Ischaemia and reperfusion injury of rat liver increases expression of glutathione S-transferase A1/A2 in zone 3 of the hepatic lobule

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Effects of ischaemia–reperfusion injury (I/R) of liver on expression of rat glutathione *S*-transferase (rGST) isoenzymes that metabolize products of oxidative stress were examined. Rats underwent lobar liver ischaemia for 30 min followed by reperfusion. In ischaemic lobes, rGSTA1/A2 transcript levels increased significantly 12 h after I/R (2.94-fold) and protein levels increased significantly at 24 h (1.45-fold); increased transcript levels were also observed in nonischaemic lobes (1.78-fold). Superoxide dismutase prevented I/R and the increases in transcript and protein levels in ischaemic and non-ischaemic lobes. By *in-situ* hybridization, increases in transcript levels at 6 h were present in zones 2 and 3 of the ischaemic lobes and peaked at 12 h (2.5-fold zone 2, 4.5-fold zone 3). Significant increases in transcript levels also were observed at 24 h in zones 2 (2.0-fold) and 3 (2.9-fold) of non-ischaemic lobes. Nuclear run-off assays showed a 1.8-fold increase in rGSTA1/A2 transcription rates in ischaemic lobes at 3 h. We conclude that I/R causes increased rGSTA1/A2 expression in the zone of the hepatic lobule most susceptible to oxidative injury and that this expression may be an important defence against injury.

INTRODUCTION

Reactive oxygen species (ROS) such as $O_2^{-\bullet}$, H_2O_2, $\cdot OH$, and their by-products are natural products of metabolism that are capable of causing oxidative damage when present in high concentrations within cells [1]. Cells contain a number of defence mechanisms against ROS including antioxidants such as vitamins C and E, GSH, and enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase, GSH peroxidase, glutathione *S*-transferases (GSTs; EC 2.5.1.18), and NAD(P)H:quinone reductase that remove ROS or their by-products, thereby limiting cellular injury [2].

Recently it has become apparent that ROS, when present in low concentrations, may affect gene expression. Increases in ROS levels within the cell appear to alter the activities of some transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1) [3,4]. Factors that activate NF-κB include cytokines, such as tumour necrosis factor α, and oxidative stress. The second messenger for activation of NF-κB by tumour necrosis factor α or okadaic acid may be H_2O_2 [3,5]. The activity of AP-1 also appears to be sensitive to oxidative stress; the generation of ROS leads to AP-1 activation and changes in the levels of expression of detoxication enzymes including some GSTs [6].

The GSTs are an important family of detoxication enzymes found in the cytosol of most cells. Isoenzymes of the cytosolic GSTs are grouped into four classes termed alpha, mu, pi and theta based on amino acid and gene sequences, substrate specificities and affinity for nonsubstrate ligands [7–12]. Different cell types contain various amounts and combinations of different GST isoenzymes. With their broad range of substrate specificities, the GSTs are thought to play a crucial role in protecting cells against injury by toxic electrophiles, especially carcinogens [10,13]. The alpha class isoenzymes have high activity against organic hydroperoxides, which include lipid hydroperoxides and, therefore, may help protect hepatocytes from damage by ROS during oxidative stress from various sources including normal metabolism and the detoxication of xenobiotics [10,14].

Recent work suggests that ROS may be important regulators of expression of some GSTs. Known *cis*-acting response elements in the 5′-flanking sequence of an alpha class GST gene include a glucocorticoid response element, an antioxidant response element [ARE, also designated electrophile response element (EpRE) in the mouse], and a xenobiotic response element. These elements mediate the changes in GST expression that are observed after exposure to a variety of drugs and naturally occurring compounds [10,15]. The ARE (EpRE) is thought to be responsible for the increase in message levels of rGSTA1/A2 (see footnote[2]) that is observed after exposure of HepG2 cells to H_2O_2, cumene hydroperoxide and menadione as well as to antioxidants such as butylated hydroxyanisole [15,16]. Pretreatment of cells with thiol-containing compounds prevents GST induction by some xenobiotics, which suggests that transcriptional activation is dependent upon the production of ROS [17]. In addition, transcriptional activation of the mouse GSTA1 gene by the antioxidants butylated hydroxyanisole and pyrrolidine dithiocarbamate was shown to be secondary to the generation of ROS during their metabolism and to subsequent increases in NF-κB and AP-1 activities [6]. Thus many xenobiotics (including antioxidants) may affect the transcription rates of GST genes by inducing oxidative stress within the cell, leading to activation of NF-κB and AP-1. The control of expression of other enzymes, such as certain forms of cytochrome *P*-450 and NAD(P)H:quinone reductase, also occurs through similar response elements,

Abbreviations used: AP-1, activator protein-1; ARE, antioxidant response element; AST, aspartate aminotransferase; EpRE, electrophile response element; GSTs, glutathione *S*-transferases; I/R, ischaemia-reperfusion injury; NF-κB, nuclear factor-κB; rGST, rat glutathione *S*-transferase; rGSTA1, A2, M1 and P1 are isoenzymes of rGST; ROS, reactive oxygen species; SOD, superoxide dismutase.

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The nomenclature used for glutathione S-transferases is defined in [7]. rGSTA1 and A2 are the same as Ya1 and Ya2 or ligandin, rGSTM1 is Yb1, and rGSTP1 is Yp (Yf).

which suggests that ROS may be important regulators of expression of detoxication enzymes [10,15,16].

Most of the above studies were performed with cultured cells using various xenobiotics and it is unclear what the importance of oxidative stress is in regulating gene expression *in vivo*. Ischaemia–reperfusion injury (I/R) of the liver is a common event following hepatic surgery and liver transplantation. The injury appears to be due, at least in part, to oxidative stress, since elevated levels of ROS can be measured in the liver after I/R and the administration of SOD minimizes the injury [18–22]. Increases in NF-κB activity also are observed following I/R [23]. Based on the above evidence and the fact that the 5′-flanking sequence of rGSTA2 isoenzyme contains an ARE that is similar to the EpRE in the mouse *Ya* gene that responds to oxidative stress [6,10], we hypothesized that oxidative stress due to I/R may affect the levels of expression of rGSTA1/A2. In addition, the rGSTA1/A2 proteins have significant enzymic activity towards products of oxidative stress, suggesting that an increase in expression could be an adaptive response to I/R. Therefore the effects of I/R on message and protein levels and on the transcriptional activity of rGSTA1/A2 were examined. The effect of administration of a long-acting form of SOD on cell injury and GST expression after I/R also was studied. In addition, changes in rGSTA1/A2 expression at the cellular level in the hepatic lobule were analyzed by *in situ* hybridization.

MATERIALS AND METHODS

All chemicals used were of analytical grade and unless otherwise stated were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.). Restriction enzymes were from Promega (Madison, WI, U.S.A.) or New England Biolabs (Beverly, MA, U.S.A.), random primer DNA labelling reagents were from Amersham (Arlington Heights, IL, U.S.A.) and radioactively labelled nucleotides ([^{32}P]dCTP and [^{35}S]UTP) were from Du Pont–NEN (Boston, MA, U.S.A.). Long acting, polyethylene glycol-treated recombinant human SOD (2500 units/mg of protein) was from DDI Pharmaceuticals Inc. (Mountain View, CA, U.S.A.). Sodium pentobarbital was from Abbott Laboratories (North Chicago, IL, U.S.A.) and phenobarbital was from Elkins-Sinn Inc. (Cherry Hill, NJ, U.S.A.). Adult male (250–300 g), pathogen-free, Sprague-Dawley rats were from Harlan Sprague-Dawley Inc. (Prattville, AL, U.S.A.), and the experimental protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Ischaemic liver model

Lobar hepatic ischaemia–reperfusion was used to generate an injury to the liver as previously described [24]. In this model an ischaemic injury to part of the liver is induced while the remaining lobules are perfused normally. Briefly, anaesthesia was induced with sodium pentobarbital (5 mg/100 g body weight intraperitoneally). A midline laparotomy was performed and the liver was gently mobilized. An atraumatic microvascular clamp (Micro-serrefines No. 18055-02, Fine Science Tools, Foster City, CA, U.S.A.) was used to interrupt the portal venous and hepatic arterial blood supply to the median and right lateral lobes of the liver. The left lateral and caudate lobes retained an intact portal and arterial inflow and venous outflow, thus minimizing intestinal venous congestion. Partial hepatic ischaemia was maintained for 30 min and then the clamp was removed to initiate reperfusion. The abdomen was closed and the animals were kept warm with a heat lamp until they were killed. Sham-operated control animals were treated in the same manner except the vascular occlusion was omitted. There was no mortality associated with the surgery. Water, but not food, was provided during the first 24 h after surgery, and then the animals were fed *ad libitum*. At 6, 12, 24 and 48 h after reperfusion, groups of three animals were anaesthetized and blood and liver samples were obtained as described below.

SOD treatment

Administration of SOD is known to prevent I/R by reducing the formation of ROS [22]. Long acting SOD (5000 units) was injected into the inferior vena cava of rats 15 min before the liver was made partially ischaemic. At 12 and 24 h after reperfusion, groups of three animals were anaesthetized and blood and liver samples were obtained as described below. Sham animals were treated in the same manner.

Phenobarbital-treated animals

Treatment of rats with agents such as phenobarbital is known to increase the expression of rGSTA1/A2 in the liver and to alter its lobular distribution [25,26]. Therefore animals were treated with phenobarbital to serve as positive controls for the *in situ* hybridization studies performed after I/R. Phenobarbital (8 mg/100 g body weight) or 0.9 % (w/v) saline for control animals was injected intraperitoneally and liver samples from the animals were obtained 24 h after injection as described below.

Tissue collection and preparation

Liver and blood samples were collected at prescribed time intervals after I/R using diethyl ether anaesthesia. A blood sample (0.5 ml) was collected into a heparinized syringe from the inferior vena cava just before removal of the liver. Blood was centrifuged and plasma was kept at −80 °C until assayed for aspartate aminotransferase (AST) activity (Sigma diagnostic kit). Small pieces of liver were cut from ischaemic (median lobe) and non-ischaemic (left lateral and caudate lobes) lobes and from the corresponding lobes of sham-treated animals. Pieces of liver from some animals were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for pathological examination. Other pieces of liver were embedded in O.C.T. compound (Miles Inc., Elkhart, IN, U.S.A.), frozen immediately at −80 °C, and used for *in situ* hybridization studies. The portal vein was then cannulated with a 20 gauge intravenous catheter and the liver was perfused with 100 ml of 0.9 % (w/v) saline. Portions of ischaemic and non-ischaemic lobes and corresponding lobes from sham-treated animals were frozen in liquid N_2, pulverized and homogenized in ice-cold guanidine thiocyanate solution (0.5 g liver/5 ml); total RNA was extracted as described previously [27].

Northern-blot analysis

Equal amounts of total RNA (estimated from $A_{1\ cm,260}$) from ischaemic, non-ischaemic and sham-treated lobes of 3 animals at each time point were pooled separately. RNA (20 μg) from each pooled sample was subjected to Northern-blot analysis as described previously [28]. The cDNAs for rGSTA2 (pGTB 38) and a mu class GST termed rGSTM1 (pGTA/C 44) were gifts from Dr. C. B. Pickett (Schering-Plough Corp. Inst., Kenilworth, NJ, U.S.A.) [25,29], and the cDNA for ribosomal protein S14 (RPS14) was from the American Type Culture Collection (A.T.C.C.). The cDNA for rGSTP1 (a pi-class enzyme) was from Dr. D. H. Barch (Northwestern University Medical School, Chicago, IL, U.S.A.) [30] and that for mouse albumin was from Dr. E. T. Morgan (Emory University, Atlanta, GA, U.S.A.). A cDNA fragment (300 bp) was purified from a *Bgl*II/*Eco*RI

digest of the rGSTA2 plasmid which hybridized with rGSTA1 and rGSTA2 transcripts, the predominant alpha class isoenzymes in rat liver [25,31]. To acknowledge that the probe hybridizes with both rGSTA1 and A2 transcripts, we use rGSTA1/A2 when referring to transcript levels. A cDNA fragment (268 bp), that hybridized with only rGSTM1 transcripts, was cut from the rGSTM1 plasmid as described previously [29]. A cDNA fragment (734 bp), which hybridized only with rGSTP1 transcripts, was purified from an *Eco*RI/*Sal*I digest of the rGSTP1 plasmid. The cDNA fragments were radioactively labelled with [^{32}P]dCTP with random primer labelling reagents (Amersham). The autoradiograms were scanned by densitometry, as described previously [28]. Blots were stripped in boiling 1% (w/v) SDS in $0.1 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl/0.015 M sodium citrate) for reprobing. Intrablot variability in RNA loading was controlled by normalizing the GST transcript signal densities to those of RPS14.

Nuclear run-off assay

Assays were performed as described previously [28]. Transcriptionally active nuclei were isolated from ischaemic and non-ischaemic liver lobes of experimental animals and from corresponding lobes of control animals at 1, 3, 6, and 12 h after I/R. Aliquots of newly synthesized, radioactively labelled RNA (3×10^7 c.p.m.) from the run-off reactions were hybridized with separate cDNA-blotted filters. The filters were exposed to film and the resulting signal bands on radioautographs were quantified by densitometry. At each time point, nuclear run-off assays were performed on two separate animals. Each experimental animal was matched with a control animal and they were killed, nuclei were isolated and run-offs performed simultaneously. The level of transcriptional activity in the ischaemic and non-ischaemic lobes was then compared with that of the sham-treated animal.

Synthesis of ^{35}S-labelled RNA probes for *in situ* hybridization

Sense (control) and anti-sense RNA probes labelled with [^{35}S]UTP for *in situ* hybridization were synthesized with Riboprobe® transcription reagents (Promega, Madison, WI, U.S.A.) [32]. A cDNA fragment (530 bp) purified from a *Pst*I digest of the rGSTA2 plasmid was subcloned into the *Pst*I site of Bluescript II KS plasmid (Stratagene, La Jolla, CA, U.S.A.) and the orientation of the cDNA was determined by sequencing the resulting construct. In order to synthesize an antisense RNA probe specific for rGSTA1/A2 transcripts but not for A3 transcripts, the plasmid was linearized with *Bgl*II, which resulted in a 377 bp RNA probe after transcription. The sense probe was synthesized after the plasmid was linearized with *Eco*RI, which resulted in a 615 bp RNA probe after transcription. The lengths of the synthesized RNA probes were confirmed by autoradiography after PAGE.

In situ hybridization

Liver sections were prepared and *in situ* hybridizations were performed as described previously [32–34]. Slides from phenobarbital-treated animals were exposed for 4 days while slides from the ischaemia-reperfusion animals were exposed for 6 days. Treatment groups and their controls were processed in the same batch to control for possible variations in thickness of the emulsions between batches. The slides were developed in Kodak D19 for 3 min, rinsed in water for 20 s, fixed in Kodak Fixer for 3 min and washed in water three times for 5 min. Sections were counterstained with haematoxylin and eosin.

Western blots

Cytosol fractions from frozen samples of ischaemic, non-ischaemic and sham-treated livers were prepared and Western blots were made as previously described [32]. Protein concentrations of the cytosol fractions were determined using the Bio-Rad protein assay with BSA as the standard. Equal amounts of cytosolic protein from liver lobes of three animals were pooled and 5 μg of total protein from the pooled cytosol fractions was loaded onto gels and subjected to SDS/PAGE. The primary antiserum used for GST detection was rabbit anti-rat GSTA1/A2/A3 antiserum (MED 26 YA, 1:3000; Biotrin, Dublin, Ireland). Bound primary antibodies were detected with enhanced chemiluminescence reagents (Amersham; Du Pont–NEN). Samples from ischaemic, non-ischaemic and sham-treated lobes from each time point were run on the same gel. Different film exposures were made and the blots selected for analysis were from exposures that were within the linear response range of the film. The density of the resultant bands was quantified by densitometry. Although the primary antibody reacts with rGSTA1, A2 and A3, complete separation of rGSTA3 from A1 and A2 is accomplished by electrophoresis [35]. Therefore the densities obtained reflect changes in rGSTA1 and A2 protein levels only.

Data analysis

Northern and Western blots are only semi-quantitative and subject to significant methodological variability. To reduce this variability, equal amounts of RNA or protein from three different animals were pooled and at least three blots were prepared from the pooled samples and probed separately. Values of Northern- and Western-blot analyses represent the mean $\pm$ S.E.M. of results from the separate blots. *In situ* hybridization sections were photographed and fields were digitized and analyzed by computer-image-analysis software (Matrox Inspector, Version 1.7; Matrox, Inc. Quebec, Canada). Briefly, microscopic fields ($\times 125$) were analyzed by dividing the hepatic lobules into equal thirds to represent zone 1 (periportal), zone 2 and zone 3 (pericentral). Exposed silver grains over three different lobules from at least four I/R animals (at least 12 lobules/time period) were counted and the mean estimated. Exposed silver grains over three different lobules from three phenobarbital-treated and saline-treated animals (nine lobules/time period) were treated similarly. Data were analyzed statistically with Student's t test for unpaired means and by analysis of variance using general linear models (SAS system software, SAS Institute, Inc., Cary, NC, U.S.A.). Statistical significance was achieved when $P \leqslant 0.05$ (2-tailed test).

RESULTS

Hepatic I/R and the effect of SOD

After I/R there was a rise in plasma levels of AST that peaked at 6 h after reperfusion (Figure 1). Histological examination of liver sections stained with haematoxylin and eosin revealed scattered areas of pericentral necrosis in the ischaemic lobes after reperfusion and normal histology in the non-ischaemic lobes and lobes taken from sham-treated animals (results not shown). Pretreatment of animals with SOD lessened the increase in plasma AST after I/R (Figure 1).

Effect of I/R on rGSTA1/A2 protein levels by Western-blot analysis

There was a significant increase in rGSTA1/A2 protein levels in ischaemic lobes 24 h after I/R. There was also an increase in the

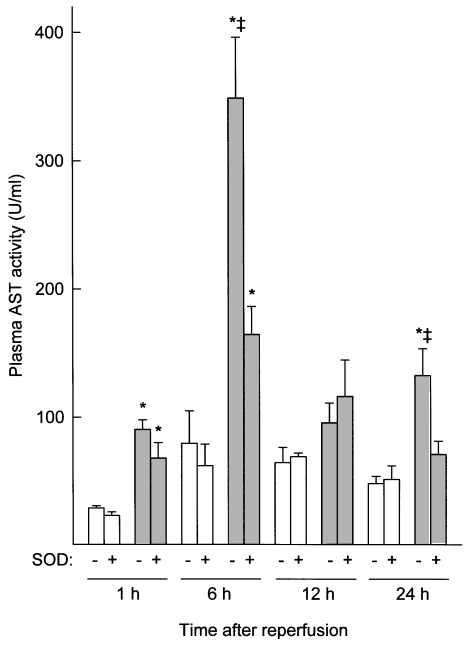

Figure 1 Effect of I/R on plasma AST levels

Plasma AST activity was measured in sham-treated animals (open bars) and in experimental animals after I/R (closed bars) with (+) and without (−) SOD. Blood was collected from the inferior vena cava after reperfusion at the times indicated. Values are means ± S.E.M. from three animals. * Significantly different from sham, $P < 0.05$; ‡different from SOD-treated, $P < 0.05$.

Table 1 Effect of I/R and I/R + SOD on rGSTA1/A2 protein levels

Protein levels were determined by Western-blot analysis using antisera against rGSTA1/A2. Samples were pooled from three animals. The data represent the mean ± S.E.M. of triplicate blots and are relative to values from sham-treated animals, which did or did not receive SOD. *Significantly different from sham-treated controls, $P < 0.05$; †significantly different from SOD-treated animals, $P < 0.05$; N.D., not determined.

		Protein levels relative to sham controls			
Sample	Time ...	6 h	12 h	24 h	48 h
Ischaemic lobes		0.89 ± 0.04	1.10 ± 0.08	1.45 ± 0.16*†	0.91 ± 0.10
Non-ischaemic lobes		0.86 ± 0.05	0.95 ± 0.08	1.25 ± 0.08†	0.90 ± 0.10
Ischaemic lobes + SOD		N.D.	1.09 ± 0.07	0.93 ± 0.05	N.D.
Non-ischaemic lobes + SOD		N.D.	1.17 ± 0.03	0.92 ± 0.04	N.D.

protein levels in the non-ischaemic lobes at 24 h. At all other time points the levels of rGSTA1/A2 proteins did not differ significantly from those of sham-treated animals. Pretreatment with long-acting SOD prevented the increases in rGSTA1/A2 protein levels that were observed in both ischaemic and non-ischaemic lobes at 24 h after I/R (Table 1).

Effect of I/R on GST message levels by Northern-blot analysis

Message levels of rGSTA1/A2 increased significantly in ischaemic lobes relative to sham-treated lobes by 6 h, peaked at 12 h and then declined towards baseline. Transcript levels of rGSTA1/A2 increased in non-ischaemic lobes at 12 and 24 h but the increases did not differ significantly from those of sham-treated animals. In contrast, there were no significant changes in the transcript levels of rGSTM1 and rGSTP1 in ischaemic and non-ischaemic lobes at any of the time points (Table 2 and Figure 2). Pretreatment of animals with long-acting SOD pre-

Table 2 Effect of I/R on mRNA levels of GSTs in ischaemic and non-ischaemic lobes

The liver was made partially ischaemic and at 6, 12, 24 and 48 h after reperfusion ischaemic and non-ischaemic lobes and lobes from sham-treated animals were removed and Northern-blot analysis was performed. Each value represents the mean ± S.E.M. of three to five blots made with pooled RNA from three animals. Transcript levels were determined by densitometry using signal from RPS14 to control for intra-blot variations in RNA loading. Transcript levels from ischaemic and non-ischaemic lobes were compared with transcript levels in identical lobes from sham-treated animals. *Significantly different from sham controls, $P < 0.05$; †significantly different from non-ischaemic lobe, $P < 0.05$.

		Transcript levels relative to sham control			
cDNA	Time ...	6 h	12 h	24 h	48 h
Ischaemic lobes					
rGSTA1/A2		1.41 ± 0.03†	2.94 ± 0.15*†	1.93 ± 0.25	1.39 ± 0.60
rGSTM1		0.77 ± 0.03	1.16 ± 0.08	1.22 ± 0.03	1.00 ± 0.14
rGSTP1		0.92 ± 0.08	1.48 ± 0.25	1.14 ± 0.12	1.69 ± 0.42
Non-ischaemic lobes					
rGSTA1/A2		0.77 ± 0.02	1.78 ± 0.08	1.52 ± 0.15	1.13 ± 0.35
rGSTM1		0.76 ± 0.04	1.36 ± 0.05	1.22 ± 0.08	0.70 ± 0.09
rGSTP1		0.74 ± 0.06	1.33 ± 0.15	1.23 ± 0.07	1.70 ± 0.51

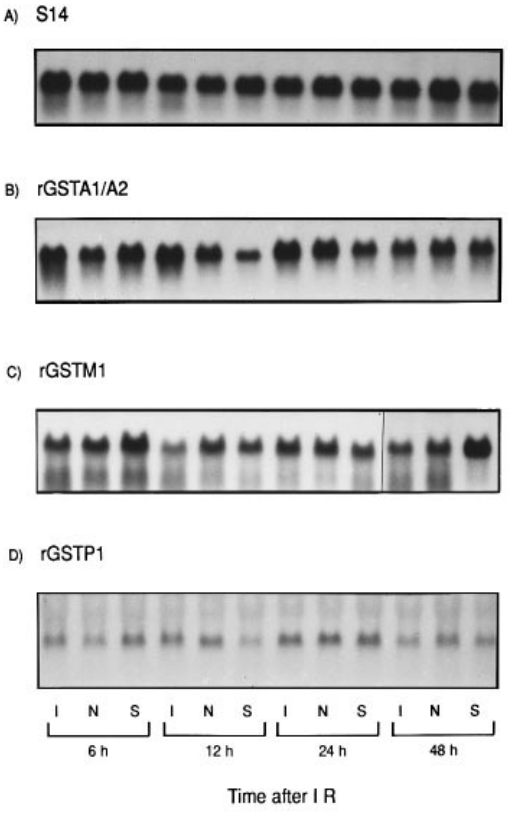

Figure 2 Northern blots of GST mRNA transcripts after I/R

Total RNA (20 μg) was loaded in each lane and probed with radioactively-labelled cDNA for (**A**) RPS14, (**B**) rGSTA1/A2, (**C**) rGSTM1 or (**D**) rGSTP1. Times indicate the period from I/R to killing of the animals. I, ischaemic; N, non-ischaemic; S, sham.

Table 3 Effect of SOD on rGSTA1/A2 transcript levels after I/R

Animals were given a long-acting form of SOD intravenously and the liver was then made partially ischaemic. After reperfusion, ischaemic and non-ischaemic lobes and lobes from sham-treated animals were removed and Northern-blot analysis was performed as described in Table 1. The results are the mean ± S.E.M. of values obtained from triplicate blots. The results obtained from treated animals were not significantly different from those of sham-treated animals, which also received SOD.

		Transcript levels relative to sham control		
Sample	Time ...	12 h	24 h	48 h
Ischaemic lobes		1.45 ± 0.20	0.50 ± 0.17	0.95 ± 0.35
Non-ischaemic lobes		0.94 ± 0.08	0.64 ± 0.18	1.00 ± 0.03

vented the increases in rGSTA1/A2 transcripts that were observed in both ischaemic and non-ischaemic lobes after I/R (Table 3).

Effect of phenobarbital on rGSTA1/A2 transcript levels

Phenobarbital treatment (8 mg/100 g body weight) increased rGSTA1/A2 transcript levels when compared with saline treatment. The effect was observed by 6 h and was maximal at 24 h (2.5-fold increase in mRNA relative to sham-treated animals). The lobular distribution of rGSTA1/A2 message was examined 24 h after phenobarbital or saline administration using the antisense probe. Transcripts for rGSTA1/A2 were uniformly distributed across the lobule in control (saline-injected) animals at all time points. Phenobarbital administration increased rGSTA1/A2 transcript levels in zones 2 and 3. Minimal uniform labelling of the hepatocytes was observed with the sense probe and there was no change in labelling after phenobarbital administration (results not shown). The confirmation of previous findings [25,26] on the induction and localization of GSTA1/A2 after phenobarbital treatment by *in situ* hybridization validates the use of the method for examining the effects of other treatments (e.g. I/R) on the lobular distribution of rGSTA1/A2 transcripts.

Effect of I/R on the zonal distribution of rGSTA1/A2 mRNA expression

The lobular distribution of rGSTA1/A2 transcripts was examined at 6, 12 and 24 h after reperfusion in I/R animals and in sham controls. There were no changes in the levels of rGSTA1/A2 transcripts across the lobule in the sham-treated animals at any time point after surgery (Figure 3A; Table 4). In contrast, rGSTA1/A2 transcript levels in the I/R animals increased in zones 2 and 3 in the ischaemic lobes at both 12 and 24 h after surgery (Figures 3C and 3D; Table 4). In zone 3 of the ischaemic lobes there was a 2.6-fold increase at 6 h relative to sham-treated animals, a 4.5-fold increase at 12 h and a 4.2-fold increase at 24 h after I/R (Table 4). Significant increases in rGSTA1/A2 transcript levels in zones 2 and 3 were also observed in the non-ischaemic lobes at 12 and 24 h after surgery (Figure 3B, Table 4).

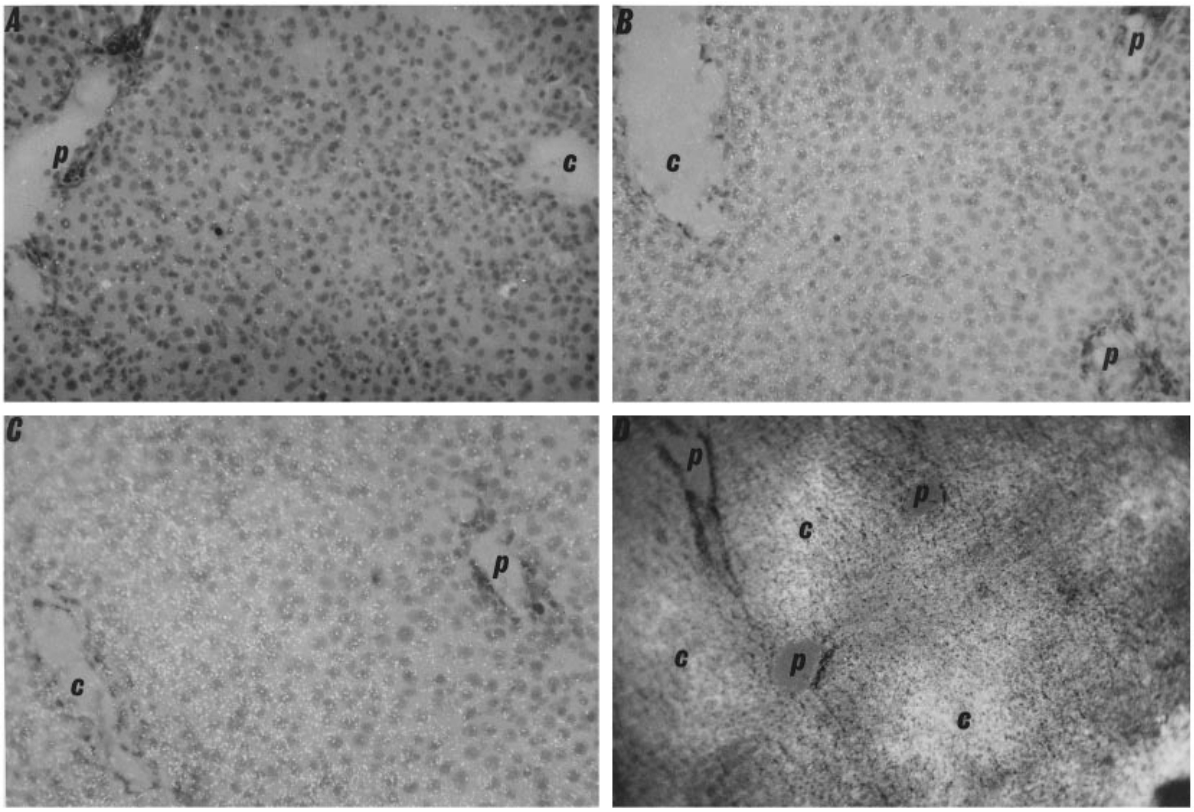

Figure 3 *In situ* hybridization of rat liver after I/R

(**A**) Sham-treated lobes 24 h after surgery (×119). (**B**) Non-ischaemic lobes 24 h after I/R. (×119). (**C**) Ischaemic lobes 24 h after I/R. (×119). (**D**) Ischaemic lobes 24 h after I/R (×47.5). Thickness of sections was 14 μm in (**A**), (**B**) and (**C**) and 40 μm in (**D**); c, central vein; p, portal area. The silver grains respresent rGSTA1/A2 transcripts in the hepatocytes.

Table 4 Effect of I/R on lobular distribution of rGSTA1/A2 transcripts

Liver sections were subjected to *in situ* hybridization and were then photographed as described in the Materials and methods section. The liver lobule was divided into three equal regions and the average number of exposed silver grains/field in each region was determined by computer image analysis. Each value represents the mean ± S.E.M. of three lobules from four different animals. *Significantly different from same zone in sham-treated lobe, $P < 0.05$; †significantly different from same zone in non-ischaemic lobe, $P < 0.05$; ‡significantly different from zone 1 in same lobe, $P < 0.05$; §significantly different from zone 2 in same lobe, $P < 0.05$.

		Number of silver grains/field		
Region	Time ...	6 h	12 h	24 h
Ischaemic lobes				
Zone 1		676 ± 90	553 ± 72	370 ± 49
Zone 2		835 ± 117	1080 ± 102*‡	1076 ± 81*‡
Zone 3		1297 ± 154*‡§	1793 ± 175*†‡§	1845 ± 133*‡§
Non-ischaemic lobes				
Zone 1		543 ± 68	298 ± 45	516 ± 77
Zone 2		757 ± 128	766 ± 59‡	1039 ± 179*‡
Zone 3		940 ± 141	923 ± 131*‡	1272 ± 209*‡
Sham-treated lobes				
Zone 1		352 ± 30	342 ± 66	409 ± 92
Zone 2		444 ± 45	424 ± 98	518 ± 108
Zone 3		496 ± 58	399 ± 84	444 ± 83

Table 5 Transcriptional rates in ischaemic and non-ischaemic lobes

Two sham-treated animals and two experimental animals were prepared at each time point and the nuclei isolated and transcriptional activity determined as described in the Materials and methods section. The results of each of two experiments are shown at each time point after surgery for non-ischaemic and ischaemic lobes. Nuclear run-off assays were performed on a total of 32 animals.

		Transcriptional rates (% of sham control)							
		Non-ischaemic lobes				Ischaemic lobes			
cDNA	Time (h) ...	1	3	6	12	1	3	6	12
rGSTA1/A2									
set 1		29	110	57	44	41	171	58	28
set 2		36	138	99	44	77	188	87	61
Average		32	124	78	44	59	180	72	44
Albumin									
set 1		93	37	48	75	126	44	33	73
set 2		116	72	116	64	111	132	97	58
Average		104	54	82	70	118	88	65	66

Effect of I/R on rate of transcription

The rate of transcription of rGSTA1/A2 increased 1.8-fold in the ischaemic lobes relative to sham 3 h after I/R and 1.2-fold in the non-ischaemic lobe at the same time point. The rates of transcription of rGSTA1/A2 in ischaemic and non-ischaemic lobes at 1, 6 and 12 h after I/R were the same or less than those in the sham-treated animals. No consistent change in the rate of transcription of albumin was observed in ischaemic and non-ischaemic lobes (Table 5).

DISCUSSION

The effect of different xenobiotics on the expression of the cytochrome *P*-450, NAD(P)H:quinone reductase and GST genes has been extensively investigated. The mechanisms of induction by xenobiotics have been well characterized and are mediated by *cis*-acting response elements, the xenobiotic response element and the ARE in the 5′-flanking sequences of these genes [7,13,36,37]. In contrast, the regulation of expression by endogenous compounds is less well understood. The rat *GSTA2* gene contains a glucocorticoid response element in its 5′-flanking sequence, which may account for the effect of glucocorticoids on levels of expression [31,38]. Cytokines also appear to affect rGSTA2 expression, again via response elements in the 5′-flanking sequence [38]. Of particular interest is the suggestion that ROS increase expression of the GSTs via the ARE. Work using GSTA2 promoter–chloramphenicol acetyltransferase (CAT) fusion constructs *in vitro* demonstrated that hydrogen peroxide increased chloramphenicol acetyltransferase activity via the ARE [15]. In other studies *in vitro* it was found that induction of mouse GSTA1 and rGSTA2 genes by a number of xenobiotics is due to oxidative stress and is mediated via the ARE (EpRE) [6,15–17]. The current study demonstrates that endogenous factors generated *in vivo* by oxidative stress induced by I/R increase the expression of rGSTA1/A2. In other studies *in vivo* it was found that oxidative stress secondary to selenium deficiency or generated by exercise also increased the expression of rGSTA1/A2 [39–41]. Therefore increases in the hepatic expression of some alpha class GSTs are a consistent feature of oxidative stress both *in vivo* and *in vitro*.

We believe that the increase in rGSTA1/A2 transcript levels is mediated, at least in part, by ROS because ROS are generated during I/R, and the administration of SOD, which prevents I/R by degrading ROS [18–22], minimized the increase in transcript levels. The effects of ROS on gene expression, however, may be mediated indirectly. The finding that rGSTA1/A2 transcripts were increased in the non-ischaemic lobe suggests that a circulating factor released from the ischaemic lobe may have affected transcript levels in the non-ischaemic lobe. I/R of the liver causes a release of cytokines [42]; ROS and cytokines can activate NF-κB, which is thought to mediate, in part, the changes in gene expression observed after oxidative stress [3]. NF-κB appears to be involved in the regulation of expression of a human GSTP gene [10]. We examined the 5′-flanking sequence of the rGSTA2 gene using the Wisconsin Sequence Analysis Package (Version 8.1) and found one NF-κB-like element at nucleotides -579 to -589. Hence, oxidative stress may lead to a cascade of events and production of factors like NF-κB that affect the expression of detoxication enzymes such as the GSTs.

The increase in rGSTA1/A2 transcript levels in zone 3 observed in ischaemic lobes after I/R is due, at least in part, to an increase in rates of transcription (Table 5). Although xenobiotics also cause an increase in rGSTA1/A2 transcript levels in zone 3 of the liver by increasing rates of transcription, the time course of induction differs from that observed following I/R. Two xenobiotics, phenobarbital and 3-methylcholanthrene, increase rGSTA1/A2/A3 transcriptional rates in a biphasic manner. For example, administration of 3-methylcholanthrene to rats caused a 3-fold increase in transcription rates of rGSTA1/A2/A3 at 4 h, a decline of rates to control levels at 6 h and then a steady rise in rates for at least 16 h following exposure to the drug [43]. In contrast, only a single, early increase in transcriptional activity was observed following I/R. We believe the early rise in transcriptional rates following the administration of 3-methylcholanthrene may be mediated by the same factors (e.g. ROS) that increase transcription rates after I/R but this remains to be proved.

The differences between the increase in message levels determined by Northern-blot analysis and in transcription rates

observed in nuclear run-off assays, and the findings by *in situ* hybridization highlight the selective nature of the induction of GSTA1/A2 in the hepatic lobule. In Northern-blot analyses and nuclear run-off assays, hepatocytes from all zones of the lobule are necessarily used. Thus the values obtained from these methods are less than those from *in situ* hybridization, which allows for the exclusion of hepatocytes from zones 1 and 2 that do not respond to the I/R. Also, the finding that increased rates of transcription shown by nuclear run-offs assays are seen at 3 h after I/R and yet message levels peak at later times suggests that other processes, such as changes in RNA processing [44], may have affected transcript levels following I/R. The half-life of rGSTA1/A2 transcripts also may have been increased after I/R due to a reduction in RNA degradation. I/R would be expected to cause liver regeneration, which is known to affect mRNA stability [45]. We believe it is unlikely, however, that changes in mRNA stability account for the observed increase because liver regeneration causes a decrease rather than an increase in rGSTA1/A2 transcript levels [28]. Also, in the current study the increase in rGSTA1/A2 transcripts in zone 3 of both ischaemic and non-ischaemic lobes is difficult to explain if this increase was secondary to an injury-mediated change in mRNA stability.

In conclusion, I/R-generated endogenous factors led to a selective increase in rGSTA1/A2 transcripts in zone 3 and to a lesser extent in zone 2 of the hepatic lobule. The increase in transcripts was seen in both ischaemic and non-ischaemic lobes and was associated with a rise in rGSTA1/A2 protein levels. These GST isoenzymes are known to metabolize products of oxidative stress and zone 3 of the hepatic lobule is the area most sensitive to ischaemic injury. Therefore increased expression of rGSTA1/A2 may be an important adaptive response to oxidative stress. An increase in rates of transcription of rGSTA1/A2 was observed after I/R, supporting the concept that induction of a number of detoxication enzymes is mediated, at least in part, by the generation of ROS.

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