipop chart of the pairwise comparisons of the utilization of energy substrates between hepatocytes incubated with TNFα+ actinomycin D in comparison to vehicle. Unlike hepatocytes treated with TNFα alone (Figure 3A), acylcarnitines were not the top utilized mitochondrial substrates in hepatocytes exposed to TNFα+ actinomycin D (Figure 4A). In these conditions, succinate replaced isocitrate as the mitochondrial substrate less utilized by these hepatocytes (Figure 4A,B). No changes in the mitochondrial utilization of isocitrate were observed (Figure 4C). These findings indicate that in the absence of albumin and under transcriptional arrest, TNFα triggers a breakpoint in the TCA cycle between succinate and fumarate leading to less utilization of succinate (Figure 4D). In contrast, in the presence of albumin, the TNFα+ actinomycin D-induced succinate breakpoint was not observed but rather the utilization of this substrate was enhanced (Figure 4E,F). Albumin did not modify the mitochondrial utilization of isocitrate (Figure 4G). Unlike intracellular, extracellular levels of succinate were reduced in hepatocytes treated with TNFα+ actinomycin D in the presence of albumin (Figure S8A,B).

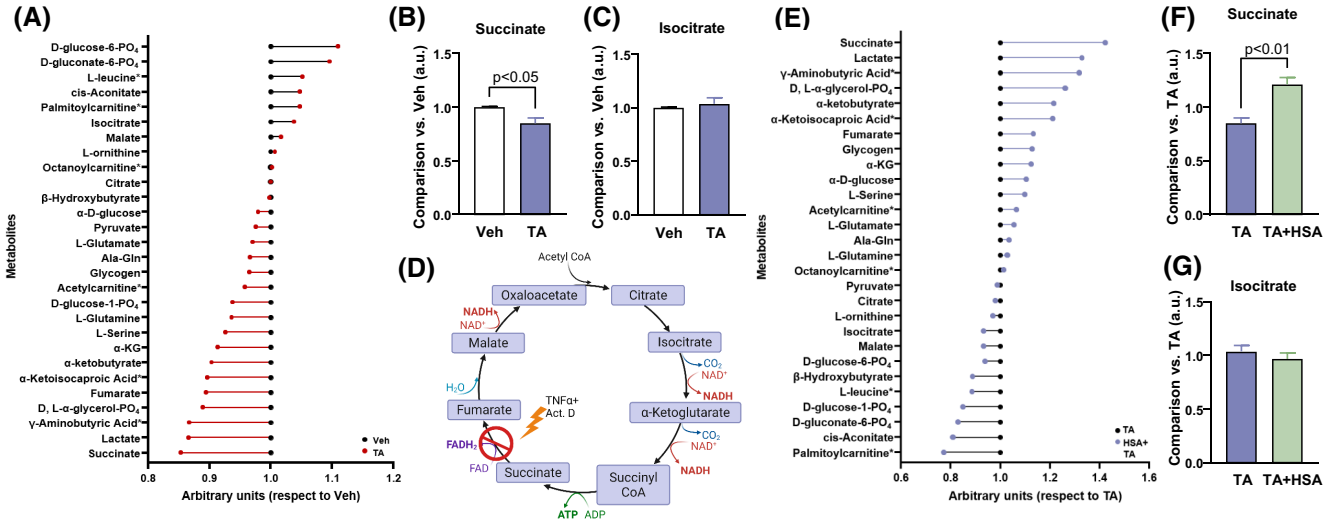

FIGURE 4 Effects of TNFα and albumin on the TCA cycle in hepatocytes growing under transcriptional arrest. (A) Utilization of different energy substrates comparing hepatocytes incubated with TNFα plus actinomycin D (TA) for 5 h from hepatocytes incubated with vehicle (Veh). (B) Succinate utilization in hepatocytes challenged with TA for 5 h in comparison to Veh. (C) Isocitrate utilization in hepatocytes incubated as described in (B). (D) Schematic diagram of TCA cycle and the damaging effects of TA. (E) Utilization of different energy substrates comparing hepatocytes incubated with TA plus albumin (HSA) for 5 h to hepatocytes incubated with TA but without HSA. (F) Succinate utilization in hepatocytes incubated with or without HSA for 30 min and then challenged with TA for 5 h. (G) Isocitrate utilization in hepatocytes incubated as described in (F). Results are from a minimum of three independent experiments assayed in duplicate and expressed as mean ± SEM.

3.6 | The homeostatic role of albumin is related to the stimulation of the antioxidant ATF3 pathway

To explore by which mechanism albumin prevents mitochondrial oxidative stress in response to TNFα, we compared the expression of antioxidant pathways, including NRF2, HO-1, and ATF3 in hepatocytes growing in the presence of albumin with respect to those growing in its absence. Expression of *Nrf2*, the gene coding for NRF2, was equal in both groups (Figure 5A). Similar findings were obtained at the protein level, in which OI was added as positive control (Figure 5B). Expression of *Hmox1*, the gene coding for HO-1, was higher in hepatocytes growing in the presence of albumin, but changes did not reach statistical significance (Figure 5C). In contrast, expression of *Atf3*, the gene coding for ATF3, was significantly higher in hepatocytes incubated with TNFα in the presence of albumin in comparison to those cultures not containing albumin in the cell media (Figure 5D). The stimulatory actions of albumin on the expression of the *Atf3* gene were confirmed at the protein level (Figure 5E). Albumin also modulated the expression of ATF3 downstream targets including *Ccl2*, *Tnf*, and *Il6* in hepatocytes exposed to TNFα (Figure 5F). Furthermore, the stimulatory actions of albumin on ATF3 and downstream targets were confirmed in vivo in mice treated with LPS and D-gal, as an experimental model of TNFα-mediated acute liver injury (Figure 5G,H). Importantly, in the in vivo model, hepatic glutathione levels were increased by albumin, indicating a reduction in oxidative stress (Figure 5I). Neither TNFα nor albumin-modified hepatic TAG levels (Figure S9). The protective role of albumin against TNFα-induced mitochondrial oxidative stress was not observed in hepatocytes under actinomycin D-induced transcriptional arrest (Figure S8C), suggesting that in our experiments the mitochondrial antioxidant properties of the albumin molecule might occur at the intracellular level. Together, these findings suggest that the presence of albumin in the culture media of hepatocytes is able to counteract TNFα-induced mitochondrial oxidative stress by mechanisms related to the activation of the ATF3 antioxidant pathway.

4 | DISCUSSION

Albumin is primarily produced by hepatocytes and is one of the most abundant proteins in the bloodstream.[6,9] Albumin is responsible for the maintenance of the oncotic pressure not only in the intravascular compartment but also in the extravascular and interstitial compartment.[6,8,9] In recent years, albumin has been described to exert pleiotropic actions beyond its oncotic properties, including immunomodulatory and cytoprotective effects.[8–10,13] In the current study, we expanded the non-oncotic properties of this protein by describing how albumin

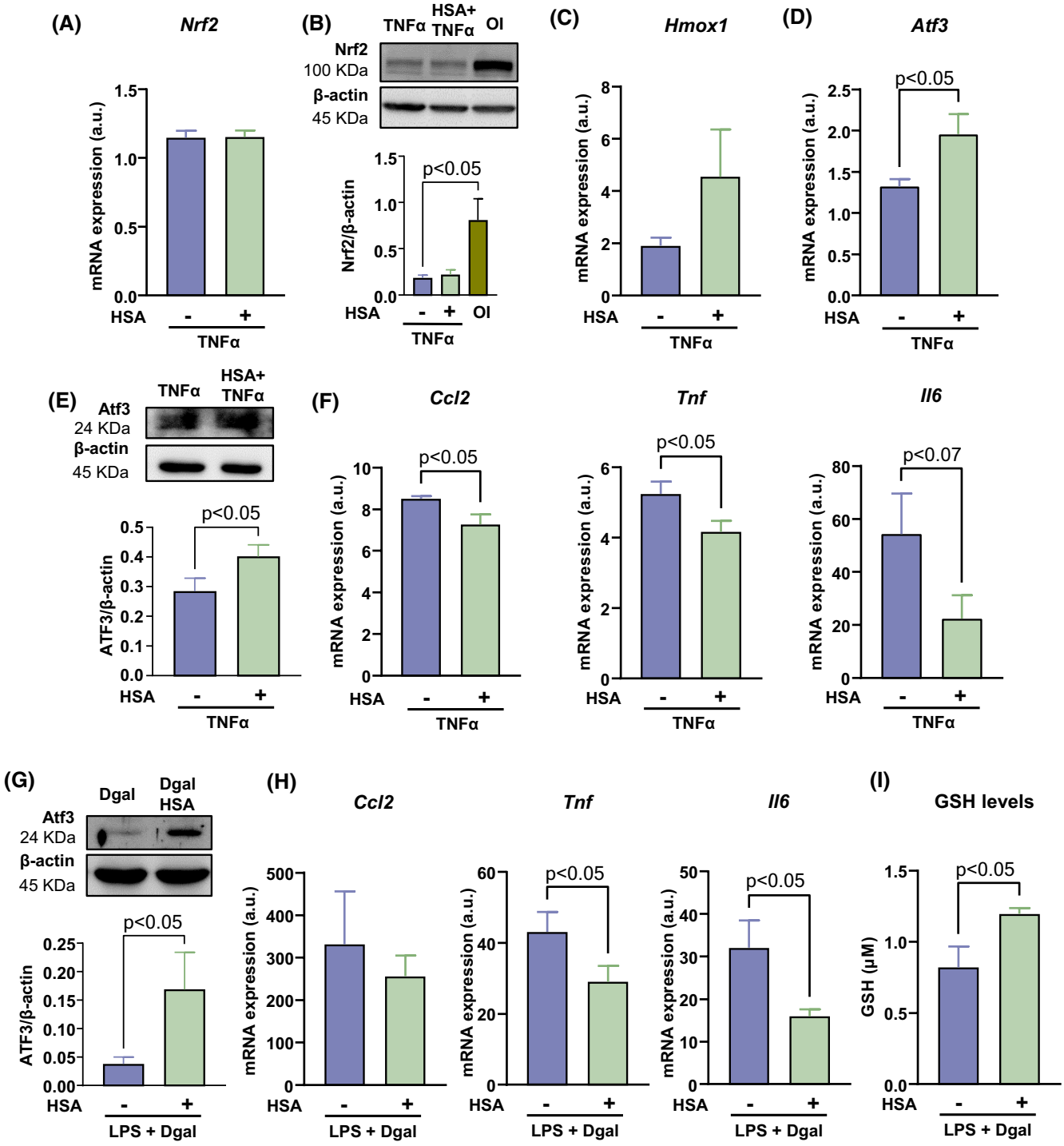

FIGURE 5 Albumin activates the antioxidant signaling ATF3 pathway in hepatocytes. (A) *Nrf2* expression in hepatocytes challenged with TNFα for 5 h and incubated with or without albumin (HSA) for 30 min. (B) Representative Western blot of NRF2 protein expression in hepatocytes incubated as described in (A). OI (125 μM, 2 h) was used as positive control. The densitometric analysis is shown below. (C) *Hmox1* expression in hepatocytes incubated as described in A. (D) *Atf3* expression in hepatocytes incubated as described in (A). (E) Representative Western blot of ATF3 protein expression in hepatocytes incubated as described in (A). The densitometric analysis is shown below. (F) *Ccl2*, *Tnf*, and *Il6* mRNA expression in hepatocytes incubated as described in (A). (G) Representative Western blot of liver ATF3 protein expression in mice treated with LPS+D-galactosamine (LPS+Dgal) receiving HSA ($n = 5$) or placebo ($n = 5$). The densitometric analysis is shown below. (H) *Ccl2*, *Tnf*, and *Il6* mRNA expression in mice treated as described in (G). (I) Glutathione levels in mice treated as described in (G). Results from hepatocytes are from a minimum of three independent experiments assayed in duplicate. All results are expressed as mean ± SEM.

preserves mitochondrial function in parenchymal liver cells and protects them from the damaging actions of pro-inflammatory and cytotoxic cytokines. Specifically, in this study, we provide evidence that albumin counteracts excessive TNFα-induced mitochondrial ROS production in ex vivo and in vitro models that mimic the extravascular and interstitial microenvironment of liver cells. TNFα is a common pro-inflammatory and cytotoxic cytokine that plays a major role in perturbing mitochondrial morphology and function during hyperinflammatory conditions.[4,5] TNFα is invariably elevated within the broad spectrum of liver diseases, from the mildest forms of non-alcoholic fatty liver disease, to the most severe, such as those manifested in patients with advanced liver cirrhosis in whom cytokine-induced tissue injury increases the risk of developing organ failure.[21,22] Therefore, our findings are of clinical relevance because mitochondrial dysfunction is a prime pathogenic factor in liver disease,[16] since these cell organelles are the main source of ROS in the cell and excessive ROS production leads to oxidative damage of cellular components such as lipids, proteins and mitochondrial DNA, promoting cytotoxicity, and tissue injury.[23] Our findings are also of relevance to the conditions in which liver patients exhibit remarkable hypoalbuminemia, especially in patients with decompensated cirrhosis with risk to progress to ACLF, in whom tissue injury and mitochondrial dysfunction leads to organ failure.[24]

Our study provides evidence that albumin is essential to preserve the antioxidant homeostasis in the liver cell microenvironment. In the peripheral circulation, albumin has long been regarded as a potent antioxidant molecule due to its free radical scavenging activities and its ability to bind pro-inflammatory cues.[9] In our study, the antioxidant properties of albumin in the liver cell microenvironment appeared to be independent of its interference with the binding of TNFα to their receptors. This view is supported by the observation that the addition of albumin 1 h after the stimulation of hepatocytes with TNFα (an approach in which TNFα has already interacted and activated its membrane receptors) produced the same protection against mitochondrial oxidative stress as pre-incubating first the hepatocytes with albumin and subsequently stimulate with TNFα. In addition, the antioxidant properties of albumin are unrelated to changes in the osmotic pressure in the cell culture since other oncotic agents such as mannitol were not able to reduce TNFα-induced mitochondrial oxidative stress in hepatocytes. Moreover, the observation that albumin produced expression changes at the gene and protein level together with the finding that the reduction in mitochondrial oxidative stress was abrogated under transcriptional arrest, suggest that the mitochondrial antioxidant properties of albumin against TNFα in liver cells occur at the intracellular level. Consistent with this, it is well known that albumin is taken up and internalized by hepatocytes[25] as well as by endothelial and immune cells.[9,10] It is also well characterized that once internalized by cells, albumin is not a mere bystander but rather an effector molecule able to block endosomal toll-like receptor signaling, lysosomal cathepsin B leakage, mitochondrial cytochrome c release and caspase-3 activity.[10,13]

Our study provides some mechanistic data underlying the antioxidant protective actions of albumin in hepatocytes. Previous studies have reported that TNFα treatment of hepatocytes leads to increased mitochondrial O_2 consumption,[4] a response likely reflecting the high energy demand required by cells exposed to pro-inflammatory stimuli.[26] However, in our experiments in which hepatocytes were incubated with albumin, the protection from TNFα-induced mitochondrial oxidative stress was not related to either changes in mitochondrial respiration or in mitochondrial membrane permeability. On the contrary, the protection from TNFα-induced mitochondrial oxidative stress by albumin was associated with a normalization of mitochondrial FAO, which when accelerated might cause excessive electron flux in ETC and ROS overproduction.[27] In addition, in these experiments, the presence of albumin prevented the formation of a breakpoint between isocitrate and α-ketoglutarate in the mitochondrial TCA cycle in response to TNFα. More importantly, following TNFα stimulation, we observed that the levels of the endogenous antioxidant ATF3 pathway were significantly higher in hepatocytes growing with albumin than in those without this molecule. Although ATF3 was initially described in macrophages, this transcription factor has been recognized in other cell types and tissues including hepatocytes and the liver, where its activation results in protection against oxidative injury.[28–30] Therefore, our data point to the common stress-responsive transcription factor ATF3 as a mechanism by which albumin preserves hepatocyte mitochondria from cytokine-induced damage.

In summary, our data provide in vitro, ex vivo, and in vivo evidence of the essential role of albumin in the preservation of liver cell mitochondria from the damaging actions of TNFα. In aggregate, our data highlight the importance of maintaining the albumin levels within the normal range not only in the intravascular compartment but also in the extravascular and interstitial space, especially in conditions such as advanced liver disease in which hypoalbuminemia is common.

AUTHOR CONTRIBUTIONS

Marta Duran-Güell and Joan Clària conceived and designed the experiments; Marta Duran-Güell, Cristina López-Vicario, Mireia Casulleras, Ingrid W. Zhang, Roger Flores-Costa, María B. Sánchez-Rodríguez, Bryan J. Contreras, and Berta Romero-Grimaldo performed

experiments; Marta Duran-Güell, Judith Cantó-Santos, and Glòria Garrabou performed and designed respirometry measurements; Raquel Horrillo, Montserrat Costa and Vicente Arroyo revised the manuscript; Marta Duran-Güell and Joan Clària wrote the manuscript.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and the online supporting information.

DISCLOSURES

RH and MC are full-time employees of Grifols.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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