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Proteasome Activity Deregulation in LEC Rat Hepatitis: Following the Insights of Transcriptomic Analysis

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ABSTRACT

LEC rats show spontaneous hepatitis and hepatocarcinoma development related to oxidative stress due to abnormal copper accumulation in the liver. We used DNA microarrays bearing 22,012 genes to investigate at the transcriptomic level the progression of the hepatitis in LEC rats in comparison to a control obtained from LEC rats treated with D-penicillamine, a copper chelating agent known to block hepatitis development. Multivariate statistical analyses as partial least square (PLS) regression between transcriptomic data and hepatitis markers in plasma led us to select 483 genes related to hepatitis development in these rats. After a complementary discriminant analysis (PLS-DA), 239 important genes for the separation between the different rat groups were selected. Gene ontology classification revealed an overrepresentation of genes involved in protein metabolism-related functions. More importantly, some genes implicated in proteasome pathway were upregulated. However, analysis of 20S proteasome activity showed that trypsin-like and peptidylglutamyl peptide hydrolase activities were diminished during hepatitis. Because oxidative stress is known to promote the inactivation of the proteasome complex, we propose the deregulation of the proteasome genes expression as a result of oxidative inactivation of proteasome activity during hepatitis in LEC rats. These results bring new insights in the hepatitis and the hepatocarcinogenesis development.

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Abbreviations: ALT; alanine aminotransferase; AST, aspartate aminotransferase; BHT, butylhydroxytoluene; BER, base excision repair; BOC-AMC, BOC-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin; ChT, chymotrypsin-like; GO, gene ontology; HNE, 4-hydroxy-2-nonenal; LEC, Long Evans cinnamon; LEA, Long Evans agouti; LLVY-AMC, N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; OSC, orthogonal signal correction; PGPH, peptidylglutamyl peptide hydrolase; PLS, partial least square; PLS-DA, partial least square discriminant analysis; ROS, reactive oxygen species; TL, trypsin-like; VIP, variable important projection; Z-LLG-NA, Z-Leu-Leu-Glu-β-naphthylamide.

INTRODUCTION

In humans, chronic liver diseases are generated by different causes. Chronic alcohol consumption, viral infections, metabolic dysfunctions, xenobiotic exposition, and metal overload are factors associated with hepatitis and liver tumor development. Oxidative stress seems to be a common mechanism involved in all these different hepatitis causes (Nagasaka et al., 2006; Seitz and Stickel, 2006; Vendemiale et al., 2001). Patients suffering from hepatitis C manifest hepatic oxidative stress, a condition that is exacerbated by alcohol consumption (Wang and Weinman, 2006). 8-Nitroguanine, a marker of DNA oxidation, is highly formed in patients with hepatitis C. Furthermore, some hepatitis C virus (HCV) carriers with normal alanine aminotransferase (ALT) levels in serum have elevated levels of lipid peroxidation products and low levels of reduced glutathione (GSH) in plasma. In these patients, a greater degree of oxidative stress markers correlates with a more severe status of the disease (Vendemiale et al., 2001). In Wilson's disease, mutations of the copper transporting ATPase *ATP7B* gene lead to decreased biliary copper excretion and accumulation of copper in liver. Copper is known to induce reactive oxygen species (ROS) production. Oxidative stress resulting from an increased production of free radicals via copper accumulation and defects in antioxidant defenses are highly relevant to the toxic process in Wilson's disease (Dalgic et al., 2005).

During oxidative stress, ROS can interact with biomolecules such as DNA, RNA, proteins, and lipids, leading to their oxidation and consequently to cellular damage, genomic instability, apoptosis, and cell cycle alterations (Valko et al., 2006). Even the evidence of the involvement of oxidative stress during liver pathologies, mechanisms in which ROS can lead to hepatitis development, are not fully understood. The aim of this study was to examine on a global scale, the gene expression changes during hepatitis development and progression in relation to oxidative stress. In this context, Long-Evans Cinnamon-like (LEC) rats have been described as a good model for studying the relation between oxidative stress, hepatitis, and hepatocarcinogenesis (Marquez et al., 2007; Mori et al., 1994). LEC rats suffer from a spontaneous copper accumulation due to a deletion in the *Atp7b* gene responsible for liver copper excretion (Wu et al., 1994). As a consequence, these rats exhibit an acute hepatitis followed by a period of chronic hepatitis and, eventually, by development of liver tumors (Li et al., 1991). Since liver pathology progression of LEC rats is comparable to that of the human Wilson's disease, these rats are considered as the biological model for studying this human liver pathology (Mori et al., 1994; Sugawara et al., 1995). D-penicillamine, a copper chelating agent, has been shown to prevent and to reverse hepatitis progression in LEC rats (Klein et al., 2000; Togashi et al., 1992). Oral administration of D-penicillamine inhibits the elevation of serum transaminases, and suppress liver histological changes in LEC rats (Togashi et al., 1992). The ability of D-penicillamine to control hepatitis initiation and progression in LEC rats makes this drug an interesting tool for studying the mechanisms involved in oxidative stress-related hepatitis. Today, DNA microarray technology is a powerful tool to determine the repertoire of genes that are differentially expressed in cells or tissues exposed to different stimuli like xenobiotic exposition or disease development. These experiments lead to a large set of expression data that are not easy to interpret in a biological context. Biological information from transcriptomic data sets can be captured by correlating them with other significant biological information using dedicated statistical methods (Hocquette, 2005; Valafar, 2002). In our study, we have used partial least square (PLS) regression and PLS-discriminant analysis (PLS-DA) either to select for genes the expression of which was significantly correlated to hepatitis symptoms or to explain the discrimination between LEC rats examined at different hepatitis stages. As a major result, this mode of data treatment enabled us to identify the proteasome system as a key target during oxidative stress-induced hepatitis.

MATERIALS AND METHODS

Animals and treatment

LEC rats were bred in our Institution animal facility from rats kindly given by Dr. Matsumoto from Tokushima University (Japan). Male LEC rats were maintained in metabolic cages from 6 weeks old until sacrifice. Experiments were done under Institutional Animal Care and Use Committee Guidelines. Rat groups ($n = 6$) were

classified according to hepatitis stage, age and treatment (Table 1). Liver disease stage was assigned according to aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, and total bilirubin (t-bil) levels in plasma and from liver histopathology. For D-penicillamine-treated rats group, D-penicillamine was administered in drinking water (100 mg/kg/day) for 6 weeks until sacrifice at 13 weeks old. D-penicillamine (Sigma, Saint-Quentin Fallavier, France) concentration was adjusted according to rats water consumption.

Hepatic function analysis

AST, ALT activities, and t-bil levels in plasma were measured spectrophotometrically by automatic biochemical analyzer at the Physiologycal Exploration platform of Toulouse Génopole (CHU Rangueil, France).

Lipid peroxidation quantification

Urine samples were collected daily from rats housed in plastic metabolic cages. 0.5 mL of a 360 mM butylhydroxytoluene (BHT) ethanolic solution was added to the urine collection tubes that were placed in a unit maintained at 0°C during urine collection. Twenty-four-hour collected urine samples were stored at −20°C until analysis.

8-Isoprostane (8-IsoPGF$_{2\alpha}$) is considered as a good marker of inflammation and oxidative stress (Liu et al., 1998). Urine 8-IsoPGF$_{2\alpha}$ was quantified by competitive enzyme immunoassay as described elsewhere (Pradelles et al., 1985). 8-Isoprostane EIA antiserum, 8-isoprostane EIA standard, and 8-isoprostane AChE tracer were obtained from Cayman (SPI-BIO, Montigny le Bretonneux, France).

Tissue preparation

Rats were sacrificed by exsanguination under ether anesthesia. Blood was taken from the aorta vein for plasma separation. Liver was excised and washed in physiological saline solution. For RNA isolation, liver samples were immersed in RNA*later* (Qiagen, Courtaboeuf, France) following manufacturer's instructions and stored at −80°C. For light microscopy examination, liver samples were fixed in 4% buffered formaldehyde, embedded in paraffin, and sections were stained with hematoxylin and eosin. For cytosol extraction, 10% (w/v) liver homogenates were prepared in 50 mM Tris-HCl pH 8.0 containing 0.1 mM EDTA and 1 mM β-mercaptoethanol. Homogenates were centrifuged at 100,000 $\times$ g for 1 h at 4°C; supernatants were stored at −80°C until use.

RNA extraction

Total RNA from RNA*later*-stabilized liver samples were performed following the instructions from Qiagen RNeasy mini kit using the On-Column DNase digestion set (Qiagen). Quality and quantity were determined by capillary electrophoresis on the bioanalyzer from Agilent (RNA 6000 Nano Assay, Agilent, Massy, France) and by spectrometric analysis on a NanoDrop ND-1000 spectrophotometer (Nyxor Biotech, Labtech, Palaiseau, France). Only samples with $\lambda_{260\ nm}/\lambda_{280\ nm}$ ratio $>$ 1.9 and $rRNA_{28S}/\ rRNA_{18S}$ ratio $>$ 1.7 were taken for DNA microarray and quantitative RT-PCR assays.

Quantitative RT-PCR (qRT-PCR)

Two micrograms of total RNA was reverse transcribed using SuperScript II-reverse transcriptase system (Invitrogen, Cergy Pontoise, France) following manufacturer's instructions. Quantitative PCR assay for cDNA was carried out using TaqMan Universal PCR master mix in a AB PRISM 7000 sequence detection system (Applied Biosystem, Courtaboeuf, France). The fluorescent probe for rat *glutathione S-transferase pi* (*GSTP*) gene, probe code: Rn00821792_g1, was used. Data were normalized against *beta actin* gene expression, probe Rn00667869-m1 (Applied Biosystem). Signals were quantified using a standard curve made by serial dilutions of a cDNA pool from all samples.

Proteasome peptidase activities

Chymotrypsin-like (ChT), trypsin-like (TL), and peptidylglutamyl peptide hydrolase (PGPH) proteasome activities were determined with fluorogenic synthetic peptides N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-

Table 1. Group Classification

	Normal 6w	*Normal 9w*	*Slight jaundice*	*Jaundice*	*D-penicillamine*	$F_{4,25}$	p *value*
Age (weeks)	6	9	11–13	11	13	—	—
Treatment	—	—	—	—	D-penicillamine 100 mg/kg/day	—	—
ALT (U/l)	62.83 (SD 13.7)[a]	66.7 (SD 7.7)[a]	584.8 (SD 120.4)[b]	1184 (SD 344.7)[c]	45.7 (SD 2.7)[a]	56	<0.0001
AST (U/l)	123.8 (SD 15.3)[a]	95.5 (SD 7.4)[a]	479 (SD 144.7)[b]	725.7 (SD 208)[c]	81.8 (SD 2.4)[a]	38.8	<0.0001
T-bilirubin (μmol/L)	2.1 (SD 1.8)[a]	1.9 (SD 0.8)[a]	107 (SD 64.7)[b]	369.7 (SD 228)[b]	2.3 (SD 0.6)[a]	13.5	<0.0001
Urinary 8-IsoPGF$_{2\alpha}$ (ng/24 h)	7.8 (SD 4.4)[a]	14.7 (SD 14)[a,b]	30.5 (SD 10.4)[b]	51 (SD 21.3)[c]	6.4 (SD 1.8)[a]	4.5	0.0106
Liver histology	Normal	Normal architecture, Slight hydropic change.	Abnormal. Conserved liver architecture; cholestasis; presence of micronecrosis and apoptosis; inflammatory infiltrates.	Abnormal. Necrosis, apoptosis, inflammation, cholestasis, nuclear polymorphism, fat droplets.	Normal. Slight hydropic change.	—	—

Values refer to means (SD = standard deviation). Means without a common letter differ as determined by ANOVA and the Bonferroni *post hoc* test.

methylcoumarin (LLVY-AMC), BOC-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (BOC-AMC) and Z-Leu-Leu-Glu-β-naphthylamide (Z-LLG-NA), respectively. Assays were performed as described elsewhere (Fataccioli et al., 1999). Briefly, cytosolic fractions containing 150 μg of protein were incubated with fluorogenic substrates (50 mM) and SDS 0.06% in 150 mM Tris-HCl pH 8.0 for 30 min at 37°C. The reaction was stopped by adding 1 mL SDS 1% and 2 mL 0.1 M sodium borate pH 9.1. The peptidase activity was determined fluorometrically by measuring the release of 7-amino-4-methylcoumarin (λ_{exc} = 370 nm, λ_{em} = 430 nm) and 2-naphthylamine (λ_{exc} = 323nm, λ_{em} = 400 nm) in a Jobin Yvon spectrofluo JY3D fluorometer (Horiba Jobin Yvon, Longjumeau, France). A standard curve of fluorescence for 7-amino-4-methylcoumarine and 2-naphthylamine was used to calculate the concentration of liberated products in the assay.

SDS-PAGE and immunoblotting

Cytosolic protein fractions were separated by SDS-PAGE using a mini-PROTEAN II electrophoresis cell (Bio-Rad, Marnes la Coquette, France). Following transfer, the nitrocellulose membranes were probed with antibodies to 20S proteasome, PROS-30 (Santa Cruz Biotechnology, Inc., Tebu-Bio, Le Perray en Yvelines, France), or α-Tubulin (Clone B-5-12) (Sigma), and visualized using the ECL chemiluminescent system from Amersham Biosciences (GE Healthcare, Ramonville Saint-Agne, France).

Microarray analysis

DNA microarray slides. DNA microarray glass slides were made at Biochips Platform of Toulouse Genopole (Toulouse, France). Rat 70mer oligos from Operon_V3_rat oligo set (Operon Biotechnologies, Cologne, Germany) were loaded at 300 pmol/well. Oligos were resuspended in 20 μL 3× SSC buffer (final concentration 20 μM). Spotting was performed as single spot per probe on Ultra GAPS glass slides (Corning, Fontainebleau, France) using Genetix Qarray mini robot (TeleChem International Inc., Saint Marcel, France). Atmosphere moisture was set at 50% and plates were cooled to 15°C to prevent drying. Spotting design was as follows: 48 grids, each grid contains 24 × 25 spots. Each slide bears 28,000 spots corresponding to 27,004 transcripts and 22,012 rat genes.

Preparation of labeled cDNA. Total RNA (5 μg) was used for cDNA preparation. Reverse transcription was made using ChipShot™ reverse transcriptase from Promega (Promega, Charbonnieres Les Bains, France) using Cy3- and Cy5-dCTP from Amersham Bioscience. cDNA purification was made by the Promega ChipShotTM labeling Clean-up system. Dye incorporation was checked by measuring the absorbance at 260 and 550 nm for Cy3 and 260 and 650 nm for Cy5 in a NanoDrop spectrophotometer (Nyxor Biotech).

Array hybridization and scanning. Equal amounts of Cy3- and Cy5-labeled cDNA from control and tested groups were hybridized for 8 h at 42°C in the automatic Hybridization Machine Discovery from Ventana (Ventana Medical Systems, Illkirch, France). After hybridization, slides were washed twice in 2× SSC (Ribowash buffer, Ventana Medical Systems) for 5 min with agitation and then once with 0.1× SSC buffer. After washing, slides were dried by centrifugation. Slides were scanned with Axon 4000A Microarray Scanner (Molecular Devices, Saint-Grégoire, France).

Data analysis and public access. All data reported here are available to public, via the NCBI-GEO platform, as both original and normalized data. Image analysis was made by Genepix Pro V6 software. Each spot was evaluated and selected to be analyzed by Bioplot software (http://biopuce.insa-toulouse.fr). Spot analysis consisted in background subtraction with Lowess allowed value 10, log transformation, and Lowess normalization. The generated data set has been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/) database (GSE7654).

Data analysis consisted of a variance analysis. The whole data set (27,004 gene probes) was subjected to an ANOVA using SPLUS 2000 software (MathSoft Inc., Seattle, WA). Significance was set as p-value <

0.05. PLS-2, PLS-DA, and orthogonal signal correction (OSC) were made using SIMCA-P 8.0 software (Umetrics, Umea, Sweden). Oligos that contribute to axis building for both PLS-2 and PLS-DA analyses were defined by the Variable Importance Projection (VIP) criterion given by the SIMCA-P 8.0 software.

For biological organization of data, gene ontology (GO) classification was performed by Gene Ontology Tree Machine from Vanderbilt University (http://bioinfo.vanderbilt.edu/gotm) (Zhang et al., 2004).

Statistical analyses

Multivariate statistical analyses are described above. Univariate statistical analysis for data from qRT-PCR, proteasome activities, and immunoblotting are expressed as means and standard deviations. Statistical differences were determined by ANOVA, followed by Bonferroni's *post hoc* test.

RESULTS

Group classification

As reported in Table 1, rats were classified into five groups according to rat disease state, age and treatment. *Normal 6w* and *Normal 9w* groups correspond to nondiseased young LEC rats (6 and 9 weeks old, respectively) that showed low level of liver enzymes ALT, AST in plasma, and a normal liver architecture. *Slight jaundice* and *Jaundice* groups are LEC rats at different hepatitis stages. These two groups showed increased levels of hepatitis markers (ALT, AST, and t-bil) in plasma, together with abnormal liver histology (i.e., necrosis, apoptosis, and nuclear polymorphism) and an increase in lipid peroxidation as measured by urinary excretion of 8-iso$PGF_{2\alpha}$. The last group corresponded to D-penicillamine-treated LEC rats. As expected, these rats showed low liver enzymes in plasma, normal liver histology, and low levels of urinary 8-iso$PGF_{2\alpha}$. Because they behaved as normal rats, *D-penicillamine*-treated rats were taken as a control group for microarrays analysis. In order to evaluate the effect of D-penicillamine in gene expression, Long-Evans rats were treated with D-penicillamine in a similar way as in *D-penicillamine* LEC group. Gene expression was compared to untreated Long-Evans rats. No significant difference between those two groups was found (data not shown).

Microarray analysis and validation

The rat pangenomic microarray used in this study was made of 27,004 oligo probes that corresponded to 22,012 rat genes. Lowess-normalized data sets with test/control (i.e., untreated/D-penicillamine-treated rats) ratios were obtained from Bioplot software. Data sets were then filtered by a one-way ANOVA analysis when considering the group factor. From 27,004 oligo probes, 696 probes with a p-value < 0.05 were considered as significant variables. In order to discriminate between genes related to hepatitis progression and those mainly explained by an age effect, a PLS-2 regression between the 696 statistically significant genes and plasma hepatitis markers ALT, AST, and t-bil was performed (Fig. 1A). The goal of this method is to analyze or predict a set of dependent variables (matrix Y, i.e., plasma hepatitis markers) from a set of independent variables (matrix X, i.e., microarray data) or predictors (Abdi, 2003). PLS-2 regression showed a projection in which the *Slight Jaundice* and *Jaundice* groups were well separated from the non diseased groups, *Normal 6w* and *Normal 9w*. However, these two latter groups could not be separated between each other using this analysis. The 696 genes were hence analyzed by a PLS-DA in order to refine the separation between all groups of rats (Fig. 1B). PLS-DA is a supervised multidimensional statistical method that uses principal component analysis (PCA) principles, that is, data projection into different dimensions, to find those components which account for differences between groups. As shown in Figure 1B, the projection in the first component (t1) separated the non diseased rats (*Normal 6w* and *Normal 9w* groups) from diseased rats, and even more, this mode of analysis was able to improve the separation between groups of rats exhibiting a slight jaundice from those with a stronger disease. The separation between groups was validated using another discriminant analysis, the factorial discriminant analysis (FDA), in which using only 10 variables selected from the first 696 ones by a specific algorithm (Dumas et al., 2002), Δ^2 Mahalanobis distances between all different groups were significantly different from zero ($p < 0.001$) (data not shown).

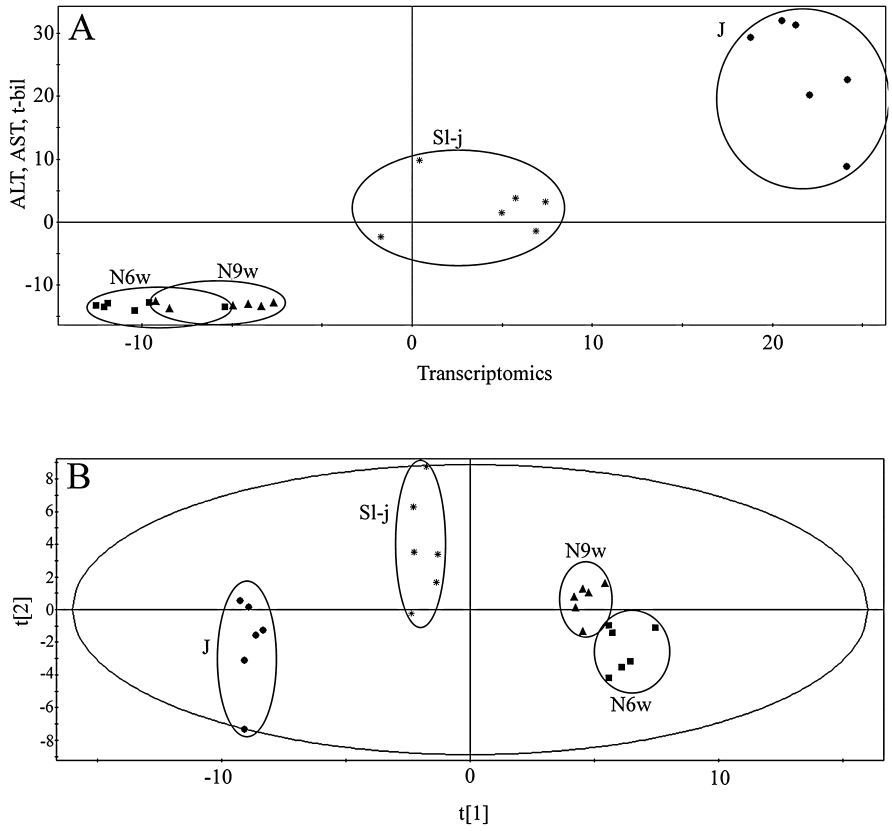


FIG. 1. Relation between hepatitis progression and gene expression. (**A**) Six hundred ninety-six statistically significant variables from transcriptomic data ($p < 0.05$) were used for a PLS-2 regression against plasma hepatitis markers, ALT, AST, and t-bil. (**B**) PLS-DA of transcriptomic data. Circles: rats within the same group. N6w: Normal 6w. N9w: Normal 9w. Sl-j: Slight jaundice. J: Jaundice.

The oligo probes that contribute to the axis building were defined using the VIP criterion given by the SIMCA-P 8.0 software. Statistical VIP criterion is a measure which quantifies the influence on the response (on the latent variable describing the projection of hepatitis development) of each variable summed over all components and categorical responses relative to the total sum of squares of the model (Perez-Enciso and Tenenhaus, 2003). It is a useful tool to select specific sets of genes that distinguishes animals at different stages of hepatitis, making it an important parameter to find possible (metabolic) pathways involved in disease development. From the 696 tested genes, 483 were considered as important for axis projection (VIP > 0.8) for the PLS-2 analysis and 239 for de PLS-DA. Fifty percent of the 239 genes found to be important for the PLS-DA were also found to be important in the PLS-2 analysis, demonstrating that the separation of groups in the PLS-DA is mainly due to hepatitis development. Table A (supplementary data) provides the list of these 239 genes ordered by decreasing importance of the VIP value.

GSTP was found among the list of genes that significantly discriminated between groups. We confirmed the expression changes of this gene by the qRT-PCR method using β-actin as the reference gene, $F(3,23) = 54.82$, $p = 0.0104$ (Fig. 2). Therefore, this data, together with the fact that this gene is considered as a tumor marker known to be expressed in LEC rats during hepatitis (Masuda et al., 1989), consolidated the validity of our transcriptomic analysis of LEC rats during hepatitis development.

Functional classification of VIP genes

To gain more biological insights of our expression data, the list of 239 genes with a significant VIP value (VIP > 0.8) were subjected to a functional classification based on GO annotation. Only 40% of the total

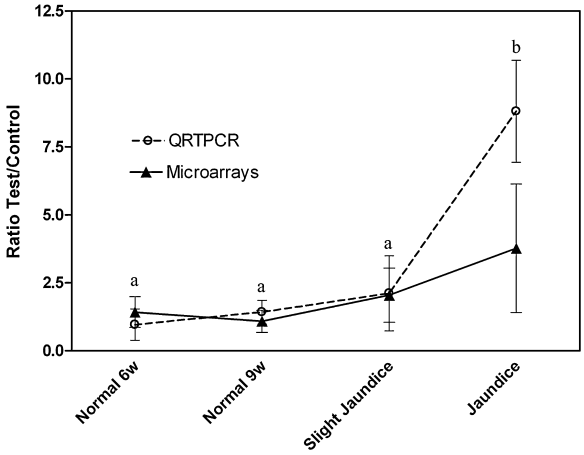
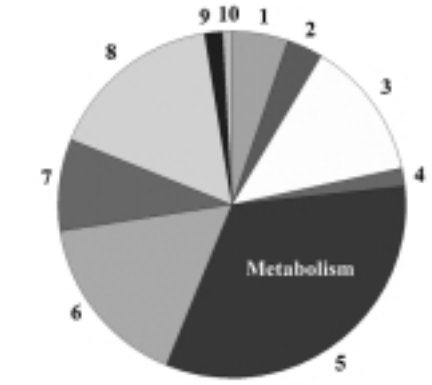


FIG. 2. Confirmation of the differential expression of GSTP by quantitative RT-PCR. Triangles: GSTP expression units from microarray data. Open circles: GSTP expression units from TaqMan QRTPCR assay. Data is expressed as ratio test/control ratio (means ± SD) where control is the D-penicillamine-treated rat group. Means without a common letter differ as determined by a one-way ANOVA and the Bonferroni *post hoc* test.



GO Biological Process (GO: 008150): 59, 100%

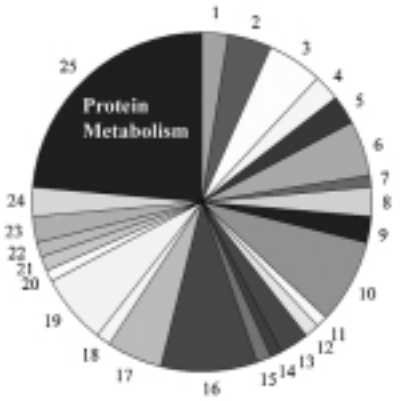
1. Death (GO: 0016265): 6, 5.2%
2. Homeostasis (GO: 0019725): 4, 3.5%
3. Localization (GO: 0051179): 15, 13.0%
4. Locomotion (GO: 0040011): 2, 1.7%
5. Metabolism (GO: 0044237): 38, 33.0%
6. Organismal Physiological Process (GO: 0032501): 19, 16.5%
7. Physiological Response to Stimulus (GO: 0050896): 10, 8.7%
8. Regulation of Physiological Process (GO: 0050794): 19, 16.5%
9. Reproductive Physiological Process (GO: 0048610): 2, 1%
10. Rhytmic Process (GO: 0048511): 1, 0.9%

FIG. 3. Gene ontology classification for 59 known genes. Classification by biological process was made (1–10). Chart data are read as: category name (accession): number of tested genes annotated in this category, percent of tested genes annotated in this category against total tested genes having a GO annotation.

rat genome has a gene ontology annotation (Lomax, 2005). This explained that only 59 genes had a GO annotation in Biological Process ontology from the 239 selected genes (Fig. 3). From these GO-annotated genes, 38 fall into Metabolism, 19 in Organismal Physiological Process, and 19 in Regulation of Physiological Process categories. A subclassification in the Cellular Metabolism category revealed that genes implicated in Protein Metabolism, and in Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolism were overrepresented (Fig. 4). Values of expression changes of these genes and their associated function are described in Tables 2 and 3.

Proteasome activity

The proteasome is a large, 26S, multicatalytic protease that degrades polyubiquitinated proteins to small peptides. It is composed of two subcomplexes: a 20S core particle that carries the catalytic activity, and the regulatory 19S particle (Ciechanover, 2005). The main function of the proteasome is to degrade superfluous and/or damaged proteins by proteolysis. Our DNA microarray data revealed that several genes in the Protein Metabolism subcategory whose expression was increased in LEC rats during hepatitis are implicated in protein degradation by the proteasome (Table 2). Furthermore, a heat map of proteasome-related genes showed a tendency of upregulation of almost all genes that encode the 19S and 20S proteasome in the *Jaundice* group of rats (Fig. 5). Among them, six genes were significantly increased. This result sug-



GO Metabolism (GO: 0044237): 38, 100%
1. Cellular Biosynthesis (GO: 0044249): 2, 2.7%
2. Lipid Biosynthesis (GO: 008610): 2, 4.0%
3. Cellular Catabolism (GO: 0044248): 4, 5.3%
4. Lipid Catabolism (GO: 0016042): 2, 2.7%
5. Macromolecule Catabolism (GO: 009057): 2, 2.7%
6. Alcohol Metabolism (GO: 006066): 4, 5.3%
7. Amine Metabolism (GO: 0009308): 1, 1.3%
8. Amino acid and Derivate Metabolism (GO: 0006519): 2, 2.7%
9. Aromatic Compound Metabolism (GO: 0006725): 2, 2.7%
10. Cellular Lipid Metabolism (GO: 0044255): 6, 8.0%
11. Cofactor Metabolism (GO: 0051186): 1, 1.3%
12. Generation of Precursor Metabolites and Energy (GO: 0043467): 1, 1.3%
13. Heterocycle Metabolism (GO: 0046483): 2, 2.7%
14. Hormone Metabolism (GO: 0042455): 1, 1.3%
15. Neurotransmitter Metabolism (GO: 0042133): 1, 1.3%
16. Nucleobase, Nucleoside, Nucleotide and Nucleic Acid Metabolism (GO: 0006139): 7, 9.3%
17. Organic Acid Metabolism (GO: 0006082): 4, 5.3%
18. Phosphorus Metabolism (GO: 0006793): 1, 1.3%
19. Regulation of Cellular Metabolism (GO: 0031323): 5, 6.7%
20. Sulfur Metabolism (GO: 0006790): 1, 1.3%
21. Vitamin Metabolism (GO: 0006766): 1, 1.3%
22. Xenobiotic Metabolism (GO: 0006805): 1, 1.3%
23. Carbohydrate Metabolism (GO: 0005975): 2, 2.7%
24. Macromolecule Catabolism (GO: 0044265): 2, 2.7%
25. Protein Metabolism (GO: 0044260): 18, 24%

FIG. 4. Metabolism ontology subclassification. Genes being part of metabolism classification were subclassified according to their specific activity (1–25). Chart data are read as: category name (accession): number of tested genes annotated in this category, percent of tested genes annotated in this category against total tested genes having a GO annotation.

Table 2. Differential Genes Classified in GO: Protein Metabolism

Description	*Symbol*	*Oligo_id*	*ENSEMBLE Gene_id*	*Genbank*	*Ratio*				p *value*
					Normal 6w	*Normal 9w*	*Slight jaundice*	*Jaundice*	
Cathepsin D precursor (EC 3.4.23.5)	Ctsd	Rn30018601	ENSRNOG00000020206	NM_134334	1.14[a]	1.09[a]	1.86[a,b]	2.44[b]	0.0207
Glycine N-methyltransferae (EC 2.1.1.20)	Gnmt	Rn30014947	ENSRNOG00000016349	X06150	0.73[a]	0.85[a,b]	0.55[b]	0.41[b]	0.0050
C-reactive protein precursor	Crp	R002545_01	ENSRNOG00000000053	DW387982	0.68[a]	0.89[a,b]	0.45[b]	0.35[b]	0.0344
H-2 class II histocompatibility antigen, gamma chain	Cd74	Rn30017236	ENSRNOG00000018735	CB576583	1.06[a]	1.15[a]	1.73[b]	2.36[c]	0.0125
Meprin A alpha-subunit precursor (EC 3.4.24.18)	Mep1a	R004430_01	ENSRNOG00000011022	BC081834	0.97[a]	0.95[a]	0.61[b]	0.92[a]	0.0351
proteasome 26S non-ATPase subunit 12	Psmd12	Rn30002766	ENSRNOG00000003117	BC083758	1.10[a,b]	1.01[a]	1.05[a,b]	1.48[b]	0.0160
Calpain small subunit 1 (CSS1)	Capns1	Rn30001369	ENSRNOG00000001503	BC098068	0.94[a,b]	0.98[a]	1.11[a,b]	1.39[b]	0.0408
Serum amyloid P-component precursor (SAP)	Apcs	R000224_01	ENSRNOG00000009086	X55761	1.01[a]	0.84[a,b]	0.41[b]	0.54[a,b]	0.0071
Complement C1q subcomponent subunit A precursor	C1qa	Rn30011801	ENSRNOG00000012807	BC086605	1.00[a]	0.95[a]	1.67[a,b]	1.91[b]	0.0439
TGFB inducible early growth response 3 (predicted)	Gna14	Rn30013625	ENSRNOG00000014840	BC090316	0.90[a]	0.96[a]	1.24[b]	0.94[a]	0.0465
Kit ligand precursor (C-kit ligand)	Kitl	R003524_01	ENSRNOG00000005386	NM_021843	1.09[a]	0.96[a]	0.90[a]	1.46[b]	0.0406
Legumain precursor (EC 3.4.22.34)	Lgmn	Rn30006415	ENSRNOG00000007089	BC087708	1.05[a,b]	0.93[a,b]	1.36[a]	1.57[b]	0.0169
Ubiquitin-conjugating enzyme E2 B (EC 6.3.2.19)	UBC2_HUMAN	Rn0004545	ENSRNOG00000005064	AF144083	1.01[a,b]	0.99[a,b]	1.17[a]	1.52[b]	0.019
Proteasome subunit beta type 8 precursor (EC 3.4.25.1)	Psmb8	R004525_01	ENSRNOG00000000456	NM_080767	1.11[a]	1.07[a]	1.16[a]	1.27[b]	0.0317
Proteasome subunit alpha type 6 (EC 3.4.25.1)	Psma6	Rn30006440	ENSRNOG00000007144	NM_017283	1.10[a]	1.01[a]	1.16[a,b]	1.44[b]	0.0132
Proteasome subunit beta type 5 precursor (EC 3.4.25.1)	Psmb5	Rn30012312	ENSRNOG00000013386	XM_341314	0.93[a]	1.05[a]	1.15[a,b]	1.43[b]	0.0213
proteasome 26S, non-ATPase regulatory subunit 6	Psmd6	Rn30006048	ENSRNOG00000006751	BC059159	1.03[a]	1.03[a]	0.89[a]	1.60[b]	0.0306
Aminopeptidase B (EC 3.4.11.6)	Rnpep	R002043_01	ENSRNOG00000006720	AY724503	0.86[a]	0.95[a]	0.62	1.11[a]	0.0465

Means without a common letter differ as determined by ANOVA and the Bonferroni *post hoc* test.

Table 3. Differential Genes Classified in GO: Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolism

Description	*Symbol*	*Oligo_id*	*ENSEMBLE Gene_id*	*Genbank*	*Ratio*				p *value*
					Normal 6w	*Normal 9w*	*Slight jaundice*	*Jaundice*	
Uricase (EC 1.7.3.3)	Uox	Rn30014939	ENSRNOG00000016339	M24396	0.69[a]	1.04[a,b]	0.57[b]	0.50[b]	0.0234
Growth arrest and DNa-damage-inducible protein GADD45 alpha	Gadd45a	R001492_01	ENSRNOG00000005615	CO396252	1.00[a]	0.94[a]	1.06[a,b]	1.49[b]	0.0055
Signal transducer and activator of transcription 3	Sta3	Rn30018174	ENSRNOG00000019742	NM_012747	0.84[a]	0.86[a]	1.56[b]	1.72[b]	0.0498
Dynamin-2 (EC 3.6.5.5)	Dnm2	Rn30006927	ENSRNOG00000007649	L25605	1.03[a]	1.06[a]	1.03[a]	1.58[b]	0.0151
Runt-related transcription factor 1, Acute myeloid leukemia 1 protein	Runx1	Rn30001533	ENSRNOG00000001704	L35271	0.93[a]	1.05[a]	1.27[b]	1.23[b]	0.0280
Cellular nucleic acid binding protein, Zinc finger protein 9	Cnbp1	R000749_01	ENSRNOG00000010239	CB743905	1.06[a,b]	1.09[a]	1.07[a,b]	1.45[a,b]	0.0298
DNA-repair protein XRCC1	Xrcc1	Rn30018333	ENSRNOG00000019915	AF290895	1.02[a,b]	0.98[a]	1.22[a,b]	1.54[a,b]	0.0338

Means without a common letter differ as determined by ANOVA and the Bonferroni *post hoc* test.

gested that the proteasome activity could be modified. To verify this hypothesis, we determined the peptidase activities of 20S proteasome using specific fluorogenic peptides as substrates in cytosolic fractions (Fig. 6). Unexpectedly, there was no significant modification in the chymotrypsin-like (ChT) proteasome activity (Fig. 6A) even when the gene encoding to the proteasome subunit responsible for this activity, the *Pmsb5* gene, was significantly upregulated (Table 2). Moreover, we found a significant decrease in trypsin-like (TL) proteasome activity in the *Slight jaundice* and *Jaundice* groups of rats, $F(4, 25) = 58.62$, $p < 0.0001$ (Fig. 6B). Likewise, the peptidylglutamyl peptide hydrolase (PGPH) proteasome activity was diminished by about 30% in the *Slight jaundice* group (Fig. 6C), but restored in the *Jaundice* rat group $F(4, 25) = 13.01$, $p = 0.0075$. Both genes responsible for TL (*Psmb2* gene) and PGPH (*Psmb1* gene) had a tendency of being overexpressed during hepatitis (Fig. 5A). In order to evaluate whether changes in proteasome activities were due to variations in proteasome protein levels, we evaluated the 20S proteasome levels in cytosol by Western blotting. As shown in Figure 7, 20S proteasome levels were increased by 1.5- and 1.8-fold, respectively, in *Slight jaundice* and *Jaundice* groups with respect to control *D-penicillamine* group, $F(4, 25) = 10.86$, $p = 0.015$. Thus, a decrease in TL and PGPH proteasome activities does not correspond to a decrease in 20S proteasome levels but rather to an inhibition of the intrinsic protein activity.

DISCUSSION

A first evaluation of gene expression in LEC rats was made by Klein et al. in 2003 (Klein et al., 2003). These authors compared the difference in gene expression patterns between female LEC rats at different hepatitis stages using Long-Evans agouti (LEA) rats as controls. Although LEA rats are often used as control rats in LEC rat studies, and derive from a common ancestor (Long-Evans rats), they may have slightly evolved differently as indicated by differences in epoxide hydrolase, gamma-glutamyltranspeptidase and UDP-glucuronyltransferase enzyme activities and in cytochrome P-450 content between LEC and LEA rats (Sugiyama et al., 1988). Here we used *D-penicillamine*-treated LEC rats as control group for transcriptomic comparisons. Histological and clinical features of these rats showed that they behave as normal rats (Table 1), according to the known effect of D-penicillamine to inhibit hepatitis and hepatocarcinogenesis in LEC rats (Klein et al., 2000; Togashi et al., 1992). Moreover, D-penicillamine might not have effect in gene transcription since normal Long-Evans rats treated with D-penicillamine showed no significant changes in gene expression in comparison with untreated Long-Evans rats. Thus, we considered *D-penicillamine*-treated LEC rats as the most suitable control for studying the mechanisms involved in hepatitis generation in LEC rats. Rat groups were defined by their liver hepatitis stage as interpreted by the plasma levels of classical hepatitis markers ALT, AST, t-bil and from liver histological observations. Oxidative stress increased with development of the hepatitis as seen by high levels of urinary 8-Iso$PGF_{2\alpha}$, a widely used marker of lipid peroxidation (Liu et al., 1998). This data confirmed that copper-induced oxidative stress might be the cause of hepatitis and hepatocarcinogenesis in LEC rats. From their transcriptomic analysis, Klein et al. have only retained genes with the highest expression changes, and from these data, concluded on a major role of oxidative stress, inflammation and DNA damage in the hepatitis progression. However, this mode of data analysis is too restrictive, and there is no obvious relationship between the levels of gene expression and the intensity of the disease, because alterations in a given cellular activity are usually not the result of a single but often depends on the coordinated effects of multiple genes (Valafar, 2002).

To optimize the exploration of large data sets such as transcriptomic data in a given biological context, multivariate statistical methods followed by the Gene Ontology classification represent powerful tools of analysis. PLS-2 analysis between microarray and plasma hepatitis markers showed a projection of group of animals regarding hepatitis, which means a strong correlation between gene expression changes and the development of hepatitis in LEC rats. This method was effective to separate between nondiseased groups from jaundice groups, but it was not pertinent enough to discriminate the two nondiseased groups (*Normal 6w* and *Normal 9w*), probably because the number of hepatitis markers is much reduced and their values are very similar between these groups. PLS-DA takes the advantage of prior class information to attempt to maximize the separation between groups of observations (Perez-Enciso and Tenenhaus, 2003). All groups were significantly separated from each other by PLS-DA. Furthermore, more than 50% of VIP-positive

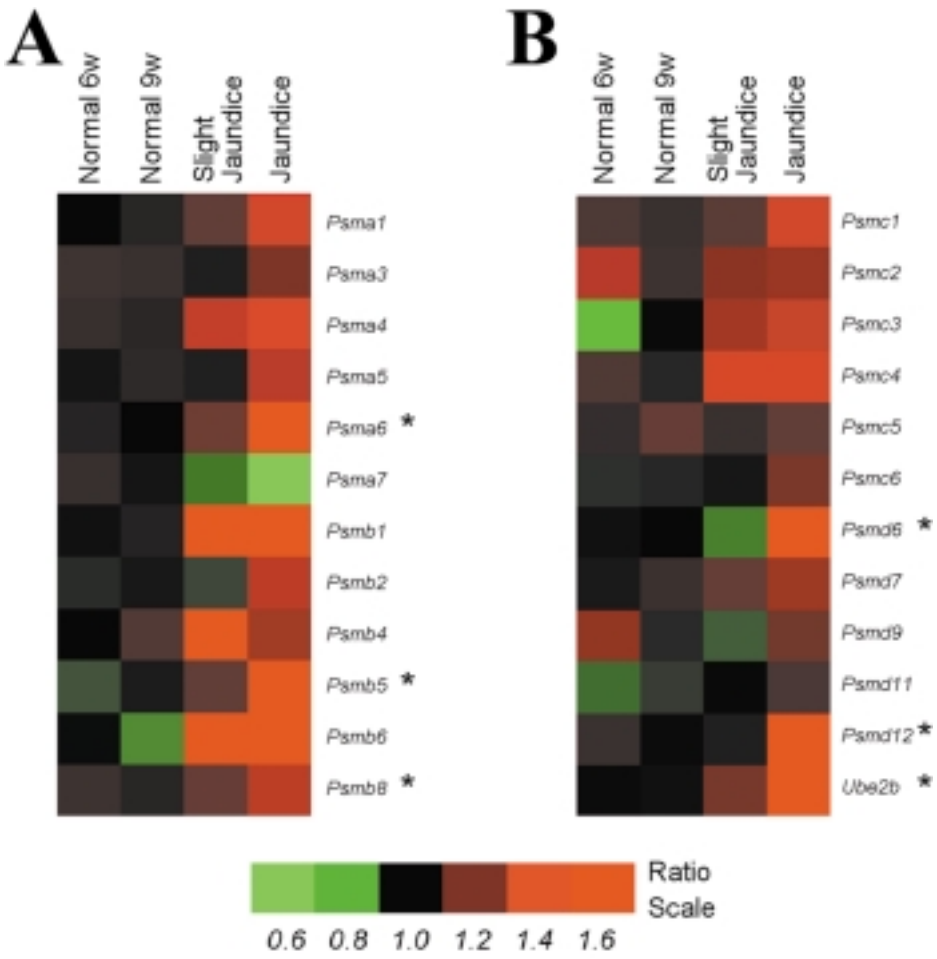


FIG. 5. Heat map of proteasome-related genes in the liver of LEC rats at different disease stages. Mean expression ratios of proteasome-related genes in each rat groups in respect to D-penicillamine-treated rats. Green and red represent downregulation and upregulation, respectively, as indicated in the "ratio scale" bar. (**A**) 20S proteasome-related genes. (**B**) 19S proteasome-related genes. $*p < 0.05$.

genes from PLS-DA were common to those coming from PLS-2 regression, demonstrating that PLS-DA group separation is also well correlated to animal hepatitis development.

Statistical VIP criterion serves as a powerful tool to select the variables that have a strong impact on the building of the axes in PLS methods. A high VIP value implies a strong influence of the variable in the axes building (Perez-Enciso and Tenenhaus, 2003). GSTP presented one of the highest VIP value (VIP value = 3.902), suggesting that the increase in expression of this gene was very important for separation between groups of rats in the PLS-DA. This finding agrees with the fact that GSTP has been found in liver of LEC rats during hepatitis (Masuda et al., 1989; Oyamada et al., 1988). Furthermore, because GSTP is considered as one of the most reliable tumor markers in liver (Sawaki et al., 1990), the idea of cancer initiation during hepatitis in LEC rats is enforced.

During hepatitis progression in LEC rats, a strong oxidative stress takes place (Yamamoto et al., 2001). Oxidation of biomolecules as DNA, lipids, and proteins has been extensively described (Marquez et al., 2007; Nair et al., 2005; Yamamoto et al., 1999; Yasuda et al., 2006). According to these observations, our analyses of microarray data show enrichment of genes that belong to Protein Metabolism and Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolism established by GO classification. Genes involved in Nucleobase, Nucleoside, Nucleotide, and Nucleic Acid Metabolism, and genes involved in DNA reparation

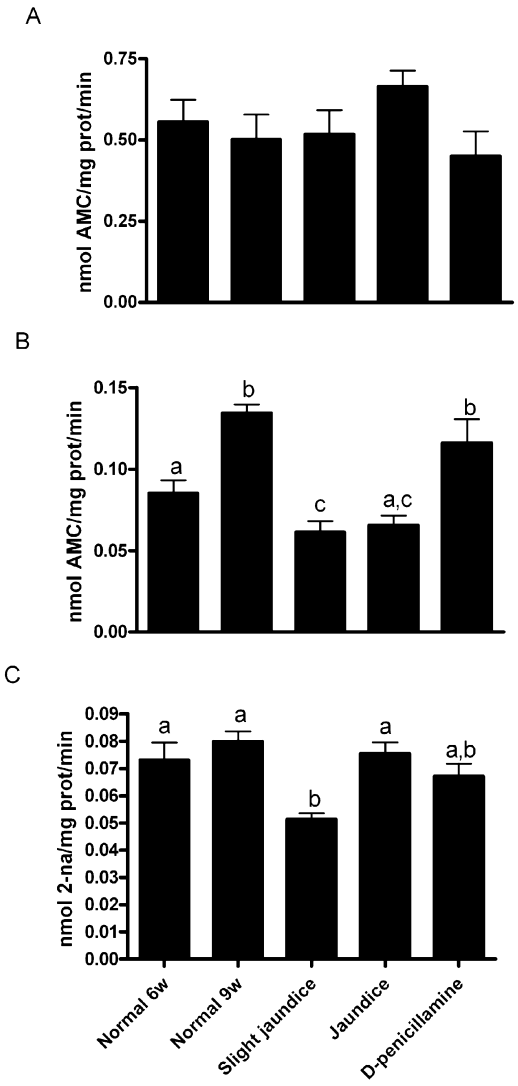


FIG. 6. Changes in proteasome activities in the liver of LEC rats at different disease stages. The proteasome activities in the liver of LEC rats in different groups were measured using the fluoropeptides, s-LLVY-AMC for the ChL activity (**A**), Boc-LSTR-AMC for the TL activity (**B**), and Z-LLE-βNA for the PGPH activity (**C**), as substrates. Each determination was performed at least in triplicate. Values refer to proteasome activity means ± SD. Means without a common letter differ as determined by a one-wayANOVA and the Bonferroni *post hoc* test.

are upregulated in the *Jaundice* group. Growth Arrest and DNA Damage 45-alpha (GADD45-alpha) is a nuclear protein involved in the maintenance of genomic stability, DNA repair, and suppression of cell growth. Overexpression of GADD45 gene has been reported in LEC rats at early stages of hepatitis suggesting important DNA damage (Klein et al., 2003). The mechanisms by which oxidized DNA bases are repaired include the base excision repair (BER) pathway. M1G, etheno–DNA adducts, 8-hydroxyguanine, and other oxidized base lesions are removed by this pathway (Valko et al., 2006). Acute hepatitis in LEC rats impairs the repair of oxidative DNA base damage by altering the activity of DNA glycosylases, endonuclease II, and 8-oxoguanine DNA-glycosylase, which initiate the BER pathway (Choudhury et al., 2003). Interestingly, our results show overexpression of the DNA-repair protein XRCC1 gene. XRCC1 protein seems to play a central role in coordinating the enzymes participating in BER pathway (Marsin et al., 2003). Moreover, the XRCC1 protein interacts with all enzymes of BER downstream of DNA-glycosylase,

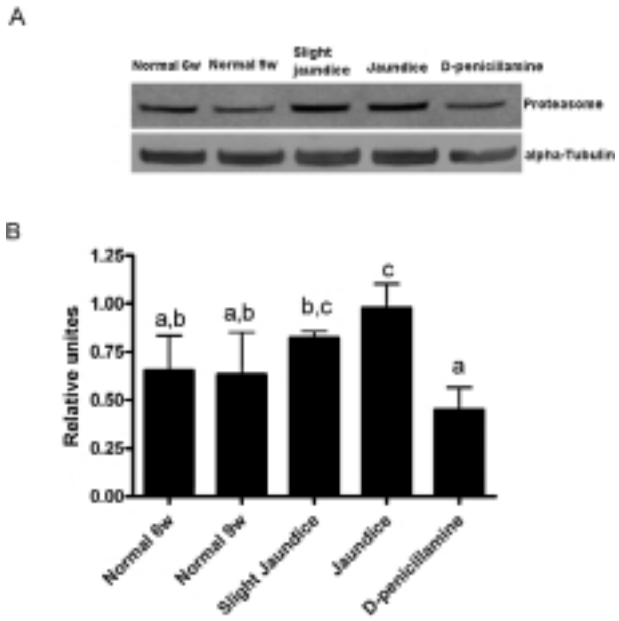


FIG. 7. Immunodetection of proteasome in liver of LEC rats at different disease stage and treatment. (**A**) Representative image of immunoblot analysis for 20S proteasome and alpha-tubulin proteins. (**B**) Densitometry analysis of immunoblot assay, values refer to proteasome/alpha-tubulin ratio means ± SD ($n = 6$). Means without a common letter differ as determined by a one-way ANOVA and the Bonferroni *post hoc* test.

leading to the stabilization of the corresponding enzyme. Hence, upregulation of XRCC1 in LEC diseased groups may contribute to BER pathway recovery in LEC rats. Further investigations are needed to determine the role of XRCC1 in the BER pathway in LEC rats. Protein Metabolism was the one category with the highest amount of associated genes. The results may indicate an increase in protein degradation, because genes falling in this category are genes related to ubiquitin–proteasome system and to other peptidases. Increase in protein degradation could reflect protein damage as protein oxidation (Grune et al., 1995). According to our results showing overexpression of genes involved in both the 19S and 20S proteasome systems, our initial hypothesis was that proteasome activity should be augmented in *Slight jaundice* and *Jaundice* groups. However, TL and PGPH 20S proteasome peptidase activities were diminished in these groups. Protein levels of proteasome were significantly increased in *Slight jaundice* and *Jaundice* groups compared to the *D-penicillamine* group, suggesting that the diminution of proteasome activities may not be due to less proteasome proteins in the cell, but rather because of a direct inhibition of protein activity.

If oxidized proteins are not eliminated through the proteasome pathway, they are able to accumulate and to aggregate (Grune et al., 2003). Oxidized protein aggregates are very difficult to eliminate because they cannot be unfolded, an important step in protein degradation, and they are able to crosslink with proteasome subunits enhancing proteasome inhibition. 4-Hydroxy-2-nonenal (HNE), a major end product of lipid peroxidation, modifies proteins leading to the formation of such protein aggregates (Grune and Davies, 2003a). Furthermore, HNE can inhibit directly the proteasome activity by covalent binding to proteasome subunits. Okada et al. (1999) have found similar results for the inhibition of TL and PGPH peptidase activities under oxidative stress conditions in the kidney. The authors concluded that the HNE–proteasome adduction was partially responsible for the proteasome inhibition under oxidative stress conditions (Okada et al., 1999). Although the detailed mechanism of inactivation of proteasome with HNE is not fully understood, the authors suggest that the active site of the enzymes (TL and PGPH) may preferentially react with HNE, leading to inactivation. These findings lead us to propose, for the first time, the inhibition of proteasome activities by oxidative stress during hepatitis progression in LEC rats.

Inhibition of proteasome activities may induce the expression of proteasome genes in a feedback mechanism that would compensate for the reduced protein degradation level. Feedback regulation of proteasome

genes have been described in *Saccharomyces cerevisiae*. Proteasome gene expression was mediated by the Rpn4 protein, which binds to PACE (proteasome-associated control element) sequences that are found in the promoters of proteasome subunits (London et al., 2004). The Rpn4 protein is actually degraded by the proteasome system; stabilization of Rpn4 by proteasome inhibition or malfunction leads to augmented transcription of proteasome subunits by a negative feedback circuit (Xie and Varshavsky, 2001). In mammal cells, Meiners et al. (2003), have studied the expression of proteasome genes after the inhibition of ChT proteasome peptidase activity by specific proteasome inhibitors. They showed that there is a concerted change in the expression of almost all the 20S proteasome subunits following proteasome activity inhibition. Our results agreed with Meiners observations, because even when not all the genes were statistically significantly overexpressed, there was a clear tendency for an upregulation of the majority of genes encoding for proteasome (Fig. 5). The implication of a common transcription factor like Rpn4 in transcriptional regulation of proteasome genes in mammal cells remains to be elucidated.

The proteasome is an essential organelle that participates in different cellular functions, like apoptosis, survival, and DNA reparation (Reed and Gillette, 2007). Alterations in proteasome activity have been related to metabolic perturbations, aging and different pathologies including Parkinson and Alzheimer diseases (Ciechanover, 2005; Hayashi and Goto, 1998; Reed and Gillette, 2007). Oxidized and damaged protein accumulation may alter cellular functions, giving rise to deleterious effects on cellular homeostasis that could be involved in initiation and progression of carcinogenesis (Cecarini et al., 2007; Friguet, 2006). Interestingly, proteasome inhibition, either by specific inhibitor or by iRNA, induces the expression of GSTP gene in epithelial liver cells. Mechanisms of GSTP induction by proteasome inhibition seem to be different in the Nrf2/Keap1 pathway (Usami et al., 2005). Further studies are necessary to know if there is an association between proteasome inhibition and GSTP upregulation in LEC rats; however, the idea of proteasome inhibition in cancer initiation is enforced.

In conclusion, using the LEC rat model, our work presents for the first time a genome-scale analysis of hepatitis development and cellular responses due to copper-induced oxidative stress. In this study, multivariate statistical analysis coupled to a biological interpretation achieved by GO classification of the transcriptomic data allowed us to focus on proteasome system and on the consequences of proteasome inhibition as a main deregulated pathway that might be a determining factor during oxidative stress-induced hepatitis and further cancer initiation. Our work shows an original and functional way of analysis that illustrates the power of multivariate statistical tools to explore microarray data to raise rational biological hypothesis.

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