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N-acetylcysteine, coenzyme Q_{10} and superoxide dismutase mimetic prevent mitochondrial cell dysfunction and cell death induced by D-galactosamine in primary culture of human hepatocytes

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ABSTRACT

D-Galactosamine (D-GalN) induces reactive oxygen species (ROS) generation and cell death in cultured hepatocytes. The aim of the study was to evaluate the cytoprotective properties of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and the superoxide dismutase (SOD) mimetic against the mitochondrial dysfunction and cell death in D-GalN-treated hepatocytes. Hepatocytes were isolated from liver resections. NAC (0.5 mM), Q_{10} (30 μM) or MnTBAP (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (1 mg/mL) were co-administered with D-GalN (40 mM) in hepatocytes. Cell death, oxidative stress, mitochondrial transmembrane potential (MTP), ATP, mitochondrial oxidized/reduced glutathione (GSH) and Q_{10} ratios, electronic transport chain (ETC) activity, and nuclear- and mitochondria-encoded expression of complex I subunits were determined in hepatocytes. D-GalN induced a transient increase of mitochondrial hyperpolarization and oxidative stress, followed by an increase of oxidized/reduced GSH and Q_{10} ratios, mitochondrial dysfunction and cell death in hepatocytes. The cytoprotective properties of NAC supplementation were related to a reduction of ROS generation and oxidized/reduced GSH and Q_{10} ratios, and a recovery of mitochondrial complexes I+III and II+III activities and cellular ATP content. The co-administration of Q_{10} or MnTBAP recovered oxidized/reduced GSH ratio, and reduced ROS generation, ETC dysfunction and cell death induced by D-GalN. The cytoprotective properties of studied antioxidants were related to an increase of the protein expression of nuclear- and mitochondrial-encoded subunits of complex I. In conclusion, the co-administration of NAC, Q_{10} and MnTBAP enhanced the expression of complex I subunits, and reduced ROS production, oxidized/reduced GSH ratio, mitochondrial dysfunction and cell death induced by D-GalN in cultured hepatocytes.

1. Introduction

Apoptosis is considered to be involved in the normal regulation of the organ size, as well as in the underlying mechanism of cell death in liver diseases. The induction of oxidative stress is a key event in the intracellular pathways leading to cellular apoptosis [1]. Several free radical generation sites, such as mitochondria or cytochrome P450-dependent metabolism, are involved in cell death [2,3]. The depletion of cellular reduced glutathione (GSH) content, as a consequence of intense intracellular oxidative stress, has been observed during cell death induced by different agents [4,5]. D-Galactosamine (D-GalN) is a suitable experimental model of human liver failure [6]. D-GalN induces oxidative stress and cell death in human and rat cultured hepatocytes [7,8]. The rapid generation of reactive oxygen species (ROS) was related to hyperpolarization of the mitochondrial membrane potential and apoptosis in D-GalN-treated human hepatocytes [9]. In addition, D-GalN-dependent cell

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necrosis was related to mitochondrial membrane depolarization [9].

Mitochondria provide most of the cell energy through the fatty acid β-oxidation, the tricarboxylic acid cycle, and oxidative phosphorylation. Endogenous compounds (such as cytokines or female sex hormones) or xenobiotics (including toxins, such as ethanol and drugs, such as aspirin, valproic acid, ibuprofen, or zidovudine) can inhibit β-oxidation directly or through a primary effect on the mitochondrial genome or the respiratory chain itself [10]. The mitochondrial ROS generation can be significantly enhanced by a rise of NADH supplementation or with the functional impairment of complexes I and III of electron transport chain (ETC) [11]. Although energy production is an important function of mitochondria, these organelles also participate in the initiation and execution of cell death, and maintaining calcium and iron homeostasis. The regulation of intracellular oxidative stress by antioxidants may determine cell fate and the mode of cell death [12]. Different antioxidant strategies have been shown to be useful to reduce oxidative stress and cell death in hepatocytes. GSH is the most abundant non-protein thiol present in mammalian cells, and acts as an essential component of the cellular antioxidant defense system. Due to its very reactive cysteine sulfhydryl moiety, GSH is enzymatically conjugated to toxic electrophiles by cellular GSH transferases. Additionally, GSH participates as a co-substrate in various GSH-dependent peroxidase reactions, protecting cells or tissues against oxidative damage by metabolizing toxic organic molecules and hydrogen peroxides. The maintenance of mitochondrial GSH content prevents cell damage in different *in vivo* [13] and *in vitro* [14] experimental models of hepatotoxicity. The administration of N-acetylcysteine (NAC), an excellent source of intracellular cysteine and free radical scavenger, has been shown to have clinical applications in HIV infection, cancer, heart disease, as well as in smoking, kidney and liver diseases [15]. The regulation of mitochondrial oxidative stress may be a useful strategy to prevent the depletion of GSH in the organelle. The aim of our study was the identification of the beneficial properties of NAC, coenzyme Q_{10} (Q_{10}) and superoxide dismutase (SOD) mimetic (MnTBAP) on oxidative stress, expression and/or activity of the mitochondrial electron transport chain (ETC) components and cell survival in D-GalN-treated hepatocytes. The study showed that the co-administration of NAC, Q_{10} and MnTBAP enhanced the expression of complex I subunits, and reduced ROS production, oxidized/reduced GSH ratio, mitochondrial dysfunction and cell death in D-GalN-treated hepatocytes.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. DME:Ham-F12 and William's E culture mediums were obtained from Sigma Chemical Co. and Applichem (Applichem GmbH, Darmstadt, Germany), respectively. Antibiotics–antimycotic solution and fetal bovine serum were obtained from Life Technologies Inc. (Paisley, UK). NAC and Q_{10} were obtained from Sigma Chemical Co. MnTBAP (Mn (III)tetrakis(4-benzoic acid) porphyrin chloride was obtained from Calbiochem (Darmstadt, Germany). The peptide-based substrate for the measurement of caspase-3 activity was N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC, Bachem AG, Bubendorf, Switzerland). The study protocol has been approved by the Ethical Committee of the Institution.

2.2. Preparation of primary human hepatocytes and cell culture

The liver biopsy was obtained from 39 patients (17 women, 22 men; 58 ± 3.8 years old) submitted to surgical resection for liver tumor after written consent of the patient. The isolation of hepatocytes was based on the two-step collagenase procedure [16]. The liver was first perfused with a non-recirculating chelating solution I containing 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetracetic acid (EGTA), 58 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma), 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min in order to remove the remaining blood. Afterwards, the liver was perfused with a non-recirculating washing solution II containing 20 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)hemisodium (HEPES), 120 mM NaCl, 5 mM KCl, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min. The liver was later perfused with a recirculating dissociation solution III containing 0.05% type IV collagenase, 20 mM HEPES, 120 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min. The cell suspension was filtered through nylon mesh (250 μm) and washed three times at $50 \times g$ for 5 min at 4 °C in the supplemented culture medium. DME–Ham-F12 and William's E mediums (1:1) were supplemented with 26 mM NaHCO, 15 mM HEPES, 0.29 g/L glutamine, 50 mg/L vitamin C, 0.04 mg/L dexamethasone, 2 mg/L insulin, 200 μg/L glucagon, 50 mg/L transferrin and 4 ng/L ethanolamine. Cell viability was consistently >85%, as determined by trypan blue exclusion. Hepatocytes (8×10^6 cells; 150,000 cells/cm^2) were seeded at confluence on type I collagen-coated dishes (Iwaki, Gyouda, Japan), and maintained in a culture medium containing 5% fetal calf serum for 12 h. Afterwards, the medium was removed and replaced by a fresh culture medium without fetal bovine serum. The study was initiated 48 h after cell seeding to ensure cell stability. Cell death was induced by D-GalN (40 mM) that has been previously shown to induce oxidative stress and cell death in hepatocytes [7]. NAC (0.5 mM) [17,18], Q_{10} (30 μM) [19] or MnTBAP (1 mg/mL) [20] was co-administered with D-GalN.

2.3. Isolation of mitochondrial fraction

Mitochondria were obtained as previously described [21] with some modifications. Cells (16×10^6) were scraped and washed twice with ice-cold phosphate buffer solution (PBS) pH 7.4 at $500 \times g$ for 5 min at 4 °C. Cells were disrupted with 9 volumes of buffer (HEPES 2 mM, pH 7.4, 0.15 mM $MgCl_2$, 10 mM KCl, 0.5 mM EGTA, 5 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) in a Dounce homogenizer. Afterwards, a concentrated sucrose solution was added to reach a final sucrose concentration of 0.32 M. Undisrupted cells and debris were removed by centrifugation at $1000 \times g$ for 5 min. The pellet was recovered and named as F1 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The supernatant was considered as the post-nuclear cell extract, and it was used to obtain crude mitochondria by centrifugation at $10{,}000 \times g$ during 10 min. The supernatant was recovered and named as F2 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The pellet was identified as the crude mitochondrial fraction, and layered onto a discontinuous sucrose gradient performed with 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose concentrations in 2 mM HEPES buffer pH 7.4. After centrifugation at $95{,}000 \times g$ for 90 min, seven fractions were obtained. The first two fractions were recovered and named as F3 and F4 fractions for the characterization of mitochondrial isolation procedure (Fig. 1). The purified mitochondrial fraction located between 1.2 and 1.4 sucrose layers (correspond to the fraction 5

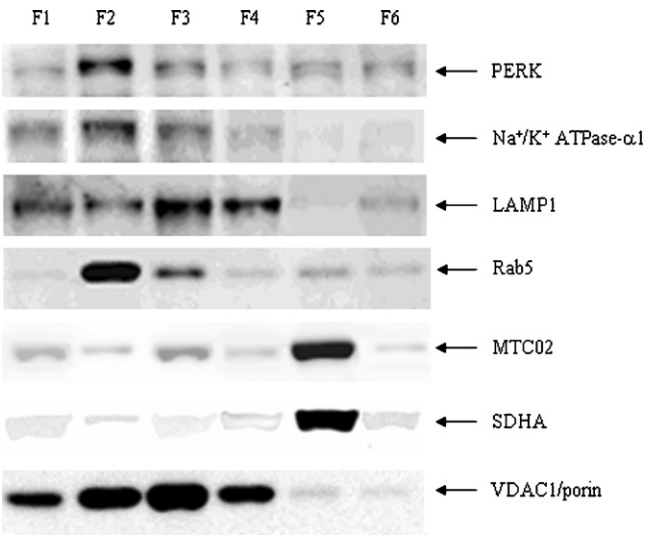


Fig. 1. Expression of mitochondria, endoplasmic reticulum, plasma membrane, lysosomes and endosomes markers in different fractions obtained during mitochondrial isolation procedure. Six fractions were isolated following the procedure described in Section 2. F1 fraction is obtained from the initial centrifugation of cell lysate at 1000 × *g*. The supernatant was submitted to centrifugation at 10,000 × *g*, and the resulting supernatant was identified as F2 fraction, and the pellet was layered onto a discontinuous sucrose gradient. After centrifugation at 95,000 × *g* for 90 min, seven fractions were obtained. The first two fractions were recovered and named as F3 and F4 fractions. The purified mitochondrial fraction located between 1.2 and 1.4 sucrose layers (correspond to the fraction 5 in the discontinuous gradient) was recovered and named as F5 fraction. The pellet obtained after ultracentrifugation was recovered and named as F6 fraction. The purity of the mitochondrial fraction was characterized in the F1–F6 fractions by the expression of voltage-dependent anion channel (VDAC1)/porin, succinate dehydrogenase (SDHA) and mitochondrial marker (MTC02). The expression of phospho-pancreatic endoplasmic reticulum kinase (PERK), Na^+/K^+ ATPase-α1, LAMP1 and Rab5A identify the expression of markers related to endoplasmic reticulum, plasma membrane, lysosomes and endosomes, respectively. The expression is assessed by SDS-PAGE followed by Western-blot analysis as described in Section 2. The images are representative of three different experiments.

in the discontinuous gradient) was recovered and named as F5 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The pellet was recovered and named as F6 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The expression of the markers in the isolated fractions (10 μg proteins) was assessed by 8–10% SDS-PAGE and Western-blot analysis. The purity of the mitochondrial fraction was characterized in the F1–F6 fractions by the expression of voltage-dependent anion channel (VDAC1)/porin (ab15895, Abcam, Cambridge, MA, USA) (1/1000), succinate dehydrogenase (SDHA) (2E3, ab14715, Abcam) (1/10,000) and mitochondrial marker (MTC02) (ab3298, Abcam) (1/200) (Fig. 1). The expression of SDHA and MTC02 was high, but very low that of VDAC1/porin, in the purified mitochondrial fraction (F5) (Fig. 1). The low expression of VDAC/porin in F5 may suggest that the outer mitochondrial has been separated from the mitochondrial inner membrane and matrix. The expression of phospho-pancreatic endoplasmic reticulum kinase (PERK) (ab65142, Abcam) (1/1000), Na^+/K^+ ATPase-α1 (ab2872, Abcam) (dilution 1/250), LAMP1 (ab25630) (1/10,000) and Rab5A (ab50523, Abcam) (1/1000) showed insignificant contamination with endoplasmic reticulum, plasma membrane, lysosomes and endosomes in the F5 fraction, respectively (Fig. 1). The endoplasmic reticulum, plasma membrane and endosomes were mostly present in F2 fraction, but lysosomes were mostly detected in the first two fractions of the discontinuous gradient (F3 and F4) (Fig. 1).

2.4. Preparation of cytoplasm and nuclear extracts

The cytoplasm and nuclear fractions from hepatocytes (3×10^6) were obtained by cell fractionation [22]. Briefly, hepatocytes were treated with lysis buffer (800 μL) containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 5 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT) and 0.6% Nonidet NP-40 for 10 min on ice. Afterwards, samples were homogenized and centrifuged at 15,000 × *g* for 3 min at 4 °C. Aliquots of the supernatant (cytoplasm fraction) were stored at −80 °C until use for the measurement of lactate dehydrogenase (LDH) release and caspase-3 activation. The pellet (nuclear faction) was discarded.

2.5. DNA fragmentation

The whole hepatocyte population (8×10^6 cells), including the floating cells obtained from the collected culture medium, was treated with lysis buffer (1 mL) containing 100 mM Trizma, 5 mM EDTA, 150 mM NaCl and 0.5% sarkosyl pH 8.0 for 10 min at 4 °C. Samples were incubated with RNAse (50 μg/mL) for 2 h at 37 °C, and with proteinase K (100 μg/mL) for 45 min at 48 °C. DNA was obtained by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Chemical Co.) extraction and precipitated with cold isopropanol (1:1) for 12 h at −20 °C. DNA was recovered by centrifugation of the sample at 20,800 × *g* during 10 min at 4 °C. Thereafter, the precipitate was washed with 70% ethanol, dried and re-suspended in Trizma–EDTA buffer (10 mM Trizma, 1 mM EDTA) at pH 8.0. Samples (100 μg DNA) were analyzed on 1.5% agarose gels with ethidium bromide (0.5 μg/mL).

2.6. Caspase-3 activation

Caspase-3 processing was assessed by Western-blot analysis in cytoplasm fraction. Proteins (50–100 μg) were separated by 14% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding primary rabbit polyclonal (1/1500) (Abcam) antibodies, and secondary goat anti-rabbit polyclonal (1/50,000) (sc-2301, Santa Cruz Biotechnology, Inc., California, USA) antibodies coupled to horseradish peroxidase, revealing the protein content by commercial enhanced chemiluminescence (ECL) assay (RPN2135, GE Healthcare, Buckinghamshire, UK). β-Actin, used as internal protein loading, was identified with commercial rabbit polyclonal antibodies (1/5000) (ab8227, Abcam). The enzymatic activities in the cytoplasm fraction (25 μg) were measured using Ac-DEVD-AFC (100 μM) in caspase-incubating buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 1 mM EDTA and 5 mM DTT) up to 100 μL total volume. The fluorescence of the sample (Ex 400, Em 505) was recorded using a GENios Microplate Reader (TECAN, Salzburg, Austria).

2.7. Measurement of lactate dehydrogenase release

LDH in the culture medium (130 μL) and cytoplasm fraction (5 μL) was assessed with 0.2 mM β-NADH and 0.4 mM pyruvic acid up to 200 μL PBS pH 7.4. LDH concentration in the sample was proportional to the rate of NADH oxidation measured by the absorbance at 334 nm (OD/min) using a GENios Microplate Reader (TECAN). LDH concentration in cytoplasm and culture medium was calculated using a commercial standard (GE Healthcare). The LDH release (%) was calculated as the ratio [(LDH culture medium)/(LDH cytoplasm + LDH culture medium)] × 100.

Table 1
Effect of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the mitochondrial complex activities in D-galactosamine (D-GalN)-treated hepatocytes.

Treatments	Complex I	Complex II	Complex III	Complexes I + III	Complexes II + III	Complex IV
Control	23.4 ± 3.4	23.0 ± 4.26	56 ± 6.3	116 ± 3.5	13.9 ± 0.51	29.2 ± 5.27
Q_{10}	63.9 ± 6.08*	36.7 ± 2.38*	150 ± 11.3*	194 ± 16.9*	22.1 ± 1.96*	48.3 ± 4.87*
NAC	45.1 ± 0.57*	19.1 ± 2.88	53 ± 5.7	124 ± 4.3	13.3 ± 0.89	30.2 ± 5.62
MnTBAP	23.5 ± 0.51	24.4 ± 1.12	67 ± 5.7	109 ± 2.0	13.1 ± 0.26	33.4 ± 3.53
D-GalN	69.5 ± 6.00*	25.7 ± 3.77	113 ± 1.5*	64 ± 3.4*	9.0 ± 0.41*	52.6 ± 2.00*
D-GalN + Q_{10}	52.8 ± 2.26*	22.0 ± 3.45	95 ± 6.5*	119 ± 11.0*	13.1 ± 0.50*	44.7 ± 1.33*
D-GalN + NAC	46.4 ± 2.60*	22.8 ± 3.01	79 ± 6.4*	85 ± 4.3*	11.8 ± 0.66*	45.2 ± 1.38*
D-GalN +MnTBAP	53.2 ± 2.50*	20.9 ± 2.31	76 ± 6.9*	87 ± 2.7*	11.9 ± 0.25*	46.0 ± 0.55*

NAC (0.5 mM), Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. The activity of complexes I, II, III, I + III, II + III and IV was measured 24 h after treatments in mitochondrial fraction following the procedure described in Section 2. All the values were expressed as a specific activity (nmol × min^{-1} × mg $protein^{-1}$). Each value represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

2.8. ATP measurement in hepatocytes

The hepatocyte population (3×10^6 cells), including the floating cells obtained from the collected culture medium, was treated with 1 mL of 85 mM perchloric acid, mixed for 20 s and centrifuged at 15,000 × *g* during 5 min at 4 °C. The samples were stored at −80 °C until use. The commercial luciferase solution (FL-AAM, Sigma) was diluted 1:25 with an ATP assay mix dilution buffer (FL-AAB, Sigma). The sample was also diluted 1:10 in 50 mM HEPES buffer solution pH 7.8. In the assay, 100 μL of luciferase was mixed with 100 μL of the sample in darkness, and the chemiluminescence of the mixture was measured using a GENios Microplate Reader (TECAN). The ATP concentration in the sample was proportional to the emitted chemiluminescence. The ATP concentration was calculated using a commercial standard (FL-AAS, Sigma).

2.9. GSH measurement in mitochondrial fraction

GSH and its oxidized form (GSSG) were measured using a commercial o-phthalaldehyde based assay (BioVision Research, Mountain View, CA, USA). The DTNB-enzyme cycling or the monochlorobimane GSH based assays can hardly detect differences between GSH + GSSG and GSH measurements. Briefly, the mitochondrial fraction (16×10^6 cells) was treated with 6N perchloric acid solution (3:1, v/v). The o-phthalaldehyde reacts with GSH, but not with GSSG, generating a fluorescent product. The specific measurement of GSSG, requires the addition of GSH quencher to the sample preventing its reaction with o-phthalaldehyde. The later addition of a reducing agent allows the removal of the excess of GSH quencher and the conversion of GSSG to GSH that will be measured by reaction with o-phthalaldehyde. The assay provides a specific procedure to eliminate protein thiol interference and to stabilize GSH and GSSG in solution. The fluorescence of the sample (Ex 340, Em 420) was recorded using a GENios Microplate Reader (TECAN). GSH concentration was obtained using commercial standards included in the assay.

2.10. Coenzyme Q_{10} determination

The content of oxidized and reduced Q_{10} in mitochondria was determined by high performance liquid chromatography (HPLC) after extraction with hexane [23]. Briefly, the mitochondrial fraction obtained from 16×10^6 cells were re-suspended in 500 μL PBS and 500 μL 10 mM butylated hydroxytoluene, and incubated for 10 min on ice. An aliquot of the sample (50 μL) was diluted with 50 μL 2% SDS and mixed by vortex during 1 min, 2 mL of ethanol/isopropanol (95:5) HPLC grade was added and mixed again for 1 min. Finally, 500 μL of hexane was added, mixed and centrifuged at 19,000 × *g* for 5 min at 4 °C. The extraction of Q_{10} with hexane was repeated twice, and the different upper organic phases were collected, pooled and dried with nitrogen. The dried residue was reconstituted with 50 μL mobile phase composed of *n*-propanol/methanol (40:60) containing 19.9 mM lithium perchlorate, and then subjected immediately to reversed phase chromatography at a flow-rate of 1 mL/min in a C18 kromasil 100 column (5-μm particle, 25 cm × 0.45 cm) (Scharlab, S.L., Sentmenat, Spain) installed in a HPLC device (Beckman-Coulter, Inc., Fullerton, USA). Monitoring was carried out with a Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) fitted with a Model 5010 analytical cell with the electrodes set at potentials of −500 mV and +300 mV. Both reduced and oxidized Q_{10} forms were detected from the electrochemical signal obtained at the second electrode. Using this protocol, reduced Q_{10} eluted at 13.5 min, whereas oxidized Q_{10} eluted at 21 min. Concentrations were calculated by integration of peak areas and comparison with external standards (oxidized or sodium borohydride-reduced Q_{10}). Pure Q_{10} was obtained from Sigma Chemicals.

2.11. Mitochondrial ETC activities and ROS production

The mitochondrial fraction obtained from 16×10^6 cells was used to measure the activity of complexes I, II, III, I + III, II + III, and IV [24]. NADH dehydrogenase (complex I) activity was determined as the rate of NADH-dependent consumption at 340 nm with coefficient of extinction or $\varepsilon = 6.81\ mM^{-1}\ cm^{-1}$ using 200 μM NADH, 1 mM NaN_3 in the presence or the absence of 10 μM rotenone. The activity of complex II (succinate dehydrogenase) was measured spectrophotometrically in the presence of 100 μM 2,6-dicholophenolindophenol, 32 mM succinate, and 50 μM Q_1. The decrease of dicholorophenolindophenol was followed at 600 nm using $\varepsilon = 20.5\ mM^{-1}\ cm^{-1}$. The complex III (ubiquinol–cytochrome *c* reductase) activity was assayed using 50 μM decilubiquinol and 50 μM cytochrome *c* as substrates. The complex III activity was measured by cytochrome *c* reduction in the presence or absence of 18.22 μM antimycin A. The reduction of cytochrome *c* was followed at 550 nm using $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$. The decilubiquinol was obtained by the chemical reduction of decilubiquinone with sodium borohydride. Cytochrome *c* oxidase (complex IV) activity was determined as the rate of oxidation of 80 μM reduced cytochrome *c* at 550 nm ($\varepsilon = 20.5\ mM^{-1}\ cm^{-1}$). Cytochrome *c* was reduced by sodium borohydride. The activities of complexes I + III and II + III consisted, respectively in the NADH- and succinate-dependent cytochrome *c* reduction assessed at 550 nm.

ROS were detected using 2,7-dichlorofluorescein diacetate dye (DCFDA; Molecular Probes Europe BV, Leiden, The Netherlands) in hepatocytes. Anion superoxide ($O_2^{\bullet -}$) production was monitored using dihydroethidium (DHE; Molecular Probes) probe in hepatocytes. The contribution of hydrogen peroxide (H_2O_2) to the oxidation of DCFDA was determined with the pre-incubation

(30 min) of the cells with catalase (500 U/mL). Hepatocytes were incubated with DHE (10 μM) or DCFDA (2 μM) for 20 and 30 min, respectively. Cells were treated with digitonin (10 μM) for 5 min in order to eliminate probe not retained in mitochondria. The culture medium was removed and replaced with PBS in order to avoid any interference with the measurement of cell fluorescence. The fluorescence emitted by DHE (Ex 510, Em 590) and DCFDA (Ex 500, Em 520) was assessed *in situ* using a GENios Microplate Reader (TECAN).

The mitochondrial transmembrane potential (MTP) was monitored using a membrane potential-sensitive fluorescent probe, such as tetramethylrhodamine methyl ester (TMR) (Molecular Probes) in hepatocytes. Cells were incubated with TMR (2 μM) for 30 min. Cells were treated with digitonin (10 μM) for 5 min in order to eliminate any probe not retained in mitochondria. The culture medium was removed and replaced with PBS in order to avoid any interference with the measurement of cell fluorescence. The fluorescence emitted by TMR (Ex 550, Em 570) was assessed *in situ* using a GENios Microplate Reader (TECAN).

2.12. Measurement of nuclear- and mitochondrial-DNA ratio

Hepatocytes were collected, washed twice with cold PBS pH7.4, re-suspended in 250 μL TE (10 mM Trizma, pH 7.5; 1 mM EDTA), 1.25 μL proteinase K (20 mg/mL), 5 μL 25% SDS and 5 μL ribonuclease A (10 mg/mL), and incubated overnight at 37 °C. DNA was extracted once with 1.5 volumes of phenol:chloroform:isoamyl alcohol (25:24:1, saturated with a solution of 10 mM Trizma pH 8, 1 mM EDTA), and twice more with chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 50 μL 7.5 M ammonium acetate and 2 volumes of cold pure ethanol for 3 h at −20 °C. DNA was recovered by centrifugation at 18,000 × *g* for 30 min, and the dry pellet was re-suspended in 10 mM Trizma buffer pH 8.0 or DNase free water (Sigma).

DNA was quantified by real-time polymerase chain reaction (RT-PCR) using the LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, USA) and QuantiTect SYBR Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany). Total cellular DNA (27 ng) was used as a template and was amplified with specific oligodeoxynucleotides for mitochondrial-encoded cytochrome oxidase subunit 2 gene (mt-Co2) (sense 5′-CGATCCCTCCCTTACCATCA-3′, and antisense 5′-CCGTAGTCGGTGTACTCGTAGGT-3′) and nuclear-encoded β-globin gene (sense 5′-GTGCATCTGACTCCTGAGGAGA-3′, and antisense 5′-CCTTGATACCAACCTGCCCAG-3′).

RT-PCR amplification was carried out in accordance with the manufacturer's recommendations in a final volume of 18 μL. The final concentrations of primers were 0.25 μM. The amplification protocol consisted of 55 cycles of incubation after the initial denaturing at 95 °C during 10 s (20 °C/s), 58 °C for 10 s (20 °C/s) and 72 °C for 10 s (2 °C/s). The melting conditions were fixed at 65 °C (0.1 °C/s). We calculated the mtDNA copy number per cell using a β-globin amplification as a reference for nuclear DNA content. The values (%) are calculated in relation to the control values of DNA copy number calculated using the formula $2 \times 2^{(\Delta Ct\ \text{nuclear-mitochondrial})}$.

2.13. Quantitative mRNA expression of nuclear-encoded complex I subunit by real-time PCR

The expression of the nuclear-encoded p17 subunit (NDUFB6 gene) was assessed with specific oligodeoxynucleotides (sense 5′-TGCTGCCCCCACAGAAGA-3′, and antisense 5′-TACCCCATGGACCATTTTCC-3′) and using the expression of the Ribosomal Protein L13A gene (sense, 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, and antisense 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′) as a reference.

RT-PCR amplification was carried out in a final volume of 18 μL. The final concentrations of primers and total RNA were 0.55 μM

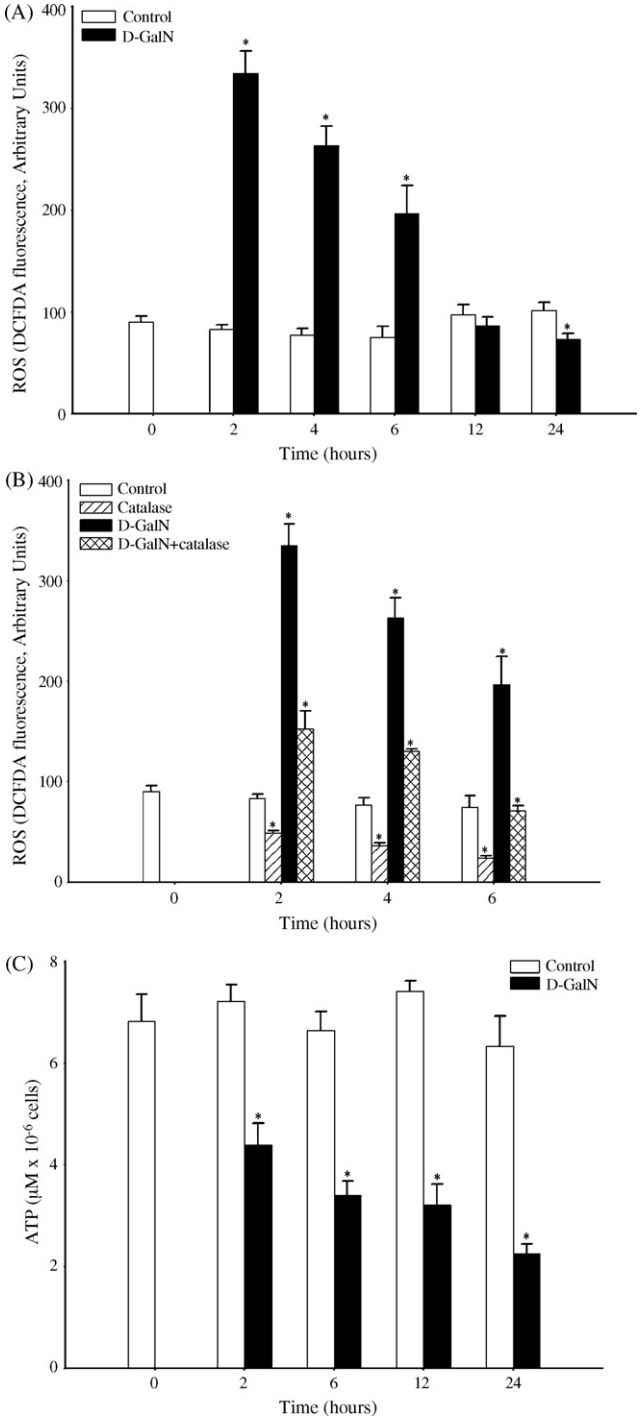


Fig. 2. Reactive oxygen species (ROS) (A) and ATP (C) in D-galactosamine (D-GalN)-treated hepatocytes. A kinetic study of D-GalN (40 mM)-induced cell death was carried out in cultured human hepatocytes. ROS were *in situ* detected using 2,7-dichlorofluorescein diacetate dye (DCFDA) in digitonin-treated cells. The contribution of H_2O_2 to the oxidation of the DCFDA was evaluated by the pre-administration (30 min) of catalase (500 U/mL) in control and D-GalN-treated hepatocytes (B). ATP was measured in acid cell extracts using luciferase reaction. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

and 5.5 ng/μL, respectively. The amplification protocol consisted of 35 cycles of denaturing at 95 °C during 15 s (20 °C/s), annealing at 60 °C for 15 s (20 °C/s) and synthesis at 72 °C for 25 s (2 °C/s). The melting conditions were fixed at 65 °C (0.1 °C/s). The used primers were tested to be optimum for quantitative analysis. To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. The melting curve analysis with the PCR products showed that there

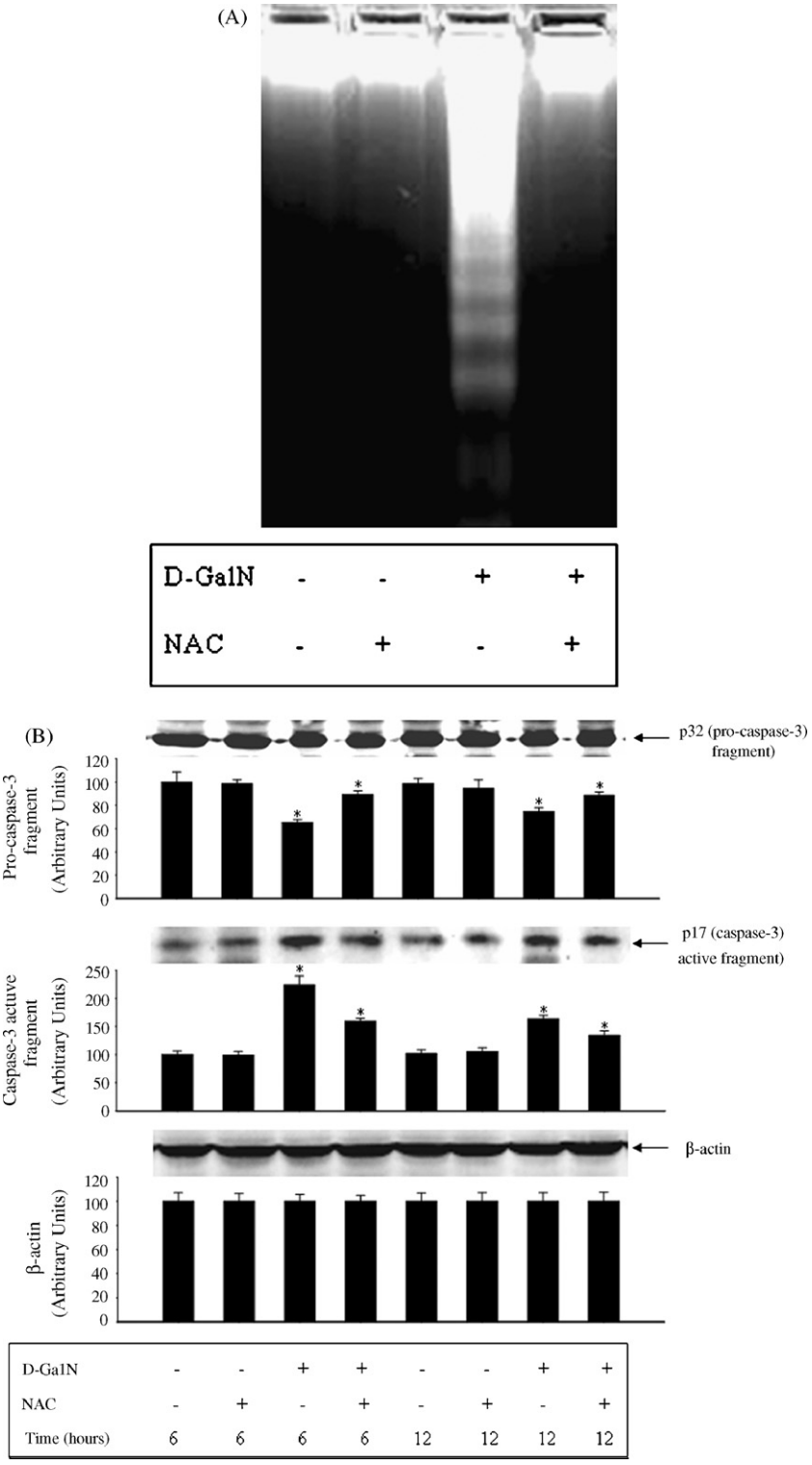


Fig. 3. Effect of N-acetylcysteine (NAC) treatment on DNA fragmentation (A), caspase-3 processing (B), caspase-3 activity (C) and lactate dehydrogenase (LDH) release (D) in D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM) was co-administered with D-GalN (40 mM) in cultured human hepatocytes. DNA fragmentation (6 h) was assessed by agarose gel electrophoresis in cell lysate including hepatocytes and floating cells present in the culture medium. The expression of pro-caspase-3 (p32) and caspase-3 active fragment (p17) was assessed by SDS-PAGE and Western-blot analysis. The statistical analysis of densitometric values of the spots is shown below each blot. β-Actin was used as internal protein loading. Caspase-3-associated activity was assessed using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) as the corresponding peptide-based substrate. LDH release represents the percentage of LDH in the culture medium in relation to total LDH. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

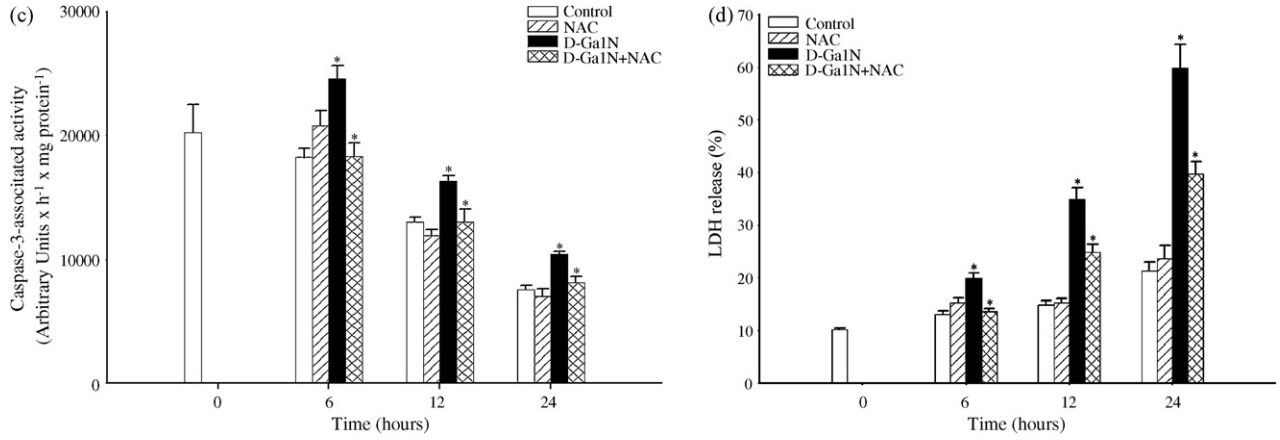


Fig. 3. (*Continued*).

was no primer dimer formation. Quantitation of relative expression was determined by the $2^{(\Delta Ct)}$.

2.14. Measurement of nuclear- and mitochondrial-encoded complex I protein expression

The protein expression of nuclear-encoded 17 kDa and mitochondrial-encoded 20 kDa subunits of mitochondrial complex I was assessed by Western-blot analysis in the mitochondrial fraction. Proteins (5 μg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding commercial primary antibodies against p17 (1/10,000) (A21359, Molecular Probes Europe BV) and p20 (1/5000) (A31857, Molecular Probes Europe BV) subunits. Secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) were used, revealing protein content by ECL.

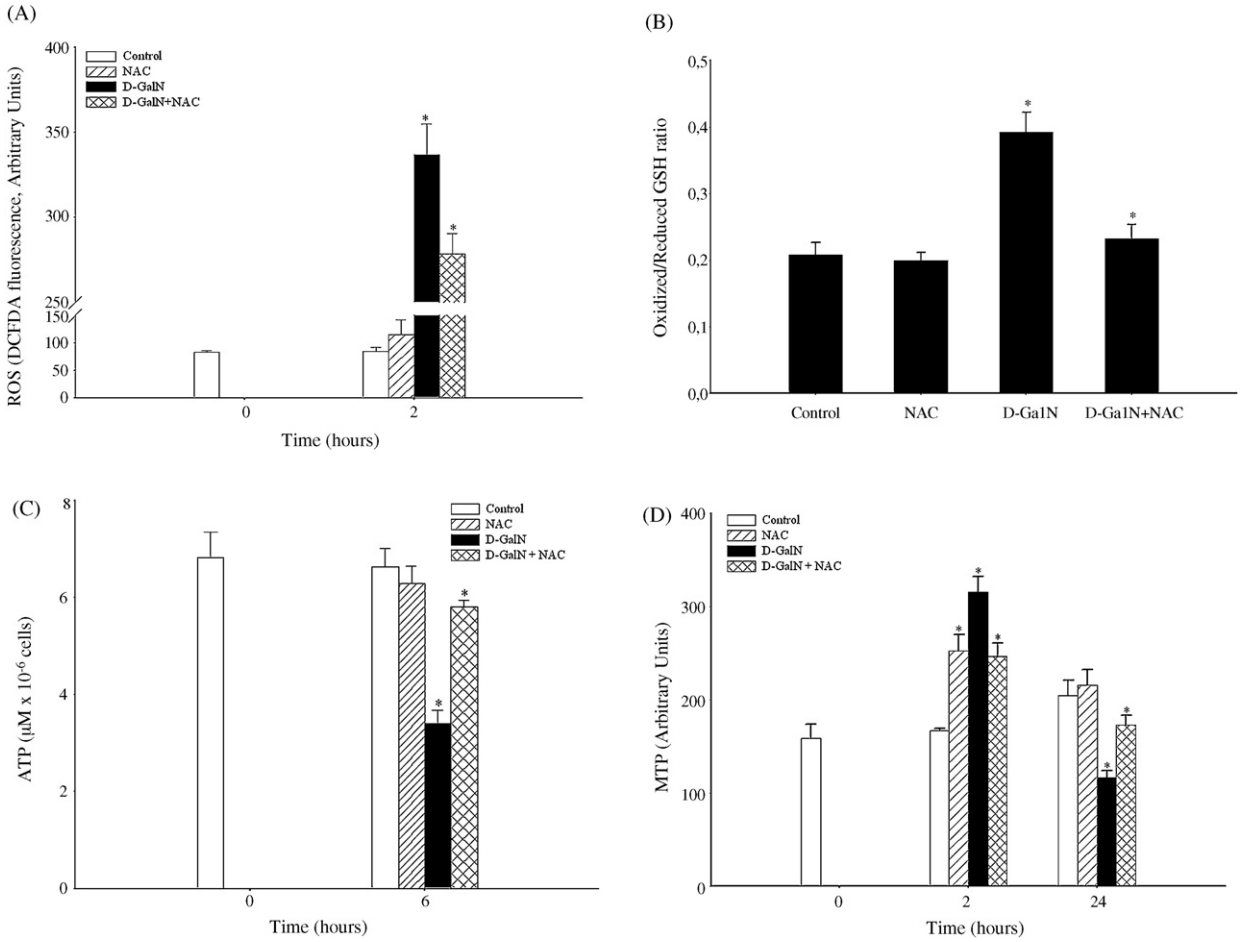


Fig. 4. Effect of N-acetylcysteine (NAC) treatment on reactive oxygen species (ROS) (A), mitochondrial oxidized/reduced glutathione (GSH) ratio (B), ATP (C) and mitochondrial transmembrane potential (MTP) (D) in D-GalN-treated human hepatocytes. NAC (0.5 mM) was co-administered with D-galactosamine (D-GalN) (40 mM) in cultured human hepatocytes. ROS were monitored *in situ* using 2,7-dichlorofluorescein diacetate dye (DCFDA) in digitonin-treated cells. GSH and its oxidized form (GSSG) were measured by the o-phthalaldehyde based assay. ATP was measured in acid cell extracts using a luciferase reaction. MTP was monitored *in situ* using a membrane potential-sensitive fluorescent probe, such as tetramethylrhodamine methyl ester (TMR), in hepatocytes. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

MTC02, used as internal protein loading, was identified with commercial primary rabbit polyclonal antibodies (1/200) (Abcam).

2.15. Statistical analysis

Results are expressed as mean ± SE of five experiments. Data were compared using the analysis of variance with the Least Significant Difference's test as post hoc multiple comparison analysis. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

3. Results

3.1. NAC reduced cell death in D-GalN-induced cytotoxicity

D-GalN induces oxidative stress and cell death in primary culture of rat and human hepatocytes [7,9]. D-GalN induced a rapid and transient induction of mitochondrial ROS generation measured by DCFDA (Fig. 2A) ($p \leq 0.05$). The contribution of hydrogen peroxide (H_2O_2) to the oxidation of the DCFDA was determined with the preincubation (30 min) of the cells with catalase (500 U/mL). Catalase showed that 50% of DCFDA oxidation was due to H_2O_2 (Fig. 2B). The profile of ROS generation mimicked the progressive reduction of cellular ATP concentration in D-GalN-treated hepatocytes (Fig. 2C). NAC administration has been shown to reduce oxidative stress, cellular GSH depletion and cell death induced by different agents in cultured rat hepatocytes [25,26]. The co-administration of NAC (0.5 mM) reduced DNA fragmentation (Fig. 3A), the expression of active caspase-3 fragment (Fig. 3B), caspase-3 activity (Fig. 3C) and cell necrosis (Fig. 3D) induced by D-GalN in hepatocytes ($p \leq 0.05$). The prevention of D-GalN-induced cell death by NAC was related to a partial reduction of ROS generation (Fig. 4A), as well as a recovery of mitochondrial oxidized/reduced GSH ratio (Fig. 4B), cellular ATP (Fig. 4C), and MTP (Fig. 4D) ($p \leq 0.05$).

3.2. Cytoprotective properties of NAC on mitochondrial function in D-GalN-treated hepatocytes

The intense oxidative stress by D-GalN did not affect the ratio of mitochondrial/nuclear DNA copy number (data not shown). Nevertheless, the alteration of ROS production (Fig. 2A), MTP (Fig. 4D) and ATP (Fig. 2C) content induced by D-GalN suggests a profound mitochondrial dysfunction. The complex I (NADH coenzyme Q reductase) and the complex III (ubiquinol cytochrome *c* reductase) are sites in which ROS production may take place [11]. In addition to the bioenergetic role of Q, as a component of the mitochondrial respiratory chain, its redox properties suggest that it exerts relevant antioxidant properties [11]. Interestingly, D-GalN increased complexes I, III and IV activities, but reduced the coupled complexes I + III and II + III activities (Table 1) ($p \leq 0.05$). The last data suggest that the toxin induced a drastic alteration of Q status in the mitochondrial membrane. In concordance, D-GalN reduced Q_{10} content (Fig. 5A) and enhanced the ratio of oxidized/reduced Q_{10} (Fig. 5B) in mitochondria ($p \leq 0.05$). Interestingly, the increase of reduced Q_{10} (Fig. 5A) content and reduction of the oxidized/reduced Q_{10} ratio (Fig. 5B) in mitochondria by NAC co-administration was related to a reduction of complexes I, II and IV activities, as well as a restoration of coupled complexes I + III and II + III activities (Table 1) from D-GalN-treated hepatocytes ($p \leq 0.05$).

3.3. The supplementation with Q_{10} or MnTBAP restored mitochondrial activities and reduced cell death in D-GalN-treated hepatocytes

The data described above showed that the induction of cell death by D-GalN was related to an increase of mitochondrial oxidative stress, depletion of mitochondrial reduced Q_{10} content and mitochondrial dysfunction in hepatocytes. The co-administration of Q_{10} could enhance reduced Q_{10} (Fig. 5A) content, but it did not recover oxidized/reduced Q_{10} ratio (Fig. 5B) in mitochondria from D-GalN-treated hepatocytes ($p \leq 0.05$). This effect of Q_{10} was related to a reduction of ROS (Fig. 6A) production measured by DCFDA fluorescence in D-GalN-treated hepatocytes ($p \leq 0.05$). The co-administration of a SOD mimetic, MnTBAP, reduced $O_2^{\bullet -}$ production measured by DHE in D-GalN-treated hepatocytes (Fig. 6A) ($p \leq 0.05$). Both experimental strategies (Q_{10} and MnTBAP) reduced mitochondrial oxidized/reduced GSH content (Fig. 6B) and complexes I, III and IV activities (Table 1), as well as increased the coupled complexes I + III and II + III activities (Table 1) in D-GalN-treated hepatocytes ($p \leq 0.05$). Q_{10} and MnTBAP reduced DNA fragmentation (Fig. 6C), caspase-3 activity (Fig. 6D), and cell necrosis (Fig. 6E) in hepatocytes treated with D-GalN ($p \leq 0.05$). The reduction of oxidative stress and the recovery of functional ETC activities by NAC, Q_{10} and MnTBAP were related to an increase of the protein expression of the p17 nuclear- and p20 mitochondria-encoded complex I subunits (Fig. 7A), as well as the mRNA expression of the p17 nuclear-encoded complex I subunit (Fig. 7B) in D-GalN-treated hepatocytes ($p \leq 0.05$).

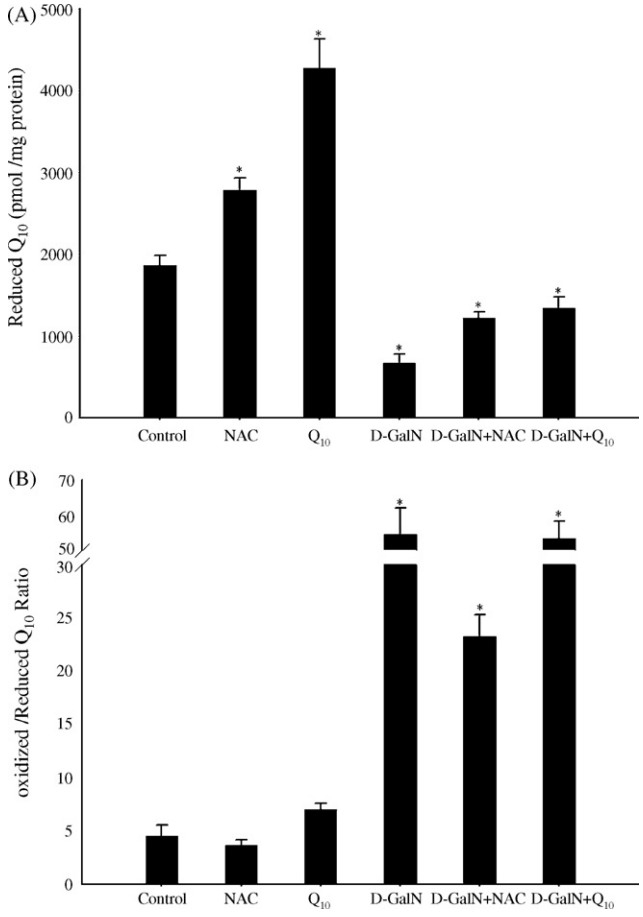


Fig. 5. Effect of N-acetylcysteine (NAC) and coenzyme Q_{10} (Q_{10}) treatments on the reduced Q_{10} content (A) and the oxidized/reduced Q_{10} ratio (B) in mitochondria from D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM) or Q_{10} (30 μM) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. Oxidized and reduced Q_{10} content (24 h) were determined by high performance liquid chromatography in mitochondria following the procedure described in Section 2. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

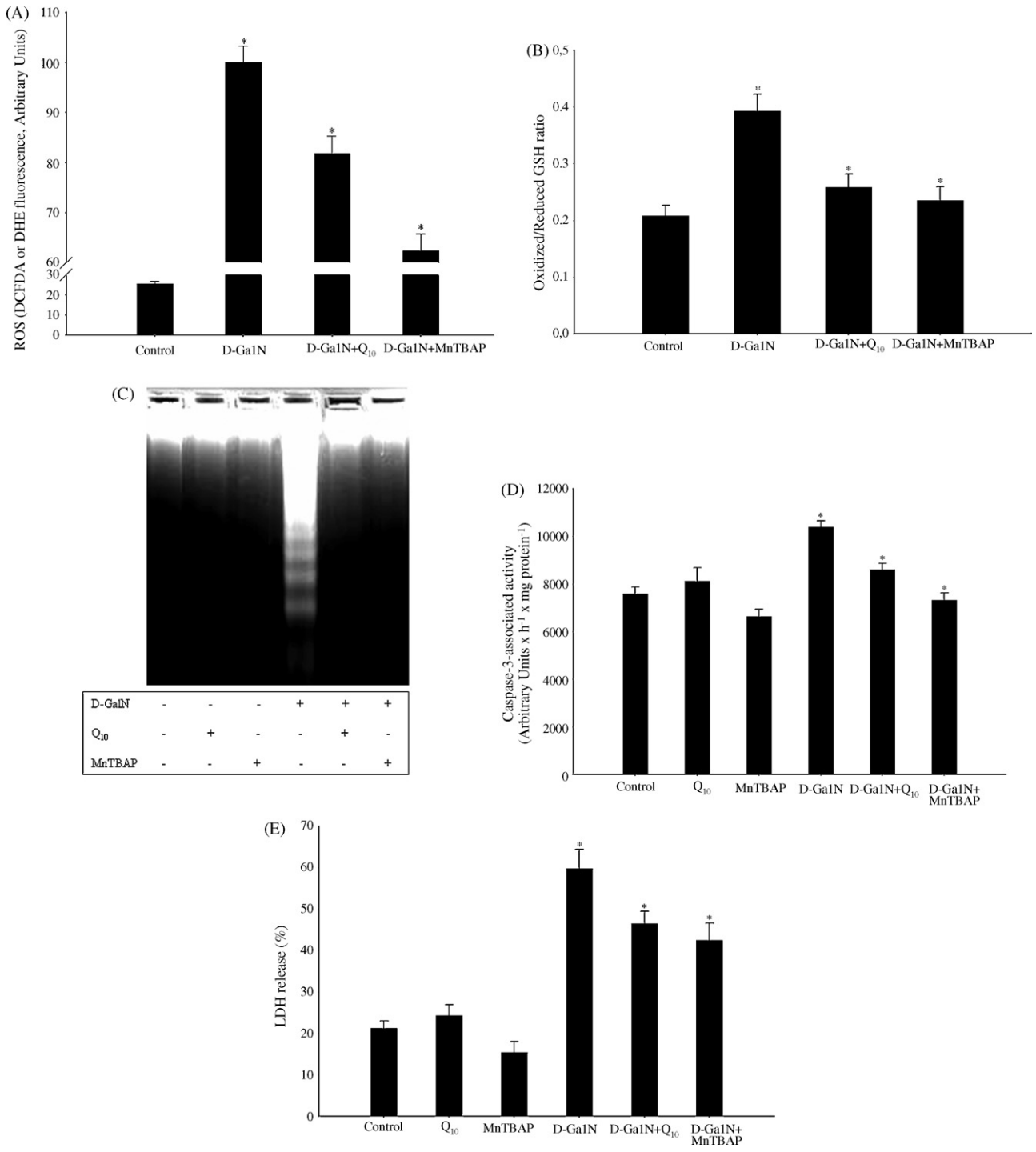
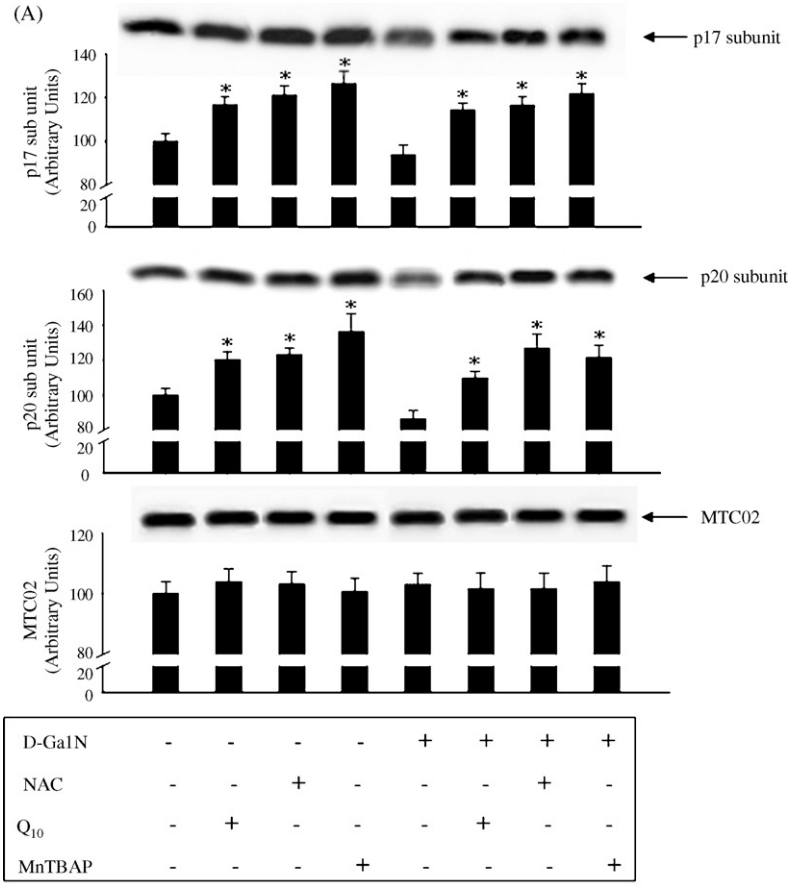
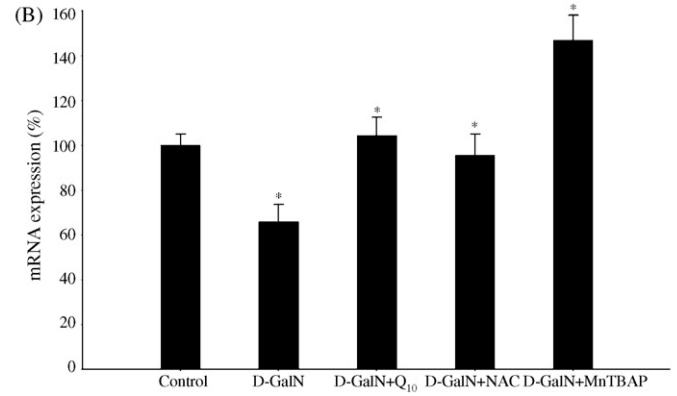


Fig. 6. Effect of coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the reactive oxygen species (ROS) (A) and the oxidized/reduced Q_{10} ratio (B) in mitochondria, DNA fragmentation (C), caspase-3 activity (D) and lactate dehydrogenase (LDH) release (E) in D-galactosamine (D-GalN)-treated human hepatocytes. Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. ROS production (2 h) was monitored *in situ* using 2,7-dichlorofluorescein diacetate dye (DCFDA) in control, D-GalN and D-GalN + Q_{10}-treated hepatocytes. Anion superoxide ($O_2^{\bullet -}$) production (2 h) was monitored *in situ* using dihydroethidium (DHE) in D-GalN and D-GalN + MnTBAP-treated hepatocytes. Oxidized and reduced Q_{10} content (24 h) were determined by high performance liquid chromatography in mitochondria following the procedure described in Section 2. DNA fragmentation (6 h) was assessed by agarose gel electrophoresis in cell lysate including hepatocytes and floating cells present in the culture medium. Caspase-3-associated activity (24 h) was assessed using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) as the corresponding peptide-based substrates. LDH release (24 h) represents the percentage of LDH in the culture medium in relation to total LDH. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

4. Discussion

Oxidative stress plays a relevant role in the induction of cell death in hepatocytes. NAC has been shown to exert cytoprotection in different thiol-depleting experimental models of cell death in hepatocytes [25,27]. D-GalN-induced ROS production, mitochondrial dysfunction and cell death in hepatocytes. The study showed that the reduction of oxidative stress, recovery of mitochondrial





Treatments	Ct mRNA RPL13A (%)
Control	100±1.6
D-GalN	106±1.9
D-GalN+Q_{10}	107±2.3
D-GalN+NAC	108±1.6
D-GalN+MnTBAP	110±3.1

Fig. 7. Effect of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the protein expression of nuclear- and mitochondria-encoded complex I subunits (A), and the mRNA expression of nuclear-encoded complex I subunit (B) in D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM), Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. The samples were obtained 24 h after D-GalN administration. The protein expression of nuclear- and mitochondrial-encoded complex I subunits (p17 and p20, respectively) was assessed by SDS-PAGE and Western-blot analysis. The statistical analysis of densitometric values of the spots is shown below each blot. Mitochondrial marker (MTC02) was used as internal protein loading. The mRNA expression of complex I subunit (p17) was assessed by RT-PCR analysis, and referred to the expression of the constitutive gene RPL13A. The statistical analysis of Ct values of all samples is shown below the mRNA expression graph. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

oxidized/reduced GSH and reduced Q_{10} content, and the restoration of ETC by antioxidants are key factors for cell survival in D-GalN-treated hepatocytes. The study also suggests that improved mitochondrial function by NAC, Q_{10} and MnTBAP was related to an increase of the expression of nuclear- and mitochondrial-encoded subunits of complex I in D-GalN-treated hepatocytes.

D-GalN has been used to induce experimental acute hepatotoxicity [6]. D-GalN is actively incorporated to hepatocytes by

carrier-mediated diffusion across the plasma membrane, phosphorylated and transformed to UDP-galactosamine by UDP-glucose:α-D-galactose-1-phosphate uridyltransferase. Consequently, the toxicity of D-GalN is related to the depletion of uridine pools and alteration of RNA and protein synthesis [6]. This experimental model is particularly interesting for the assessment of mitochondrial protective agents. The UTP synthesis also affects the activity of a mitochondrial enzyme, dihydroorotate dehydrogenase, which catalyzes the oxidation of dihydroorotate to orotate with the reduction of Q. In this sense, the activity of dihydroorotate dehydrogenase requires a functional mitochondrial chain activity [23].

D-GalN induces apoptosis and necrosis in liver tissue and cultured hepatocytes [7,9,28]. The intracellular ROS generation is associated with cell death in cultured hepatocytes [2,3,29]. The complexes I and III are major sites of mitochondrial ROS production [11]. The rapid hyperpolarization of mitochondrial membrane by D-GalN was related to an increase of complexes I, III and IV activities, as well as a rapid and transient ROS production. The generation of ROS reduces antioxidant content and may damage mitochondrial components through lipid peroxidation products, protein oxidation and nitrosylation, as well as mtDNA mutations in the liver [11,30–32]. The induction of oxidative stress by D-GalN was associated with a sharp increase of mitochondrial oxidized/reduced GSH and Q_{10} ratios, and reduction of coupled complexes I + III and II + III activities, expression complex I subunits and cellular ATP content. Oxidative stress [7,12] and ATP depletion [33] are related to a shift from an apoptotic to a necrotic cell death pathway. The rapid induction of ROS production (4.3-folds at 2 h) may be responsible for the early presence of apoptotic and necrotic cell death markers in D-GalN-treated hepatocytes. The great reduction of ATP content at advanced stages of the study (24 h) was associated with more prominent hepatocellular necrosis.

The maintenance of mitochondrial GSH content prevents cell damage in different *in vivo* [13] and *in vitro* [14] experimental models of hepatotoxicity. The depletion of intracellular GSH by diethylmaleate exacerbates D-GalN-induced cell death in hepatocytes [34]. The administration of NAC, as a precursor of GSH and free radical scavenger, has beneficial clinical implications in HIV infection and cancer, as well as in heart, kidney and liver diseases [15]. In particular, NAC has been shown to improve the hemodynamic and metabolic parameters in patients with fulminant hepatic failure [35], as well as reduces cellular GSH depletion during the induction of cell death in cultured rat hepatocytes [25,26]. The alteration in the mitochondrial Q_{10} content has also been related to severe dysfunction in muscle and nervous system [36,37]. The supplementation with Q_{10} or analogues, such as idebenone, improves the neurodegenerative disorders [38–40], and reduces ROS generation and apoptosis induced by bile acid in hepatocytes [41]. In our study, the reduction of ROS generation and cell death by NAC or Q_{10} administration was related to a recovery of the oxidized/reduced GSH ratio, reduced Q_{10} content, and the coupled complexes I + III and II + III activities in mitochondria in D-GalN-treated hepatocytes.

The direct involvement of ROS production in mitochondrial function and cell death was addressed using a SOD mimetic. The administration of MnTBAP reduces liver injury during acute liver failure induced by Fas in mice [20]. The administration of MnTBAP reduced mitochondrial $O_2^{\bullet -}$ production, recovered mitochondrial oxidized/reduced GSH ratio and coupled complexes I + III and II + III activities, and reduced cell death induced by D-GalN in hepatocytes.

The administration of NAC, Q_{10} and MnTBAP enhanced the protein expression of nuclear- and mitochondria-encoded complex I subunits in control and D-GalN-treated hepatocytes. It appears that this effect was related to an early increase of MTP (NAC) and complex I-associated activity (NAC and Q_{10}) in control cells. Nevertheless, the increase of complex I subunit expression by antioxidants was related to a reduction of complex I activity, restoration of complexes I + III and II + III activities and reduction of cell death in D-GalN-treated hepatocytes. These results suggest a relevant role of antioxidants in the fine regulation of complex I expression and activity that may reduce the potential deleterious effect of ETC-derived ROS production and cell death induced by D-GalN. The improvement of the mitochondrial function with up-regulation of protein components of ETC complex has been observed in a comparative study using different neuronal cell types [42], as well as during the aging studies in which the compensatory mechanism is sustained until advanced stages of the process [43].

In conclusion, D-GalN-induced cell death was related to a rise of oxidative stress, depletion of mitochondrial reduced GSH and Q_{10} contents and mitochondrial dysfunction. The reduction of mitochondrial oxidative stress by NAC, Q_{10} and MnTBAP enhanced mitochondrial GSH and reduced Q_{10} content, improved mitochondrial function and prevented cell death. In addition, it was particularly interesting the beneficial properties of antioxidants on the expression of nuclear- and mitochondrial-encoded complex I subunits that may affect the stability and/or efficiency of ETC in hepatocytes submitted to toxic agents.

Conflict of interest

None declared.

Acknowledgments

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