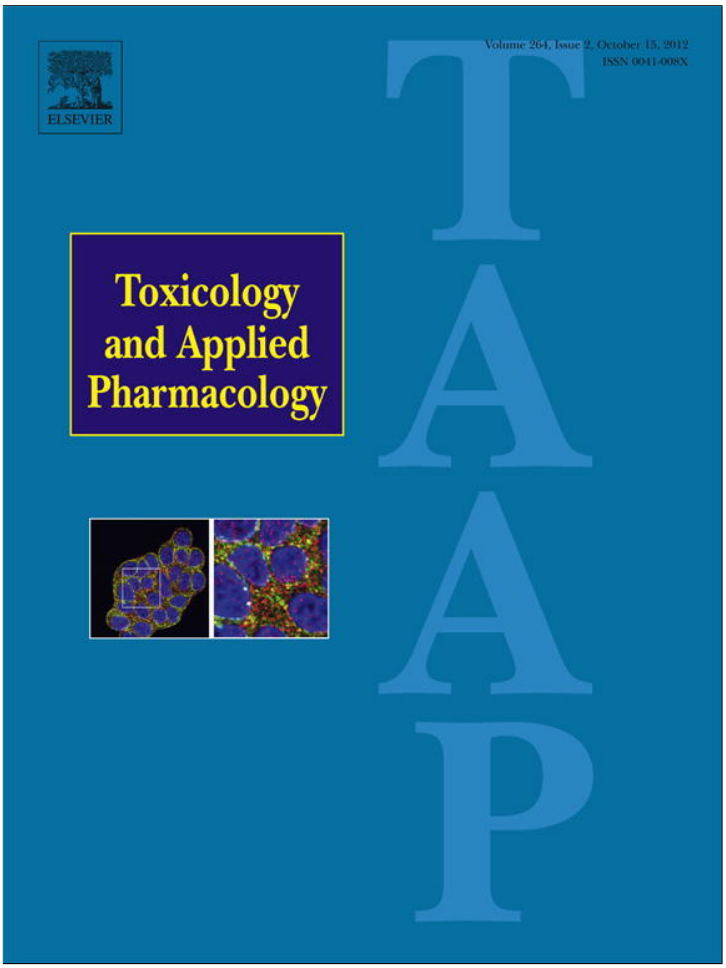

Toxicology and Applied Pharmacology 264 (2012) 246–254

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

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

Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells causes ROS-induced mitochondrial depolarization and loss of viability

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ARTICLE INFO

Article history:
Received 4 April 2012
Revised 6 July 2012
Accepted 6 August 2012
Available online 15 August 2012

Keywords:
Mitochondria
Aquaporin-8
Hydrogen peroxide
ROS
HepG2

ABSTRACT

Human aquaporin-8 (AQP8) channels facilitate the diffusional transport of H_2O_2 across membranes. Since AQP8 is expressed in hepatic inner mitochondrial membranes, we studied whether mitochondrial AQP8 (mtAQP8) knockdown in human hepatoma HepG2 cells impairs mitochondrial H_2O_2 release, which may lead to organelle dysfunction and cell death. We confirmed AQP8 expression in HepG2 inner mitochondrial membranes and found that 72 h after cell transfection with siRNAs targeting two different regions of the human AQP8 molecule, mtAQP8 protein specifically decreased by around 60% ($p<0.05$). Studies in isolated mtAQP8-knockdown mitochondria showed that H_2O_2 release, assessed by Amplex Red, was reduced by about 45% ($p<0.05$), an effect not observed in digitonin-permeabilized mitochondria. mtAQP8-knockdown cells showed an increase in mitochondrial ROS, assessed by dichlorodihydrofluorescein diacetate (+120%, $p<0.05$) and loss of mitochondrial membrane potential (−80%, $p<0.05$), assessed by tetramethylrhodamine-coupled quantitative fluorescence microscopy. The mitochondria-targeted antioxidant MitoTempol prevented ROS accumulation and dissipation of mitochondrial membrane potential. Cyclosporin A, a mitochondrial permeability transition pore blocker, also abolished the mtAQP8 knockdown-induced mitochondrial depolarization. Besides, the loss of viability in mtAQP8 knockdown cells verified by MTT assay, LDH leakage, and trypan blue exclusion test could be prevented by cyclosporin A. Our data on human hepatoma HepG2 cells suggest that mtAQP8 facilitates mitochondrial H_2O_2 release and that its defective expression causes ROS-induced mitochondrial depolarization via the mitochondrial permeability transition mechanism, and cell death.

Introduction

H_2O_2 is a major reactive oxygen species (ROS) which can be formed in mitochondria as a by-product of aerobic metabolism. Within the electron transport chain, complex I and in less proportion complex III, have been identified as the sites of ROS (H_2O_2) generation (Andreyev et al., 2005; Miwa and Brand, 2003).

Hepatic mitochondria, including those of HepG2 cells, are important sources for ROS generation and also key targets for their potential damage (Bai et al., 1999). H_2O_2 can cause loss of mitochondrial membrane potential and mitochondrial dysfunction with the consequent triggering of cell death mechanisms (Ott et al., 2007). Under physiological conditions, H_2O_2 moves out of the mitochondria into the cytoplasm where it participates in signal transduction pathways (Cadenas, 2004; Dickinson and Chang, 2011; Rigoulet et al., 2011). H_2O_2 has been long thought to be freely diffusible across cellular membranes. Nevertheless, this notion has been challenged by the existence of H_2O_2 gradients across plasma and peroxisomal membranes (Bienert et al., 2006) and the finding that membrane permeability is a rate-limiting factor in H_2O_2 elimination by mammalian cells (Makino et al., 2004). There is also experimental evidence for limited diffusion of H_2O_2 across mitochondrial membranes (Grivennikova et al., 2010). However, the molecular pathways for H_2O_2 membrane permeation across mitochondria are unknown. H_2O_2, like water, cannot freely diffuse through membranes (Antunes and Cadenas, 2000). Also, both molecules have similar size, dielectric properties, bipolar momentum, and capacity to form hydrogen bonds (Bienert et al., 2006), all determining characteristics of passage through channel membrane proteins such as AQPs. Accordingly, recent studies indicate that a member of the mammalian AQP family, i.e., human AQP8, facilitates the H_2O_2 passage across plasma membranes of reconstituted yeast (Bienert et al., 2007), and transfected mammalian cells (Miller et al., 2010).

Interestingly, AQP8 was found to be expressed in the inner mitochondrial membranes (IMM) of some cells, including hepatocytes

Abbreviations: ROS, reactive oxygen species; AQP, aquaporin; AQP8, aquaporin-8; mtAQP8, mitochondrial aquaporin-8; SCR, scrambled $siRNA_1$ sequence; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; TMRM, tetramethylrhodamine methyl ester; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; CsA, cyclosporin A; DCF, 2′,7′-dichlorofluorescein; MPTP, mitochondrial permeability transition pore; IMM, inner mitochondrial membranes; OTC, ornithine transcarbamylase; siRNA, short interfering RNA.

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http://dx.doi.org/10.1016/j.taap.2012.08.005

(Calamita et al., 2005; Ferri et al., 2003). Thus, it is conceivable that human mitochondrial AQP8 (mtAQP8) may participate in mitochondrial H_2O_2 release and that its defective expression may lead to oxidative stress-induced mitochondrial dysfunction and, eventually, cell death.

Accordingly, in this study we evaluated in a human hepatocarcinoma-derived cell line HepG2, the effect of mtAQP8 gene silencing on mitochondrial H_2O_2 release and ROS content as well as on mitochondrial membrane potential and cell viability.

Materials and methods

Materials and reagents

Dulbecco's modified Eagle medium, L-glutamine, Pen-Strep antibiotic mixture, 0.25% trypsin-EDTA, and Lipofectamine 2000 reagent were all purchased from Invitrogen Corp., CA, USA. Non-essential amino acids and fetal calf serum were obtained from PAA Laboratories GmbH, Linz, Austria. Silencer siRNA construction kit was from Ambion, TX, USA. Amplex™ Red hydrogen peroxide/peroxidase assay kit, and Cell Titer 96 nonradioactive cell proliferation assay kit were from Promega, WI, USA. The ATP determination kit was from Molecular Probes, OR, USA. Anti-AQP8 antibodies, as well as MitoTempol, were from Santa Cruz Biotechnology Inc., CA, USA, while anti-prohibitin was from Abcam, Cambridge, UK. The protease inhibitors phenyl-methylsulfonyl fluoride and leupeptin, DCFH-DA, cyclosporin A, digitonin, trypan blue dye, and antibodies against OTC (ornithine transcarbamylase) were all from Sigma, MO, USA. Sucrose was purchased from Merck Chemicals, Darmstadt, Germany. Materials for immunoblotting were obtained as follows: polyscreen PVDF transfer membrane from Perkin Elmer Life and Analytical Sciences, MA, USA; Pierce ECL Western blotting substrate from Thermo Scientific, IL, USA; and Kodak XAR film from Eastman Kodak, NY, USA. The LDH assay kit was from Wiener Lab, Rosario, Argentina.

HepG2 culture and incubation

HEPG2 clone C3A (ATCC CRL-10741) was cultured in T75 flasks in Dulbecco's modified Eagle medium (110 mg/l, high glucose formulation), supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10% heat-inactivated fetal calf serum and 100 I.U. penicillin/100 μg streptomycin at 37 °C in a 5% CO_2 atmosphere. Media were changed every other day and cells were trypsinized after reaching confluency (Larocca et al., 2009).

Synthesis of short interfering RNA (siRNA)

The 21-nucleotide RNA duplexes were synthesized using the Silencer siRNA kit following the manufacturer's directions, with oligonucleotides synthesized by Invitrogen as templates. Two sequences targeting different regions of the human AQP8 molecule were chosen following the guidelines described by Elbashir et al. (2001), and they induced a significant decrease in AQP8 expression, as analyzed by immunoblotting. $siRNA_1$ was specifically targeted at nucleotides 177–197 (AACGGTTTG TGCAGCCATGTC) and $siRNA_2$ at nucleotides 749–769 (AACCACTGGAA CTTCCACTGG) of human AQP8. The control scrambled siRNA (SCR) was designed by randomly scrambling the nucleotides of $siRNA_1$ (AATGTG TCCGTGAGCACGTCT).

AQP8 knockdown

For siRNA transfection, cells were trypsinized and plated in 35-mm plastic petri dishes at a density of 5×10^5 cells/dish in antibiotic-free medium. After 18 h of culture, HepG2 cells were transfected with AQP8 siRNAs by use of Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions. mtAQP8 protein expression was analyzed in the cells 24, 48, and 72 h after by immunoblotting.

Mitochondrial H_2O_2 release in isolated mitochondria

H_2O_2 release from isolated mitochondria from transfected HepG2 cells was measured by use of the Amplex™ Red-horseradish peroxidase assay kit as has been previously described (Muller et al., 2004). This assay utilizes horseradish peroxidase to catalyze the H_2O_2-dependent oxidation of non-fluorescent Amplex™ Red to fluorescent resorufin red and detects only the release of hydrogen peroxide, since the size of HRP prevents it from entering the mitochondria. Briefly, 50 μM Amplex™ Red reagent and 0.1 U/ml peroxidase were added to the mitochondria (50 μg protein) in 200 μl of mitochondrial respiration buffer: 10 mM Tris HCl, 0.32 M mannitol, 8 mM inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/ml bovine serum albumin (pH 7.4) (Santiago et al., 2008). Absorbance was followed at a 565 nm wavelength every 3 min for 30 min at 37 °C in an automatic microplate reader (Beckman Coulter DTX 880 Multimode Detector) equipped with a thermally controlled compartment. For each assay, wells also contained buffer with substrates only or buffer with mitochondria to estimate background absorbance values and auto-oxidation rates of Amplex Red. Mitochondrial H_2O_2 release was determined in the absence of exogenous substrates (State 1) and the slopes of the increase in absorbance vs. time were calculated. H_2O_2 was quantified by a standard curve of known H_2O_2 concentrations.

Furthermore, to evaluate the rate of H_2O_2 release in mitochondria without the barrier that the IMM constitute for said release, 72 h after transfection, mitochondria were isolated and incubated with 0.2% digitonin at 0 °C for 30 min to render them permeable. Digitonin is a steroid glycoside which is most effective in solubilizing sterol-rich membranes such as the mitochondrial outer membrane, and only at higher concentrations does it solubilize the sterol-poor IMM (Uren et al., 2005). After this treatment, H_2O_2 release from these mitochondria was determined with the Amplex™ Red-horseradish peroxidase assay kit.

Mitochondrial ROS in HepG2 cells

Mitochondrial ROS levels in HepG2 cells were monitored using the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which readily diffuses through cell membranes and is hydrolyzed by nonspecific esterases in intracellular compartments, including the mitochondria (Mattiasson, 2004a), to nonfluorescent, impermeable DCFH, which in turn is oxidized to fluorescent DCF (2′,7′-dichlorofluorescein) by ROS (Degli Esposti, 2002). Briefly, 24, 48, and 72 h after siRNA transfection, HepG2 cells were incubated with 5 μM DCFH-DA for 20 min at 37 °C in the dark. The cells were then washed in PBS, scraped, and resuspended in 0.3 M sucrose containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM leupeptin. After sonication, they were subjected to low-speed centrifugation to obtain postnuclear supernatants. These supernatants were centrifuged at $6200\times g$ for 10 min, yielding the mitochondrial HepG2 fraction where the intensity of fluorescence was immediately read in a fluorescence microplate reader (Beckman Coulter DTX 880 Multimode Detector) at an excitation wavelength of 488 nm and emission wavelength of 525 nm.

ROS production in isolated mitochondria

Analysis of ROS total production was carried out in isolated mitochondria, incubated with DCFH-DA, from control and mtAQP8-knockdown cells at 72 h after transfection. DCFH-DA crosses the mitochondrial membranes and is activated by ROS inside the organelle and in the buffer outside, thus detecting intra- and extramitochondrial ROS (Mattiasson, 2004b). Briefly, isolated mitochondria (50 μg protein) were suspended in 200 μl of respiration buffer (10 mM Tris HCl, 0.32 M mannitol, 8 mM

inorganic phosphate, 4 mM $MgCl_2$, 0.08 mM EDTA, 1 mM EGTA and 0.2 mg/ml bovine serum albumin, pH 7.4) and 1 μM DCFHDA (Degli Esposti, 2002). The fluorescent signal from DCF (excitation 488 nm, emission 525 nm) was registered every 3 min up to 30 min of incubation at 37 °C in a fluorescence microplate reader (Beckman Coulter DTX 880 Multimode Detector). Background fluorescence (determined by analyzing a sample without mitochondria) was subtracted before comparison.

MitoTempol treatment

To evaluate the role of oxidative stress in mtAQP8-knockdown HepG2 cells, 72 h after transfection cells were treated for 1 h at 37 °C with MitoTempol 100 μM. MitoTempol is a mitochondria-targeted derivative of TEMPOL, which rapidly traverses biological membranes, accumulates several hundred-fold in mitochondria and has been proven to protect mitochondria from the oxidative damage they accumulate (Liang et al., 2010; Trnka et al., 2008). After MitoTempol treatment, mitochondrial ROS levels in HepG2 cells were measured using the probe DCFH-DA as described above. Also, mitochondrial membrane potential was monitored by tetramethylrhodamine methyl ester (TMRM) fluorescence microscopy and imaging; cell viability was analyzed by use of the MTT assay and trypan blue exclusion test as will be explained below.

Inhibition of the mitochondrial permeability transition pore (MPTP) by cyclosporin A (CsA)

To assess the role of mitochondrial permeability transition in the mitochondrial membrane potential of mtAQP8-knockdown HepG2 cells, 48 h after transfection cells were treated for 24 h at 37 °C with CsA 1.5 μM to bind to the MPTP protein cyclophilin D, thus blocking said pore (Kroemer et al., 1997). After the 24 h treatment, hence, at 72 h post-transfection, mitochondrial ROS levels were evaluated using the probe DCFH-DA as previously described. Also, after CsA treatment, mitochondrial membrane potential was analyzed by TMRM-coupled fluorescence microscopy and imaging as described and cell viability was measured by MTT assay, lactate dehydrogenase (LDH) assay, and trypan blue exclusion, as will be described next.

Mitochondrial membrane potential

To monitor mitochondrial transmembrane potential in whole cells, HepG2 cells were loaded with TMRM, which is a cationic fluorophore that accumulates electrophoretically in normal mitochondria because of their negative membrane potential (Ehrenberg et al., 1988) and can be used in unfixed live cells and visualized under fluorescent microscopy. In brief, transfected HepG2 cells were first loaded with 8 μM TMRM in complete culture medium at 37 °C for 20 min 24, 48 or 72 h post-transfection. After staining, cells were washed twice and 8 nm TMRM was added to the medium as to preserve dye equilibrium. Cells were then visualized under a Nikon Eclipse TS100 inverted microscope with epifluorescence attachment using the adequate filter setting and microscopic images were collected with a Nikon Coolpix 990 camera. Fluorescence intensity was quantified by image analysis with ImageJ Software (Rasband, 1997–2011).

Measurement of ATP levels

Cellular ATP levels were quantified using a luciferin- and luciferase-based assay. 72 h after transfection, cells were rinsed with PBS, scraped, resuspended in 0.3 M sucrose + protease inhibitors and sonicated. An aliquot of the lysate was taken for the determination of ATP concentrations using an ATP determination kit according to the manufacturer's directions. Luminescence from the samples was read using a luminescence microplate reader (Beckman Coulter LD400) (Lee et al., 2009).

Cellular viability: MTT assay

The effect of mtAQP8 knockdown on the activity of mitochondrial complex II/succinate dehydrogenase was estimated 24, 48 and 72 h post-transfection by use of the MTT assay (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1983), with the Cell Titer 96 Nonradioactive cell proliferation assay kit as recommended by the manufacturer. The absorbance of the reaction solution at 570 nm was recorded with a Perkin Elmer Lambda XLS + spectrophotometer.

Cellular viability: LDH assay

LDH activity was used as a measure of cell viability as previously reported (O'Brien et al., 2000). The enzyme's leakage was measured using the LDH assay kit as advised by the manufacturer. 24, 48 and 72 h after transfection, the culture medium was aspirated and centrifuged at 500×*g* for 5 min in order to obtain a cell-free supernatant. The percentage of LDH release was determined by dividing the LDH released into the media by the total LDH (released plus cellular). Cellular LDH samples were obtained by lysis of the same cells by sonication. Aliquots of media or lysate and warm reagent were mixed in a 96-well plate and absorbance at 340 nm was recorded using a microplate analysis spectrometer (Beckman Coulter DTX 880 Multimode Detector).

Cellular viability: trypan blue exclusion assay

At 72 h after transfection, HepG2 cells were trypsinized and collected by centrifugation. An aliquot of collected cells was combined with trypan blue dye at a concentration of 0.04% (w/v) and analyzed microscopically on a Neubauer counting chamber. Blue cells were counted as nonviable, and cells excluding the dye were counted as viable. Cell viability was expressed as percentage of viable cells (Bai and Cederbaum, 2006).

Preparation of mitochondrial and submitochondrial fractions

Sonicated HepG2 cells were subjected to low-speed centrifugation to obtain postnuclear supernatants which were then centrifuged at 6200×*g* for 10 min, yielding the mitochondrial fraction. Mitochondria were washed twice before being resuspended in the appropriate buffers or were used to obtain IMM as described by Ragan et al. (1987). First, mitoplasts were prepared by using the detergent approach. Briefly, digitonin was added to a suspension of mitochondria (100 mg of protein/ml) to a final concentration of 0.6% w/v in 0.3 M sucrose and incubated for 15 min on ice under gentle stirring. After dilution with 3 volumes of 0.3 M sucrose, the suspension was centrifuged at 15,000×*g* for 10 min at 4 °C. The resulting pellet (mitoplasts) was resuspended in isolation medium at a protein concentration of 15 mg/ml before being sonicated. Mitoplasts were then diluted with an equal volume of 0.3 M sucrose and centrifuged at 15,000×*g* for 10 min at 4 °C. The resulting pellet was resuspended in 10 volumes of 0.3 M sucrose and centrifuged again at 100,000×*g*; this process was repeated twice. The final pellet (i.e., IMM) was resuspended in 0.3 M sucrose with protease inhibitors. Protein content was determined according to Lowry et al. (1951).

Immunoblotting

The different HepG2 fractions were prepared as described above and were heated for 15 min at 90 °C in sample buffer (20 mM Tris, pH 8.5, 1% SDS, 400 μM DTT, 10% glycerol), subjected to 12% SDS-PAGE and transferred to polyvinyl difluoride membranes. After blocking for a minimum of 2 h and washing, blots were incubated overnight at 4 °C with goat antibodies against human AQP8 (0.4 μg/ml). As loading/enrichment control, membranes were also incubated with 0.1 μg/ml rabbit antibodies against prohibitin (a marker of IMM) or against OTC (a matrix protein, negative marker for IMM). The blots were then washed extensively and

incubated with the horseradish peroxidase-conjugated corresponding secondary antibodies; bands were detected by the enhanced chemiluminescence detection system (ECL) and autoradiographs were obtained by exposing PVDF membranes to radiographic film. Densitometric analysis of the developed bands was performed using ImageJ Software (Rasband, 1997–2011). Under the working conditions used, there was a linear range of response of the films.

Statistical analysis

Data are means ± SE (standard error). Significance was determined using 1-way ANOVA, Tukey test. $p<0.05$ was considered as statistically significant.

Results

mtAQP8 expression and knockdown in HepG2 cells

AQP8 is present in IMM of rodent hepatocytes (Calamita et al., 2005; Ferri et al., 2003). We confirmed the expression of AQP8 in IMM of HepG2 cells by mitochondrial subfractionation and immunoblotting studies using a commercially available affinity-purified antibody raised against human AQP8. Mitochondrial subfractionation showed a band of an expected size (28 kDa) that was strongly detected in highly purified IMM of HepG2 cells (Fig. 1A). As expected, the inner mitochondrial membrane protein prohibitin was enriched in IMM, whereas the matrix protein ornithine transcarbamylase was not detected in IMM (Fig. 1A). The 36 and 56 kDa glycosilated forms of AQP8 present in plasma membranes of HepG2 cells (Larocca et al., 2009) were not detected in mitochondrial fractions. Similar results were obtained in immunoblotting studies using N-terminal anti-AQP8 antibodies generated in Dr Calamita's laboratory (Calamita et al., 2005; Ferri et al., 2003; not shown). Furthermore, the specificity of the AQP8 immunoblot was verified by the decrease of the intensity of the immunodetected band in samples from AQP8-knockdown cells (Fig. 1B, see below for explanation).

To induce a decrease in mtAQP8 expression, siRNAs containing sequences specific for human AQP8 were designed and synthesized. HepG2 cells were then transfected with those siRNAs, and mtAQP8 expression was checked 24, 48 and 72 h after by immunoblotting. Fig. 1C shows that 24 or 48 h after transfection, mtAQP8 expression was unaffected. However, 72 h after transfection, siRNAs 1 and 2 induced a significant decrease in mtAQP8 expression. Densitometric analysis of mtAQP8 levels related to prohibitin (an IMM protein) indicated a decrease in mtAQP8 expression of 53 and 65% in $siRNA_1$ and $siRNA_2$-treated cells, respectively at 72 h. In HepG2, AQP8 is also expressed on canalicular plasma membranes (Larocca et al., 2009). Contrary to mtAQP8, plasma membrane AQP8 was earlier knocked-down (i.e. 70% at 48 h), as we previously described (Larocca et al., 2009). As short RNA duplexes inhibit

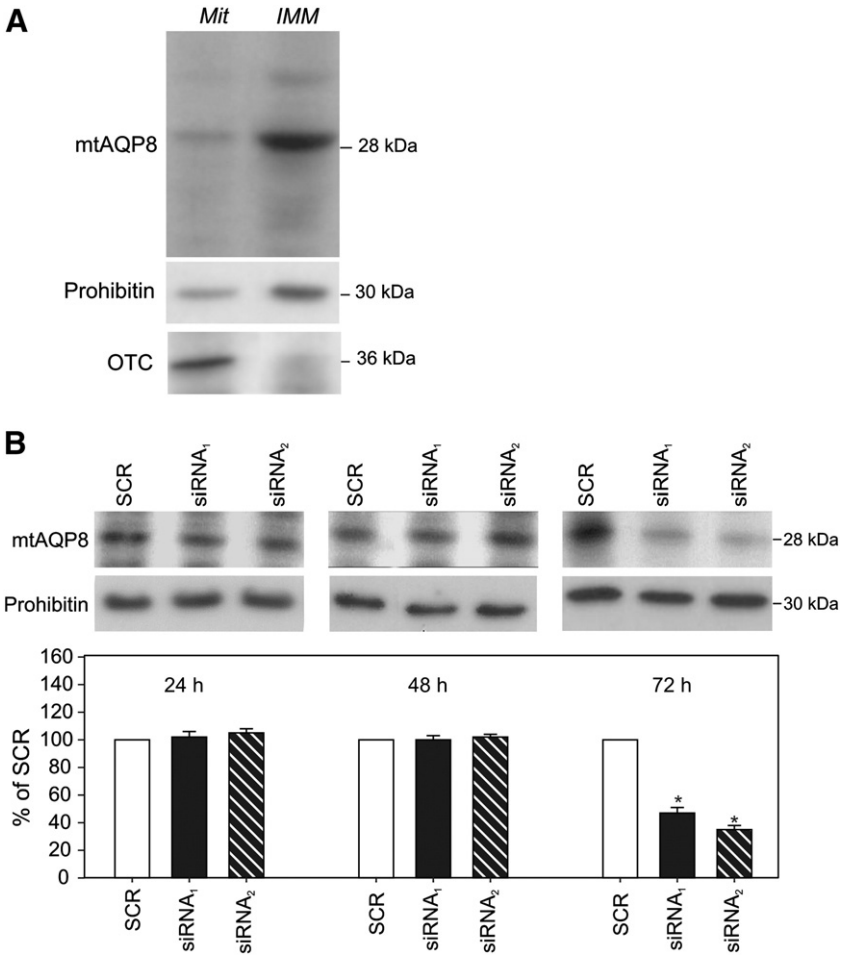

Fig. 1. Expression of mtAQP8 in HepG2 cells and its knockdown by RNA interference. (A) Immunoblotting for AQP8 in mitochondrial (Mit) and inner mitochondrial membrane (IMM) fractions. Each lane was loaded with 20 μg of protein. Bands migrated to approximately 28 kDa, showing enrichment from Mit to IMM. Immunoblottings were reprobed for prohibitin (IMM marker) and OTC (ornithine transcarbamylase, matrix protein) as controls. (B) Representative immunoblottings for mtAQP8 in mitochondrial fractions at 24, 48, and 72 h post-transfection with siRNAs or control SCR, and corresponding densitometric analysis related to prohibitin. Each lane was loaded with 20 μg of protein. Data are percentages of SCR and represent means ± SE of three independent experiments. *$p<0.05$ from SCR.

protein expression either by decreasing mRNAs levels or by diminishing their translation, dissimilar protein translation or half life may account for this difference.

Impaired release of H_2O_2 from mtAQP8-knockdown mitochondria

We measured H_2O_2 release in mitochondria isolated from HepG2 cells transfected with $siRNA_1$, $siRNA_2$ or SCR at 24, 48, and 72 h after transfection using the Amplex-Red/peroxidase system. We performed these studies in the absence of exogenous substrates (i.e., the respiratory state 1). State 1 is considered to reflect H_2O_2 production/release with true endogenous substrate (Boveris and Chance, 1973; Mansouri et al., 2006). Given the enhanced sensitivity of the Amplex Red assay, we were able to detect a release as low as approximately 50 pmol H_2O_2/min/mg protein in the control cells (i.e., cells transfected with SCR). This is a similar value to that reported previously for mitochondria in respiratory state 1 (Mansouri et al., 2006). The rate of H_2O_2 release showed a non-significant increase at 24 h and a significant reduction of about 45% in mitochondria from mtAQP8-knockdown HepG2 cells (i.e., at 72 h) (Fig. 2A). In digitonin-permeabilized mitochondria, the rate of H_2O_2 release did not differ among groups (Fig. 2B). Together, these data suggest that mtAQP8 knockdown impairs the normal, but membrane-conditioned, out-diffusion of H_2O_2 across HepG2 mitochondrial membranes.

Increased mitochondrial ROS in mtAQP8-knockdown HepG2 cells

Mitochondrial ROS in HepG2 cells were monitored using the fluorescent probe DCFH-DA. As shown in Fig. 3, there was no alteration of mitochondrial fluorescence in siRNA-treated cells at 24 or 48 h; nevertheless, at 72 h, when mtAQP8 knockdown was evident, the compound's fluorescence was significantly enhanced by about 120%. After treatment with the mitochondria-targeted antioxidant MitoTempol fluorescence did not differ among groups. As also shown in Fig. 3, pre-treatment of cells with the MPTP inhibitor CsA did not affect the increase in mitochondrial ROS observed in mtAQP8-knockdown cells.

ROS production assessed in isolated mitochondria using DCFH-DA (see Materials and Methods) showed a tendency to increase, although it was not statistically significant, i.e., mitochondrial ROS production (arbitrary fluorescence units/min/mg mitochondrial protein, % SCR): 100.0 (SCR) vs. 124.7 ± 15.3 ($siRNA_1$) and 168.3 ± 42.0 ($siRNA_2$).

These data suggest mitochondrial ROS accumulation and outline an oxidative stress scenario in mtAQP8-knockdown cells.

Dissipation of mitochondrial membrane potential in mtAQP8-knockdown HepG2 cells

Mitochondrial membrane potential was assessed using the red-fluorescence marker TMRM. At 72 h after transfection with the siRNAs, the red TMRM fluorescence clearly declined (Figs. 4A and 5A). This was later quantified by image analysis showing a fluorescence decrease of around 80% (Figs. 4B and 5B). TMRM fluorescence in $siRNA_1$ and $siRNA_2$-treated cells at 24 or 48 h was not significantly altered (data not shown). Mitochondrial depolarization was restored by MitoTempol (Fig. 4), thus suggesting an oxidative stress-induced loss of mitochondrial membrane potential in mtAQP8-knockdown HepG2 cells. As shown in Fig. 5, pre-treatment for 24 h with 1 μM CsA prevented mitochondrial depolarization in mtAQP8-knockdown HepG2 cells suggesting the involvement of the mitochondrial permeability transition mechanism. In separate experiments, 5 μM CsA treatment for 1 h at 37 °C was also able to fully restore mitochondrial membrane potential (data not shown).

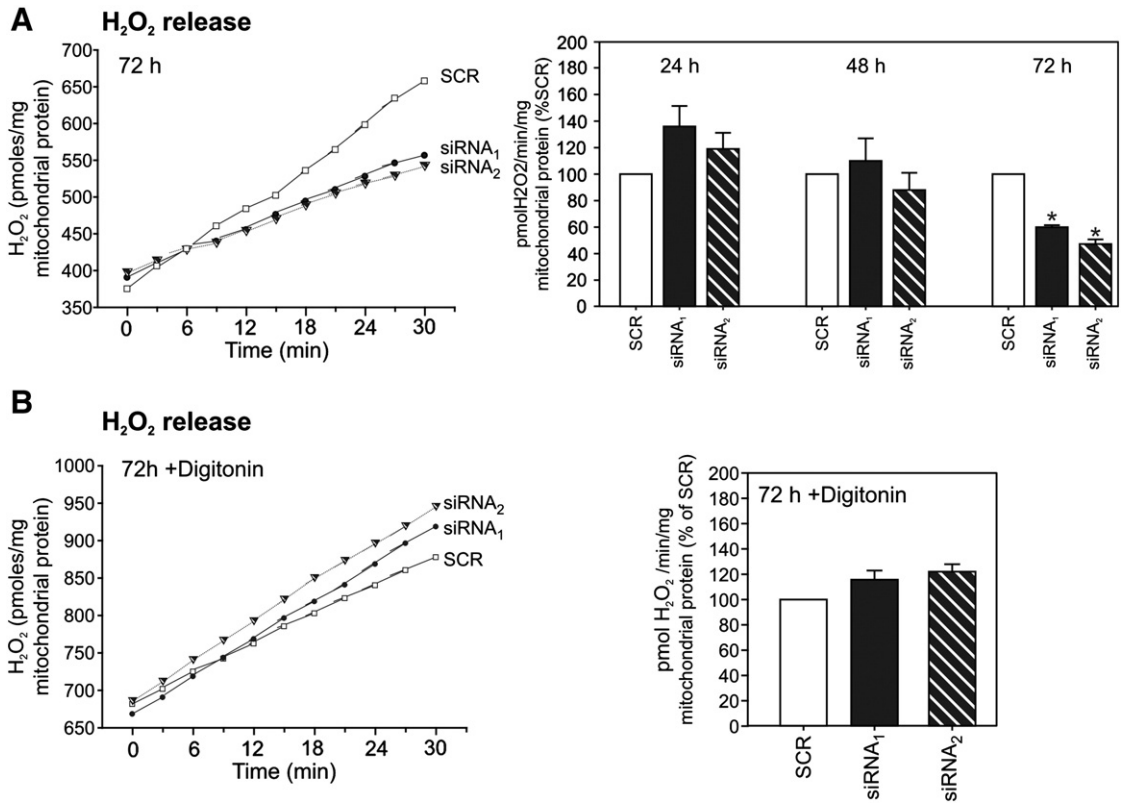

Fig. 2. H_2O_2 release in mtAQP8-knockdown mitochondria. Mitochondria from transfected HepG2 cells were isolated and used for H_2O_2 release experiments. (A) *Left*: representative time course of mitochondrial H_2O_2 release at 72 h post-transfection. *Right*: rate of mitochondrial H_2O_2 release at 24, 48, and 72 h. Data are means ± SE of three independent experiments. *$p<0.05$ from SCR. (B) *Left*: representative time course of H_2O_2 release from digitonin-permeabilized mitochondria (72 h). *Right*: rate of mitochondrial H_2O_2 release at 72 h. Data are means ± SE of three independent experiments.

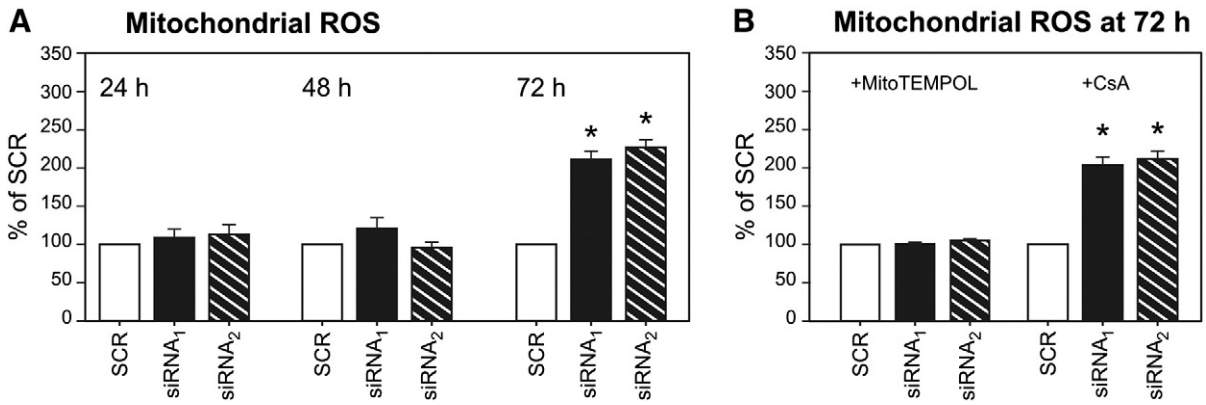

Fig. 3. Mitochondrial ROS in mtAQP8-knockdown HepG2 cells. HepG2 cells were transfected with $siRNA_1$, $siRNA_2$ or a scrambled $siRNA_1$ sequence (SCR) for 24, 48, and 72 h and then incubated with 5 μM DCFH-DA. Mitochondria were isolated and the intensity of fluorescence was immediately read (A). Also, pre-treatments with MitoTempol or CsA were carried out prior to DCFH-DA incubation and mitochondrial fluorescence determinations (at 72 h only, B). Values are arbitrary fluorescence units (AFU) per mg protein expressed as the percentage of SCR controls. Data are means ± SE. of three independent experiments. *$p<0.05$ from SCR.

Reduced viability in mtAQP8-knockdown HepG2 cells

The activity of mitochondrial succinate dehydrogenase (respiratory chain complex II), assayed by MTT, was found to be significantly reduced by around 50% in either $siRNA_1$ or $siRNA_2$-treated cells at 72 h (Fig. 6A). MitoTempol and CsA treatment prevented the MTT-assessed mitochondrial dysfunction (Fig. 6B).

LDH leakage assay was also used to examine the effect of mtAQP8 knockdown on the viability of HepG2 cells. As shown in Fig. 7A, LDH significantly increased in cultured media 72 h after transfection with both siRNAs. No significant changes, however, were obtained at shorter times of transfection. The loss of cell viability at 72 h of siRNA treatment was also revealed using trypan blue exclusion (Fig. 7C).

The data indicate that mtAQP8 knockdown led to plasma membrane damage and loss of viability of HepG2 cells. When cells were pre-treated for 24 h with CsA, HepG2 viability was fully or partially restored (Figs. 7B and D).

Discussion

Our major aim was to study in human hepatoma HepG2 cells the effect of mtAQP8 knockdown on mitochondrial H_2O_2 release and ROS content as well as on mitochondrial membrane potential and cell viability. After confirming the expression of mtAQP8 in HepG2 cells, we provided experimental evidence suggesting that i) the knockdown of mtAQP8 expression impaired H_2O_2 mitochondrial release and increased mitochondrial ROS, and ii) the mtAQP8 knockdown also caused ROS-induced mitochondrial depolarization via the mitochondrial permeability transition mechanism, and cell death.

AQP8 expression in hepatocyte mitochondria has been reported in rodent cells (Calamita et al., 2005; Ferri et al., 2003). By mitochondrial subfractionation and immunoblotting studies, we now show the expression of AQP8 in the IMM of the human hepatocyte cell line HepG2 (Fig. 1). Besides, we provide the first evidence for AQP8-mediated mitochondrial transport of H_2O_2. Our studies on cultured HepG2 cells and on isolated mitochondria suggest that the knockdown of mtAQP8 impairs H_2O_2 release and increases ROS mitochondrial content (Figs. 2 and 3).

Mitochondrial accumulation of ROS is well known to induce deleterious effects on the organelle function (Ott et al., 2007). Mitochondrial membrane depolarization is one of the expected events that occur as a response to oxidative stress (Orrenius et al., 2007). Such oxidative stress markedly sensitizes mitochondria toward the opening of nonspecific pores in the IMM, i.e., the mitochondrial permeability transition pore (MPTP; Ott et al., 2007), which leads to membrane depolarization. Consistently, mtAQP8 knockdown cells showed a significant loss of mitochondrial potential which could be prevented by the mitochondria-targeted antioxidant MitoTempol (Fig. 4) and the MPTP-blocker CsA (Fig. 5). Disruptions to the transmembrane potential have severe consequences on mitochondrial respiration and energy production since the proton gradient across the inner membranes enables the energetically-unfavorable production of ATP. In fact, ATP levels in HepG2 cells with mtAQP8 silencing (i.e., at 72 h) significantly decreased by around 40%. ATP levels, expressed as arbitrary luminescence units per mg of protein and normalized to SCR cells were: 65.8 ± 1.7% ($p<0.05$) and 54.6 ± 0.3%

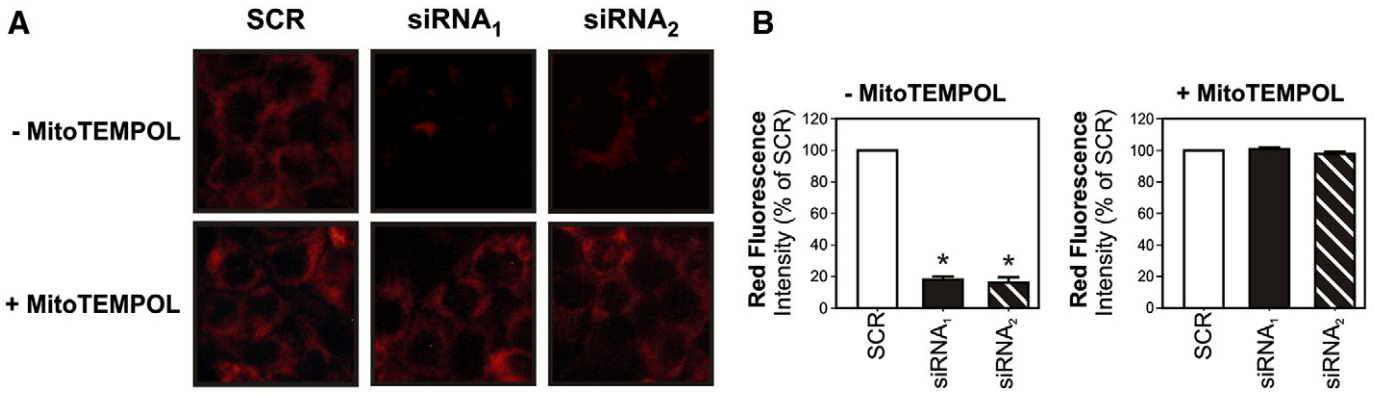

Fig. 4. Mitochondrial membrane potential in mtAQP8-knockdown HepG2 cells. Effect of MitoTempol. (A) 72 h post-transfection, HepG2 cells were treated or not treated with MitoTempol before TMRM staining. (B) Quantification of the TMRM-fluorescence decrease in mtAQP8-knockdown cells (*left*) and of MitoTempol-induced mitochondrial membrane potential restoration (*right*). Data are means ± SE of measurements from 500 cells per group. *$p<0.05$ from SCR.

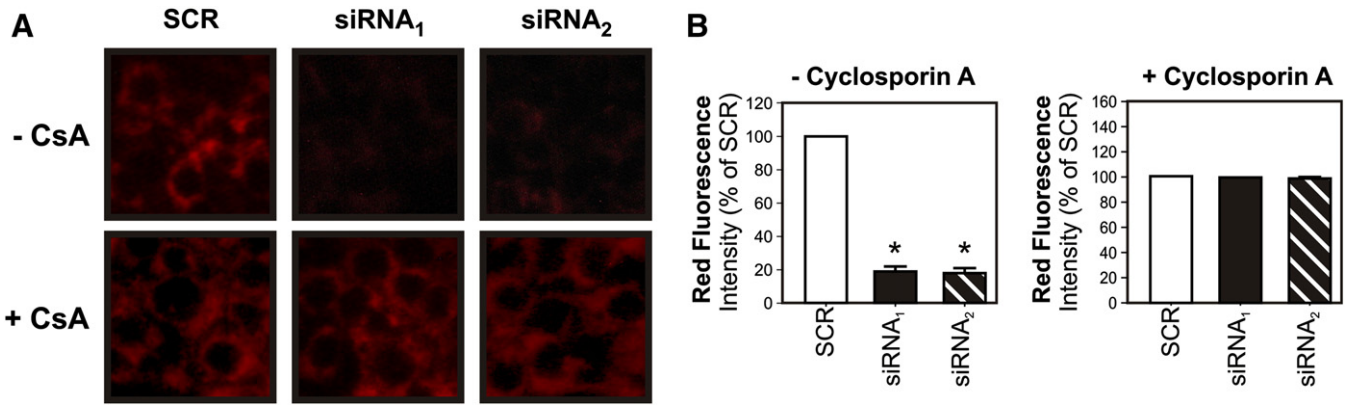

Fig. 5. Mitochondrial membrane potential in mtAQP8-knockdown HepG2 cells. Effect of CsA. (A) 72 h post-transfection, cells were treated or not treated with CsA before TMRM staining. (B) Quantification of the TMRM-fluorescence decrease in mtAQP8-knockdown cells (*left*) and of CsA-induced mitochondrial membrane potential restoration (*right*). Data are means ± SE of measurements from 500 cells per group. $^{*}p<0.05$ from SCR.

($p<0.05$) in $siRNA_1$ and $siRNA_2$-treated cells, respectively. ATP content in control cells (SCR-treated) was 16.6 pmol/mg protein.

These alterations of normal mitochondrial function are expected to have an impact on cell viability. Thus, the activity of mitochondrial complex II/succinate dehydrogenase, assayed by MTT, was significantly reduced in mtAQP8 knockdown cells (Fig. 6A). This is consistent with a mitochondrial dysfunction in HepG2 cells induced by ROS accumulation and the permeability transition pore mechanism this entails, since MitoTempol and CsA treatment prevented the MTT-assessed mitochondrial dysfunction (Fig. 6B). Necrotic or late apoptotic cells lose their plasma membrane integrity, thus allowing intracellular constituents, such as LDH, to leak out of the cell into the culture media, as we observed in the mtAQP8-knockdown cells (Fig. 7A). Trypan blue data in Fig. 7C are also consistent with an alteration of cell membrane integrity and, together with LDH release data, a clear evidence for loss of viability in mtAQP8-knockdown HepG2 cells. The prevention by CsA (Figs. 7B and D) further supports the above suggested mechanism for mitochondrial dysfunction.

Mitochondrial permeability transition may precede cell death by necrosis, apoptosis or autophagy (Elmore et al., 2001; Kowaltowski et al., 2001). Additional studies are required to understand the mechanisms that actually cause the death of mtAQP8-knockdown HepG2 cells.

In HepG2 cells, AQP8 is also expressed at the canalicular plasma membrane domain (Larocca et al., 2009). We reported that canalicular AQP8 works as a water channel facilitating the movement of water molecules coupled to osmotic gradients, a process that may play a role in hepatocyte bile formation (Marinelli et al., 2011). Interestingly, mtAQP8 was found to have no major relevance to the facilitated transport of water across IMM (Gena et al., 2009), thus the functional role of mtAQP8 in hepatic cells is still under study. AQP8 is able to facilitate the transport of ammonia across mitochondrial membranes (Soria et al., 2010), a process that may play a role in hepatic mitochondrial ammonia detoxification via ureagenesis. Nevertheless, this process cannot take place in HepG2 cells since they lack a functional urea cycle (Mavri-Damelin et al., 2008). Thus, it is tempting to speculate that in HepG2 cells, mtAQP8 would play a role as peroxiporin facilitating H_2O_2 release from mitochondria. Whether this also applies to normal human and rodent hepatocytes as well as to other hepatoma cells is a matter of further detailed studies.

In conclusion, our data on human hepatoma HepG2 cells suggest that mtAQP8 plays a role in mitochondrial H_2O_2 release and support the notion that defective mtAQP8 expression causes ROS-induced mitochondrial depolarization via the permeability transition pore mechanism, and cell death. The mtAQP8-mediated mitochondrial H_2O_2 release in HepG2 may have key implications in processes modulated by redox signaling pathways, such as cell survival or death. On the other hand, as oxidative stress-induced mitochondrial dysfunction is linked to a variety of therapeutic strategies against hepatoma cells, our data may also contribute to this purpose.

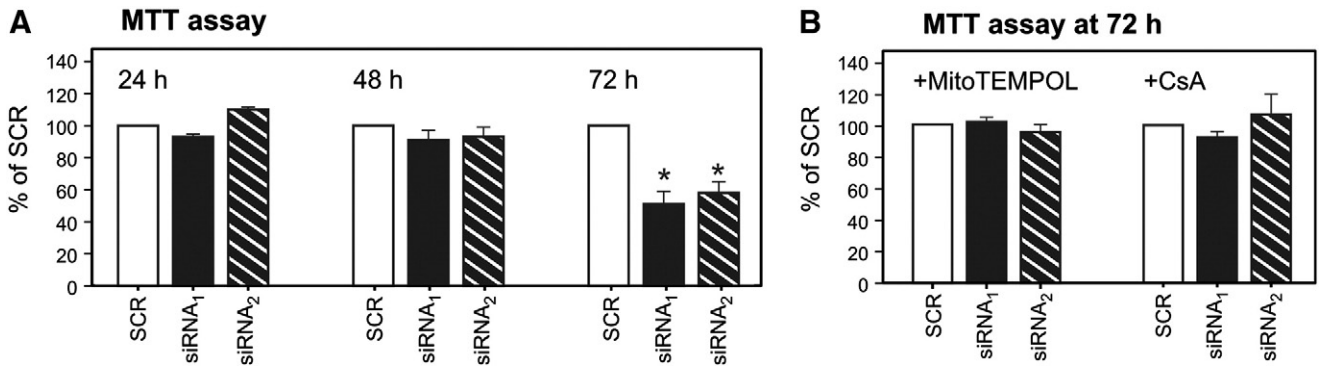

Fig. 6. MTT-assessed viability of mtAQP8-knockdown HepG2 cells. 24, 48, and 72 h post-transfection, mitochondrial succinate dehydrogenase activity was measured in HepG2 cells by the MTT assay (A). The same was performed with MitoTempol or CsA pre-treatment at 72 h (B). Data are percentages of SCR controls and represent means ± SE of three independent experiments. $^{*}p<0.05$ from SCR.

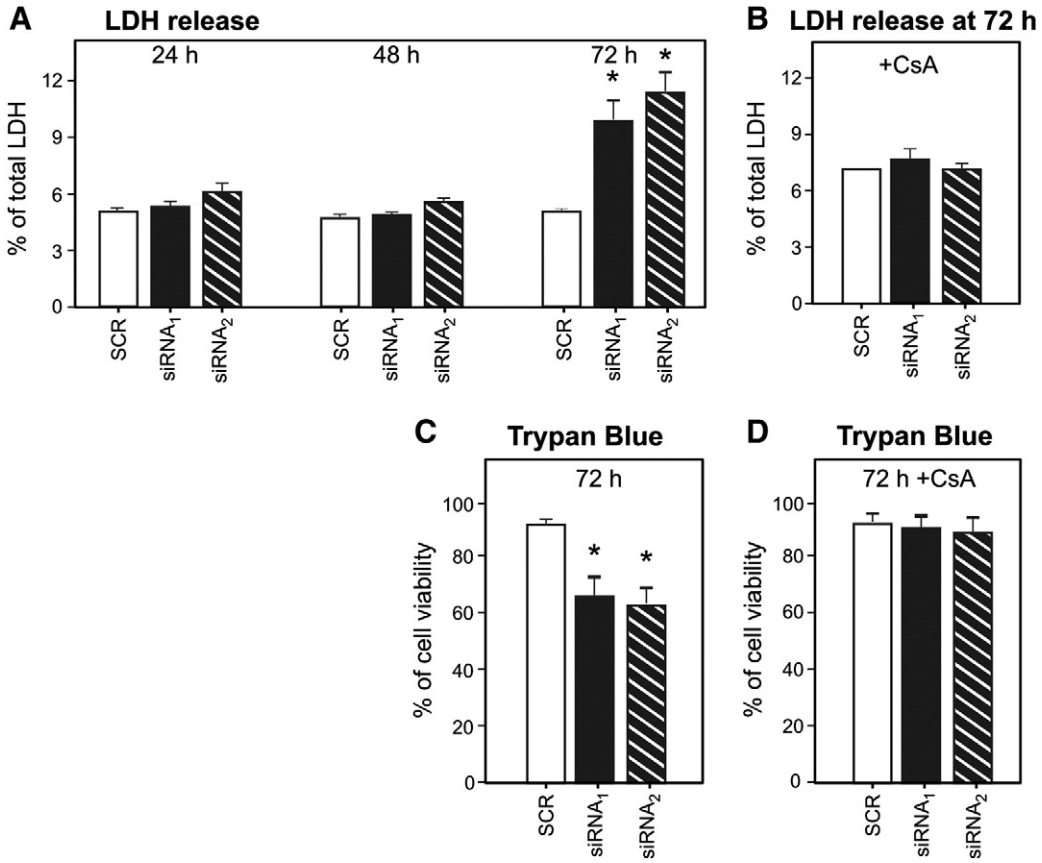

Fig. 7. LDH and trypan blue-assessed viability of mtAQP8-knockdown HepG2 cells. Leakage of cytosolic LDH was assessed 24, 48, and 72 h post-transfection (A), and at 72 h the same measurements were performed with CsA pre-treatment (B). Data represents LDH released as a percentage of the total LDH activity recovered from the same sonicated cells and represent means ± SE of three independent experiments. $^{*}p<0.05$ from SCR. Cell viability evaluated by trypan blue exclusion test in HepG2 cells at 72 h after siRNA treatment (C) and at 72 h after siRNA treatment and CsA pre-treatment (D). Cell viability is presented as the percentage of viable cells. Data are the mean values of three independent experiments. $^{*}p<0.05$ from SCR.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by grant PICT 0946 (R.A. Marinelli) from Agencia Nacional de Promoción Científica y Tecnológica, and by grant PIP 1563 (R.A. Marinelli) from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors would like to thank JM Pellegrino for his assistance with the microscopic image acquisition and analysis.

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