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Changes in Glucose-6-Phosphate Dehydrogenase and Malic Enzyme Gene Expression in Acute Hepatic Injury Induced by Thioacetamide

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ABSTRACT. NADPH-generating enzymes, glucose-6-phosphate dehydrogenase (G6PDH), and malic enzyme (ME) were studied in rat liver when necrosis and regeneration were induced by a single sublethal dose of thioacetamide (6.6 mmol/kg). Both enzyme activities decreased sharply at 12–24 hr of treatment and increased thereafter. These biphasic changes are related to the sequential processes of liver injury and hepatocellular regeneration. Expression of mRNA for G6PDH decreased at 12 hr following thioacetamide injection and increased during liver regeneration, reaching its highest levels of expression at 48 hr (247% of the control), parallel to the peak of DNA synthesis. Expression of ME decreased at 12–24 hr and increased during the postnecrotic regenerating process, reaching only half of the control value at 96 hr. A relationship between mRNA G6PDH gene expression, oxidative stress (detected by the GSH/GSSG ratio and malondialdehyde (MDA) concentration), and DNA synthesis is proposed. BIOCHEM PHARMACOL 51;9:1159–1163, 1996.

KEY WORDS. gene expression; NADPH-generating enzymes; oxidative stress; necrosis; regeneration

G6PDH† is a key enzyme that catalyzes the production of ribose-5-phosphate for nucleic acid synthesis and reducing power in the form of NADPH. ME, another NADPH-generating enzyme that catalyzes the oxidative decarboxylation of L-malate to yield CO_2, pyruvate, and NADPH, is considered to be a lipogenic enzyme whose activity correlated with *de novo* fatty acid synthesis [1–3]. Reducing NADPH equivalents are involved in three main cellular mechanisms: reductive synthesis, maintenance of the cell redox state, and the microsomal reactions of drug detoxification [4]. The induction of G6PDH activity by agents that induce oxidative stress and lipoperoxidation [5] led us to consider whether or not the G6PDH gene responds to the oxidative attack by providing protection in the form of NADPH to maintain the cellular redox state [6]. Furthermore, G6PDH activity, through the generation of ribose-5-phosphate, is involved in nucleic acid synthesis and repair, because hepatocyte growth is stimulated following necrosis and the expression of this enzyme increased, thereby favouring NADPH and pentose-phosphate generation [7–9].

The formation of reactive metabolites by the microsomal oxidizing mechanism is enhanced by NADPH, because NADPH-generating systems are required for microsomal biotransformation of xenobiotics [4, 6]. The present study was, therefore, undertaken to determine G6PDH and ME activities and gene expression at the mRNA level in liver during the necrosis and regeneration induced by thioacetamide administration to rats. The results obtained show that the noticeable increase in the activity of the G6PDH enzyme is due both to an enhancement in the respective gene expression, as a part of the coordinated cellular events against oxidative stress, and to the increased rate of cell proliferation, which depends on the elevated values in the hepatocyte population involved in DNA replication. However, the values of ME enzyme activity and mRNA gene expression suggest that ME is not involved in the regenerating mechanisms.

MATERIALS AND METHODS

Animals and Treatment

Adult male Wistar rats aged 2 months (180 to 220 g), supplied with food and water *ad lib.* and exposed to a 12-hr light-dark cycle, were given i.p. a necrogenic dose of thioacetamide (6.6 mmol/kg body wt) freshly dissolved in 0.9% NaCl. To avoid diurnal variations, rats were sacrificed by cervical dislocation between 9.00 and 9.30 AM. Livers were quickly removed and freeze-clamped. All experiments were performed against time-matched controls injected with 0.5 mL of 0.9% NaCl. Samples were obtained at 3, 12, 24, 48, 72, and 96 hr of hepatotoxin administration [10]. Fetuses were obtained from pregnant albino Wistar rats by cesarean section at the 22nd day of gestation [11]. Each experiment

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† *Abbreviations:* G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); ME, malic enzyme (EC 1.1.1.40); MDA, malondialdehyde.

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was repeated 4 times and followed the international criteria for the use and care of experimental animals in research.

Isolation of Hepatocytes and Flow Cytometry Analysis

Hepatocytes were isolated according to the classic collagenase perfusion method; cell viability, determined by Trypan Blue exclusion, was greater than 90%. The analysis of DNA content, 1×10^6 viable cells, was carried out following the Vindelov multistep procedure [12]. The emitted fluorescence of the DNA-propidium iodide complex was assayed in a FACScan flow cytometer (Becton-Dickinson).

Enzyme Activities and Metabolite Determination

Enzymatic determinations were carried out in the soluble fraction of liver homogenates under optimal conditions of pH and temperature and with substrates and cofactors at saturation. G6PDH was determined spectrophotometrically at 340 nm [13], and malic enzyme activity was assayed in the same fraction by the method previously described [14]. Proteins were evaluated by the Bradford method [15]. Reduced (GSH) and oxidized glutathione (GSSG) were determined according to Hissin and Hilf [16]. Hepatic levels of MDA were obtained as described by Nieaus *et al.* [17].

Northern Blot Analysis

Fifty milligrams of liver were lysed with guanidinium thiocyanate/phenol reagent for RNA isolation [18]. Total cellular RNA (20 μg) was submitted to Northern blot analysis, electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to GeneScreenTM membranes and cross-linked to the membranes by UV light. Hybridization was in 0.25 mM $NaHPO_4$ pH 7.2, 0.25 M NaCl, 100 μg/mL denatured salmon sperm DNA, 7% SDS and 50% deionized formamide, containing denatured ^{32}P-labeled cDNA (10^6 cpm/mL) for 40 hr at 42°C as described [19]. cDNA labeling was carried out with $\alpha^{32}P$-dCTP using a multiprimer DNA-labeling system kit (Amersham, Buckinghamshire, U.K.). The resulting membranes were subjected to autoradiography for 1–3 days. Relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Finally, the filters were hybridized with an 18S rRNA probe for RNA normalization. The analysis of the Northern blot was performed in duplicate from two livers. The variability in the measurement of fold increase in mRNA, after quantification by scanning densitometry from the filters, was not greater than 15%.

TABLE 1. Concentration of GSH and GSSG and the GSH/GSSG ratio in liver of thioacetamide-treated rats

Thioacetamide treatment (hr)	GSH (nmol/g liver)	GSSG (nmol/g liver)	GSH/GSSG
Control	9200 ± 100	310 ± 29	29.6 ± 3.0
3	8970 ± 90	320 ± 28	28.0 ± 2.8
12	6237 ± 61*	310 ± 26	20.1 ± 1.9*
24	5336 ± 48*	380 ± 41	14.0 ± 1.3*
48	6360 ± 52*	394 ± 41	16.1 ± 1.6*
72	7764 ± 69	343 ± 32	22.6 ± 2.1
96	8556 ± 91	291 ± 27	29.4 ± 2.8

The results are calculated as nmol of GSH per g of fresh liver and are the mean ± SD of 4 different observations. *$P < 0.001$ vs control. Thioacetamide was administered i.p. in a single dose of 6.66 mmol/kg body wt and control animals received an i.p. injection of 0.5 mL of 0.9% NaCl.

TABLE 2. Time-course of malondialdehyde (MDA) levels in liver of thioacetamide-treated rats

Thioacetamide treatment (hr)	MDA (nmol/g liver)	Protein (mg/g liver)	MDA (pmol/mg protein)
Control	2.6 ± 0.2	92 ± 8	28.2 ± 3
3	2.7 ± 0.2	93 ± 9	26.2 ± 3
12	3.8 ± 0.4*	79 ± 7	48.4 ± 5*
24	4.8 ± 0.5*	75 ± 6	64.8 ± 7*
48	4.1 ± 0.3*	75 ± 6	54.1 ± 5*
72	3.2 ± 0.3	93 ± 8	34.2 ± 3
96	2.8 ± 0.3	92 ± 9	30.0 ± 3

The results are expressed as nmol per g of liver and pmol per mg of protein and are the mean ± SD of 4 different observations. *$P < 0.001$. Thioacetamide was administered i.p. in a single dose of 6.66 mmol/kg body wt and control animals received an i.p. injection of 0.5 mL of 0.9% NaCl.

RESULTS AND DISCUSSION

Thioacetamide is a hepatotoxic compound that induces necrosis in the perivenous space of the liver acinus, as detected by the measurements of parameters of cellular injury, such as the activity of serum alanine aminotransferase that peaked at 24 hr with values 17-fold of the control [10]. To observe whether or not the hepatic metabolism of thioacetamide produces oxidative stress, the GSH/GSSG redox state, an indicator of reactive oxygen production, as well as the levels of MDA as a marker of lipoperoxidation, were determined in liver following acute intoxication. As Table 1 shows, the GSH/GSSG ratio markedly decreased at 12, 24, and 48 hr after the treatment, reaching values of 67%, 47%, and 54% of the control, respectively. From this point on, until the end of the experiment, hepatic GSH/GSSG increased towards the control value. Table 2 shows the hepatic concentration of MDA, which increases progressively, reaching a two-fold peak at 24 hr of thioacetamide administration.

Because the coordinated cellular response against oxidative stress involves a higher requirement of cell-reducing power, the systems that generate reducing equivalents are expected to be enhanced. Accordingly, the time-course of G6PDH and ME activities was assayed in the soluble fraction of liver homogenates obtained from thioacetamide-treated rats. These results are summarized in Fig. 1, in

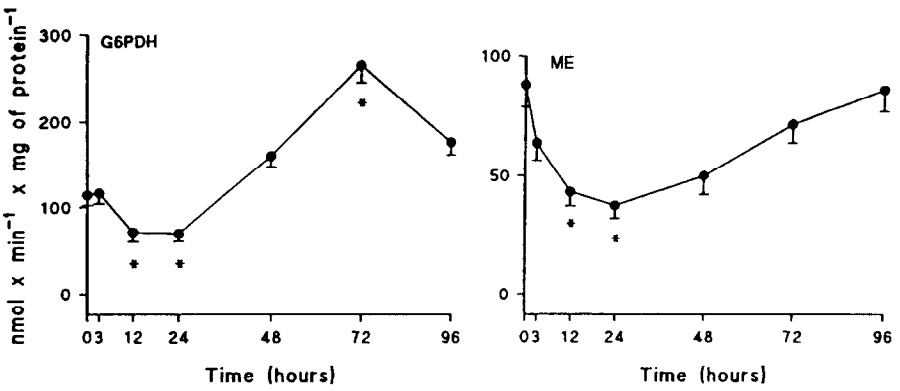

FIG. 1. Time-course of G6PDH (EC 1.1.1.49) and ME (EC 1.1.1.40) enzyme activities in the soluble fraction of rat liver during the necrosis and regeneration induced by thioacetamide. The activities were assayed in the soluble fraction of liver samples from rats at 0, 3, 12, 24, 48, 72, and 96 hr after thioacetamide treatment. The results are expressed as m units (nmol × min^{-1}) × mg of $protein^{-1}$ and are means ± SD of 4 experimental observations. *P < 0.001 vs control.

which both enzymes, G6PDH and ME, display a progressive and statistically significant decrease in their activities, reaching values of 60% and 42% at 24 hr, respectively. From 48 hr, an increase in ME activity towards the initial values was observed. However, in the same period, a sharp increase over the control in G6PDH activity was detected. The values obtained for G6PDH activity with respect to the control were 139%, 230%, and 152% at 48, 72, and 96 hr, respectively.

In a previous study, the intraacinar location of liver necrosis induced by a sublethal dose of thioacetamide was characterized by light microscopy in the acinar region surrounding the venous terminal [10]. Considering the heterogeneous distribution of NADPH-generating enzymes [20], which are mainly located in the injured perivenous area, the destruction of the hepatocytes of this acinar space may explain the decreased activities of these two enzymes at 24 hr of thioacetamide administration.

The changes in G6PDH and ME activities, described above, led us to study the expression of G6PDH and ME genes. Figure 2 illustrates the Northern blot analysis of the mRNA content of G6PDH and ME, indicating that the gene expression of these enzymes was very different; thus, the postnecrotic induction of G6PDH gene expression started at 24 hr of thioacetamide injection, reaching a peak at 72 hr. ME mRNA gene expression remained low in the period between 12 and 48 hr and slightly increased towards half of the initial values at 72–96 hr.

Considering the alterations in enzyme activities, together with the noticeable induction of G6PDH mRNA gene expression and its involvement in *de novo* nucleic acid synthesis and cell growth, we studied the extent of DNA replication by means of flow cytometry in isolated hepatocytes. Table 3 shows the time course of the hepatocyte population in phase S (DNA synthesis) of the cell cycle. It is well known that most of the hepatocytes in normal liver

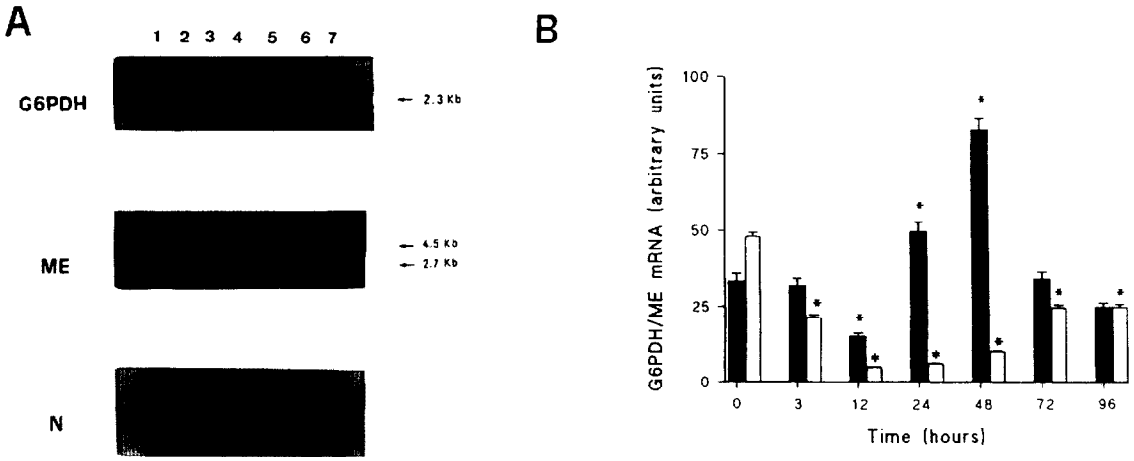

FIG. 2. Northern blot analysis of G6PDH and ME mRNA in liver of rats after thioacetamide treatment. Panel A shows a representative Northern blot from 2 independent experiments with 18S rRNA probe for RNA normalization (N). RNA were isolated from livers from control and TAM-treated rats at 0 (1), 3 (2), 12 (3), 24 (4), 48 (5), 72 (6), and 96 (7) hr. Panel B shows the quantification of the G6PDH and ME mRNAS after correction with 18S rRNA. Dashed bars, G6PDH; open bars, ME. The data are media and standard deviation. *P < 0.001 vs control.

TABLE 3. Quantitative analysis of hepatocyte population in phase S of the cell cycle (DNA replication)

Thioacetamide treatment (hr)	Phase S (%)
Fetal	7.1 ± 0.8
Control	0.74 ± 0.1
3	0.68 ± 0.08
12	0.48 ± 0.06
24	1.30 ± 0.09
36	11.8 ± 1.4*
48	12.2 ± 1.4*
72	3.1 ± 0.4
96	1.1 ± 0.1

The values are expressed as the hepatocyte population (%) in S phase that corresponds to cells synthesizing DNA from G_1 to G_2 (2N → 4N). Thioacetamide was administered i.p. in a single dose of 6.66 mmol/kg body wt and control animals received an i.p. injection of 0.5 mL of 0.15 M NaCl. Results are the mean ± SD of 4 experimental observations. *$P < 0.001$ vs control. Fetal liver cells were obtained from fetuses after 22 days of gestation.

are in the quiescent state, and fewer than 1% undergo DNA replication. However, when an aggressive attack occurs and cells die, the remaining cells dedifferentiate and divide [10]. In the present investigation, postnecrotic hepatocyte DNA replication increased markedly at 48 hr of thioacetamide intoxication when compared to the control. In agreement with the present results, it has been shown [7] that ME gene expression in primary cultures of adult hepatocytes does not respond to the mitogenic effects of growth factors (EGF), which seems to indicate that ME activity is not involved in the mechanisms of cell growth.

From our experiments, we conclude that the sharp decrease in both enzyme activities parallel to the peak of necrosis may be due to the heterogeneous acinar distribution of NADPH-generating enzymes, which are located mainly in the perivenous space. Furthermore, our results show that biotransformation of thioacetamide produces oxidative stress detected at 12–24 hr by a decrease in the GSH/GSSG ratio and an increase in MDA concentration. We, therefore, propose that the mRNA expression for G6PDH is induced, under our experimental conditions, in response to oxidative stress. The quantitative increase in the activity and mRNA gene expression of G6PDH may be a consequence of the dual role played by this enzyme system in providing either NADPH for maintenance of the cell redox state or pentose-phosphates for DNA synthesis. However, changes in the activity and mRNA ME gene expression are apparently not involved in these processes, the time-course of the profile of this enzyme being a clear example of hepatotoxic-induced damage and postnecrotic restoration of liver function. Finally, the fact that oxidative stress parallels the alterations in the activity of NADPH-generating enzymes in thioacetamide-induced liver disease convinces us that future studies should be performed on the basis of pharmacological protective intervention of vitamins C and E to normalize the altered parameters of gene expression found in the present investigation.

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