# Elevated serum β-glucuronidase reflects hepatic lysosomal fragility following toxic liver injury in rats

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**Abstract:** The level of serum β-glucuronidase increases in various pathological conditions, including liver disorders. The aim of this investigation was to study the changes in liver lysosomal membrane stability during experimentally induced hepatic fibrosis that may result in the elevation of serum β-glucuronidase. Liver injury was induced by intraperitoneal injections of *N*-nitrosodimethylamine (NDMA) in adult male albino rats over 3 weeks. The progression of fibrosis was evaluated histopathologically as well as by monitoring liver collagen content. Lipid peroxides and β-glucuronidase levels were measured in the liver homogenate and subcellular fractions on days 0, 7, 14, and 21 after the start of NDMA administration. Serum β-glucuronidase levels were also determined. A significant increase was observed in β-glucuronidase levels in the serum, liver homogenate, and subcellular fractions, but not in the nuclear fraction on days 7, 14, and 21 after the start of NDMA administration. Lipid peroxides also increased in the liver homogenate and the lysosomal fraction. The measurement of lysosomal membrane stability revealed a maximum lysosomal fragility on day 21 during NDMA-induced fibrosis. In vitro studies showed that NDMA has no significant effect on liver lysosomal membrane permeability. The results of this investigation demonstrated that lysosomal fragility increases during NDMA-induced hepatic fibrosis, which could be attributed to increased lipid peroxidation of lysosomal membrane. In this study, we also elucidated the mechanism of increased β-glucuronidase and other lysosomal glycohydrolases in the serum during hepatic fibrosis.

*Key words:* lysosomal fragility, lipid peroxidation, oxidative stress, β-glucuronidase, *N*-nitrosodimethylamine, hepatic fibrosis.

**Résumé :** Les niveaux de β-glucoronidase sérique augmentent dans différentes conditions pathologiques dont les maladies hépatiques. Le but de cette recherche était d'étudier les altérations de la stabilité de la membrane lysosomale du foie qui pourraient conduire à une élévation de β-glucuronidase sérique lors d'une fibrose hépatique induite expérimentalement. Le dommage hépatique a été induit par des injections intrapéritonéales de *N*-nitrosodiméthylamine (NDMA) chez des rats mâles adultes albinos pendant trois semaines. La progression de la fibrose a été évaluée par histopathologie et en surveillant le contenu de collagène du foie. Les niveaux de lipides peroxydes et de β-glucuronidase ont été mesurés dans l'homogénat de foie et dans les fractions subcellulaires, 0, 7, 14 et 21 jours après le début de l'administration de NDMA. Les niveaux de β-glucuronidase sérique ont aussi été déterminés. Une augmentation significative des niveaux de β-glucuronidase du sérum, de l'homogénat de foie et des fractions subcellulaires a été observée, contrairement à la fraction nucléaire, 7, 14 et 21 jours après de début de l'administration de NDMA. Les lipides peroxydes ont aussi augmenté dans l'homogénat de foie et dans la fraction lysosomale. La mesure de la stabilité de la membrane lysosomale a révélé que la fragilité était maximale au jour 21 de la fibrose induite par NDMA. Des études *in vitro* ont montré que le NDMA n'avait pas d'effet significatif sur la perméabilité de la membrane lysosomale. Les résultats de l'étude présente ont démontré qu'il y avait une augmentation de la fragilité lysosomale lors de la fibrose hépatique induite par NDMA, ce qui pourrait été attribuable à l'augmentation de la peroxydation lipidique de la membrane lysosomale. L'étude présente a aussi élucidé le mécanisme responsable de l'augmentation de la β-glucuronidase et des autres glycohydrolases lysosomales dans le sérum lors de la fibrose hépatique.

*Mots-clés :* fragilité lysosomale, peroxydation lipidique, stress oxydatif, β-glucuronidase, *N*-nitrosodiméthylamine, fibrose hépatique.

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## Introduction

Hepatic fibrosis is a dynamic process that involves the interplay of different cell types in the hepatic tissue (George and Tsutsumi 2007; Wynn 2008). The pathogenesis of hepatic fibrosis is mediated through oxidative stress and hepatocyte injury and is always accompanied by impaired hepatic metabolism and deposition of connective tissue components, especially collagen and hyaluronan in the liver (George et al. 2004; George and Stern 2004).

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Lysosomes are a distinct group of cell organelles that contain a variety of acid hydrolases (Saftig 2005). The stability and integrity of lysosomal membrane is important to maintain normal levels of lysosomal enzymes in tissues and body fluids. An increase in serum acid hydrolases, including β-glucuronidase, has been reported in a number of pathological conditions, such as arthritis (Sandya et al. 2007), myocardial infarction (Rajadurai and Prince 2007), and Hansen's disease (George et al. 1990; Nandan et al. 2007). Lysosomal membrane plays a vital role in the regulation of lysosomal enzyme secretion in pathophysiology (Pillay et al. 2002; Thoene 1992) and in various inflammatory processes (Ignarro 1974). Degradation of connective tissue constituents, such as collagen (Katz 2005), glycoproteins (George and Chandrakasan 1996*a*), and glycosaminoglycans (Shikhman et al. 2000) are brought about by enzymes that primarily originate from lysosomes. Increased activity of lysosomal glycohydrolases and matrix metalloproteinases (MMPs) may disrupt normal levels of collagen and other connective tissue constituents of the liver, which can exacerbate tissue damage (Pott et al. 1982).

Changes in lysosomal stability have been reported in connective tissue disorders such as rheumatoid arthritis (Reddy and Dhar 1987) and myocardial infarction (Ravichandran et al. 1990). Increased lysosomal glycohydrolases have been reported in acute alcoholism (Hauge et al. 1998) and various experimental liver injuries (Slater and Greenbaum 1965). However, lysosomal membrane stability has not been examined in any liver disorder, including hepatic fibrosis. The aim of this investigation was to study the changes in liver lysosomal stability during *N*-nitrosodimethylamine (NDMA)-induced hepatic fibrosis in adult male albino rats. We previously demonstrated that NDMA-induced hepatic fibrosis in rats is a suitable and reproducible animal model for studying pathophysiological and biochemical changes associated with the development of hepatic fibrosis and alcoholic cirrhosis of human beings (George and Chandrakasan 1997; George et al. 2001). This model has also been shown to produce many decompensating features of human hepatic fibrosis, such as portal hypertension, ascites, hypoproteinemia, and biochemical abnormalities (Jenkins et al. 1985; George and Chandrakasan 2000). Because β-glucuronidase is used as a reporter gene to monitor gene expression and is also very rich in liver lysososmes compared with all other glycohydrolases, we selected it as a marker enzyme to study changes in lysosomal membrane stability during NDMA-induced hepatic fibrosis. The subcellular distribution of β-glucuronidase and its rate of release from the lysosomes were measured at various time points, and β-glucuronidase levels were also monitored in serum samples from both control and experimental animals. Furthermore, the effect of NDMA on lysosomal membrane stability was studied in vitro at various time points.

## Materials and methods

### Chemicals

*N*-nitrosodimethylamine, L-hydroxyproline, chloramine-T, bovine serum albumin, *p*-nitrophenol, *p*-nitrophenyl β-D-glucuronide, and Triton X-100 were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Ethylene glycol monomethyl ether (methyl cellosolve) was procured from Fluka AG (Switzerland) and *p*-dimethylaminobenzaldehyde was purchased from E. Merck (Darmstadt, West Germany). Trichloroacetic acid, potassium sodium tartarate, and Folin-Ciocalteau's phenol reagent were obtained from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

### Animals

The animal protocol was approved by the Central Leather Research Institute (Adyar, Madras, India) animal care and use committee for the maintenance and use of laboratory animals, and followed in accordance with the *Guide for the Care and Use of Laboratory Animals*. Adult male albino Wistar rats, approximately 3 months old and weighing between 180 and 200 g, were bred and maintained in polypropylene animal cages with a wire mesh top and a hygienic bed of husk. They were provided with commercial rat feed pellets (Hindustan Lever, Mumbai, India) and water available ad libitum.

### Induction of hepatic fibrosis

Hepatic fibrosis was induced by intraperitoneal injections of NDMA in 1 μL doses (diluted to 1:100 with 0.15 mol/L sterile NaCl) per 100 g body mass, as described previously (George 2006). The injections were given on 3 consecutive days of each week over a period of 3 weeks. Control animals also received the same volume of 0.15 mol/L NaCl without NDMA. The injections were given without anesthesia. Animals were observed for morphological and behavioral changes. Treated animals were sacrificed on days 7, 14, and 21 from the beginning of exposure. Some of the control animals were sacrificed at the beginning of the experiment, and others were sacrificed with the treated animals on days 7, 14, and 21; the pooled value was used as the control. The control and day 7 groups comprised 9 rats each, whereas the day 14 and day 21 groups consisted of 7 and 6 rats, respectively. All animals were anesthetized with diethyl ether before the sacrifice. Blood was collected from a deep cut made with a scalpel blade in the right jugular vein of the neck. The blood was allowed to clot at 30 °C for 1 h and the serum was separated by centrifugation at 2000*g* for 10 min. The serum was not stored, and the enzyme assay was carried out immediately after the collection.

### Assessment of hepatic fibrosis

The clinical indices of hepatic fibrosis were evaluated histopathologically as well as by quantifying total collagen content in the liver. The liver sections were stained with hematoxylin and eosin, examined using an Olympus BH2 microscope, and photographed.

The total collagen content of the liver tissue was measured as a biochemical parameter to assess the progression of fibrosis. The collagen content in the liver was determined by estimating hydroxyproline, a characteristic imino acid present in collagen. All samples (100 mg wet liver tissue) were hydrolyzed in 6 mol/L HCl in sealed tubes at 110 °C for 16 h. The hydrolyzed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was redissolved in distilled water and made up to a known volume. It was then treated with activated charcoal and fil-

tered through Whatman No.1 filter paper. The clear filtrate was used for the determination of hydroxyproline, as described previously (George and Chandrakasan, 1996*b*). In brief, 1 mL of the filtrate was mixed with 1 mL of freshly prepared chloramine-T solution and allowed to stand for 20 min. It was further mixed with 1 mL of 3.15 mol/L perchloric acid and left for 5 min. Finally, 1 mL of freshly prepared *p*-dimethylaminobenzaldehyde was added and mixed well, and the mixture was incubated in a water bath at 60 °C for 20 min. The absorbance of the solution was determined with a spectrophotometer at 560 nm. The total collagen content in the liver tissue was calculated by multiplying the hydroxyproline content by the factor 7.46.

### Separation of subcellular fractions

The livers were rinsed in ice-cold 0.15 mol/L NaCl to remove blood-borne contaminants and blotted. All liver specimens were weighed, and a portion was homogenized using a Polytron homogenizer (Kinematica AG, Switzerland) in ice-cold 0.25 mol/L sucrose solution at 4 °C. The final concentration of the homogenate was adjusted to 100 mg liver tissue/mL and used for the enzyme assay. A portion of the enzyme preparation was used to determine the total β-glucuronidase activity. Another portion was centrifuged at 600*g* for 10 min in a Hitachi refrigerated centrifuge (Himac SCR 20B) at 4 °C. The sediment, containing nuclei, unbroken cells, and plasma membrane (nuclear fraction), was suspended in 0.1 mol/L acetate buffer (pH 5.0) prepared in 0.25 mol/L sucrose solution. The supernatant was centrifuged at 16 000*g* for 30 min at 4 °C (Hitachi Himac SCR 20B), and the sediment (lysosomal fraction) and supernatant (soluble fraction) were collected separately. The nuclear, lysosomal, and soluble fractions were used to determine β-glucuronidase activity.

### Rate of release of β-glucuronidase from the lysosomal fraction

Since β-glucuronidase is a characteristic lysosomal enzyme, its activity in various subcellular fractions and its rate of release from the lysosomal fraction were taken as a measure of lysosomal membrane stability. The activity of β-glucuronidase in the supernatant, i.e., the proportion of lysosomal enzyme activity remaining in the supernatant after resedimentation of lysosomes, was taken as an index of lysosomal membrane stability (Wilson et al. 1992). The rate of release of β-glucuronidase from the lysosomes, measured at various time points, was considered a measure of lysosomal fragility. The lysosomal fraction at 16 000*g* was used to study the rate of release of β-glucuronidase from the lysosomes.

The lysosomal fraction from the cell preparation was suspended in 0.1 mol/L acetate buffer (pH 5.0) in 0.25 mol/L sucrose solution. It was divided into 5 sets of 2 mL each and incubated at 37 °C. At 0, 15, 30, 45, and 60 min, the tubes were removed and centrifuged at 27 000*g* for 15 min in a Hitachi refrigerated centrifuge (Himac SCR 20B) at 4 °C. The supernatants were collected and stored at 4 °C until the enzyme assay was performed. The enzyme activity in the supernatants, collected at the various time points, was represented as the percentage of total activity prior to centrifugation. The total β-glucuronidase activity in the lysosomal fraction was determined after the addition of 0.2% Triton X-100 (final concentration, *v*/*v*).

We also investigated the effect of NDMA, at various time points, on control rat liver lysosomes. The incubation mixture was incorporated with NDMA at concentrations of 10 μg/mL and the experiment was performed as described above. Since the blood level of NDMA was <10 μg/mL after injection (the concentration of NDMA was 1 mg/μL), the effect of higher concentrations of NDMA was not studied.

### Assay of β-glucuronidase

β-Glucuronidase activity was determined according to the method of Koldovsky (Koldovsky 1971), with *p*-nitrophenyl β-D-glucuronide as a substrate. The assay mixture contained 0.5 mL of freshly prepared 4 mmol/L *p*-nitrophenyl β-D-glucuronide (final concentration 2 mmol/L) in 0.1 mol/L sodium acetate buffer (pH 5.0). Next, 100 μL serum, 25 μL of 1:10 diluted liver homogenate (250 μg of original liver tissue), or 25 μL of undiluted subcellular fraction was made up to 1.0 mL with assay buffer and incubated at 37 °C for 1 h. The reaction was stopped by adding 3 mL of 0.2 mol/L glycine–NaOH buffer (pH 11.7). The enzyme was added to the controls after adding glycine buffer. The absorbance was measured at 410 nm using a Shimadzu UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). The enzyme activity was determined from a standard curve plotted with *p*-nitrophenol. The activity was expressed as nmol *p*-nitrophenol liberated/(h·mL serum) and μmol *p*-nitrophenol liberated/(h·100 mg protein), for serum and liver fractions, respectively.

### Determination of lipid peroxides in the liver homogenate and lysosomal fraction

Lipid peroxides present in the liver homogenate and lysosomal fraction were determined by the thiobarbituric acid reaction method described by Ohkawa et al. (1979), with tetramethoxypropane as a standard. An aliquot of 0.5 mL of liver homogenate or lysosomal fraction was treated with 3.5 mL of ice-cold 10% trichloroacetic acid and mixed well. The tubes were centrifuged at 3000 r/min (4500*g*) for 10 min, and 2 mL of the supernatant was mixed with 2 mL of 0.6% thiobarturic acid reagent (prepared freshly by dissolving in 0.25 mol/L HCl with gentle warming). The supernatant mixture was covered with glass marbles and placed in a boiling water bath for 15 min. The absorbance of the resultant brilliant pink color was measured on a Shimadzu UV-260 spectrophotometer at 532 nm. The levels of lipid peroxides present in the liver homogenate and lysosomal fraction were expressed as nmol malondialdehyde liberated/100 mg protein.

### Estimation of total protein

Total proteins were determined by the method of Lowry et al. (1951). Bovine serum albumin in 0.1 mol/L NaOH solution was used as the standard.

### Statistical analysis

The results were statistically evaluated using a one-way analysis of variance (ANOVA). The arithmetic mean and standard deviation and (or) error were calculated for the

data. The least significant difference method was used to compare mean values for the control samples with those of the treated samples obtained from days 7, 14, and 21 of the experiment. Student's *t* test was used to compare β-glucuronidase activities in samples from day 14 of the experiment with those from day 21. Pearson and Lee's correlation coefficient was used to study the correlation between β-glucuronidase levels in the serum and levels in the liver homogenate and subcellular fractions. The correlation analysis was also used to elucidate the correlation between serum β-glucuronidase and lipid peroxidation in the liver and lysosomal fraction during the progression of NDMA-induced hepatic fibrosis. Furthermore, the correlation analysis was used to assess the linear curve fitting of the *p*-nitrophenol standard curve used for the β-glucuronidase assay, and the correlation coefficient was >0.999.

## Results

### Assessment of hepatic fibrosis

The histopathology of hepatic damage is depicted in Figs. 1A–1D. The control liver showed normal lobular architecture, with central vein and radiating hepatic cords (Fig. 1A). On day 7, there was severe centrilobular congestion and marked dilatation of central veins and sinusoids, as well as massive hemorrhagic necrosis and multifocal collapse of the liver parenchyma (Fig. 1B). On day 14, multifocal hepatocyte necrosis and neutrophilic infiltration were observed. Well developed fibrosis and early cirrhosis were prominent. Bridging necrosis and apoptosis were also present in certain cases (Fig. 1C). On day 21, the liver sections demonstrated clear cirrhosis, with collagen fibers, intense neutrophilic infiltration, and regeneration of hepatocytes (Fig. 1D). Hydropic and focal fatty changes were also seen.

### Total collagen in the liver

The results of the biochemical evaluation of hepatic fibrosis carried out by determining the total collagen content of the liver tissue iare shown in Fig. 2. The total collagen content in the liver significantly increased ($p < 0.001$) after the start of NDMA administration. The maximum increase was on day 21, and was about 4-fold that of the untreated (control) livers. The 4-fold increase in total collagen on day 21 coincided with the histopathological observation of well developed fibrosis and deposition of collagen fibers in the liver.

### β-Glucuronidase activity in the liver and subcellular fractions

Changes in β-glucuronidase levels during the progression of NDMA-induced hepatic damage are presented in Table 1. The total activity of β-glucuronidase in the liver increased significantly ($p < 0.001$) on all days after the start of NDMA administration. The maximum activity was observed on day 14, which was about 2.5-fold that of the control value. There was a significant decrease ($p < 0.001$, Student's *t* test) in β-glucuronidase activity on day 21 compared with that on day 14, probably due to a reduction in the rate of neutrophilic infiltration. The level of β-glucuronidasein the nuclear fraction did not change. β-Glucuronidase activity increased markedly ($p < 0.001$) in the lysosomal fraction on all days during the progression of NDMA-induced hepatic fibrosis. The maximum increase was on day 14, which was about 2.2-fold that of the untreated (control) animals. The β-glucuronidase activity in the soluble fraction also increased significantly ($p < 0.001$) on all days after the start of NDMA administration. Unlike the pattern of total and lysosomal activities, the maximum activity in the soluble fraction occurred on day 21, indicating increased lysosomal fragility. The ratio of soluble (free) to lysosomal (bound) enzyme activity, as well as the ratio of soluble to total enzyme activity, also showed significant increases from days 7 to 21. In both cases, the maximum increase occurred on day 21, indicating increased enzyme leakage on day 21.

### Rate of release of β-glucuronidase from rat liver lysosomes

The effect of NDMA on control rat liver lysosomes and the rate of release of β-glucuronidase from NDMA-treated rat liver lysosomes, measured at various time points, are presented in Table 2. There was no significant change in the rate of release of β-glucuronidase measured at various time points after NDMA incorporation in the assay, indicating that the NDMA has no significant role in lysosomal membrane stability changes in vitro. The rate of release of β-glucuronidase showed significant increases at various time points on all days following the start of NDMA treatment. The highest increase was on day 21, with the maximum percentage activity at 60 min. We noticed that the rate of release of β-glucuronidase tended to increase during the progression of hepatic fibrosis.

### Lipid peroxides in the liver and lysosomal fraction

Lipid peroxide content in both the whole liver homogenate and liver lysosomal fraction is shown in Fig. 3. Lipid peroxides, expressed as nmol malondialdehyde/100 mg protein, increased significantly ($p < 0.001$) in the liver homogenate and lysosomal fraction on all days following the start of NDMA treatment. The increase was gradual and remarkable in both the whole liver and lysosomal fraction, from day 0 to day 21. In both cases, the maximum increase was on day 21. The increase in liver lipid peroxides correlated positively ($r = 0.998$) with the elevation of β-glucuronidase activity in the soluble fraction of the cell preparation. Similarly, the rise in lysosomal lipid peroxides correlated significantly ($r = 0.996$) with β-glucuronidase activity in the soluble fraction. The marked increase in lipid peroxides in both liver homogenate and the lysosomal fraction during NDMA treatment indicates the formation of reactive oxygen species, which play a significant role in cell injury and pathogenesis of hepatic fibrosis (George 2003).

### Serum levels of β-glucuronidase

Changes in serum levels of β-glucuronidase during the progression of NDMA-induced hepatic fibrosis are shown in Fig. 4. Serum β-glucuronidase activity increased significantly ($p < 0.001$) on all days measured following the start of NDMA treatment. The maximum increase was on day 14, which was about 2.5-fold that of the control value. A

**Fig. 1.** Hematoxylin and eosin staining of rat liver during the progression of *N*-nitrosodimethylamine (NDMA)- induced hepatic fibrosis (×40). (A) Normal liver. (B) Day 7 of NDMA treatment; note massive hepatic necrosis, multifocal collapse of the liver parenchyma (arrows), severe centrilobular congestion, and dilatation of sinusoids with focal hemorrhage. (C) Day 14 of NDMA treatment; note well developed fibrosis and early cirrhosis with multifocal hepatocyte necrosis and neutrophilic infiltration (arrows). (D) Day 21 of NDMA treatment; note well developed cirrhosis with collagen fibers (arrows).

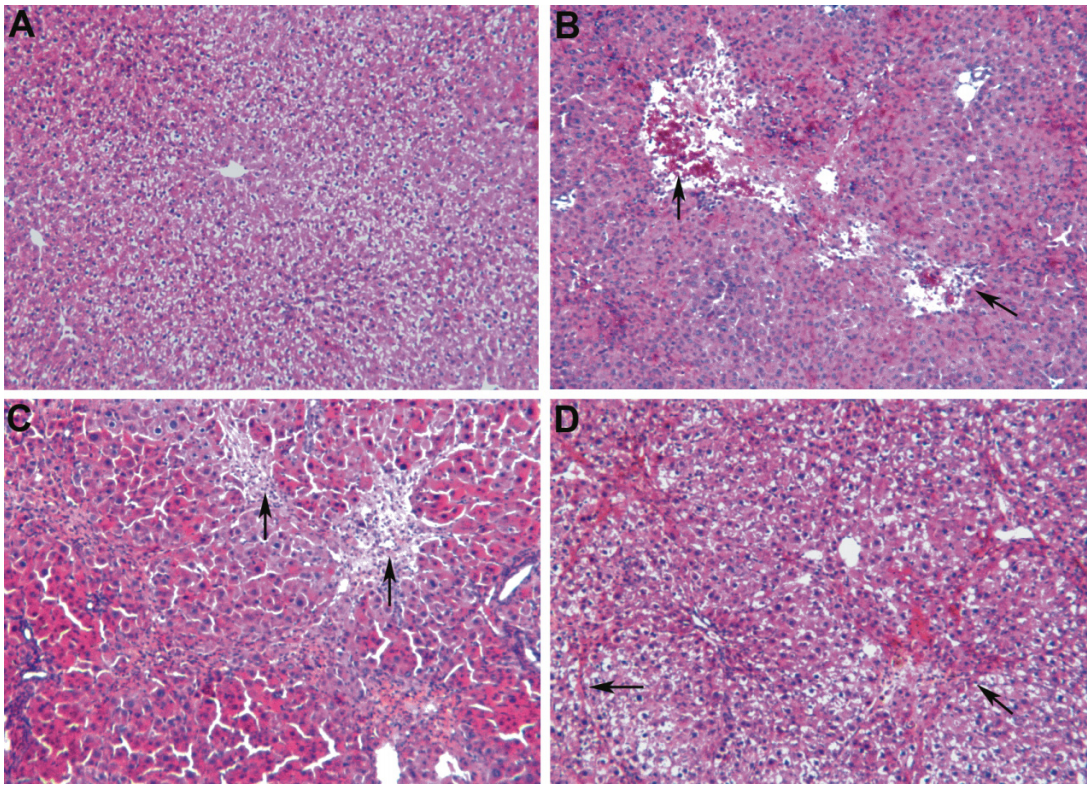
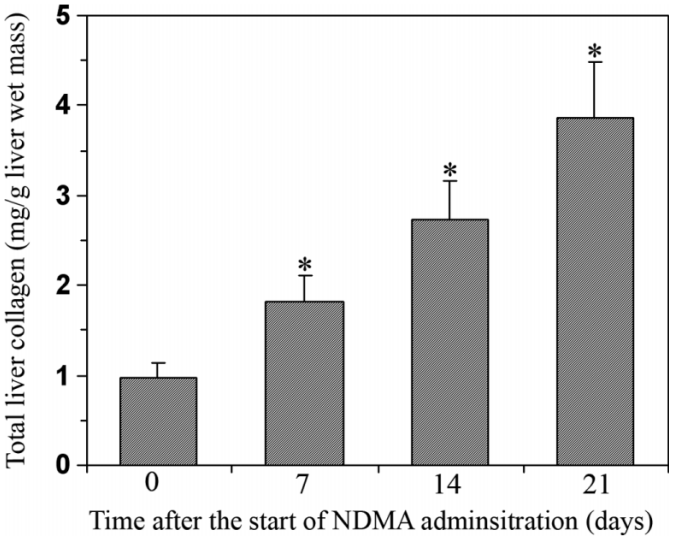



**Fig. 2.** Total collagen content in the liver during *N*-nitrosodimethylamine (NDMA)-induced hepatic fibrosis in rats. The values given are the means ± SD (*, $p < 0.001$ with an ANOVA). The collagen content in the liver tissue was determined by estimating the level of hydroxyproline, a characteristic imino acid present in collagen. The total collagen content was calculated by multiplying the hydroxyproline content by the factor 7.46.




correlation analysis revealed that the rise in serum β-glucuronidase correlated positively ($r$ = 0.995) with the elevation of liver lipid peroxides during the progression of NDMA-induced hepatic fibrosis. Similarly, serum β-glucuronidase correlated significantly ($r$ = 0.994) with lipid peroxides in the lysosomal fraction. Finally, the increase in β-glucuronidase activity in the serum correlated positively ($r$ = 0.999) with that in the lysosomal soluble fraction during NDMA administration.

## Discussion

This study is the first to demonstrate increased lysosomal fragility during the pathogenesis of NDMA-induced hepatic fibrosis and its correlation with increased lipid peroxidation. The integrity of the lysosomal membrane is crucial to the maintenance of normal levels of lysosomal glycohydrolases and cathepsins in tissues and bodily fluids. A compromise of lysosomal membrane integrity may lead to an undesirable elevation of lysosomal enzymes in both intra- and extracellular space, which could pave the way for cellular and tissue disorders, including apoptosis. Although the regulation of the release of lysosomal enzymes from lysosomes is not completely understood, it is likely that lysosomal membrane stability might be a major contributing factor. The release of cysteine cathepsins and the aspartic protease cathepsin D into the cytosol through lysosomal permeabilization can trigger cell death by different mechanisms (Stoka et al. 2007). Since β-glucuronidase is considered to

**Table 1.** Subcellular distribution of liver β-glucuronidase during the progression of *N*-nitrosodimethylamine-induced hepatic fibrosis in rats.

| | β-Glucuronidase activity, μmol *p*-nitrophenol liberated/(h·100 mg protein) | | | |
|---|---|---|---|---|
| | Control ($n$ = 9) | Day 7 ($n$ = 9) | Day 14 ($n$ = 7) | Day 21 ($n$ = 6) |
| **Fraction** | | | | |
| Whole liver (total activity) | 85.30±3.44 | 157.28±4.08** | 215.96±6.71** | 178.71±5.92** |
| Nuclear | 28.25±1.12 | 31.74±1.29 | 32.39±1.65 | 32.45±1.71 |
| Lysosomal | 122.73±4.14 | 223.75±6.39** | 268.46±8.90** | 241.12±7.62** |
| Soluble | 60.86±2.62 | 125.14±3.92** | 210.84±6.98** | 230.48±7.65** |
| **Ratio** | | | | |
| Soluble (free) to lysosomal (bound) activity | 0.496 | 0.559* | 0.785** | 0.955** |
| Soluble (free) to total activity | 0.713 | 0.795* | 0.976** | 1.289** |

**Note:** Values represent the means ± SE vs the control. Values for the ratios represent the means. Statistical significance was determined with an ANOVA. *, Significant at $p < 0.05$; **, significant at $p < 0.001$.

be a marker enzyme for lysosomes, the release of β-glucuronidase is frequently used as an index of lysosomal membrane integrity (Michihara et al. 2005).

Previous reports regarding β-glucuronidase levels in the liver during NDMA-induced hepatic fibrosis are not available. However, increased β-glucuronidase activity in the liver has been noticed in rats with carbon tetrachloride-induced liver cirrhosis (Melen et al. 1985; Hultberg et al. 1988). In this study, we also found significantly elevated levels of β-glucuronidase activity in the liver on all days following the start of NDMA treatment. It has been suggested that the increase in lysosomal enzymes in the liver during carbon tetrachloride-induced cirrhosis was mainly due to an increased synthesis of the enzymes in the hepatocytes because they are the predominant cell type in the liver (Hultberg et al. 1988; Holmberg et al. 1986). The increased levels of β-glucuronidase in the liver during NDMA treatment could be due to either enhanced synthesis of the enzyme or to a contribution from the infiltrated neutrophils. In this study, intense neutrophilic infiltration occurred on each day following the start of NDMA treatment. The major cause of the increase in β-glucuronidase levels in the liver is the upregulation of the enzyme's synthesis during fibrosis. Various cytokines, growth factors, and NDMA itself could serve as stimulants for this increased enzyme production. The synthesis and deposition of connective tissue components during the pathogenesis of hepatic fibrosis could also upregulate the production of lysosomal enzymes. Macrophages can also serve as the cellular source for the increased lysosomal enzymes, because macrophages respond to various stimuli during the pathogenesis of hepatic fibrosis. In liver diseases, a variety of substances known to be elevated in circulation, such as immune complexes, cytokines, and ammonia, are known to stimulate macrophages (Skudlarek et al. 1984). The observed decrease in β-glucuronidase activity in the liver on day 21 compared with that on day 14 was probably due to a reduction in the rate of neutrophilic infiltration on day 21, as evidenced by histopathology. The increased lysosomal fragility on day 21 could have facilitated the leakage of the enzyme into the blood stream, which may also have contributed to a decrease in β-glucuronidase activity.

There are no available data on the subcellular activities of β-glucuronidase in the liver either in experimentally induced or human hepatic fibrosis. The increased β-glucuronidase activity observed in the lysosomal fraction following NDMA administration reflects the elevation of the enzyme in the liver. The significant rise in the enzyme's activity in the soluble fraction, especially on day 21, indicates increased lysosomal fragility after NDMA administration. This coincides with the increased lipid peroxidation observed on day 21 in both liver and lysosomes. Significantly higher ratios of enzyme activity in both free-to-bound and free-to-total activity again substantiate increased lysosomal fragility during the pathogenesis of NDMA-induced hepatic fibrosis.

No previous study has reported on the rate of release of β-glucuronidase from liver lysosomes during hepatic fibrosis. However, increased lysosomal fragility has been reported in experimental hepatic injury induced by copper overload in rats (Myers et al. 1993). The increased rate of release of β-glucuronidase observed in our investigation indicates increased lysosomal fragility in the rat liver during the pathogenesis of hepatic fibrosis. The absence of the effect of NDMA on rat liver lysosomes in vitro suggests that the increased lysosomal fragility during NDMA-induced fibrosis is not due to the toxicity of NDMA but rather, to other factors.

Increased oxidative stress and lipid peroxidation have been reported in NDMA-induced hepatic fibrosis (Vendemiale et al. 2001). In this study, we also found that lipid peroxides were elevated in the hepatic tissue as well as in the lysosomal fraction of the cell preparation. During NDMA administration and pathogenesis of hepatic fibrosis, the liver tissue was subjected to increased oxidative stress, which results in the formation of ROS, mostly of mitochondrial origin, including oxygen ions, free radicals, and peroxides. The ROS reacts with the lipid bilayer of intracellular organelles, including lysosomes, which destabilizes lysosomal membrane and results in the rupture of lysosomes. It was reported that exposure of mammalian cells to oxidant stress causes early lysosomal rupture followed by apoptosis or necrosis of the cell (Zhao et al. 2003). It has also been suggested that the increased lysosomal fragility in iron-induced liver injury is due to elevated lipid peroxidation (Britton et al. 2002). In the present study, we observed

**Table 2.** Effect of *N*-nitrosodimethylamine (NDMA) on liver lysosomal membrane permeability and the rate of release of β-glucuronidase from liver lysosomes during the progression of NDMA-induced hepatic fibrosis in rats.

| | | Rate of release β-glucuronidase, % total activity | | | | |
|---|---|---|---|---|---|---|
| Group | Total lysosomal activity, μmol *p*-nitrophenol liberated/(h·100 mg protein) | 0 min | 15 min | 30 min | 45 min | 60 min |
| Control ($n$ = 9) | 122.73±4.14 | 15.36±0.72 | 17.54±0.93 | 20.62±0.98 | 22.62±1.06 | 23.32±1.15 |
| Control + NDMA treated ($n$ = 9) | 128.46±5.65 | 13.21±0.68 | 18.36±0.88 | 21.26±1.08 | 23.13±1.15 | 25.36±1.31 |
| NDMA treated, day 7 ($n$ = 9) | 223.75±6.39 | 16.62±0.76 | 20.26±1.24 | 25.61±1.56 | 28.17±1.96* | 36.85±2.37** |
| NDMA treated, day 14 ($n$ = 7) | 314.46±10.90 | 15.31±0.81 | 23.95±1.35** | 32.56±2.21*** | 43.39±2.58*** | 49.48±2.87*** |
| NDMA treated, day 21 ($n$ = 6) | 261.12±8.62 | 17.92±0.92 | 26.86±1.56*** | 35.72±2.42*** | 48.16±2.72*** | 56.26±3.16*** |

**Note:** Values represent the means ± SE vs the control. Statistical significance was determined with an ANOVA. *, Statistically significant at $p < 0.05$; **, statistically significant at $p < 0.01$; ***, statistically significant at $p < 0.001$.

**Fig. 3.** Lipid peroxide in the whole liver homogenate and lysosomal fraction during *N*-nitrosodimethylamine-induced hepatic fibrosis in rats. The data represent the means ± SD. *, Statistically significant at $p < 0.001$ with an ANOVA compared with control values. Lipid peroxide determined by the thiobarbituric acid reaction method using tetramethoxypropane as a standard.

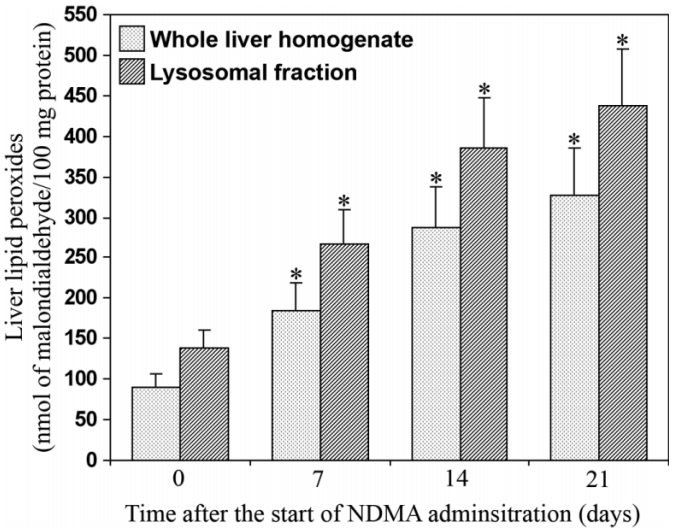
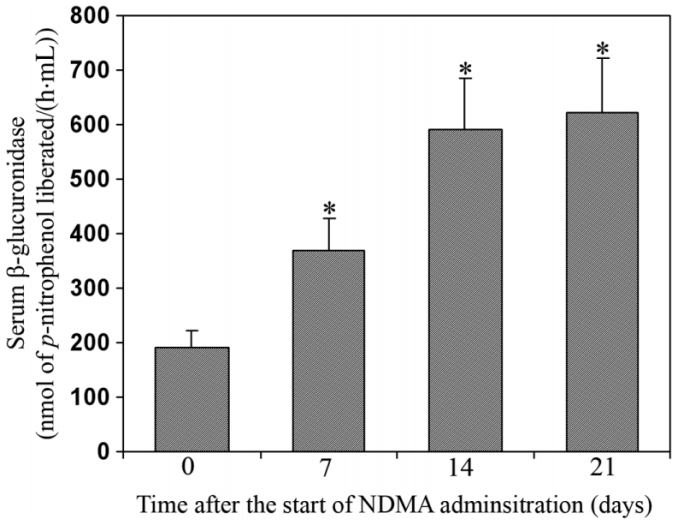



**Fig. 4.** Serum β-glucuronidase levels during NDMA-induced hepatic fibrosis in rats. Values represent the means ± SD. *, Statistically significant at $p < 0.001$ with an ANOVA. Serum β-glucuronidase activity was determined by spectrophotometry using *p*-nitrophenyl β-D-glucuronide as a substrate.




positive correlations between increased lipid peroxidation in the liver and elevated β-glucuronidase activity in the soluble fraction of the cell preparation. Thus, our study indicates that lipid peroxidation is the cause of increased lysosomal fragility during NDMA-induced hepatic fibrosis.

Elevated levels of serum β-glucuronidase were observed in rats with carbon-tetrachloride-induced liver cirrhosis (Melen et al. 1985; Hultberg et al. 1988). We also noticed significantly increased levels of β-glucuronidase in the serum. The early increase in β-glucuronidase in the circulation mainly reflects hepatocellular necrosis, cell release, and apoptosis during NDMA-induced fibrosis. Furthermore, the increased circulation of acid hydrolases could trigger cell damage and apoptosis, which in turn raise β-glucuronidase levels in the serum. However, the increase in serum β-glucuronidase in advanced fibrosis is mostly due to the decrease of liver lysosomal stability and the subsequent release of lysosomal enzymes into the blood stream due to the enhanced lipid peroxidation in the hepatic tissue. This is corroborated with the significant positive correlation between elevated serum β-glucuronidase and increased liver lipid peroxidation. The increased rate of release of β-glucuronidase from the lysosomes provided evidence for increased lysosomal fragility during the pathogenesis of the NDMA-induced hepatic fibrosis.

The results of this investigation demonstrated enhanced lysosomal activity and increased serum β-glucuronidase levels during the pathogenesis of NDMA-induced hepatic fibrosis. We also demonstrated an elevation of lipid peroxides in the liver during NDMA administration, as well as decreased lysosomal stability and an increased rate of release of β-glucuronidase during hepatic fibrosis. Moreover, this study showed that NDMA has no significant effect on rat liver lysosomal membrane stability in vitro, and provided

evidence that the elevation of serum β-glucuronidase during the pathogenesis of NDMA-induced hepatic fibrosis was due to increased lipid peroxidation, the result of which was lysosomal fragility and the subsequent release of lysosomal enzymes into the blood stream. Taken together, this study provides evidence that lipid peroxidation induced by oxidative stress is the mechanism of increased lysosomal fragility during the pathogenesis of NDMA-induced hepatic fibrosis.

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