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S-nitrosation of proteins during D-galactosamine-induced cell death in human hepatocytes

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Abstract

Nitric oxide (NO) participates in the cell death induced by D-Galactosamine (D-GalN) in hepatocytes, and NO-derived reactive oxygen intermediates are critical contributors to protein modification and hepatocellular injury. It is anticipated that *S*-nitrosation of proteins will participate in the mechanisms leading to cell death in D-GalN-treated human hepatocytes. In the present study, D-GalN-induced cell death was related to augmented levels of NO production and *S*-nitrosothiol (SNO) content. The biotin switch assay confirmed that D-GalN increased the levels of *S*-nitrosated proteins in human hepatocytes. *S*-nitrosocysteine (CSNO) enhanced protein *S*-nitrosation and altered cell death parameters that were related to *S*-nitrosation of the executioner caspase-3. Fifteen *S*-nitrosated proteins participating in metabolism, antioxidative defense and cellular homeostasis were identified in human hepatocytes treated with CSNO. Among them, seven were also identified in D-GalN-treated hepatocytes. The results here reported underline the importance of the alteration of SNO homeostasis during D-GalN-induced cell death in human hepatocytes.

Keywords: *Hepatocyte, D-galactosamine, apoptosis, necrosis,* S*-nitrosation, proteomics*

Introduction

Hepatic injury induced by D-Galactosamine (D-GalN) is a suitable experimental model of hepatocellular injury. D-GalN depletes the intracellular pool of UTP [1] causing a transient arrest in the cellular transcription and protein synthesis, that alters hepatocellular function. Nevertheless, the exact molecular mechanisms responsible for D-GalN-induced apoptosis and/or necrosis in hepatocytes are not well understood. We have previously shown that nitric oxide (NO) mediates D-GalN-induced cell death in rat and human hepatocytes [2–5]. NO is a diatomic free radical that plays an important role in the homeostatic regulation of the central nervous, immune, and cardiovascular systems [6–9]. In addition to its interaction with guanylate cyclase, which results in the production of the second messenger cyclic GMP, there is now a large body of evidence indicating that *S*-nitrosation of cysteine thiols constitutes a significant route through which NO bioactivity is transduced. *S*-nitrosothiols (SNOs), derived from proteins, peptides and aminoacids, supply cellular compartments and extracellular fluids with NO bioactivity. *S*-nitrosation of the thiol group of cysteine has emerged as the prototype redox-based post-translational modification that is

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increasingly becoming recognized as a ubiquitous regulatory reaction comparable to phosphorylation [10–13]. It has been proposed that disruption or deregulation of SNOs signalling leads to impairment of cellular function and disease [12].

Both cytoprotective and cytotoxic effects of NO have been demonstrated in the liver and *S*-nitrosation of proteins appears to be involved in some of the mechanisms responsible for these dual roles. Thus, NO can induce apoptosis through the accumulation of pro-apoptotic factors like p53, ceramide or the activation of c-jun *N*-terminal kinase/stress-activated protein kinase and p38 kinase [14]. However, NO can inhibit apoptosis in multiple types of cells, including hepatocytes, inhibiting caspase activity through *S*-nitrosation of the active site cysteine residue [15–16]. The present study was aimed to explore the role of protein *S*-nitrosation during D-GalN-induced cell death in human hepatocytes. In addition, using a proteomic approach, we were able to identify 15 *S*-nitrosated proteins, participating in metabolism, antioxidative defense and cellular homeostasis, in human hepatocytes treated with the cell permeable nitrosothiol CSNO. Among them, seven were also identified in hepatocytes treated with D-GalN.

Materials and methods

Materials

All reagents were from Sigma Chemical Co. (St Louis, Missouri, TX, 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. CSNO was synthesized as described elsewhere [17] by incubation of L-cysteine with acidified sodium nitrite and quantification by absorbance at 334 nm using a molar absorption coefficient of $0.74\,mM^{-1}\,cm^{-1}$. Antibiotics–antimycotic solution and fetal bovine serum were from Life Technologies Inc. (Paisley, UK). The study protocols comply with the Institution's guidelines.

Preparation of primary human hepatocytes, cell culture and sample preparation

Liver resections were obtained after written consent from 13 patients (6 women, 7 men; 45 ± 16 years old) submitted to surgical intervention for primary or secondary liver tumor. Cell isolation was carried out through *ex vivo* collagenase perfusion as described by Ferrini et al. [18]. Liver was first perfused with a non-recirculating washing solution I (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 0.5% glucose, 100 μM sorbitol, 100 μM manitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) pH 7.4 at a flow of 75 ml/min in order to remove blood cells. Afterwards, liver was perfused with a non-recirculating chelating solution II (0.5 mM EGTA, 58.4 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 25 mM *N*-tris(hydroxymethyl(methylglycine, 100 μM sorbitol, 100 μM manitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) pH 7.4 at a flow of 75 ml/min. Liver was further perfused with recirculating isolation solution III (0.050% collagenase, 20 mM HEPES, 120 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5% glucose, 100 μM sorbitol, 100 μM manitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) pH 7.4 at a flow of 75 ml/min. Cell suspension was filtered through nylon mesh (250 μ) and washed three times at 50*g* 5 min 4°C in supplemented culture medium. DEM:Ham-F12 and William's E mediums (1:1) were supplemented with 26 mM $NaHCO_3$, 15 mM HEPES, 0.292 gr/l glutamine, 50 mg/l vitamin C, 0.04 mg/l dexamethasone, 2 mg/l insulin, 200 μg/l glucagon, 50 mg/l transferrin, 4 ng/l ethanolamine). Cell viability was consistently >85%, as determined by trypan blue exclusion. Hepatocytes (150,000 cells/cm^2) were seeded in type I collagen-coated dishes (Iwaki, Gyouda, Japan) and cultured in culture medium containing 5% fetal calf serum for 4 h. Afterwards, the medium was removed and replaced by fresh culture medium without fetal bovine serum. The study was initiated 24 h after seeding of the cells to allow stabilization of the culture. A kinetic study (0–48 h) of cell damage by D-GalN (40 mM) was carried out in cultured hepatocytes. Culture medium was collected and stored at −80°C for the measurement of lactate dehydrogenase (LDH) activity. Cells were washed with PBS, scraped and resuspended in non-denaturing lysis solution (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1 mM neocuproine, 1% Triton X-100 and 1 mM PMSF plus aprotinin and leupeptin), incubated on ice for 15 min, and centrifuged at 10,000*g*, 4°C for 15 min. Supernatant was collected and protein was quantified with Bradford reagent (Bio-Rad, Hercules, CA, USA).

Apoptotic cell death assay

Apoptosis was measured with cell death detection enzyme-linked immunosorbent assay plus (Roche Diagnostic, Barcelona, Spain) according to the manufacturer's instruction. Briefly, cell lysates equivalent to 10^3 cells were incubated with both anti-histone antibody labelled with biotin and anti-DNA antibody conjugated with peroxidase in streptavidin-coated microplates for 2 h. Microplate wells were washed and incubated with substrate for colorimetric measurement at wavelength 405 nm with reference at 490 nm.

Measurement of lactate dehydrogenase release

LDH was measured by modification of a colorimetric routine laboratory method (17). Briefly, a volume of culture medium or cell lysate ranging from 50–200 μl was incubated with 0.2 mM β-NADH and 0.4 mM pyruvic acid diluted in PBS pH 7.4. LDH concentration in the sample was proportional to the linear decrease in the absorbance at 334 nm. LDH concentration was calculated using a commercial standard.

Measurement of NO production and SNO content

The hepatocyte NO production was estimated by measuring its stable end products, nitrite plus nitrate, in culture medium, using a NO analyzer (NOA 280i; Sievers Instruments, Boulder, CO, USA). Briefly, a volume (200 μl) of culture medium was deproteinated and injected into a purge vessel where nitrite–nitrate content was reduced back to NO with a vanadium reducing mixture at 90°C. NO was carried on a constant current of nitrogen gas to a reaction chamber, and the light emitted from the chemiluminescence reaction between NO and ozone was detected by a photomultiplier tube. Calibration of the magnitude of NO production was determined from the signal obtained from nitrite concentration standards. High molecular weight SNOs in hepatocyte lysates were also detected by chemiluminescence using a NO analyzer, following the method described by Feelisch et al. [19]. Briefly, proteins were precipitated with acetone for 20 min at −20°C, the pellet was resuspended in non-denaturing lysis solution, and 50 μl of this suspension was injected in the purge vessel containing 5 ml of 45 mM potassium iodide (KI) and 10 mM iodine (I_2) in glacial acetic acid, at 60°C, purged continuously with nitrogen. As a control for detection of *S*-nitrosated proteins, lysates were incubated with 4.5 mM $HgCl_2$, which selectively cleavages S—NO bonds, for 10 min at room temperature followed by 20 min incubation at 4°C, before acetone precipitation. All steps were carried out avoiding light exposure. A stock solution of nitrosoglutathione (GSNO) was used to generate a standard curve.

Detection of protein S*-nitrosation by the biotin switch method*

The procedure was performed according to the protocol by Jaffrey et al. [20]. Briefly, hepatocyte lysates were incubated with 20 mM methyl methanethiosulfonate (Sigma) followed by acetone precipitation. Precipitates were centrifuged and resuspended in HENS buffer (250 mM HEPES pH 7.7, 1 mM EDTA, 0. 1 mM neocuproine, and 1% SDS) and then incubated with 1 mM ascorbic acid and 4 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Biotin–HPDP, Pierce, Rockford, IL, USA) for 1 h. Because biotin–HPDP is cleavable under the reduced conditions, prepared samples were loaded onto SDS-PAGE gels without dithiothreitol. All steps preceding SDS-PAGE were carried out in the dark. Biotinylated samples were then detected by Western Blot analysis using a primary monoclonal anti-biotin antibody (Sigma), a secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the ECL advance detection system (Amersham Biosciences, Uppsala, Sweden).

Purification of S*-nitrosated proteins*

Hepatocyte lysates (3–4 mg protein) were biotinylated as described above. Biotin–HPDP was removed by acetone precipitation and centrifugation, and the pellet was resuspended in HENS buffer as above. Two volumes of neutralization buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 0.5% Triton X-100) was added, and EZview™ Red streptavidin–agarose (Sigma) was added to purify biotinylated proteins. Biotinylated proteins were incubated with the resin for 1 h at room temperature, washed five times with neutralization buffer adjusted to 600 mM NaCl, and then incubated with elution buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 100 mM 2-mercaptoethanol) for 20 min at 37°C with gentle agitation to recover the bound protein. Supernatants were collected and proteins were separated in 10% SDS-PAGE gels, which were stained with Sypro Ruby protein stain (Biorad).

Western blot analysis for caspase-3 processing

Proteins (100 μg) were separated in 12% SDS-PAGE and transferred to nitrocellulose. The expression of caspase-3 active fragment was determined by Western-blot analysis by the incubation of membranes with the corresponding commercial primary and secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and revealed using the ECL Advance detection system (Amersham Biosciences, Uppsala, Sweden).

Assay for caspase-3-like activity

Caspase-3-like activity in lysates (25 μg) was measured using the corresponding peptide-based substrate (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 increase in fluorescence (Ex 400 nm/Em 505 nm) of enzymatically released AFC was recorded using a GENios Reader (TECAN, Salzburg, Austria).

Protein identification

Gel bands were excised using a robotic workstation (Investigator™ Propic™, Genomics Solutions, Ann Harbor, MI, USA) and were trypsin-digested using a robotic digestion system (ProGest™, Genomic Solutions). Tryptic digests were then analyzed on a MALDI-ToF/ToF 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Mass spectrometry data were searched against the human protein database from MSDB using Mascot search engine (Matrix Science Inc., Boston, MA, USA).

For the ESI–MS/MS analysis, microcapillary reversed phase LC was performed with a CapLCTM (Waters, Milford, MA, USA) capillary system. Reversed phase separation of tryptic digests were performed with an Atlantis, C18, 3 μm, 75 μm × 10 cm Nano Ease™ fused silica capillary column (Waters) equilibrated in 5% acetonitrile, 0.2% formic acid. After injection of sample (18 μl) the column was washed during 5 min with the same buffer

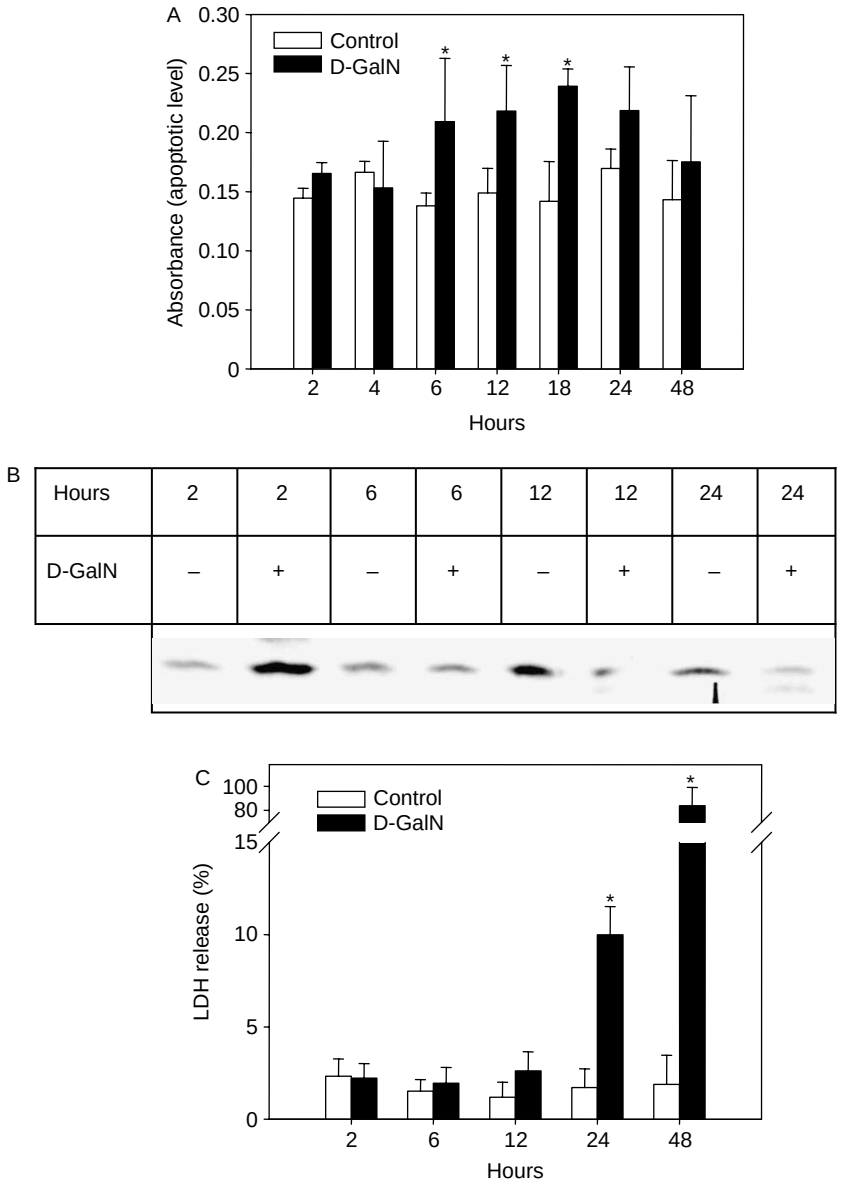

Figure 1. D-GalN induces apoptosis followed by necrosis in human primary hepatocytes. *Panel A*, hepatocytes were treated with 40 mM D-GalN for the indicated hours and the apoptotic level was measured by enzyme-linked immunosorbent assay for cytosolic histone-associated DNA fragment. Using this assay, an apoptotic level of 0.478 ± 0.054 was observed in hepatocytes treated for 24 h with 10 ng/ml TNF-alpha and 15 ng/ml Actynomicin D. Data are the mean ± SE of 5 different experiments. *Panel B*, the caspase-3 active fragment was detected by Western blotting from the hepatocytes treated with 40 mM D-GalN for the indicated times. The image is representative of five independent experiments. *Panel C*, cell necrosis was determined by measuring the release of LDH activity from hepatocytes into the culture medium. Data are the mean ± SE of five different experiments.

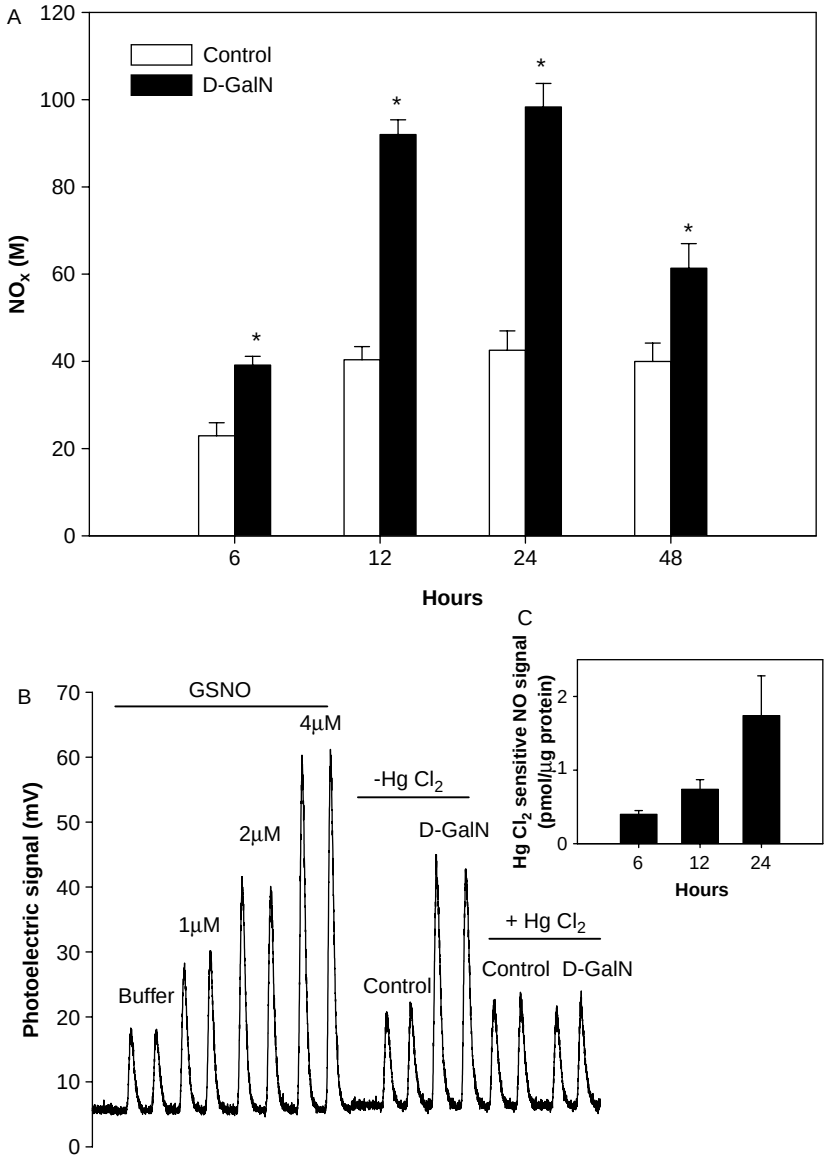

Figure 2. Treatment of human primary hepatocytes with D-GalN increases NO production and SNO levels. *Panel A*, hepatocytes were treated with 40 mM D-GalN for the indicated hours and the NO production was estimated by measuring its stable end products, nitrite plus nitrate, in culture medium. The treatment of hepatocytes with interleukin 1-beta (10 ng/ml), interferon gamma (25 ng/ml) and lipopolysaccharide (2 μg/ml) as a control produced a peak level of nitric oxide production (430 ± 28 μM NO_x) at 24 h. Data are the mean ± SE of 5 different experiments. *Panel B*, hepatocyte lysates were incubated in presence or absence of $HgCl_2$ and subsequently subjected to acetone precipitation. The NO released following reduction in acidic KI/I_2 was detected by chemiluminescence. *S*-GSNO was used to generate an standard curve. *Panel C*, the $HgCl_2$ sensitive NO signal was quantified as a measurement of *S*-nitrosated proteins in hepatocytes treated for the indicated hours with D-GalN.

and the peptides were eluted using a linear gradient of 5–50% acetonitrile in 30 min at a constant flow rate of 0.2 μl/min. The column was coupled online to a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80°C and the spray voltage was 1.8–2.2 kV. MS/MS data were collected in an automated data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by collision-induced dissociation (CID) using an isolation width of 2.5 and the relative collision energy of 35%. Data processing was performed with MassLynx 4 and ProteinLynx Global Server 2 (Waters).

Statistical analysis

Results are expressed as means with their corresponding standard errors. Comparisons were made using ANOVA with least significant difference (LSD) test. Statistical significance was set at $p \leq 0.05$.

Results

D-GalN induced apoptosis followed by necrosis in human hepatocytes

D-GalN has been previously shown to induce apoptosis and necrosis in cultured rat hepatocytes

[21]. The concentration of D-GalN (40 mM), previously described to be able to enhance intracellular oxidative stress [21], was selected. D-GalN enhanced apoptosis (Figure 1A,B) and necrosis (Figure 1C) in primary culture of human hepatocytes. Treatment of human hepatocytes with D-GalN induced a clear apoptotic response at the 6, 12, and 18-h points, decreasing at the latest time points of the study (Figure 1A). The cell death assay detects only the end point of apoptosis, so we did not observe significant increases of cell death at earlier time points. However, a pro-apoptotic signal was already evident 2 h after D-GalN administration, as shown by the presence of the active fragment of caspase-3 (Figure 1B), although this activation was not maintained during later time points of the study. The active fragment of caspase-3 was detected in all samples, and we cannot exclude low levels of caspase-3 activation slightly increasing with culture time in control hepatocytes. However, D-GalN treated samples showed a much lower active fragment band intensity compared to their respective controls at 12 and 24 h. This reduction of the apoptotic response was related to the high necrotic effect of D-GalN observed at the longest time points of the study (24–48 h) (Figure1A,C).

D-GalN augmented the levels of nitric oxide and high molecular weight S*-nitrosothiols in human hepatocytes*

Treatment of hepatocytes with D-GalN resulted in an enhanced level of NO production, as indicated by the higher levels of its stable end products, nitrite plus nitrate, in culture medium (Figure 2A). Furthermore, D-GalN treatment also increased the high molecular weight SNO content in hepatocytes. Cell lysates were incubated in presence or absence of $HgCl_2$ and subsequently subjected to acetone precipitation. The NO released following reduction in acidic KI/I_2 was detected by chemiluminescence. Results in Figure 2B,C, demonstrate increases in the NO signal in hepatocytes treated with D-GalN, that were sensitive to the incubation with $HgCl_2$, consistent with the formation of *S*-nitrosated proteins. Using this approach, we were not able to detect SNO in control hepatocytes.

D-GalN increased the levels of S*-nitrosated proteins in human hepatocytes*

We next evaluated whether the biotin-switch method might detect changes in the *S*-nitrosated protein pattern of hepatocytes after D-GalN treatment. As shown in Figure 3, using this method, the incubation of hepatocytes lysates with GSNO, a reagent that can transnitrosate protein cysteines, resulted in biotinylated proteins that were detected by western blot. This method was also capable to detect the increase in protein *S*-nitrosation in D-GalN treated hepatocytes. This increase was observed after 12 and 24 h of treatment with the hepatotoxin (Figure 3). Endogenously biotinylated proteins were detected in the absence of ascorbate or HPDP–biotin. Importantly, the increase in *S*-nitrosated proteins after D-GalN treatment was abolished if hepatocytes were pretreated with L-NAME, a competitive inhibitor of the nitric oxide synthase activity (Figure 3).

S*-nitrosocysteine also enhanced protein* S*-nitrosation and altered cell death parameters in human hepatocytes*

To address whether the *S*-nitrosation of proteins might be responsible for the alteration of cell death parameters observed in D-GalN-treated hepatocytes, we tested if the treatment of human hepatocytes with CSNO could mimic this effects. CSNO is a membrane-permeable physiological nitrosothiol [22] that is capable to participate in transnitrosation reactions. Hepatocytes treated with CSNO, in the absence or presence of D-GalN, showed an increase in *S*-nitrosated proteins that were detected using chemiluminescence (data not shown) and the biotin switch method (Figure 4). Using this latter method the pattern of *S*-nitrosated proteins in CSNO-treated

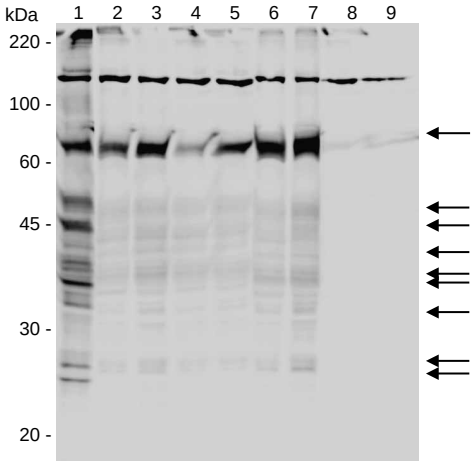

Figure 3. Detection of *S*-nitrosated proteins in human hepatocytes. Hepatocyte lysates were subjected to the biotin switch assay to detect *S*-nitrosated proteins. L-NAME (0.5 mM) was administered 2 h before D-GalN (40 mM) to cultured hepatocytes. Lane 1, 12 h control hepatocytes lysate pre-incubated 20 min with 200 μM *S*-GSNO; lanes 2 and 3, lysates from 12 h control and D-GalN-treated hepatocytes, respectively; lanes 4 and 5, lysates from L-NAME-pretreated 12 h control and D-GalN-treated hepatocytes, respectively; lane 6 and 7, lysates from 24 h control and D-GalN-treated hepatocytes, respectively. In order to establish the specificity of the assay and to detect endogenous biotinylated proteins, lysates from 24 D-GalN treated hepatocytes were processed in the absence of ascorbate (lane 8) or in the absence of HPDP–biotin (lane 9). Endogenous biotinylated proteins with molecular weight higher than 100 kDa were detected in all samples. The remaining specific nitrosated proteins in the blots are indicated with arrows. The image is representative of three different experiments.

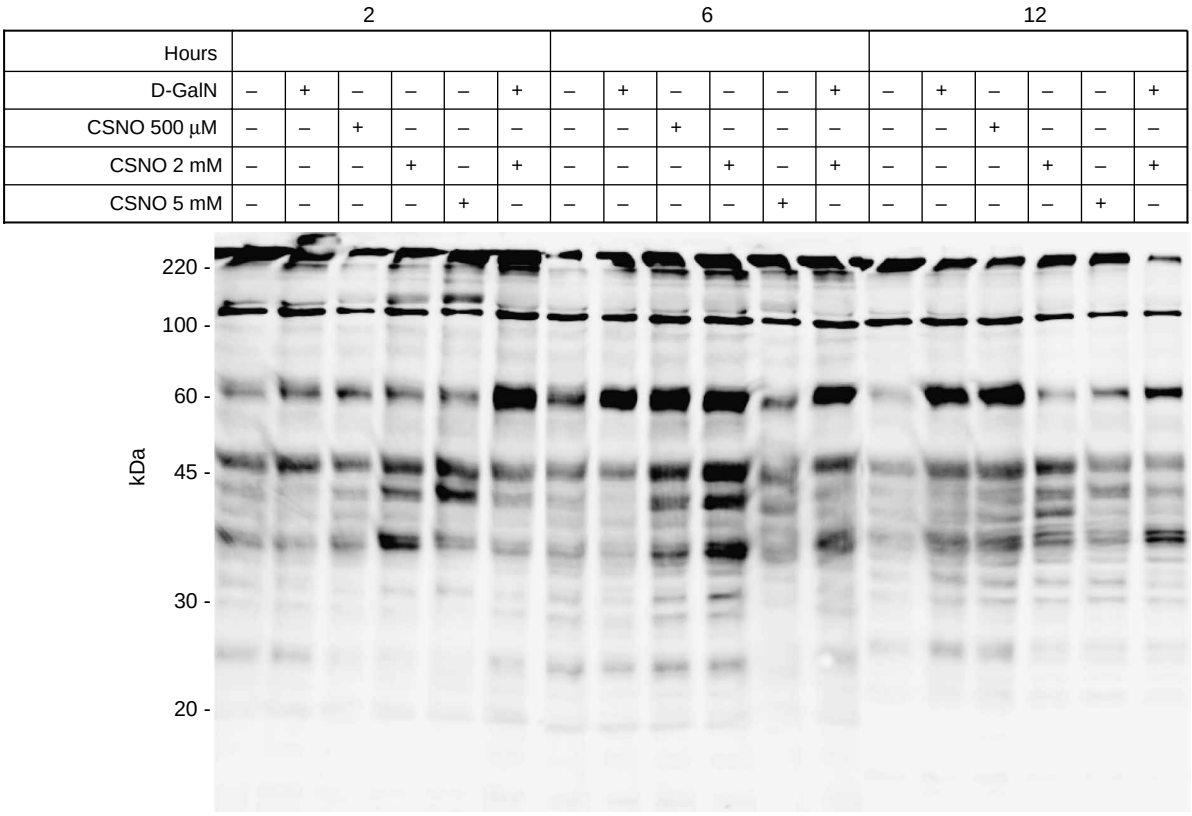

Figure 4. *S*-nitrosation of proteins in human hepatocytes after treatment with D-GalN and/or CSNO. Hepatocytes were treated for the indicated times with 40 mM D-GalN and/or different concentrations of CSNO and the cell lysates were subjected to the biotin switch assay to detect *S*-nitrosated proteins. Endogenous biotinylated proteins with molecular weight higher than 100 kDa were detected in all samples. The image is representative of three different experiments.

hepatocytes was strikingly similar to that obtained in D-GalN-treated hepatocytes. The increase in *S*-nitrosation of proteins in hepatocytes was related to CSNO concentration (Figure 4). CSNO also altered cell death parameters in human hepatocytes, as shown in Figure 5. The nitrosothiol not only did not induce apoptosis in human hepatocytes but abolished the apoptotic response in D-GalN-treated hepatocytes (Figure 5A). This effect was related to an important increase in the necrotic response observed at the late times (12 h) of the study. As shown in Figure 5B, CSNO induced a pronounced concentration-dependent necrotic effect, in the absence or the presence of D-GalN.

S-*nitrosation of caspase-3*

NO interferes with apoptotic responses through *S*-nitrosation of the active site cysteine of members of the caspase family of proteins [23–28]. Therefore, we next investigated if the alteration of cell death parameters by treatment of human hepatocytes with D-GalN or CSNO could be related to the *S*-nitrosation of executioner caspase-3 and the concomitant inhibition of its catalytic activity. Treatment of human hepatocytes with D-GalN or CSNO resulted in inhibition of caspase-3 like activity. Furthermore, this inhibition was partially reverted when lysates from CSNO-treated hepatocytes were preincubated with DTT (Figure 6A). However, in lysates from D-GalN-treated hepatocytes, this improvement after preincubation with the thiol reductant, was not significant (Figure 6A). In agreement with these results, caspase-3 was clearly detected among the purified *S*-nitrosated proteins in lysates from hepatocytes after treatment with 5 mM CSNO, but only a faint caspase-3 band was observed in the case of 24 h treatment with D-GalN (Figure 6B).

Identification of other S-*nitrosated proteins*

The results described above suggested the *S*-nitrosation of caspase-3 in human hepatocytes when cultured in the presence of CSNO. However, the *S*-nitrosation of caspase-3 was not so evident in D-GalN-treated hepatocytes. Thus, it was necessary to identify the *S*-nitrosated proteins arising after the addition of the hepatotoxin. As the biotin switch assay is amenable to a proteomic analysis, experiments were performed to identify *S*-nitrosated proteins in human hepatocytes treated with D-GalN or CSNO. Hepatocyte lysates were subjected to the biotin-switch assay and the

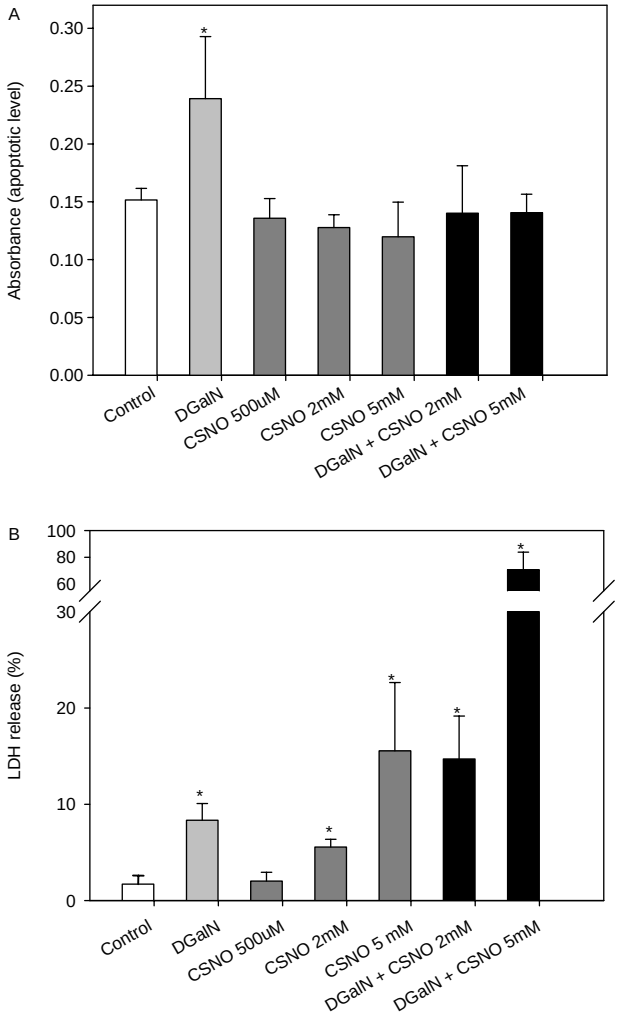

Figure 5. CSNO altered cell death in human hepatocytes. *Panel A*, hepatocytes were treated with 40 mM D-GalN or the indicated concentrations of CSNO for 6 h and the apoptotic level was measured by enzyme-linked immunosorbent assay for cytosolic histone-associated DNA fragment. Data are the mean ± SE of 5 different experiments. *Panel B*, the necrotic level of cell death was determined by measuring the release of LDH activity from hepatocytes (12 h) into the culture medium. Data are the mean ± SE of five different experiments.

resulting biotinylated proteins purified by immobilization in streptavidin-agarose and elution with 2-mercaptoethanol. As the biotin is incorporated via a disulfide bond and elution is carried out in reducing conditions, endogenously biotinylated proteins are not purified. The purified proteins were separated by SDS-PAGE, and the coomassie-stained bands (Figure 7) were excised from the gel, trypsin-digested and the corresponding proteins identified by MALDI-TOF and peptide mass fingerprinting. Alternatively, the mixture of proteins eluted from the streptavidin–agarose were directly analyzed using ESI–MS/MS, obtaining protein sequence tags for the identification. The *S*-nitrosated proteins identified using both methods are listed in Table I. Fifteen *S*-nitrosated proteins, participating in metabolism, antioxidative defense and cellular homeostasis, were identified in human hepatocytes treated with CSNO. Among them, seven were also identified in hepatocytes treated with D-GalN (Table I).

Discussion

The damage induced by D-GalN in cultured hepatocytes is an appropriate experimental model of hepatocellular injury. We have previously shown that oxidative stress and nitric oxide-derived reactive intermediates are critical contributors to protein modification and hepatocelular injury [2,4,21]. In this sense, the treatment of human hepatocytes with D-GalN induced the tyrosine nitration of several proteins involved in cellular energy content and homeostasis [4]. We hypothesized that other NO-related post-translational modifications, such as *S*-nitrosation, might also participate in the mechanisms leading to cell death in D-GalN-treated human hepatocytes. D-GalN caused apoptosis followed by necrosis, and this induction of cell death by the hepatotoxin was accompanied by an enhancement of nitric oxide production in human hepatocytes. Notably, D-GalN-treated hepatocytes also showed augmented levels of detectable high molecular weight SNOs. Furthermore, using the biotin-switch assay, we were able to detect increased levels of *S*-nitrosated proteins in the hepatocytes treated with D-GalN. The abolition of this increase in protein *S*-nitrosation in L-NAME pre-treated hepatocytes also suggested the participation of nitric oxide synthase activity.

S-nitrosation of several critical factors in D-GalN treated hepatocytes may be important for cell survival. We tested whether the treatment with a physiological nitrosothiol could also increase protein *S*-nitrosation and alter death cell parameters in human hepatocytes. CSNO is readily taken up by cells via the amino acid transporter system L [22], and can transfer the bioactivity of the *S*-nitroso functional group from the extracellular to the intracellular space through its participation in transnitrosation reactions. Treatment of hepatocytes with CSNO resulted in increased intracellular SNO formation and higher *S*-nitrosated protein levels. This impairment in intracellular SNO homeostasis may interfere with the apoptotic machinery. In this sense, we observed that CSNO inhibited the apoptotic response in D-GalN treated hepatocytes. Furthermore, a pronounced necrotic effect was observed in the hepatocytes treated with this nitrosothiol. Therefore, CSNO treatment mimicked in some extent the effects of D-GalN in human hepatocytes and aggravated the apoptosis to necrosis shift observed in the presence of this hepatotoxin. *S*-nitrosation of several protein targets of the apoptotic machinery may be responsible for the inhibition of apoptosis and the shift into necrosis [27,29].

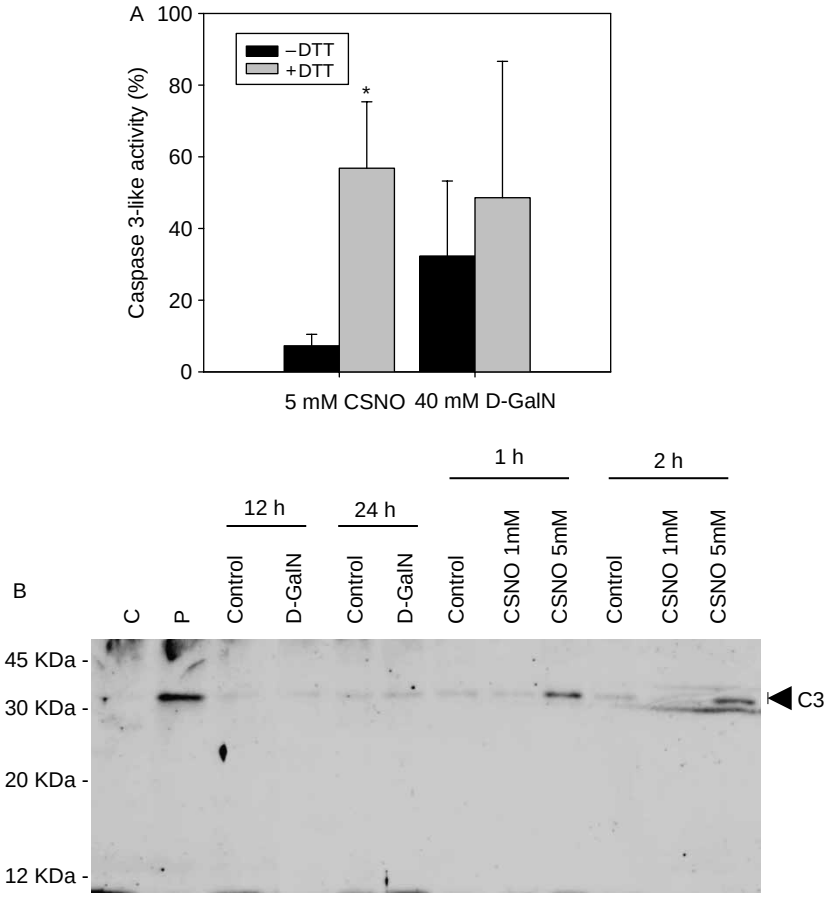

Figure 6. *S*-nitrosation of caspase-3 and inhibition of its related enzymatic activity in cultured human hepatocytes. *Panel A*, Effect of DTT on caspase-3 like activity. Hepatocytes were treated or not for 2 h with 5 mM CSNO or for 12 h with 40 mM D-GalN. Cell lysates were incubated with or without 10 mM DTT on ice for 30 min and caspase-3-like protease activity was measured with Ac-DEVD-AFC using a fluorometric assay. The caspase-3 like activity is represented as percentage related to the corresponding controls without treatment. Results are mean ± SE of three different experiments. *Panel B*, Detection of caspase-3 among the *S*-nitrosated proteins purified from human hepatocytes. Hepatocytes were treated for the indicated times with 40 mM D-GalN or different concentrations of *S*-CSNO and the cell lysates were sujected to the biotin switch assay. Afterwards, biotinylated proteins were purified using streptavidin–agarose, separated by SDS-PAGE, and caspase-3 protein was detected by Western blot. C and P, control hepatocyte lysate; pre-incubated without and with 1 mM *S*-GSNO, respectively. The image is representative of three different experiments.

Inhibition of apoptosis by *S*-nitrosation of the essential cysteine residue at the active site of caspase-3 has been demonstrated in liver [23–24], myocardial [25] and endothelial cells [28]. In our study, we found an inhibition of caspase-3 associated activity in CSNO-treated human hepatocytes that was partially recovered by a thiol reductant. Besides, the caspase-3 zymogen was detected among the purified *S*-nitrosated proteins in hepatocytes treated with CSNO. Although the results obtained in D-GalN treated hepatocytes were not so clear, the *S*-nitrosation of caspase-3 cannot be ruled out in hepatocytes treated with the hepatotoxin.

Protein targets other than caspases could be *S*-nitrosated in CSNO and D-GalN-treated hepatocytes. The biotin switch assay, yielding biotin-labeled proteins that can be further purified, provides a useful platform for a proteomic analysis of *S*-nitrosated proteins [30–31]. Using this approach, we were able to identify 15 *S*-nitrosated proteins in human primary hepatocytes after treatment with CSNO. These proteins included metabolic enzymes, antioxidant defenses and other proteins involved the response to stress and maintenance of cellular homeostasis. Notably, roughly half of these proteins were also identified as *S*-nitrosated in D-GalN treated hepatocytes. It is interesting to note the identification of several proteins that participate in the processing of proteins, including proper folding and transport (GRP94, GRP75, Hsp60, protein disulfide isomerase (PDI)) and degradation (proteasome activator protein). This can be related to the preferential localization of *S*-nitrosated proteins to mitochondria and the endoplasmic reticulum, with a comparatively more appropriate environment favouring *S*-nitrosation reactions and SNOs stability than cytosol [32].

Being central regulators of assembly, transport, and folding of other proteins, heat shock proteins (hsp) play a major role in apoptotic signalling events [33]. Hsp60 participates in the folding of mitochondrial

Figure 7. Purification of *S*-nitrosated proteins in human hepatocytes. Hepatocytes were treated with 5 mM *S*-nitrosocysteine (CSNO) for 1 h or with 40 mM D-GalN for 12 h, and cell lysates were subjected to the biotin switch assay. Proteins biotinylated after the assay were immobilized with streptavidin-agarose, eluted with 2-mercaptoetanol, and separated by SDS-PAGE. The protein bands were trypsin-digested and the corresponding proteins were identified by MALDI-TOF and peptide mass fingerprinting. Numbered arrows refer to the corresponding proteins as listed in Table I.

proteins and facilitates the proteolytic degradation of misfolded or denatured proteins in an ATP-dependent manner [34]. A pro-apoptotic role for mitochondrial Hsp60 has been demonstrated in HeLa and Jurkat cells. Both *in vitro* and *in vivo* Hsp60 was shown to associate with procaspase-3 and to favour its activation [35,36].Recently, it has been shown in endothelial cells that *S*-nitrosation of Hsp90 inhibited its ATPase activity [37]. The *S*-nitrosation of some hsp in CSNO or D-GalN-treated hepatocytes can compromise their connection with the apoptotic machinery, preventing apoptosis and shifting the cell death pathway to necrosis.

PDI is found primarily in the endoplasmic reticulum where it catalyzes protein thiol exchange reactions, contributing to proper folding of nascent proteins [38]. This enzyme is secreted and may also localize to the cell surface of several cell types including platelets, megakaryocytes, and pancreatic cells [39], where it catalyzes, through *S*-denitrosation activity, the transfer of NO from extracellular SNOs into the cytosol [39,40]. Recently, it has been reported that PDI can be *S*-nitrosated and, acting as a NO sink, could also be implicated in the efflux of SNO-bound NO from cells [41]. The enzymes enolase and aldolase B have been reported to be *S*-nitrosated in liver [42–43] and the NO inhibition of alcohol dehydrogenase

Table I. *S*-nitrosated proteins in *S*-nitrosocysteine-treated human primary hepatocytes.

Protein name*	NCBI accession number	D-GalN†	Function comments	Location
1. Carbamoyl phosphate synthase	sp: P31327	+	Removes excess ammonia from the cell	Mit
2. GRP94 (endoplasmin)	sp: P14625	–	Processing and transport of secreted proteins	ER
3. Far upstream element binding protein 2	sp: Q92945	–	Exon inclusion in tissue-specific alternative splicing	Nuc
4. GRP75 (Mortalin)	sp: P38646	–	Control of cell proliferation and cellular aging	Mit
5. Hsp60	sp: P10809	+	Mitochondrial protein import and macromolecular assembly	Mit
6. Protein disulfide isomerase	sp: P07237	+	Catalyzes the rearrangement of -S—S-bonds in proteins	ER
7. Alpha enolase	sp: P06733	–	Glycolysis, growth control, hypoxia tolerance and allergic responses	Cit, Nuc
8. Alcohol dehydrogenase (beta chain)	sp: P00325	+	Alcohol metabolism	Cit
9. Fructose-bisphosphate aldolase B	sp: P05062	–	Glycolysis	Cit
10. Proteasome activator protein beta (PA28)	sp: Q9UL46	+	Proteasome assembly	Cit, Nuc
11. Mitochondrial adenylate kinase	sp: P54819	–	Maintenance and cell growth	Mit
12. Enoyl CoA hydratase	sp: P30084	–	Fatty acid beta-oxidation cycle	Mit
13. Glutathione *S*-transferase A1	sp: P08263	+	Detoxification of exogenous and endogenous hydrophobic electrophiles	Cit
14. Manganese superoxide dismutase	sp: P04179	+	Antioxidative defense in mitochondria	Mit
15. Interleukin-19 precursor	sp: Q9UHD0	–	Inflammatory responses. Up-regulates IL-6 and TNF-alpha and induces apoptosis	ER

*Numbers designate the corresponding bands in Figure 7; †Indicates whether the protein was also identified as *S*-nitrosated (+)or not (–) in D-GalN-treated human hepatocytes.

activity in rat hepatocytes has been shown to be associated with the disruption of the zinc/thiolate active center due to *S*-nitrosation [44]. *S*-nitrosation by NO donors has also been reported to be responsible for the inhibition of enzyme activity of glutathione transferase cytosolic isoforms in rat liver [45]. Caspase-3 was not among the *S*-nitrosated proteins identified in CSNO-treated hepatocytes, probably indicating that less abundant proteins may not be identified using this proteomic approach. However, this approach has been a useful tool for detection and identification of several proteins that were *S*-nitrosated in both CSNO and D-GalN-treated human hepatocytes, arguing for the importance of this post-translational modification in this experimental model of hepatocellular injury. In this sense, we have shown elsewhere [5] that administration of prostaglandin E1 during advanced D-GalN cytotoxicity exacerbated nitrosative stress and *S*-nitrosation of proteins, losing its cytoprotective properties in cultured human hepatocytes. The results of the present study suggest that the *S*-nitrosation of proteins in D-GalN-treated hepatocytes is responsible for the shift from apoptosis to necrosis. However, this post-translational modification could also participate in the mechanisms leading to apoptosis in this experimental model.

In summary, this study underscores the importance of the alteration of SNO homeostasis during D-GalN-induced cell death in human hepatocytes, in agreement with our previous studies showing a critical role of NO mediating D-GalN-induced cell death in rat and human hepatocytes [2–5]. In addition to identifying novel targets of *S*-nitrosation, our results provide a more complete description that should facilitate future research to define specificity and functional consequence of this post-translational modification in human hepatocytes.

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References

[1] Keppler DO, Pausch J, Decker K. Selective uridine triphosphate deficiency induced by D-galactosamine in liver and reversed by pyrimidine nucleotide precursors. Effect on ribonucleic acid synthesis. J Biol Chem 1974;249:211–216.

[2] Siendones E, Fouad D, Abou-Elella AM, Quintero A, Barrera P, Muntane J. Role of nitric oxide in D-galactosamine-induced cell death and its protection by PGE1 in cultured hepatocytes. Nitric Oxide 2003;8:133–143.

[3] Siendones E, Fouad D, Diaz-Guerra MJ, De la MM, Bosca L, Muntane J. PGE1-induced NO reduces apoptosis by D-galactosamine through attenuation of NF-kappaB and NOS-2 expression in rat hepatocytes. Hepatology 2004;40:1295–1303.

[4] Rodriguez-Ariza A, Lopez-Sanchez M, Gonzalez R, Corrales FJ, Lopez P, Bernardos A, Muntane J. Altered protein expression and protein nitration pattern during D-galactosamine-induced cell death in human hepatocytes: A proteomic analysis. Liver Int 2005;25:1259–1269.

[5] Ranchal I, Gonzalez R, Lopez-Sanchez LM, Barrera P, Lopez-Cillero P, Serrano J, Bernardos A, De la Mata M, Rodriguez-Ariza A, Muntane J. The differential effect of PGE(1) on D-galactosamine-induced nitrosative stress and cell death in primary culture of human hepatocytes. Prostaglandins Other Lipid Mediat 2006;79:245–259.

[6] Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 1989;86:3375–3378.

[7] Knowles RG, Palacios M, Palmer RM, Moncada S. Formation of nitric oxide from *l*-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci USA 1989;86:5159–5162.

[8] Stuehr DJ, Gross SS, Sakuma I, Levi R, Nathan CF. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. J Exp Med 1989;169:1011–1020.

[9] Moncada S, Rees DD, Schulz R, Palmer RM. Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. Proc Natl Acad Sci USA 1991;88:2166–2170.

[10] Stamler JS, Lamas S, Fang FC. Nitrosylation: The prototypic redox-based signaling mechanism. Cell 2001;106:675–683.

[11] Mannick JB, Schonhoff CM. Nitrosylation: The next phosphorylation? Arch Biochem Biophys 2002;408:1–6.

[12] Foster MW, McMahon TJ, Stamler JS. *S*-nitrosylation in health and disease. Trends Mol Med 2003;9:160–168.

[13] Martinez-Ruiz A, Lamas S. *S*-nitrosylation: A potential new paradigm in signal transduction. Cardiovasc Res 2004;62:43–52.

[14] Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. Biochem Biophys Res Commun 2001;282:1075–1079.

[15] Li J, Billiar TR. Nitric Oxide. IV. Determinants of nitric oxide protection and toxicity in liver. Am J Physiol 1999;276:G1069–G1073.

[16] Clemens MG. Nitric oxide in liver injury. Hepatology 1999;30:1–5.

[17] Shah CM, Locke IC, Chowdrey HS, Gordge MP. Rapid *S*-nitrosothiol metabolism by platelets and megakaryocytes. Biochem Soc Trans 2003;31:1450–1452.

[18] Ferrini JB, Ourlin JC, Richard L, Fabre G, Maurel P. Human hepatocyte culture. Methods Mol Biol 1998;107:341–352.

[19] Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourd'Heuil D, Kelm M. Concomitant *S*-, *N*-, and heme-nitros(yl)ation in biological tissues and fluids: Implications for the fate of NO *in vivo*. FASEB J 2002;16:1775–1785.

[20] Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. Nat Cell Biol 2001;3:193–197.

[21] Quintero A, Pedraza CA, Siendones E, Kamal ElSaid AM, Colell A, Garcia-Ruiz C, Montero JL, De la MM, Fernandez-Checa JC, Mino G, Muntane J. PGE1 protection against apoptosis induced by D-galactosamine is not related to the modulation of intracellular free radical production in primary culture of rat hepatocytes. Free Radic Res 2002;36:345–355.

[22] Zhang Y, Hogg N. The mechanism of transmembrane *S*-nitrosothiol transport. Proc Natl Acad Sci USA 2004;101:7891–7896.

[23] Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 1997;272: 31138–31148.
[24] Li J, Bombeck CA, Yang S, Kim YM, Billiar TR. Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. J Biol Chem 1999;274:17325–17333.
[25] Maejima Y, Adachi S, Morikawa K, Ito H, Isobe M. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via *S*-nitrosylation. J Mol Cell Cardiol 2005;38: 163–174.
[26] Mannick JB, Schonhoff C, Papeta N, Ghafourifar P, Szibor M, Fang K, Gaston B. *S*-Nitrosylation of mitochondrial caspases. J Cell Biol 2001;154:1111–1116.
[27] Melino G, Catani MV, Corazzari M, Guerrieri P, Bernassola F. Nitric oxide can inhibit apoptosis or switch it into necrosis. Cell Mol Life Sci 2000;57:612–622.
[28] Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, Dimmeler S. Nitric oxide inhibits caspase-3 by *S*-nitrosation *in vivo*. J Biol Chem 1999;274:6823–6826.
[29] Nicotera P, Merlino G. Regulation of the apoptosis–necrosis switch. Oncogene 2004;23:2757–2765.
[30] Kuncewicz T, Sheta EA, Goldknopf IL, Kone BC. Proteomic analysis of *S*-nitrosylated proteins in mesangial cells. Mol Cell Proteomics 2003;2:156–163.
[31] Martinez-Ruiz A, Lamas S. Detection and proteomic identification of *S*-nitrosylated proteins in endothelial cells. Arch Biochem Biophys 2004;423:192–199.
[32] Yang Y, Loscalzo J. *S*-nitroso protein formation and localization in endothelial cells. Proc Natl Acad Sci USA 2005;102:117–122.
[33] Sreedhar AS, Csermely P. Heat shock proteins in the regulation of apoptosis: New strategies in tumor therapy: A comprehensive review. Pharmacol Ther 2004;101:227–257.
[34] Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem Biophys Res Commun 2003;304:505–512.
[35] Samali A, Cai JY, Zhivotovsky B, Jones DP, Orrenius S. Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. EMBO J 1999;18:2040–2048.
[36] Xanthoudakis S, Roy S, Rasper D, Hennessey T, Aubin Y, Cassady R, Tawa P, Ruel R, Rosen A, Nicholson DW. Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. EMBO J 1999;18: 2049–2056.
[37] Martinez-Ruiz A, Villanueva L, de Orduna CG, Lopez-Ferrer D, Higueras MA, Tarin C, Rodriguez-Crespo I, Vazquez J, Lamas S. *S*-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. Proc Natl Acad Sci USA 2005;102:8525–8530.
[38] Creighton TE, Hillson DA, Freedman RB. Catalysis by protein-disulfide isomerase of the unfolding and refolding of proteins with disulfide bonds. J Mol Biol 1980;142:43–62.
[39] Zai A, Rudd MA, Scribner AW, Loscalzo J. Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. J Clin Invest 1999;103:393–399.
[40] Ramachandran N, Root P, Jiang XM, Hogg PJ, Mutus B. Mechanism of transfer of NO from extracellular *S*-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. Proc Natl Acad Sci USA 2001;98:9539–9544.
[41] Sliskovic I, Raturi A, Mutus B. Characterization of the *S*-denitrosation activity of protein disulfide isomerase. J Biol Chem 2005;280:8733–8741.
[42] Gao CJ, Guo HT, Wei JP, Mi ZY, Wall PY, Kuo PC. Identification of *S*-nitrosylated proteins in endotoxin-stimulated RAW264.7 murine macrophages. Nitric Oxide 2005;12:121–126.
[43] Shi Q, Lou YJ. Microsomal glutathione transferase 1 is not *S*-nitrosylated in rat liver microsomes or in endotoxin challenged rats. Pharmacol Res 2005;51:303–310.
[44] Gergel D, Cederbaum AI. Inhibition of the catalytic activity of alcohol dehydrogenase by nitric oxide is associated with *S*-nitrosylation and the release of zinc. Biochemistry 1996;35:16186–16194.
[45] Ji Y, Toader V, Bennett BM. Regulation of microsomal and cytosolic glutathione *S*-transferase activities by *S*-nitrosylation. Biochem Pharmacol 2002;63:1397–1404.