CMLS, Cell. Mol. Life Sci. 61 (2004) 220–229
1420-682X/04/020220-10
DOI 10.1007/s00018-003-3357-4

CMLS Cellular and Molecular Life Sciences

Research Article

Mitochondrial respiratory function and antioxidant capacity in normal and cirrhotic livers following partial hepatectomy

S. Yang [a], T. M. C. Tan [a,*], A. Wee [b] and C. K. Leow [c]

[a] Department of Biochemistry, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Fax: +65 67791453, e-mail: bchtant@nus.edu.sg
[b] Department of Pathology, National University of Singapore, MD7, Singapore S117597
[c] Department of Surgery, National University of Singapore, MD7, Singapore S117597

Received 15 September 2003; received after revision 26 October 2003; accepted 19 November 2003

Abstract. For many liver malignancies, major hepatectomy is the usual therapy. Although a normal liver has a tremendous capacity for regeneration, liver hepatectomy in humans is usually carried out on a diseased liver and, in such cases, liver regeneration takes place in a cirrhotic remnant. Mitochondrial function in cirrhotic livers shows a variety of changes compared to control livers. This study investigated how mitochondrial respiratory function and antioxidant capacity change following partial hepatectomy of cirrhotic livers, because liver regeneration requires greater energy demands and control of oxidative stress. Cirrhosis was induced in male Wistar-Furth rats by administration of thioacetamide. NADH-cytochrome c reductase activity, mitochondrial glutathione peroxidase activity and mitochondrial GSH levels were all significantly lowered in cirrhotic livers and in the cirrhotic remnants up to 72 h after 70% hepatectomy when compared to the corresponding controls. Lower respiratory control ratios with succinate as substrate were also observed from 6 to 48 h post-hepatectomy. At 24 h post-hepatectomy, higher levels of lipid peroxidation were observed. We conclude that, compared to the controls, cirrhotic livers have diminished oxidative phosphorylation capabilities due to changes in NADH and $FADH_2$-linked respiration as well as impaired antioxidant defenses following partial hepatectomy. Both of these factors, if critical, could then impede liver regeneration.

Key words. Cirrhosis; partial hepatectomy; mitochondria; oxidative phosphorylation; oxidative stress.

The normal liver has a phenomenal capacity to regenerate following insults such as viral infections or surgical resection. This allows for the complete restoration of liver mass and tissue-specific functions following injury or hepatectomy [1]. Many factors including cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), hepatocyte growth factor (HGF) and transforming growth factor-alpha (TGF-α) are necessary to induce quiescent remnant liver cells to proliferate and regenerate [1, 2]. In addition, the regenerative process itself places a high energy demand on the hepatocytes [3–6]. The energy required is supplied by the mitochondria through the process of oxidative phosphorylation and reactive oxygen species are formed as byproducts. In a normal liver, the levels of reactive oxygen species are low and antioxidant defenses are adequate to prevent oxidative damage [7]. Animal studies on mitochondrial function, oxidative stress and antioxidant defenses in the normal liver following partial hepatectomy (PH) have been performed. During the regeneration process, increases in respiratory control and phosphorylation rates together with changes in the activities of the complexes involved in the electron transport chain have been described [3, 4, 8–10]. Mitochondrial glutathione (GSH) has been observed to decrease after

* Corresponding author.

PH [11, 12] while mitochondrial oxidant production is increased [13]. However, there are conflicting data on the levels of mitochondrial lipid peroxidation. Increased and unchanged malondialdehyde production had been described in two different studies [11, 14].

A diseased liver can become increasingly fibrotic and eventually cirrhotic. Patients with cirrhosis have an increased risk of developing hepatocellular carcinoma, the third most common cause of death due to cancer worldwide [15]. For many liver malignancies including hepatocellular carcinoma [15, 16], the main therapeutic intervention is surgical resection. Fibrotic or cirrhotic livers do not tolerate insults well and experience delayed regeneration following surgery. Several studies using whole cirrhotic rat livers have demonstrated that the capacity of cirrhotic livers to respond to oxidative stress [17, 18] and to carry out oxidative phosphorylation [19, 20] is impaired. We postulate that these two factors may in turn have an adverse effect on liver regeneration after hepatectomy leading to inadequate responses to increased energy demand and oxidative stress. To verify this, liver cirrhosis was induced by thioacetamide administration to rats and the changes in mitochondrial oxidative function and antioxidant defenses were examined before and after partial hepatectomy. Our results concur with our postulation, and differences in mitochondrial oxidative function and antioxidant defenses were observed between normal and cirrhotic livers after PH.

Materials and methods

Materials

Sucrose and the Bio-Rad protein assay dye were obtained from Bio-Rad (Hercules, Calif.). Glutathione was from ICN (Aurora, Ohio). Tris-(hydroxymethyl) aminomethane was from Baker (Phillipsburg, N. J.). Sodium dithionite, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, hydrochloric acid and sulfuric acid were purchased from Merck (Darmstadt, Germany). Monochlorobimane (MCB) was purchased from Molecular Probes (Eugene, Ore.). All other chemicals were purchased from Sigma (St. Louis, Mo.).

Induction of cirrhosis

Ten-week-old male Wistar-Furth rats were kept on standard rat chow and tap water ad libitum under 12-h light-dark cycles. Cirrhosis was induced by intraperitoneal injection of 300 mg thioacetamide/kg thrice weekly for 10 weeks as previously described with slight modifications [21, 22]. Healthy, control animals were kept under the same conditions without any treatment. All animals received humane care in compliance with the International Guiding Principles for Animal Research. After 1 week acclimation to allow for the washout of thioacetamide, all animals underwent 70% PH. Two experimental groups were studied. In group 1, healthy, control rats were submitted to PH, while in group 2, cirrhotic animals were submitted to PH. Within each group, six rats were sacrificed at 3, 6, 24, 48 and 72 h after surgery.

Partial hepatectomy

The rats were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) followed by ether inhalation. Through a midline incision, a 70% hepatectomy was performed by removing the median and left lateral lobes of the liver as described by Higgins and Anderson [23]. The wound was closed with catgut suture and the rats allowed to recover. At 3, 6, 24, 48 and 72 h after surgery, the rats were anesthetized with ether and killed by exsanguination after the drawing of blood from the heart. The remnant livers were then removed and a part of the left lateral lobe was used for histological examination. Cirrhosis was confirmed by gross inspection and histological examination.

Preparation of mitochondria

Mitochondria were prepared from the excised and the remaining resected liver as previously described with slight modifications [24]. The liver was chopped into small pieces and homogenized in 5 vol of buffer containing 0.25 M sucrose, 5 mM HEPES and 0.5 mM EGTA, pH 7.5. The homogenate was centrifuged at 585 g at 4 °C for 10 min. This was repeated twice and 2 ml of the supernatant was stored at –80 °C and used for determination of hepatic GSH. The remainder was centrifuged at 12,100 g at 4 °C for 10 min. The pellet was washed twice with buffer containing 0.25 M sucrose and 5 mM HEPES, pH 7.5. The mitochondria pellet was then resuspended and the protein concentration was determined with the Bio-Rad protein assay dye reagent using bovine serum albumin as the standard. Freshly prepared mitochondria were used to determine respiratory control and ADP/O ratios while submitochondrial particles were used for all other assays. Submitochondrial particles were prepared by freezing and thawing the mitochondrial suspension.

Determination of the respiratory control and ADP/O ratios

These were determined polarographically using a Clark oxygen electrode (Hansatech, Pentney, UK) [25]. A mitochondria suspension containing 2.5 mg protein was added to 2 ml of buffer containing 0.1 M KCl, 10 mM Tris-Cl, 12.5 mM KH_2PO_4 and 12.5 mM potassium succinate, pH 7.6. Oxygen consumption was measured in the absence (giving the state 4 activity) and presence (giving the state 3 activity) of 0.25 mM ADP. The respiratory control ratio (RCR) was expressed as the ratio of state 3 to state 4 respiration, while the ADP/O ratio was expressed

as the ratio of ADP added to that of oxygen atoms consumed during state 3 respiration.

Mitochondrial NADH-cytochrome c reductase activity

NADH-cytochrome c reductase (NCCR) activity was measured as previously described [26]. Five micrograms of submitochondrial particles was added to 1 ml of buffer containing 25 mM potassium phosphate, pH 7.4, 2.5 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 10 µM ferricytochrome c and 2 mM KCN. The reaction was started by the addition of 25 µM NADH. Reduction of ferricytochrome c was followed at 25 °C at 550 nm for 3 min. The extinction coefficient of cytochrome c is 19 $mM^{-1}\,cm^{-1}$ [8].

Mitochondrial succinate-cytochrome c reductase activity

Succinate-cytochrome c reductase (SCCR) activity was measured as previously described [26]. Five micrograms of submitochondrial particles was added to 1 ml of 50 mM potassium phosphate, pH 7.4, 20 mM succinate, 10 µM ferricytochrome c, 2 µg/ml rotenone and 2 mM KCN at room temperature. The absorbance change at 550 nm was recorded at 25 °C for 5 min. The extinction coefficient of cytochrome c is 19 $mM^{-1}\,cm^{-1}$ [8].

Mitochondrial cytochrome c oxidase activity

Cytochrome c oxidase (CCO) activity was measured as previously described [26]. Five micorgrams of submitochondrial particles was added to 1 ml of 50 mM potassium phosphate, pH 7.4 and 10 µM ferrocytochrome c (reduced by sodium dithionite at a weight ratio of 2:7, the solution was then shaken vigorously for 2 min to remove excess sodium dithionite). The absorbance change at 550 nm was recorded at 25 °C for 5 min. The extinction coefficient of cytochrome c is 19 $mM^{-1}cm^{-1}$ [8].

Mitochondrial ATPase activity

This was determined by measuring the release of inorganic phosphate [27]. The reaction was initiated by addition of 250 µg of submitochondrial particles to 0.25 ml of buffer containing 1 mM ATP, 100 mM NaCl, 10 mM KCl and 3 mM $MgCl_2$, pH 7.4. After incubation at 37 °C for 5 min, the reaction was stopped by addition of 2 ml of 0.1 M ice-cold HCl. A portion (0.2 ml) of the reacted mixture or standards corresponding to 0, 0.02, 0.04, 0.06, 0.08 and 0.1mM KH_2PO_4 were added to 0.8 ml of 5 mM H_2SO_4. One hundred and sixty microliters of 2.5% ammonium molybdate and 40 µl of 1% stannous chloride were then added. After incubation at 25 °C for 15 min, the absorbance at 680 nm was read.

Hepatic and mitochondrial GSH determination

GSH levels were measured by using the specific reaction of MCB with GSH. MCB, itself nonfluorescent, forms a stable, fluorescent adduct, bimane-GS with GSH in a reaction catalyzed by GSH S-transferases (GSTs) [28]. The endogenous GST was first removed by boiling the sample for 3 min. To quantify the amount of GSH, 0.2 U of equine liver GST was added to 250 µl of phosphate-buffered saline, 0.1 mM MCB and GSH standards corresponding to 0–0.04 mM GSH or to the sample. Following incubation at 37 °C for 15 min, the fluorescence of all samples was read using an excitation wavelength of 385 nm and an emission wavelength of 478 nm in a Gemini XS microplate spectrofluorometer from Molecular Devices (Sunnyvale, Calif.). Following this, a calibration curve was obtained and the fluorescence of the samples was then correlated with the curve.

Mitochondrial superoxide dismutase activity

This was measured using the method of Flohé and Ötting [29]. Solution A (1.45 ml; containing 0.76 mg xanthine dissolved in 10 ml 0.001 N NaOH and 24.8 mg cytochrome c mixed with 100 ml 50 mM KH_2PO_4-Na_2HPO_4, 0.1 mM EDTA, pH 7.8) was pipetted into a cuvette. The suspension of submitochondrial particles was spun and 25 µl of the supernatant (0.5 mg protein) was added to solution A. The reaction was started by addition of 25 µl of solution B [freshly prepared xanthine oxidase in 0.1 mM EDTA to produce a rate of cytochrome c reduction of 0.025 absorbance units/min in the control without superoxide dismutase (SOD)]. The absorbance change at 550 nm was recorded at 25 °C for 5 min.

Mitochondrial glutathione peroxidase activity

This was measured using the method of Flohé and Günzler [30]. The following solutions were pipetted into a cuvette: 500 µl of buffer containing 0.1 M potassium phosphate/1 mM EDTA, pH 7.0, 100 µl submitochondrial particles (50 µg protein), 100 µl (0.24 U) glutathione reductase (freshly prepared in 0.1 M potassium phosphate/0.1 mM EDTA, pH 7.0) and 100 µl of 10 mM GSH. The mixture was preincubated for 10 min at 25 °C. One hundred microliters 1.5 mM NADPH in 0.1% $NaHCO_3$ was then added. The reaction was started by adding 100 µl of 12 mM t-butyl hydroperoxide. The decrease in absorption at 340 nm was monitored for 5 min. The extinction coefficient of NADPH is 6.2 $mM^{-1}\,cm^{-1}$.

Mitochondrial glutathione reductase activity

This was measured as previously described [31]. One hundred and fifty micrograms of submitochondrial particles was added to 1 ml of 50 mM potassium phosphate, pH 7.6, 0.1 mM NADPH, 1 mg bovine serum albumin and 3.25 mM oxidized glutathione. The oxidation of NADPH was monitored at 340 nm at 25 °C for 5 min in a spectrophotometer. The extinction coefficient of NADPH is 6.2 $mM^{-1}\,cm^{-1}$.

Measurement of malondialdehyde

The extent of lipid peroxidation was determined by the thiobarbituric acid (TBA) test [32]. Fifty microliters of 8.1% SDS, 375 µl of 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 375 µl of 0.6% aqueous solution of TBA were added to 200 µl of 0.4 mg/ml mitochondria suspension. The mixture was heated in a water bath at 95 °C for 60 min. After cooling, 1 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added and mixed vigorously. After centrifugation at 4000 g for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. Results were expressed in malondialdehyde equivalents using 1,1,3,3,-tetraethoxypropane as the standard.

Statistical analysis

Data are expressed as means ± SE and were subjected to ANOVA analysis followed by a Tukey post hoc test. A p-value of less than 0.05 was considered significant. Two sets of comparisons were carried out. The first was within group 1 for the healthy controls before and post-PH. The second comparison was between group 1 and group 2 at all time points of the study.

Results

Respiratory enzymes activities in normal and cirrhotic livers

The livers of all thioacetamide treated animals were grossly cirrhotic. Similar macronodular cirrhosis had been previously observed in rats subsequent to continuous oral or intraperitoneal administration of thioacetamide [21, 22, 33]. Liver mitochondria were isolated from control and cirrhotic rats. Using succinate as the substrate, there were no differences in state 3 respiration, state 4 respiration, the respiratory control and the ADP/O ratios between the two groups of animals. Activities of the respiratory chain enzymes and the mitochondrial ATPase were also examined. There were no significant differences in the SCCR, CCO and ATPase activities. However, NCCR activity was significantly lowered in cirrhotic livers (table 1).

Mitochondrial antioxidant capacity in normal and cirrhotic livers

Within the mitochondria, enzymes involved in antioxidant defenses include SOD and glutathione peroxidase. The activities of these enzymes as well as that of glutathione reductase and the mitochondrial GSH levels were analyzed. In cirrhotic livers, total hepatic GSH levels were not significantly different from controls but mitochondrial GSH levels were significantly lower. There was no significant difference in the mitochondrial SOD activity but mitochondrial glutathione peroxidase was significantly lowered in the cirrhotic group. In contrast, mitochondrial glutathione reductase activity was significantly higher (table 2).

Table 1. Mitochondrial function in control (group 1) and cirrhotic (group 2) rats.

	Group 1	Group 2
State 3 respiration (nmol O atom/ min per milligram protein)	116.6 ± 5.1	113.4 ± 5.0
State 4 respiration (nmol O atom/ min per milligram protein)	23.9 ± 1.1	28.9 ± 0.9
Respiratory control ratio	4.4 ± 0.1	4.0 ± 0.2
ADP/O ratio	1.9 ± 0.1	1.9 ± 0.1
ATPase activity (nmol/min per milligram protein)	62.2 ± 1.6	66.5 ± 1.6
NCCR activity (nmol/min per milligram protein)	673.4 ± 27.5	427.8 ± 23.5*
SCCR activity (nmol/min per milligram protein)	104.3 ± 6.3	88.1 ± 5.5
CCO activity (nmol/min per milligram protein)	130.2 ± 5.3	174.2 ± 7.7

Data are presented as the mean ± SE of 30 animals in each group. State 3 respiration, state 4 respiration, respiratory control ratio and ADP/O ratio were determined in the presence of succinate. Submitochondrial particles were used to determine the mitochondrial ATPase, NCCR, SCCR and CCO activities. * Compared with group 1, $p<0.05$ (Student's t test).

Changes in mitochondrial respiratory enzymes and ATPase following hepatectomy

To examine how PH influences mitochondrial respiratory activity, liver mitochondria were isolated at various time points post-PH. At each time point, mitochondria were isolated from six cirrhotic rats and six control rats. Mitochondria from each animal were assayed individually to determine activities related to the respiratory chain and oxidative phosphorylation. Activities from the excised

Table 2. Antioxidant status in control (group 1) and cirrhotic (group 2) rats.

	Group 1	Group 2
Hepatic GSH (nmol/mg protein)	82.0 ± 2.8	98.8 ± 2.7
Mt GSH (nmol/mg protein)	7.6 ± 0.2	1.9 ± 0.1*
Mt GPx activity (nmol/ min per milligram protein)	301.7 ± 11.3	189.1 ± 7.6*
Mt GRd activity (nmol/ min per milligram protein)	23.2 ± 1.1	36.3 ± 2.0*
Mt SOD activity (U/mg protein)	18.5 ± 0.8	15.0 ± 1.3

Data are presented as the mean ± SE of 30 animals in each group. Liver homogenates were used to determine the hepatic GSH level. Submitochondrial particles were used to determine the mitochondrial GSH level, glutathione peroxidase (GPx), glutathione reductase (Mt) (GRd) and SOD activities. * Compared with group 1, $p < 0.05$ (Student's t test).

livers of control rats were used as the basis for comparison and set at 100%. Comparisons were first carried out to determine if there were significant changes in controls (group 1 rats) following PH and, second, to determine if there were significant differences between the control (group 1) rats and the cirrhotic (group 2) rats during the entire course of the experiment.

Respiration using succinate was first examined. Six hours after PH, state 3 and state 4 respiration were reduced to 65% ± 7% and 65% ± 3% respectively in the control group while the RCR was significantly increased at 24 h post-PH (fig. 1A–C). In contrast, for the cirrhotic rats, the RCRs were significantly lower when compared to the corresponding controls from 6 to 48 h post-PH (fig. 1C). This concurs with the SCCR activities over the same time points. SCCR activity was lowered from 6 to 72 h post-PH although statistical significance was only observed at 48 h (fig. 2A). The ADP/O ratios were not affected except at 6 h where the ratio for the cirrhotic group was significantly lowered when compared to the corresponding controls (fig. 1D). Increased ATPase activity at 3 and 6 h post-PH was also observed in mitochondria from cirrhotic rats when compared to controls (fig. 3).

Mitochondria isolated from control rats showed no changes in NCCR activity during the regenerative process. However, NCCR activity at all time points was significantly reduced in mitochondria from cirrhotic animals when compared to controls (fig. 2B). Following PH, the CCO in controls was significantly increased from 24 to 72 h post-PH. CCO activity in mitochondria from cirrhotic animals was not significantly different from that of the control group during the course of the study (fig. 2C).

Changes in mitochondrial antioxidant capacity following partial hepatectomy

Following PH, mitochondrial GSH levels decreased at 3 and 6 h but returned to pre-hepatectomy levels at 24 h in controls (fig. 4A). A transient drop at 3 h followed by a peak at 24 h in total GSH levels was also observed for the controls (fig. 4B). In the cirrhotic group, mitochondria GSH levels were consistently lower than those of controls at all time points (fig. 4A) even though there were no significant differences in total hepatic GSH levels between

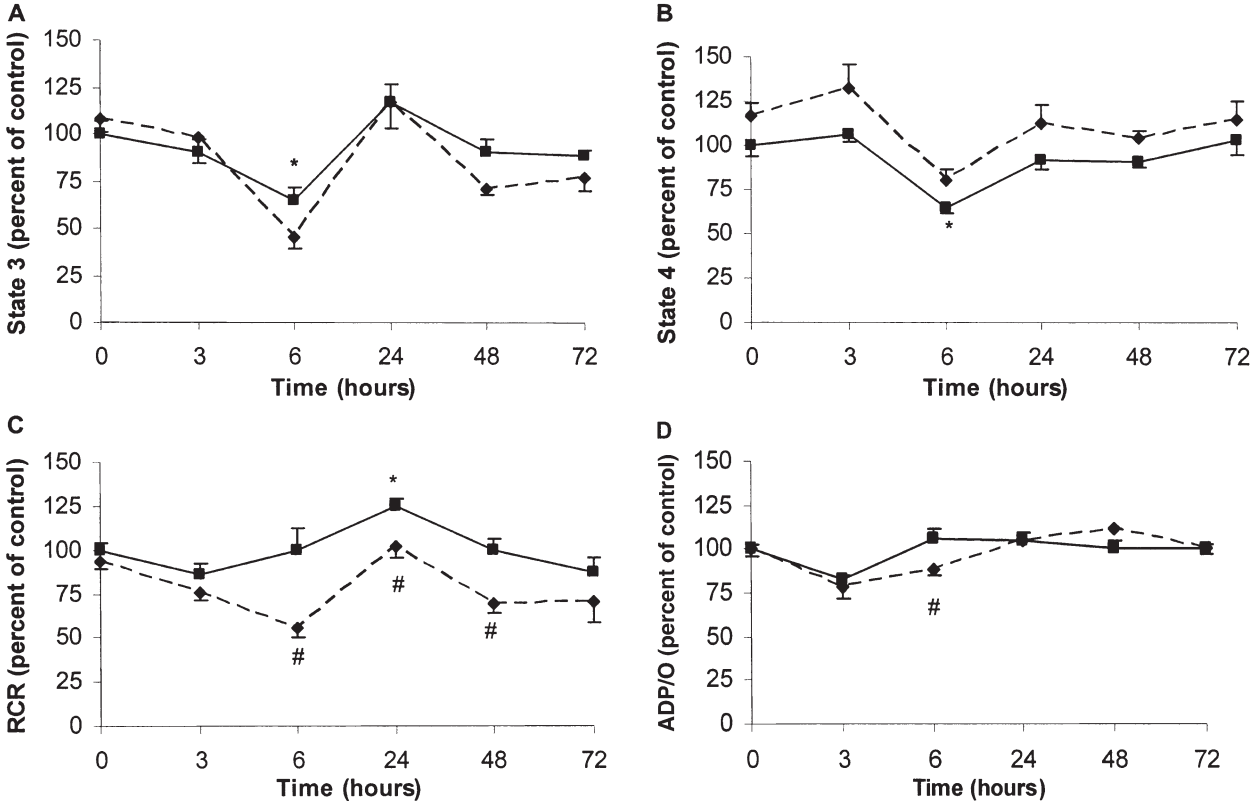

Figure 1. Changes in liver mitochondrial respiration of control (solid line) and cirrhotic rats (broken lines) following partial hepatectomy. State 3 respiration (*A*), state 4 respiration (*B*), respiratory control ratio (RCR) (*C*) and ADP/O ratio (*D*) were measured using succinate as the substrate. Results (mean ± SE from six rats in each group) are expressed as percent of control activity at 0 h (100%). Control values for state 3 and state 4 respiration, RCR and ADP/O ratio were 116.6 ± 5.1 nmol O atom/min per milligram mitochondrial protein, 23.9 ± 1.1 nmol O atom/min per milligram mitochondrial protein, 4.4 ± 0.1 and 1.9 ± 0.1, respectively, and were determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. *$p < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

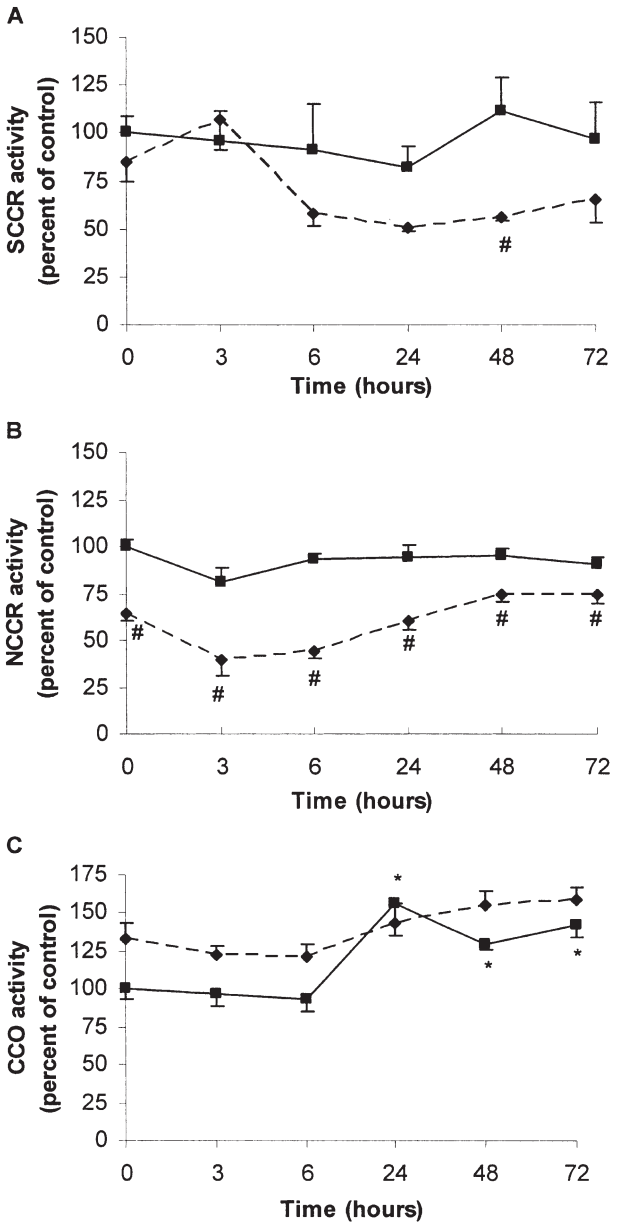

Figure 2. Time-dependent changes SCCR (*A*), NCCR (*B*) and CCO (*C*) activities from control (solid line) and cirrhotic rats (broken lines) after PH. Results (mean ± SE from six rats in each group) are expressed as percent of control activity at 0 h (100%). Control values for SCCR, NCCR and CCO were 104.3 ± 6.3 nmol/min per milligram mitochondrial protein, 673.4 ± 27.5 nmol/min per milligram mitochondrial protein and 130.2 ± 5.3 nmol/min per milligram mitochondrial protein, respectively, and were determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. *$p < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

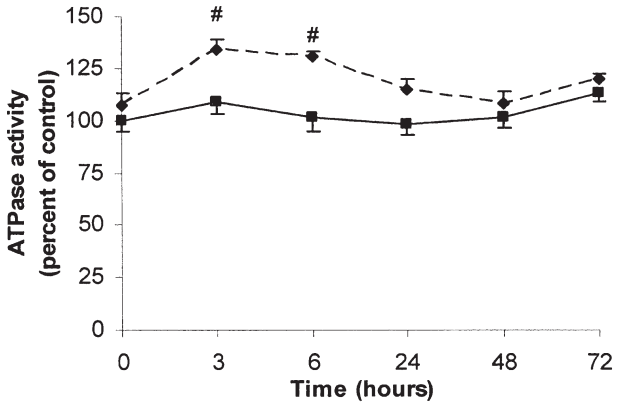

Figure 3. Mitochondrial ATPase activity from control (solid line) and cirrhotic rats (broken lines) after PH. Results (mean ± SE from six rats in each group) are expressed as percent of control activity at 0 h (100%). The control activity was 62.2 ± 1.6 nmol/min per milligram mitochondrial protein and was determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

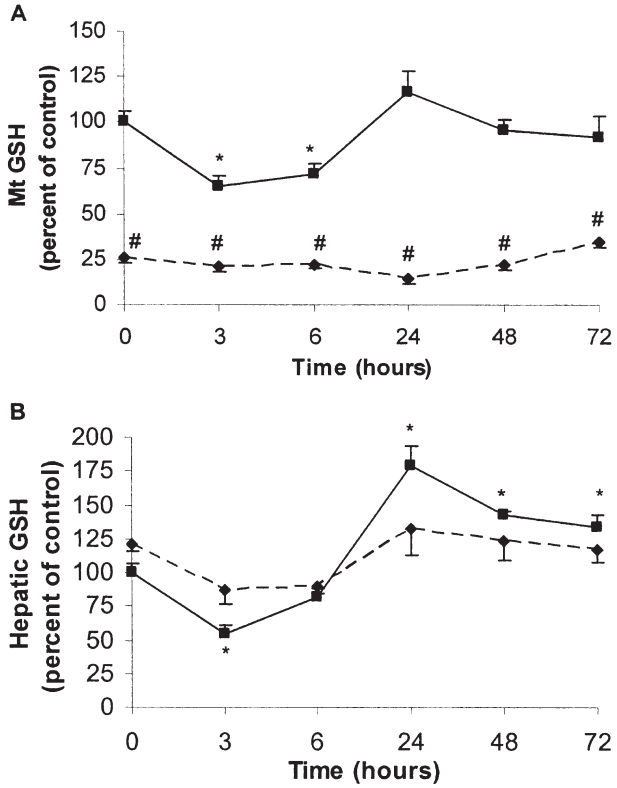

Figure 4. Mitochondrial GSH (*A*) and hepatic GSH (*B*) content in control (solid line) and cirrhotic rats (broken lines) after PH. Results (mean ± SE from six rats in each group) are expressed as percent of control at 0 h (100%). Control GSH content was determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. The control mitochondrial GSH level was 7.6 ± 0.2 nmol/mg protein while that for hepatic GSH was 82.0 ± 2.8 nmol/mg protein. *$p < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

the two groups of animals throughout the experimental time frame (fig. 4B).

Mitochondria glutathione peroxidase activities in the cirrhotic animals were also consistently lower than those of controls during the entire experimental time frame, while mitochondria glutathione reductase activity was comparable or even higher than that for controls (fig. 5A–B).

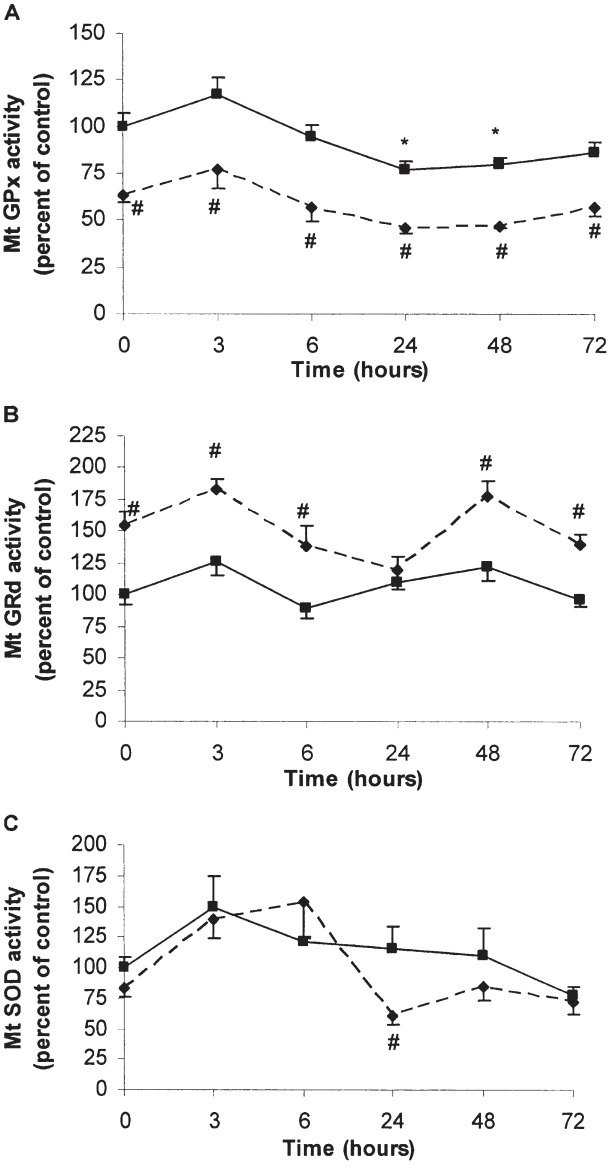

Figure 5. Mitochondrial peroxidase GPx (*A*), glutathione reductase (GRd) (*B*) and SOD (*C*) activities from control (solid line) and cirrhotic rats (broken lines) after PH. Results (mean ± S.E.M. from six rats in each group) are expressed as percent of control activity at 0 h (100%). The control activities were determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. Control values were 301.7 ± 11.3 nmol/min per milligram protein, 23.2 ± 1.1 nmol/min per milligram protein and 18.5 ± 0.8 U/mg protein for GPx, GRd and SOD, respectively. *$p < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

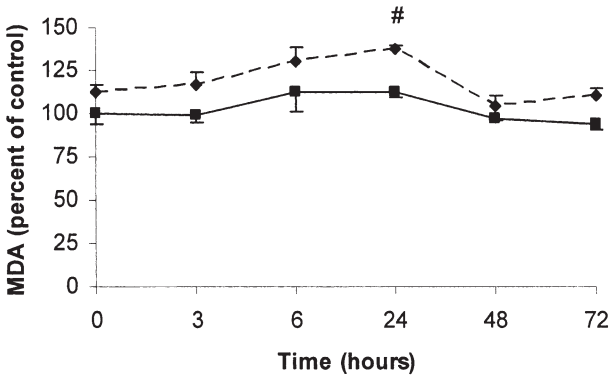

Figure 6. Mitochondrial TBARS level from control (solid line) and cirrhotic rats (broken lines) after PH. Results (mean ± SE from six rats in each group) are expressed as percent of control activity at 0 h (100%). The control activity was 1.84 ± 0.05 nmol malondialdehyde (MDA)/mg mitochondrial protein and was determined from the excised livers (at 0 h) of all control rats (n = 30) used in the study. #$p < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

Lipid peroxidation was assessed using the TBA-reacting substances (TBARS) assay. The only significant rise was observed in the mitochondria of cirrhotic rats 24 h post-PH (fig. 6). This coincides with a significantly lowered mitochondria SOD activity at the same time point (fig. 5C).

Discussion

In a healthy adult liver, hepatocytes are quiescent. However, following hepatic resection or injury, the liver has the capacity to regenerate fully. In humans, hepatic resection is often carried out on a diseased, cirrhotic liver which may result in delayed regeneration or even failure to regenerate. To enable us to examine the changes that occur following resection of cirrhotic livers, the thioacetamide-induced rodent model of liver cirrhosis was employed in this study.

In a healthy liver, energy required for all cellular processes is supplied by the mitochondria through the process of oxidative phosphorylation. A series of enzyme complexes are involved in this process which couples the oxidation of the electron donors, NADH or $FADH_2$, with ATP synthesis from ADP and inorganic phosphate. Using rodent models of cirrhosis, changes in mitochondrial functions have been observed in cirrhotic livers. These include the loss of respiratory control in the presence of an NADH-linked substrate, glutamate [20, 34], or an $FADH_2$-linked substrate, succinate [34]. In this study, liver cirrhosis in rats was induced with thioacetamide and the activities of respiratory enzyme complexes were first examined. In cirrhotic livers, NCCR activities were significantly reduced, suggesting that NADH-linked oxida-

tion may be impaired as previously reported [20, 34]. However, in our study, succinate-linked respiration was not affected by cirrhosis. In agreement with this observation, all complexes involved in $FADH_2$-linked oxidative phosphorylation, namely SCCR, CCO and ATPase activities, were also unchanged. This is in contrast to previous observations [34] and may be due to the rats' access to food prior to sacrifice. Feeding leads to a greater degree of coupling of oxidative phosphorylation [35], and in this study, the animals were allowed free access to food. Similar observations have been made in the carbon tetracholoride-induced model of cirrhosis [20]. The RCR was reduced in mitochondria from cirrhotic livers with β-hydroxybutyrate as substrate but not with succinate. This also concurs with previous observations that complex I is more susceptible to damage than complex II and complex III [36, 37].

The process of oxidative phosphorylation can result in the generation of reactive oxygen species as byproducts. Within the mitochondria, the reaction catalyzed by SOD (also known as Mn-SOD) is the first step in dealing with oxidative stress. This removes the superoxide anion and generates hydrogen peroxide which is then reduced to water by glutathione peroxidase, a selenoprotein, using GSH as the cosubstrate. The oxidized glutathione is then regenerated following reduction by glutathione reductase [for a review see ref. 7]. In this thioacetamide-induced model of cirrhosis, the mitochondrial antioxidant defenses were impaired although mitochondrial lipid peroxidation was not significantly increased, as observed in the study by Herńandez-Muñoz et al. [38]. Although mitochondrial SOD activity was not affected, mitochondrial glutathione peroxidase activity was significantly decreased in the cirrhotic liver. This decrease may be due to the loss of selenium from the liver following chronic thioacetamide treatment [18, 39]. Mitochondrial GSH levels in the cirrhotic liver were significantly lower than in the control. The decreased mitochondrial GSH level was not due to impaired regeneration of oxidized GSH, because mitochondrial glutathione reductase was significantly higher than or similar to controls. In addition, similar to previous observations [38, 40], total hepatic GSH levels were not different from controls. Thus, the lowered mitochondrial GSH levels may be attributed to an impaired transport of GSH from the cytoplasm into the mitochondria or an increased utilization of GSH to counter oxidative stress.

Following hepatic resection, ATP demand increases due to loss of liver mass and the needs of the regenerative process. In normal rat liver remnants, the energy charge potential as well as liver ATP levels have been shown to decrease significantly at 24 h post-PH [41, 42]. Changes in mitochondrial functions have also been observed. These include changes in RCR [3, 4, 8–10]. In this study, as in earlier studies using rats [10, 41, 42], a peak in RCR was observed at 24 h post-PH in normal liver remnants. CCO activities were also enhanced from 24 to 72 h post-PH and this may represent the initial steps in mitochondria biogenesis [43]. Both these changes will lead to enhancement of phosphorylation rates, which were also maximal at 24 h post-PH [41]. The increase in the RCR and CCO are probably adaptive measures to enhance mitochondrial ATP-synthesizing activity, because the increase in RCR and phosphorylation rate coincides with low levels of liver ATP [41, 42].

A key difference between the normal and cirrhotic livers post-PH was the significantly lowered NCCR activity both before and after hepatectomy, which would diminish NADH-linked respiration. In contrast to normal liver remnants, the RCR in the cirrhotic group was significantly lower [this study and ref. 42] than that of the corresponding controls indicating a higher degree of uncoupling of oxidative phosphorylation. Taken together, this would account for the absence of enhancement of phosphorylation rates in cirrhotic livers after partial hepatectomy [41] and would contribute to the depressed energy status and low ATP levels after hepatectomy [6, 41, 42]. This will in turn result in a less efficient regeneration process and may even cause liver failure if the degree of histologic damage in the remnant liver is high and oxidative phosphorylation is greatly impaired.

An increase in oxygen free radicals has been shown to occur during liver regeneration after PH [8]. In addition, TNF-α, which has growth-stimulatory effects after hepatectomy, also increases mitochondrial oxidant production [13]. In the early phase of regeneration in normal remnants (before 24 h post-PH), decreased mitochondrial and hepatic GSH levels were observed (fig. 4) [11] probably as a consequence of the increased oxidant production [8, 13]. Following an initial drop in hepatic GSH levels at 3 h post-PH, increases were observed from 24 to 72 h post-PH. Both a normal baseline hepatic GSH level and a subsequent increase in GSH following PH are necessary for normal regeneration [44]. The mechanism for this is currently not known [44] although increased GSH levels have been shown to be associated with an early proliferative response and are essential for the cell to enter S phase [45, 46]. Despite the initial decrease in GSH levels after PH in normal livers, there was no increase in lipid peroxidation in the mitochondria, similar to observations made in an earlier study [14]. Mitochondrial SOD and glutathione reductase activities were not affected by PH. However, decreased mitochondrial glutathione peroxidase activities at 24 h and 48 h post-PH were observed. Although the reason for this is not clear, glutathione peroxidase can be inactivated by a variety of physiological substances including nitric oxide. Thus, the decreased activity may be the result of inactivation of the peroxidase by nitric oxide which is released in the regenerating liver after PH [47, 48].

Cirrhotic livers had significantly lower mitochondrial GSH as well as lower mitochondrial glutathione peroxidase activities. Following hepatectomy, hepatic GSH levels were not different from controls but both mitochondrial GSH and glutathione peroxidase activity remained depressed up to 72 h post-PH. In addition, a significant decrease in mitochondrial SOD activity at 24 h post-PH together with increased lipid peroxidation was also observed in the cirrhotic remnants (this study) [41]. Thus unlike the control, the cirrhotic remnant is unable to provide sufficient antioxidant defense (in the form of GSH and glutathione peroxidase) in the mitochondria following PH.

We conclude that the impaired mitochondrial antioxidant system and reduced complex I activity in cirrhotic livers may in turn generate more oxidative stress and also adversely affect the ability of the mitochondria to carry out oxidative phosphorylation following PH. The diminished ATP production, if critical, would then impede the liver regeneration process resulting in liver failure. Indeed, diminished ATP production has been observed not only in rodent experimental models [41, 42] but also during the regenerative process of cirrhotic livers in humans [6].

Acknowledgement. This study was supported by research grants from the National Medical Research Council, Singapore (awarded to T. Tan as PI and C. K. Leow as co-PI) and from the Singapore Cancer Society. We thank Dr M. Whiteman for use of the Germini XS microplate spectrofluorometer.

1 Fausto N. (2000) Liver regeneration. J. Hepatol. **32 (suppl. 1):** 19–31
2 Court F., Wemyss-Holden S., Dennison A. and Maddern G. (2002) The mystery of liver regeneration. Br. J. Surg. **89:** 1089–1095
3 Kamiyama Y., Ozawa K. and Honjo I. (1976) Changes in mitochondrial phosphorylation activity and adenylate energy charge of regenerating rabbit liver. J. Biochem. **80:** 875–881
4 Inomoto T., Tanaka A., Mori S., Jin M., Sato B., Yanabu N. et al. (1994) Changes in the distribution of the control of the mitochondrial oxidative phosphorylation in regenerating rabbit liver. Biochim. Biophys. Acta **1188:** 311–317
5 Guerrieri F., Muolo L., Cocco T., Capozza G., Turturro N., Cantatore P. et al. (1995) Correlation between rat liver regeneration and mitochondrial energy metabolism. Biochim. Biophys. Acta **1272:** 95–100
6 Mann D., Lam W., Hjelm M., So N., Yeung D., Metreweli C. et al. (2001) Human liver regeneration: Hepatic energy economy is less efficient when the organ is diseased. Hepatology **34:** 557–565
7 Ferńandez-Checa J., Kaplowitz N., García-Ruiz C., Colell A., Miranda M., Marí M. et al. (1997) GSH transport in mitochondria: defense against TNF-induced oxidative stress and alcohol-induced defect. Gastrointest. Liver Physiol. **36:** G7–G17
8 Tsai J., King K., Chang C. and Wei Y. (1992) Changes of mitochondrial respiratory functions and superoxide dismutase activity during liver regeneration. Biochem. Inter. **28:** 205–217
9 Satoh S., Tanaka A., Hatano E., Inomoto T., Iwata S., Kitai T. et al. (1996) Energy metabolism and regeneration in transgenic mouse liver expressing creatine kinase after major hepatectomy. Gastroenterol. **110:** 1166–1174
10 Herńandez-Muñoz R., Śanchez-Sevilla L., Marínez-Gómez A. and Dent M. (2003) Changes in mitochondrial adenine nucleotides and in permeability transition in two models of rat liver regeneration. Hepatology **37:** 842–851
11 Guerrieri F., Vendemiale G., Grattagliano I., Cocco T., Pellecchia G. and Altomare E. (1999) Mitochondrial oxidative alterations following partial hepatectomy. Free Radic. Biol. Med. **26**: 34–41
12 Vendemiale G., Guerrieri F., Grattagliano I., Didonna D., Muolo L. and Altomare E. (1995) Mitochondrial oxidative phosphorylation and intracellular glutathione compartmentation during rat liver regeneration. Hepatology **21:** 1450–1454
13 Lee F., Li Y., Zhu H., Yang S., Lin H., Trush M. et al. (1999) Tumor necrosis factor increases mitochondrial oxidant production and induces expression of uncoupling protein-2 in the regenerating mice liver. Hepatology **29:** 677–687
14 Aguilar-Delfín I., López-Barrera F. and Herñandez-Muñoz R. (1996) Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CCl_4 administration. Hepatology **24:** 657–662
15 Parkin D., Bray F. and Devesa S. (2001) Cancer burden in the year 2000 – the global picture. Eur. J. Cancer. **37**: S4–S66
16 Kanematsu T., Takenaka K., Matsumata T., Furuta T., Sugimachi K. and Inokuchi K. (1984) Limited hepatic resection effective for selected cirrhotic patients with primary liver cancer. Ann. Surg. **199**: 51–56
17 Cabré M., Camps J., Paterńain J., Ferré N. and Joven J. (2000) Time-course of changes in hepatic lipid peroxidation and glutathione metabolism in rats with carbon tetrachloride-induced cirrhosis. Clin. Exp. Pharmacol. Physiol. **27:** 694–699
18 Balkan J., Doğru-Abbasoğlu S., Kanbağli Ö., Çevikbaş U., Aykaç-Toker G. and Uysal M. (2001) Taurine has a protective effect against thioacetamide-induced liver cirrhosis by decreasing oxidative stress. Hum. Exp. Toxicol. **20:** 251–254
19 Moller B. and Dargel R. (1984) Structural and functional impairment of mitochondria from rat livers chronically injured by thioacetamide. Acta Pharmacol. Toxicol. **55:** 126–132
20 Krähenbühl S., Stucki J. and Reichen J. (1989) Mitochondrial function in carbon tetrachloride-induced cirrhosis in the rat: qualitative and quantitative defects. Biochem. Pharmacol. **38:** 1583–1588
21 Chijiiwa K., Nakano K., Kameoka N., Nagai E. and Tanaka M. (1994) Proliferating cell nuclear antigen, plasma fibronectin, and liver regeneration rate after seventy percent hepatectomy in normal and cirrhotic rat. Surgery **116:** 544–549
22 Zhao G., Nakano K., Chijiiwa K., Ueda J. and Tanaka M. (2002) Inhibited activities in CCAAT/enhancer-binding protein, activating protein-1 and cyclins after hepatectomy in rats with thioacetamide-induced liver cirrhosis. Biochem. Biophys. Res. Commun. **292:** 474–481
23 Higgins G. and Anderson R. (1931) Experimental pathology of the liver: restoration of the white rat following partial surgical removal. Arch. Pathol. **12:** 186–202.
24 Johnson D. and Lardy H. (1967) Isolation of liver or kidney mitochondria. Methods Enzymol. **10:** 94–96
25 Estabrook R. (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol. **10:** 41–47
26 Boffoli D., Scacco S., Vergari R., Solarino G., Santacroce G. and Papa S. (1994) Decline with age of respiratory chain activity in human skeletal muscle. Biochim. Biophys. Acta **1226:** 73–82
27 Penney C. and Bolger G. (1978) A simple microassay for inorganic phosphate. II. Anal. Biochem. **89:** 297–303
28 Fernandez-Checa J. and Kaplowitz N. (1990) The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal. Biochem. **190:** 212–219
29 Flohé L. and Ötting F. (1984) Superoxide dismutase assays. Methods Enzymol. **105:** 93–104

30 Flohé, L. and Günzler, W. (1984) Assays of glutathione peroxidase. Methods Enzymol. **105:** 114–121
31 Racker E. (1955) Glutathione reductase from bakers' yeast and beef liver. J. Biol. Chem. **217:** 855–865
32 Ohkawa H., Ohishi N. and Yagi K. (1979) Assays for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95:** 351–358
33 Li X., Benjamin I. and Alexander B. (2002) Reproducible production of thioacetamide-induced macronodular cirrhosis in the rat with no mortality. J. Hepatol. **36:** 488–493
34 Nozu F., Takeyama N. and Tanaka T. (1992) Changes of hepatic fatty acid metabolism produced by chronic thioacetamide administration in rats. Hepatology **15:** 1099–1106
35 Soboll S. and Stucki J. (1985) Regulation of the degree of coupling of oxidative phosphorylation in intact rat liver. Biochim. Biophys. Acta **807:** 245–254
36 Cederbaum A., Lieber C. and Rubin E. (1974) Effects of chronic ethanol treatment on mitochondrial functions damage to coupling site I. Arch. Biochem. Biophys. **165:** 560–569
37 Bottenus R., Spach P., Filus S. and Cunningham C. (1982) Effect of chronic ethanol consumption on energy-linked processes associated with oxidative phosphorylation: proton translocation and ATP-Pi exchange. Biochem. Biophys. Res. Commun. **105:** 1368–1373
38 Herńandez-Muñoz R., Díaz-Muñoz M., López V., López-Barrera F., Yáñez L., Vidrio S. et al. (1997) Balance between oxidative damage and proliferative potential in an experimental rat model of CCl_4-induced cirrhosis: protective role of adenosine administration. Hepatology **26:** 1100–1110
39 Al-Bader A., Abul H., Hussain T., Al-Moosawi M., Mathew T. and Dashti H. (1998) Selenium and liver cirrhosis. Mol. Cell. Biochem. **185:** 1–6
40 Muller D., Sommer M., Kretzschmar M., Zimmermann T., Buko V., Lukivskaya O. et al. (1991) Lipid peroxidation in thioacetamide-induced macronodular rat liver cirrhosis. Arch. Toxicol. **65:** 199–203
41 Morimoto T. and Isselhard W. (1992) Absence of mitochondrial enhancement in the remnant liver after partial hepatectomy in cirrhotic rats. Res. Exp. Med. (Berl) **192:** 89–97
42 Kurokawa T., Nonami T., Sugiyama S., Ozawa T. and Takagi H. (1991) Effects of long-acting superoxide dismutase on liver metabolism and major hepatic resection in rats with cirrhosis. Eur. Surg. Res. **23:** 65–72
43 Krähenbühl S. and Reichen J. (1992) Adaptation of mitochondrial metabolism in liver cirrhosis: different strategies to maintain a vital function. Scand. J. Gastroenterol. Suppl. **193:** 90–96
44 Huang Z., Chen C., Zeng Z., Yang H., Oh J., Chen L. et al. (2001) Mechanism and significance of increased glutathione level in human hepatocellualar carcinoma and liver regeneration. FASEB J **15**:19–21
45 Messina J. and Lawrence D. (1989) Cell cycle progression of glutathioine-depleted human peripheral blood mononuclear cells is inhibited at S phase. J. Immunol. **143:** 1974–1981
46 Poot M., Teubert H., Rabinovitch P. and Kavanagh T. (1995) De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. J. Cell. Physiol. **163:** 555–560
47 Hortelano S., Dewez B., Genaro A., Diaz-Guerra M. and Bosca L. (1995) Nitric oxide is released in regenerating liver after partial hepatectomy. Hepatology **21:** 776–786
48 Miyamoto Y., Koh Y., Park Y., Fujiwara N., Sakiyama H., Misonou Y. et al. (2003) Oxidative stress caused by inactivation of glutathione peroxidase and adaptive responses. Biol. Chem. **384:** 567–574