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Basic Studies

Altered protein expression and protein nitration pattern during D-galactosamine-induced cell death in human hepatocytes: a proteomic analysis

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Abstract: *Background/Aims:* Hepatic injury by D-galactosamine (D-GalN) is a suitable experimental model of hepatocellular injury. The induction of oxidative and nitrosative stress participates during D-GalN-induced cell death in cultured rat hepatocytes. This study aimed to identify protein expression changes during the induction of apoptosis and necrosis by D-GalN in cultured human hepatocytes. *Methods:* A proteomic approach was used to identify the proteins involved and those altered by tyrosine nitration. A high dose of D-GalN (40 mM) was used to induce apoptosis and necrosis in primary culture of human hepatocytes. Cellular lysates prepared at different times after addition of D-GalN were separated by two-dimensional electrophoresis. Gel spots with an altered expression and those matching nitrotyrosine-immunopositive proteins were excised and analyzed by mass spectrometry. *Results:* D-GalN treatment upregulated microsomal cytochrome b5, fatty acid binding protein and manganese superoxide dismutase, and enhanced annexin degradation.
D-GalN increased tyrosine nitration of four cytosolic (Hsc70, Hsp70, annexin A4 and carbonyl reductase) and three mitochondrial (glycine amidinotransferase, ATP synthase β chain, and thiosulfate sulfurtransferase) proteins in human hepatocytes. *Conclusions:* The results provide evidences that oxidative stress and nitric oxide-derived reactive oxygen intermediates induce specific alterations in protein expression that may be critical for the induction of apoptosis and necrosis by D-GalN in cultured human hepatocytes.

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Key words: apoptosis – D-galactosamine – hepatocyte – necrosis – nitric oxide – nitrotyrosine – oxidative stress

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Hepatic injury induced by D-galactosamine (D-GalN) is a suitable experimental model of hepatocellular injury. D-GalN is metabolized to UDP-hexosamines and UDP-*N*-acetylhexosamines in hepatocytes. This effect depletes the intracellular pool of UTP (1) causing a transient arrest in the cellular transcription and protein synthesis that alters hepatocellular function. However, the exact molecular mechanisms responsible for D-GalN-induced apoptosis and/or necrosis in hepatocytes are not well understood. Diverse intracellular regulatory signals affect the induction of apoptosis and necrosis (2). Nowadays, there is growing evidence that changes in intracellular redox state appear to regulate critical biological responses. Reactive oxygen species influence signal transduction and transcription factors, and play a central role in the pathophysiology of liver injury (3). In this sense, we (4) and others (5) have shown that oxidative stress shifts cell death from apoptotic to necrotic pathways in D-GalN-treated rat hepatocytes. Mitochondria, as the most important cellular sources of free radicals, play an essential role in the development of cell death by apoptosis or necrosis.

We have previously shown that nitric oxide (NO) mediates apoptosis by D-GalN in primary culture of rat hepatocytes (6, 7). NO has been recognized as a critical mediator in normal hepatocyte

function as well as in the development of liver diseases (8, 9). In addition, NO participates in numerous biological processes, including blood vessel relaxation (10, 11), neurotransmission (12) and host defense (13). Some chemical aspects of this molecule determine its free radical capacity to modify protein targets that may mediate cell signaling. NO generated by iNOS has been shown to exert a noxious effect in the initiation and progression of cell death, specifically through the reaction with superoxide to form peroxynitrite (14). This reactive nitrogen species may modulate NO signaling functions and have direct cytotoxic effects through protein tyrosine nitration (15).

The purpose of the present study was the analysis of apoptosis and necrosis induced by D-GalN using a proteomic approach. In particular, the study will identify the alteration of the expression of specific proteins and the presence of tyrosine-nitrated proteins during the cell death by D-GalN in human cultured hepatocytes.

Methods

Materials

All reagents were from Sigma Chemical Co. (St. Louis, MO) 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 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 resection was obtained after written consent from four patients (two women, two men; 61 ± 10 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. (16). 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 μm) and washed three times at 50g for 5 min at 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 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, 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 (0–24 h) of cell damage by D-GalN (40 mM) was carried out in cultured hepatocytes. The hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 ml of lysis buffer (urea 7 M, thiourea 2 M, 4% CHAPS, 1% DTT, 0.5% Pharmalyte 3–10). The sample was centrifuged at 14 000g for 5 min at 4 °C and the supernatant was stored at −80 °C before proteomic analysis. Culture medium was also stored at −80 °C for the measurement of lactate dehydrogenase (LDH) release.

DNA fragmentation

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

Measurement of LDH 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 to 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.

Two-dimensional (2D) electrophoresis

IPG strips (17 cm, pH range 3–10, BioRad, Hercules, CA) were passively rehydrated with 150 µg (analytical gels) or 400–600 µg (preparative gels) of protein lysate in 300 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% Triton X-100, 0.5% pharmalyte 3–10 and 0.001% bromophenol blue) for 12 h. Isoelectrofocusing was carried out at 20 °C, using a PROTEAN IEF system (BioRad). Focusing was started with a conditioning step of 250 V for 15 min, followed by a voltage ramping step to 10 000 V for 3 h, and a final focusing step of 60 000 V h. Thereafter, the strips were soaked in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.001% bromophenol blue) containing 7.5 mg/ml DTT for 15 min and then in equilibration solution containing 45 mg/ml iodoacetamide for 15 min later. Second dimension was carried out in 12% polyacrylamide gels at 35 mA/gel (PROTEAN Xi Cell, BioRad).

Gel staining and image analysis

The analytical gels were silver stained according to the procedure described by Rabilloud et al. (18). The preparative gels used for peptide identification were stained with colloidal Coomassie stain (BioSafe, BioRad). Gel images were obtained using a GS-800 imaging densitometer (BioRad) and analyzed with the PDQuest 2D analysis software (BioRad). To accurately compare spot quantities between gels, image spot quantities were normalized dividing the raw quantity of each spot in a gel by the total quantity of all the valid spots in that gel.

Protein identification

Protein spots of interest were manually excised from preparative gels, transferred to Eppendorf tubes and subjected to MS analysis.

For the matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS) analysis, the Coomasie-stained gel specimens were destained with 50 mM ammonium bicarbonate, 50% acetonitrile. Then, proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate and alkylated with 55 mM iodoacetamide in the same buffer. In-gel protein digestion was performed with 6 ng/µl trypsin in 50 mM ammonium bicarbonate, for 5 h at 37 °C. The resulting peptides were extracted with 1% formic acid, 2% acetonitrile. Finally, 1.2 µl sample were mixed with 1.2 µl of a saturated solution of α-cyano-4-hydroxy-*trans*-cinaminic acid in 0.1% TFA and 50% acetonitrile and spotted into a MALDI target plate. Tryptic digests were then analyzed on a MALDI-TOF-GL-REF mass spectrometer (Waters, Milford, MA). Data processing was performed with MassLynx 4 and database searching (SWISSPROT, TREMBL, ENSEMBL) to identify the proteins of interest from their peptide fingerprint was performed with ProteinLynx Global Server 2 (Waters).

For the LC-MS/MS analysis, microcapillary reversed phase LC was performed with a CapLCTM (Waters) capillary system. Reversed phase separation of tryptic digests were performed with an Atlantis, C18, 3 µm, 75 µm × 10 cm Nano EaseTM-fused silica capillary column (Waters) equilibrated in 5% acetonitrile, 0.2% formic acid. After injection of 6 µl of sample, the column was washed during 5 min with the same buffer 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 using an isolation width of 2.5 and a relative collision energy of 35%. Data processing was performed with MassLynx 4 and ProteinLynx Global Server 2 (Waters).

Western blot analysis

The samples (600 µg protein) separated by 2D electrophoresis were transferred to a nitrocellulose membrane and tyrosine nitrated proteins were selectively detected using polyclonal antibodies raised against nitrotyrosine (Sigma Chemical Co.). A secondary antibody-horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and the ECL Advance detection system (Amersham Biosciences, Uppsala, Sweden) were used to visualize immunoreactive spots.

Statistical analysis

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

Results

Induction of apoptosis and necrosis by D-GalN in human hepatocytes

D-GalN has been shown to induce apoptosis and necrosis in cultured rat hepatocytes (4). In our conditions, D-GalN was also able to induce apoptosis (Fig. 1A) and necrosis (Fig. 1B) in primary culture of human hepatocytes. Interestingly, a rapid proapoptotic signal was already evident 2 h after D-GalN administration (Fig. 1A). As shown in Fig. 1B, D-GalN significantly induced cell necrosis at the latest time points of the study (12–24 h, $P \leq 0.05$). A reduction of apoptosis was related to the high necrotic effect of D-GalN observed at the longest time points of the study (18–24 h) (Fig. 1A and B).

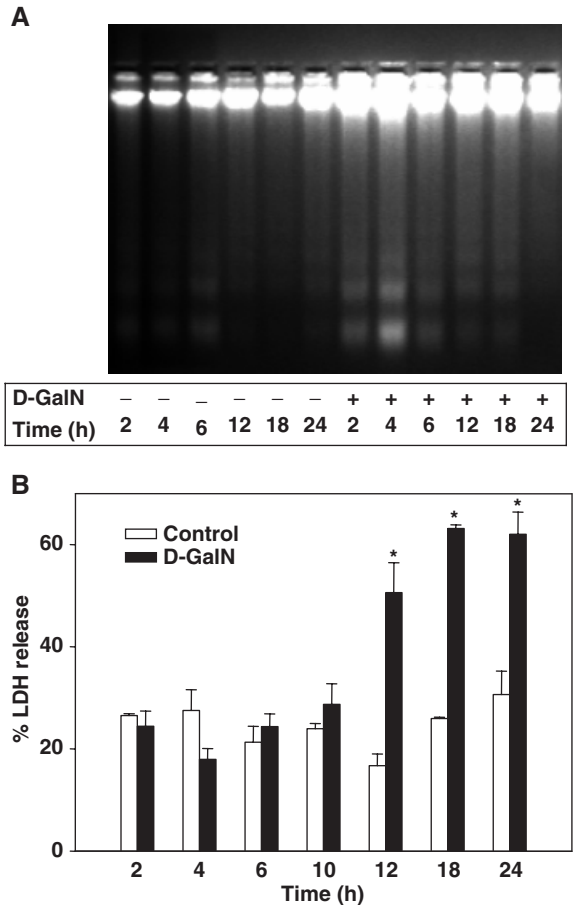

Fig. 1. Effect of D-galactosamine (D-GalN) on the induction of DNA fragmentation (A) and lactate dehydrogenase release (B) in human cultured hepatocytes. D-GalN (40 mM) induced apoptosis followed by necrosis in cultured hepatocytes. Data are the mean ± SD of four different experiments.

Modification of protein expression in D-GalN-treated hepatocytes

To analyze protein expression profiles, cell lysates were obtained at different times after D-GalN administration in cultured human hepatocytes. Cell protein extracts were separated by 2D electrophoresis. Samples from control and D-GalN-treated hepatocytes obtained in the same culture were run in parallel in order to search for a consistent altered protein expression pattern during D-GalN-induced cytotoxicity. Figure 2 shows a representative silver-stained gel obtained from 6 h control (A) and 40 mM D-GalN-treated hepatocytes (B). This analysis revealed a subset of spots (shown as insets in Fig. 2) that showed an altered expression with D-GalN treatment. These spots were excised from the corresponding Coomassie preparative gels, digested in-gel with trypsin, and tryptic peptides were analyzed by MALDI-TOF-MS. Database searching with the peptide masses identified the selected proteins (Table 1). A low-molecular number of tryptic peptides was obtained from spots 2 – 5 and the corresponding proteins were analyzed by electrospray ionization coupled to tandem mass spectrometry (ESI-MS/MS) to obtain sequence tags that allowed the identification of these proteins (Table 1).

Our study showed that D-GalN upregulated (1.5–5-fold) the expression of the reticulum endoplasmic form of cytochrome b5 (spot 1), annexin A5 fragments (spots 2 and 3), protein disulfide isomerase fragment (spot 4) and annexin A6 fragment (spot 5) during the early stages of the cytotoxicity (1–6 h) (Fig. 3). A sharp rise of the expression of all spots was observed at the necrotic phase of cytotoxicity (10–24 h) (Fig. 3). The analysis of the sequence tags obtained by ESI-MS/MS form spots 2 and 3, when compared with the complete sequence of human annexin A5, provided additional information. Thus, the sequence tags from spot 2 aligned to the N-terminal domain sequence, while the sequence tags from spot 3 aligned to the C-terminal domain sequence. Therefore, spots 2 and 3 could arise after a single proteolytic cleavage event on annexin A5. A weak PEST region (Fig. 4), as a proteolytic recognition site, has been previously shown to be present in annexin A5 (19). Experimental molecular weights of proteins in spots 2 (16 kDa) and 3 (17 kDa) agree with such a single proteolytic cleavage of the 35.8 kDa annexin 5 protein. The sequence tags from the annexin A6 fragment suggest that spot 6 may also arise from

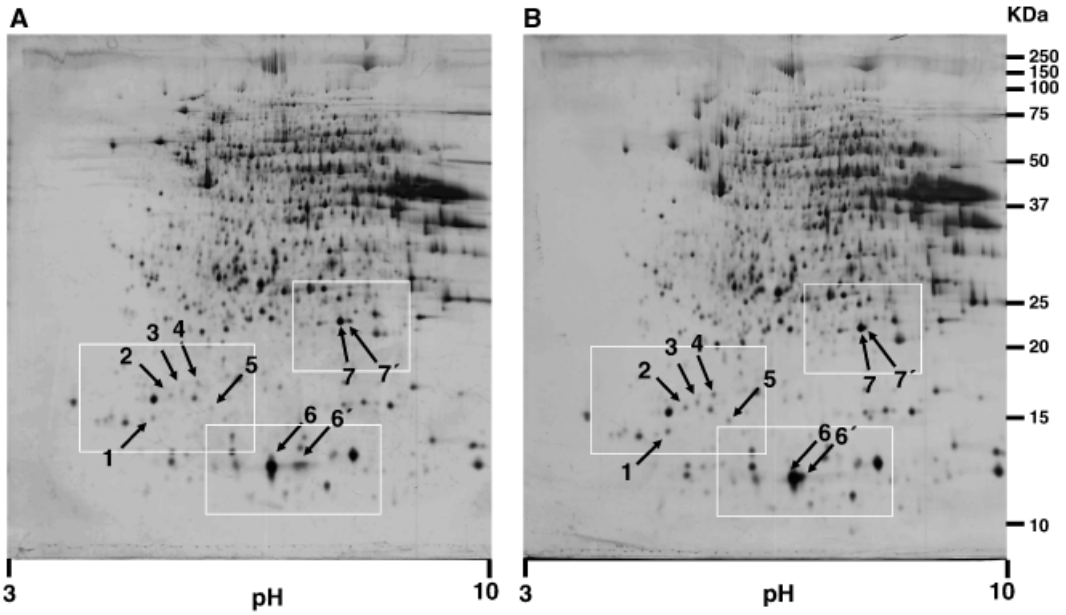

Fig. 2. Two-dimensional (2D)-electrophoresis analysis of control (A) and D-galactosamine (D-GalN)-treated (B) human hepatocytes. Cell lysates (150 μg protein) from 6 h control and D-GalN-treated hepatocytes were subjected to 2D electrophoresis and silver stained. The spots with a consistent altered expression pattern throughout the kinetic study are indicated by arrows. They were excised from the corresponding Coomassie preparative gels, digested in-gel with trypsin and analyzed by mass spectrometry. The corresponding identified proteins are detailed in Table 1. The image is representative of four different experiments.

Table 1. MS-analyzed proteins (spots 1–7) with altered expression pattern in D-galactosamine (D-GalN)-treated human hepatocytes

Spot	Accession number	Protein	MW	pI	Matched peptides	Sequence coverage (%)	Sequence tags
1	P00167	Cytochrome b5 (endoplasmic reticulum form)	15189	4.91	8	65	
2	P08758	Annexin A5 (fragment)	35783	4.95	5	17	VLTEIIASR GTVTDFPGFDER
3	P08758	Annexin A5 (fragment)	35783	4.95	–	–	SEIDLFNIR GAGTDDHTLIR SEIDLFNIRK
4	P07237	Protein disulfide isomerase (fragment)	57116	5.94	–	–	LKAEGSEIR EADDIVNWLK VDATEESDLAQQYGVR
5	P08133	Annexin A6 (fragment)	75695	5.42	–	–	DAISGIGTDEK EAILDIITSR DLEADIIGDTSGHFQK
6	P07148	Fatty acid binding protein (FABP1)	14208	6.60	7	61	–
6′	P07148	Fatty acid binding protein (FABP1)	14208	6.60	10	74.	–
7	P04179	Mn-SOD	24722	8.35	9	50	–
7′	P04179	Mn-SOD	24722	8.35	9	21	–

a proteolytic cleavage at a PEST region localized to the N-terminal domain of annexin A6 (data not shown). Spots 6 and 6′ were identified as fatty acid binding protein (FABP1) (Table 1). Figure 5 shows that D-GalN enhanced FABP1 expression (1.5–3-fold) at the necrotic phase of cytotoxicity (10–24 h). Figure 6 shows changes observed in spots 7 and 7′ that were identified as the mitochondrial antioxidant enzyme manganese superoxide dismutase (Mn-SOD). It was also observed a raise on Mn-SOD expression 10–18 h after D-GalN administration.

Protein nitration in D-GalN-treated hepatocytes

We have previously shown that NO generation mediates the induction of apoptosis by D-GalN in primary culture of rat hepatocytes (6, 7). In addition, D-GalN induces an intense oxidative stress in hepatocytes (4). The reaction of NO and superoxide anion generates peroxynitrite (14). This highly reactive nitrogen species may exert its toxic effects through protein tyrosine nitration (15). We therefore investigated the changes in tyrosine-nitrated proteins in D-GalN-treated hepatocytes.

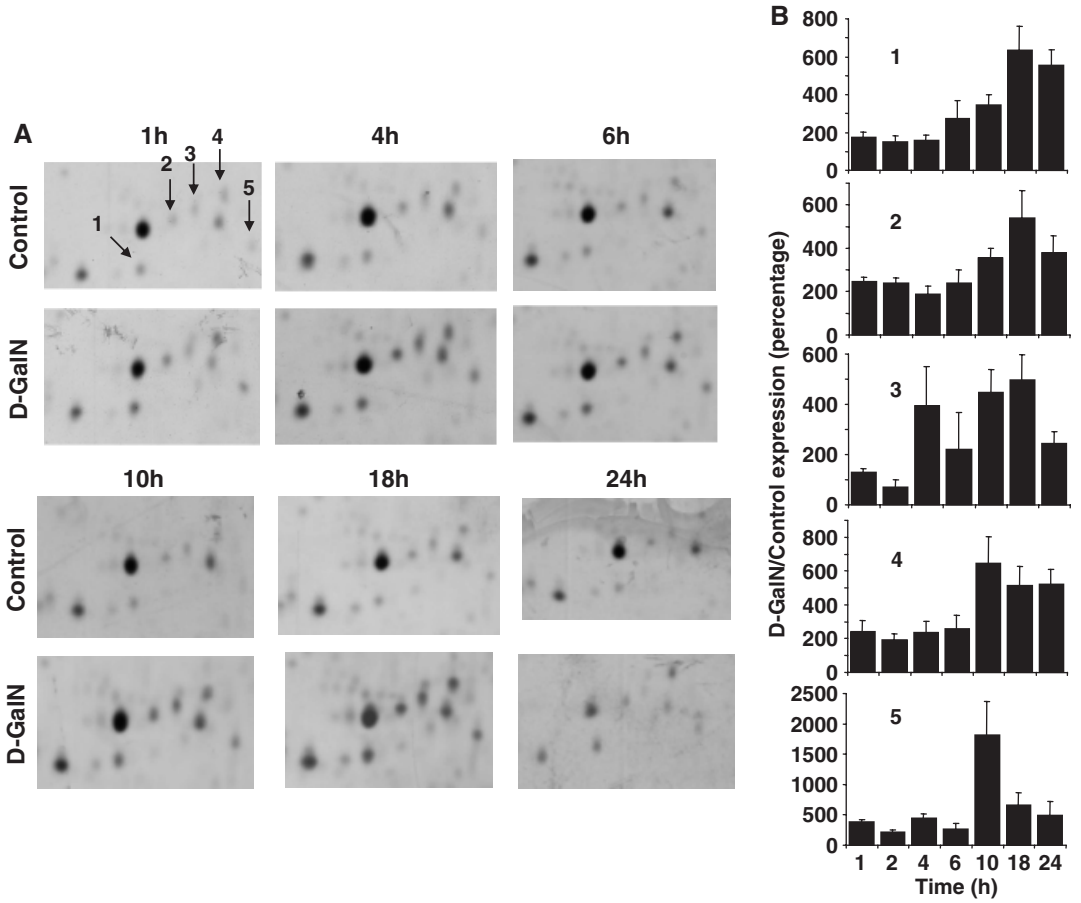

Fig. 3. Effect of D-galactosamine (D-GalN) on the expression of cytochrome b5 (spot 1), annexin A5 fragments (spots 2 and 3), protein disulfide isomerase fragment (spot 4) and annexin A6 fragment (spot 5) in cultured human hepatocytes. The corresponding gel areas after 2D electrophoresis of cell lysates (150 μg protein) from control and D-GalN-treated hepatocytes at different are shown in (A). The increase (percentage) of the corresponding protein expression in D-GalN vs control hepatocytes is shown in (B). The image is representative of four different experiments. Data are the mean ± SEM ($n = 4$).

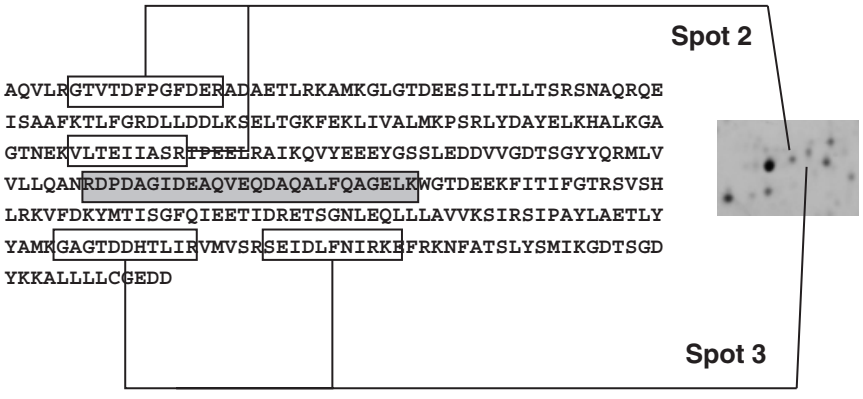

Fig. 4. Alignment of the human annexin A5 sequence and four sequence tags (white boxes) obtained from spots 2 and 3 (Figs 2 and 3) using LC-MS/MS analysis. The gray box indicates a weak PEST region.

Proteins from cell lysates were separated by 2D electrophoresis, partially transferred onto nitrocellulose membranes and immunodetected using a polyclonal antibody. D-GalN (6 h) enhanced the levels of eight nitrotyrosine immunopositive spots in cultured human hepatocytes (Fig. 7). The immunopositive proteins were excised from the parent acrylamide gel and digested in-gel with trypsin and tryptic peptides were analyzed by MALDI-TOF spectrometry. Database searching with the peptide masses identified the immunopositive proteins. The immunoreactive proteins

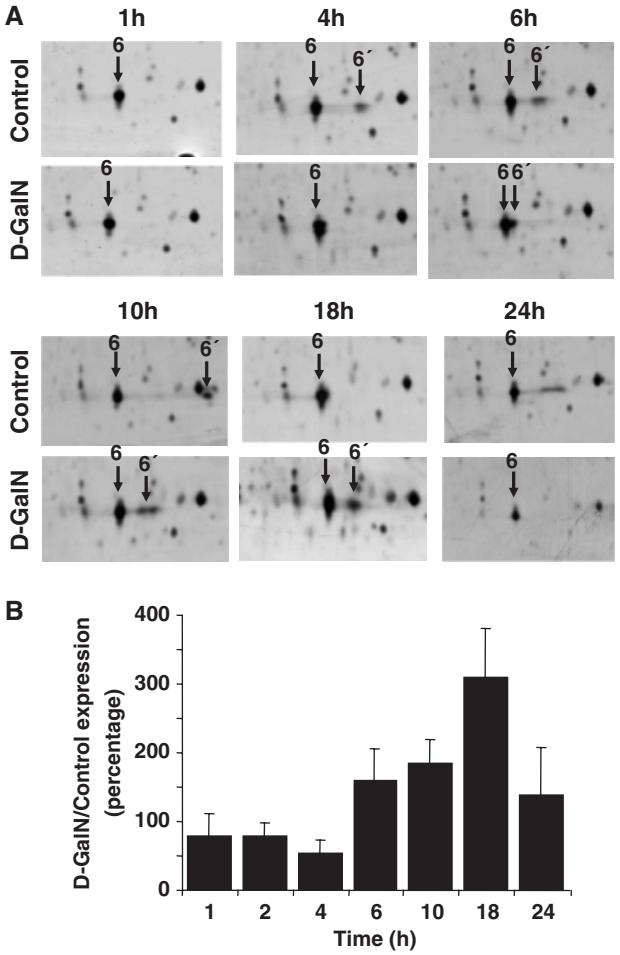

Fig. 5. Effect of D-galactosamine (D-GalN) on the expression of fatty acid binding protein 1 (FABP1) (spots 6 and 6′) in cultured human hepatocytes. The corresponding gel areas after two-dimensional electrophoresis of cell lysates (150 μg protein) from control and D-GalN-treated hepatocytes at different times are shown in (A). The increase (percentage) of the corresponding protein expression in D-GalN vs control hepatocytes is shown in (B). The image is representative of four different experiments. Data are the mean ± SEM ($n = 4$).

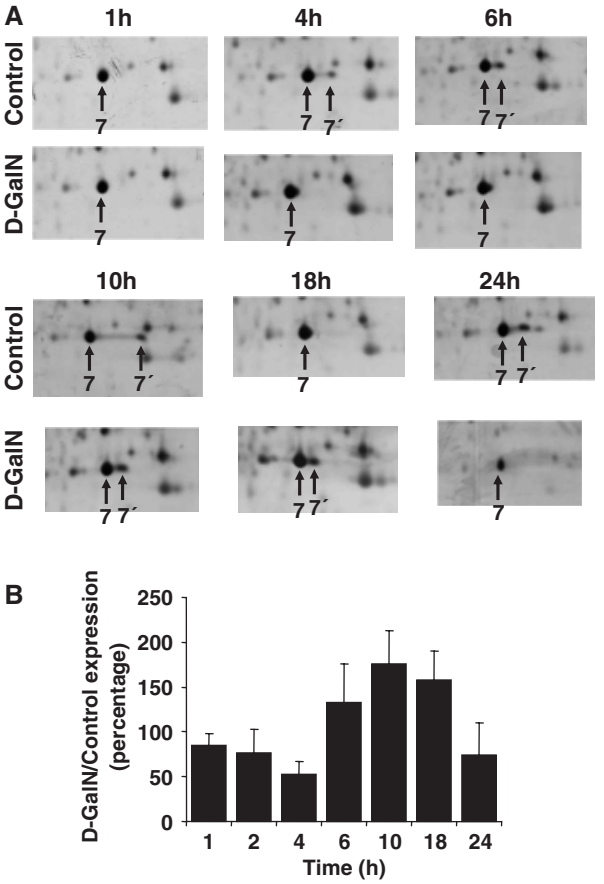

Fig. 6. Effect of D-galactosamine (D-GalN) on the expression of manganese superoxide dismutase (Mn-SOD) (spots 7 and 7′) in D-GalN-treated human hepatocytes. The corresponding gel areas after two-dimensional electrophoresis of cell lysates (150 μg protein) from control and D-GalN-treated hepatocytes at different times are shown in (A). The increase (percentage) of the corresponding protein expression in D-GalN vs control hepatocytes is shown in (B). The image is representative of four different experiments. Data are the mean ± SEM ($n = 4$).

identified are listed in Table 2. Four of them (Hsc70, heat shock protein (Hsp)70, annexin A4 and carbonyl reductase) were cytosolic, while the other three (glycine amidinotransferase, ATP synthase β chain, and thiosulfate sulfurtransferase) were mitochondrial.

Discussion

Hepatic injury induced by D-GalN is a suitable experimental model of human liver failure (1). This study showed that D-GalN-induced apoptosis followed by necrosis in cultured human hepatocytes. In this sense, we (4) and others (5) have shown that oxidative stress can transfer cell death from apoptotic to necrotic pathways in D-GalN-treated rat hepatocytes. The identification of proteins with altered expression or modifications during the cell death induced by D-GalN in human hepatocytes can help in the insight of the causal molecular mechanisms responsible for the induction of apoptosis and its shift to necrotic cell death pathways.

In this study, the proteomic approach allowed the identification of several proteins upregulated during D-GalN-induced cell death. The expression of some proteins (cytochrome *b*5, annexin A5, and A6 fragments and protein disulfide isomerase fragment) was rapidly enhanced at the early stages of apoptosis induced by D-GalN. It is well known the ability of cytochrome *b*5 to form tight complexes with cytochrome *c* (20). Although its pathophysiological role is still unclear it has been suggested that cytochrome *b*5 can be considered as a competitor for apoptogenic interactions between cytochrome *c* and the Apaf-1–caspase-9 complex (21). In this sense, its upregulation by D-GalN may reduce the

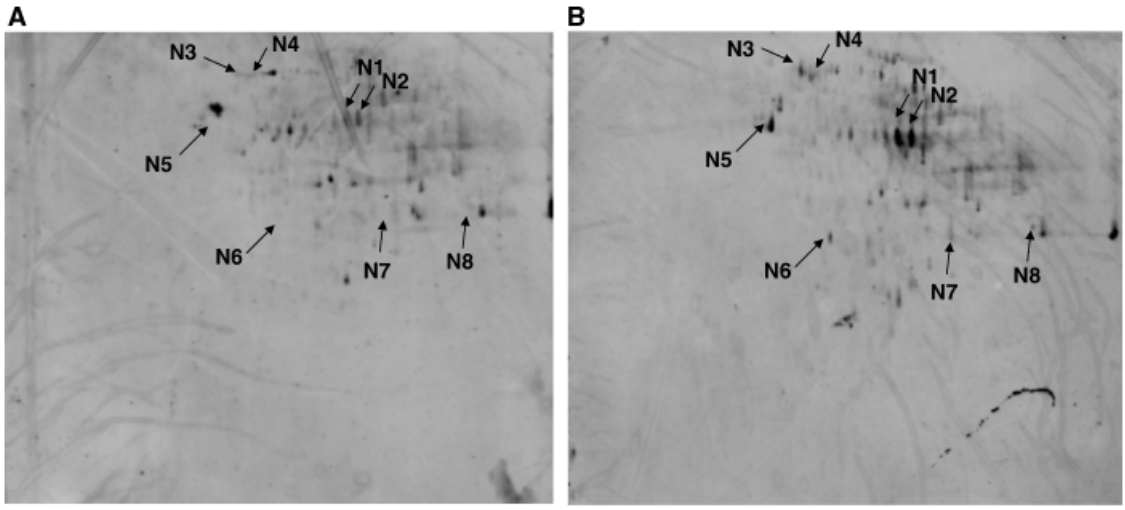

Fig. 7. Effect of D-galactosamine (D-GalN) on the levels of anti-nitrotyrosine immunopositive proteins in cultured human hepatocytes. After two-diemnsional electrophoresis of cell lysates (600 μg protein), proteins were partially transferred to nitrocellulose membranes and anti-nitrotyrosine inmunopositive spots were detected. A representative Western blot of 6 h control (A) and D-GalN-treated (B) hepatocytes is shown. Matching spots in the corresponding Coomassie-stained polyacrylamide gels were excised, digested in-gel with trypsin and analyzed by mass spectrometry. The corresponding identified proteins are detailed in Table 2.

Table 2. Identified anti-nitrotyrosine immunopositive proteins in D-galactosamine (D-GalN)-treated human hepatocytes

Spot	Accession number	Protein	Matched peptides	Sequence coverage (%)	Function. comments	Location
N1	P50440	Glycine amidinotranferase	26	58	Creatine biosynthesis; first (rate-limiting) step.	Mit
N2			35	66		Mit
N3	P11142	Heat shock cognate 71 kDa	33	51	Chaperone. Folding, transport and assembly of newly synthesized polypeptides.	Cyt
N4	P08107	Heat shock protein 70 kDa	15	27	Chaperone. Folding, transport and assembly of newly synthesized polypeptides	Cyt
N5	P10719	ATP synthase beta chain	16	42	ATP production. The β chain has been also detected as Tyr-nitrated protein in liver of LPS-treated rats (31)	Mit
N6	P09525	Annexin A4	12	38	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. This protein has been also detected as Tyr-nitrated protein in liver of LPS-treated rats (31)	Cyt
N7	Q16762	Rhodanese (thiosulfate sulfurtransferase)	13	47	Mitochondrial thiosulfate sulfurtransferase. Has been also detected as Tyr-nitrated protein in liver of LPS-treated rats (31)	Mit
N8	P16152	Carbonyl reductase	15	59	Catalyzes the reduction of a wide variety of carbonyl compounds	Cyt

proapoptotic cell death pathway related to cytochrome *c* release. D-GalN also induced a higher degradation rate of annexins. These proteins bind phospholipids in a calcium-dependent manner and participate in the regulation of membrane organization, membrane traffic, and the regulation of calcium transport across membranes and calcium concentration within cells (22). In our study the two identified fragments of annexin A5 seem to be the consequence of a single proteolytic cleavage event, in the weak PEST region observed in the sequence of human annexin A5 that may act as a degradative signal (19). A proteolytic cleavage recognizing a N-terminal PEST region in annexin A6 may also be responsible for the high levels of the annexin A6 fragment detected in D-GalN-treated hepatocytes. It is interesting to note that rates of synthesis of annexins are not acutely regulated (22) and mechanisms other that mRNA levels might contribute to altered protein expression or activities under different conditions. Our study showed that different degradation rates of annexins can be a relevant response to D-GalN treatment in human hepatocytes.

Other subset of proteins (FABP1 and Mn-SOD) enhanced their expression during the necrotic phase of D-GalN-induced cell death. FABP1 is an abundant constituent of the cytoplasm and regulates lipid transport and metabolism. This protein binds free fatty acids, their CoA derivatives, bilirubin,

organic anions, and other small molecules. In addition, FABP1 is required for cholesterol synthesis and metabolism. Several posttranslational modifications, including covalent modifications by cysteine and glutathione, have been suggested to explain the existence of different isoforms of FABP in bovine, rat and human liver (23, 24). However, little is known about the significance of these modifications in the ability of FABP to bind endogenous ligands. In our conditions, D-GalN clearly altered the isoelectric point of FABP1 in 2D electrophoresis in hepatocytes. Additionally, it has been recently shown that FABP in hepatocytes plays a role in fatty acid and drug signaling to nucleus (25). After binding the signaling molecules, this low-molecular protein can freely diffuse to the nucleus and activate gene expression through direct interaction with peroxisome proliferated-activated receptors (PPARs). The altered pattern of FABP expression in human hepatocytes after D-GalN treatment suggests an altered function of this important protein in the metabolism and regulation of fatty acid metabolism in the latest stages of cytotoxicity.

Mn-SOD was also identified as a protein upregulated in D-GalN-treated hepatocytes. Mn-SOD is a vital antioxidant enzyme localized in the mitochondrial matrix and plays an important role in the cellular defense against superoxide produced by the mitochondrial electron transport chain during normal cellular metabolism. It is known that Mn-SOD expression is induced by a variety of stimuli including reactive oxygen species, ceramide, and the proinflammatory cytokines TNF-α, IFN-γ, and IL-1β (26). However, the molecular intracellular pathways and the nature of the induction of Mn-SOD by inflammatory mediators are still being unravelled. Recently, Mn-SOD has been identified as an NO-regulated gene in mesangial cells (27). In our study, the upregulation of Mn-SOD may be a consequence of oxidative stress (4) or NO synthesis (7). The upregulation of this pivotal antioxidant defense during the necrotic phase of D-GalN cytotoxicity may intend to counteract the oxidative stress-dependent shift from apoptotic to necrotic cell death pathways (4, 5). Interestingly, it has been observed that PPARs ligands increase the expression of Cu, Zn-SOD in primary endothelial cells (28). Moreover, treatment of these cells with monounsaturated and polyunsaturated fatty acids enhanced the expression of this antioxidant defense. It is feasible that the enhancement of FABP1, as a coactivator of PPARs, might be related to the enhanced expression of Mn-SOD during the late stages of D-GalN-induced cytotoxicity in cultured human hepatocytes.

The reaction of NO and superoxide anion generates peroxynitrite (14). This highly reactive nitrogen species exerts its toxic effects through protein tyrosine nitration (15). Nitration in tyrosine residues has been suggested to possess properties for consideration as a redox-based signaling mechanism. In addition, this posttranslational modification may interfere with cellular processes relying on tyrosine phosphorylation and dephosphorylation (29). A number of studies have pointed out that nitration of proteins could significantly alter protein function and may target modified proteins for degradation (30). The detection of an increase in nitrotyrosine-immunopositive proteins after D-GalN treatment in human hepatocytes was in agreement with previous studies showing that NO mediates apoptosis by this hepatotoxin in cultured rat hepatocytes (6, 7). It is important to remark the mitochondrial location of three of these nitrotyrosine proteins. Since the electron transport chain is the major cellular source of superoxide, mitochondria are expected to be a major site of peroxynitrite formation and nitrotyrosine protein formation. Many of the proteins nitrated in tyrosine identified in this study (Table 2) had been previously shown to be also present in livers from animals with septic shock (31). Glycine amidinotransferase is a mitochondrial enzyme catalyzing the rate-limiting step in creatine biosynthesis. The creatine/creatine-phosphate system plays an important role in the storage and transmission of phosphate-bound energy. Other protein involved in mitochondrial energy production that also appeared nitrated was the β chain of ATP synthase. Nitration of these and other proteins in mitochondria after D-GalN treatment could affect the energy balance in the hepatocytes, limiting the apoptotic vs necrotic cell death pathway.

Hsp function as molecular chaperones in regulating cellular homeostasis and promoting cell survival. Therefore, it is not surprising that Hsp have an extremely complex role in the regulation of apoptosis. Various levels of chaperone overload may have an important contribution to the signals directing the cell to senescence, apoptosis, or necrosis (2). On the other hand, Hsp70 has been shown to inhibit apoptosis by preventing the recruitment of procaspases 9 and 3 to the apoptosome complex, thereby preventing the assembly of a functional apoptosome (32). Tyrosine nitration of Hsc70 and Hsp70 in hepatocytes treated with D-GalN may result not only in a fail to maintain cell homeostasis but also in a hampered capability of hepatocytes to execute an appropriate apoptotic response, hence resulting in a pro-necrotic effect.

In summary, the proteomic approach has been proven to be a very useful tool for the identification of changes in protein expression and modification in a model of hepatocellular injury. The identification of oxidative stress and NO-derived reactive oxygen intermediates as critical contributors to protein modification and hepatocellular injury, provides potential targets for therapeutic intervention. In the present study we showed that the increase of the selected protein expression is associated with the induction of apoptosis or necrosis during D-GalN-related cytotoxicity. In addition, the tyrosine nitration of proteins involved in cellular homeostasis and energy content might also influence the shift from an apoptotic to necrotic cell death pathway by D-GalN in cultured human hepatocytes. The role of all these proteins and their modifications in the initiation of apoptosis and/or necrosis in hepatocytes after D-GalN treatment are currently under investigation.

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