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Assessment of in Vivo Oxidative Lipid Metabolism Following Acute Microcystin-LR-induced Hepatotoxicity in Rats

RHEAL A. TOWNER*, SHARELLE A. STURGEON and KRISTIN E. HORE

Department of Physiology and Pharmacology, School of Biomedical Sciences, North Queensland Magnetic Resonance Centre, James Cook University, Townsville, Qld, Australia

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Oxidative lipid metabolism as a result of acute cyanobacterial toxin-induced hepatotoxicity was monitored in male Sprague–Dawley rats using electron spin resonance (ESR) spectroscopy and image-guided proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. ESR spectroscopy, coupled with spin trapping, was used to trap and detect lipid-derived radicals, formed in rat livers after acute in vivo exposure (LD_{50}) to the cyanobacterial toxin, microcystin-LR (MCLR). A statistically significant increase in the levels (spectral peak integrals) of lipid radicals was detected in MCLR-treated livers ($p < 0.05$) ($n = 8$), in comparison to control livers ($n = 6$). In order to monitor lipid metabolism, before and for a period of 3 h, following toxin exposure, in vivo proton image-guided NMR spectroscopy was used. A statistically significant decrease in the levels of lipid methylene hydrogen resonances (spectral peak integrals) was observed from MCLR-treated livers ($n = 6$) 2 and 3 h post-exposure ($p < 0.05$), in comparison to controls ($n = 6$). Image-guided NMR spectroscopy was also used to detect significant decreasing levels of in vivo glutamine/glutamate, following exposure to MCLR. Biochemical assessment of perchloric extracts of liver glutamine and glutamate levels correlated with NMR spectroscopy results. Lactate levels measured as perchloric acid extracts, were also found to significantly decrease. In addition, assessment of serum enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were used to confirm hepatotoxicity ($n = 20$). This study strongly suggests that oxidative stress related processes are involved in in vivo microcystin-induced hepatotoxicity in mammals, and may play an integral role in MCLR-induced toxicity.

Keywords: Electron spin resonance (ESR) spectroscopy; Spin trapping; Free radicals; Nuclear magnetic resonance (NMR) spectroscopy; Hepatotoxicity; Microcystins

INTRODUCTION

Bloom-forming cyanobacteria in fresh- and brackish-waters are widely recognised for their toxin production, which can be hazardous to a number of mammalian species.[1,2] Most of the toxic cyanobacteria are found in freshwater, and most poisonings occur among terrestrial animals, particularly ruminants, after drinking algal infected freshwater supplies.[2,3] The most frequent bloom-forming cyanobacteria in Australian freshwater tributaries and reservoirs are *Anabaena circinalis* and *Microcystis aeruginosa*.[4] *M. aeruginosa* produce cyclic hepta-peptide toxins referred to as either cyanoginosins or microcystins.[4] The most toxic microcystin, microcystin-LR (MCLR), has an LD_{50} value of 65 μg/kg i.p. in mice[1,5]. Microcystins are potent hepatotoxins in mammals, causing widespread hepatocellular failure and necrosis.[4–6] Hepatic lesions are initially observed 10 min post-administration of MCLR, and consist of centrilobular widening of hepatocyte intracellular spaces.[5] The worst incident involving human exposure occurred in 1996, in Caruaru, Brazil, when haemodialysis patients were exposed to dialysate fluid contaminated with cyanobacteria.[7] Of the 126 patients dialysed, 85% developed severe liver injury, and 60 of the patients died.[7]

The primary mechanism for the toxicity of microcystins involves the inhibition of protein phosphatases 1 and 2A, which are responsible for

*Corresponding author. Tel.: +61-7-4781-4448. Fax: +61-7-4779-1526. E-mail: rheal.towner@jcu.edu.au.

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maintaining cellular homeostasis by participating in carbohydrate and lipid metabolism, signal transduction, maintenance of cytoskeletal structure, suppression of cell transformation and regulation of apoptosis and cell division rates.[4–6] Studies in the last 10 years have shown that the toxicity of microcystins may also be mediated by membrane alterations via oxidative processes.[8,9] Increased lipid peroxidation was observed in hepatocytes[10] and hepatic microsomes[8] following exposure to microcystins or lyophilised cyanobacteria extracts. MCLR is thought to either directly or indirectly affect the function and alter the membrane structure of smooth endoplasmic reticulum.[8] In addition, membrane-active antioxidants, such as vitamin E and the flavone silymarin (extracted from milk thistle *Silybum marianum*), as well as glutathione (GSH) and the monoethyl ester of GSH, have shown significant protection from lethality due to MCLR in mice.[9] It has also been shown that microcystin toxicity results in a decrease in GSH levels.[11] Recently GSH conjugates of microcystins have been isolated in rat liver by HPLC.[12,13] Collectively, these results indicate indirectly that oxidative stress may play a role in the toxicity of MCLR, in addition to or as a result of protein phosphatase inhibition.

The aim of this study was to use electron spin resonance (ESR) spin trapping spectroscopy to detect and characterise free radical metabolites as a result of *in vivo* MCLR-induced hepatotoxicity in rats, and utilise *in vivo* image-guided proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to confirm alterations in rat liver lipid levels of oxidative lipid metabolism.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (150–200 g) were obtained from the James Cook University breeding colony and housed in the James Cook University animal facility ($n = 46$). Rats were fed with commercial rat chow and water *ad libitum*, and kept on a 12:12 h light / dark cycle. Animals were fasted 18 h prior to all experiments. Ethics approval was obtained from the Experimentation Ethics Review Committee of James Cook University (ethics approval numbers A404 and A532).

Toxins

Lyophylized MCLR (0.50 mg) was obtained from Calbiochem–Novabiochem (PO Box 140, Alexandria, NSW, Australia) and stored at 0°C. An export license was required from the United States Department of Commerce, Bureau of Export Administration, Washington, DC, to purchase MCLR (export license no. D264325). The toxin was dissolved in saline prior to use to produce stock solutions with a concentration of 1 mg / ml. $LD_{50} = 50\ \mu g/kg$ rat.

ESR Spectroscopy Studies

Rats were anaesthetised with isoflurane (2.0%) via inhalation at a rate of 500 ml O_2 / min. Treated rats ($n = 8$) were exposed to MCLR for 2 h via an intraperitoneal (i.p.) injection of a LD_{50} of toxin ($LD_{50} = 50\ \mu g$ toxin/kg rat) made up to a 500 ml aliquot with PBS. Control rats ($n = 6$) were treated with 500 µl PBS for 2 h via an i.p. injection. Each rat was administered PBN (α-phenyl-*tert*-butyl nitrone; i.p. injection; 125 mM / kg rat in 500 µl PBS and 500 µl olive oil to make an emulsion), a lipid-soluble spin-trapping compound, 1.5 h after toxin or saline treatment for 30 min. After the 2 h treatment period, the anaesthetised rats were euthanised via cervical dislocation. The livers were removed, homogenised (Plytron PT 1200 Kenematica AG hand held homogeniser) at 4°C and then centrifuged (Hettich Universal 16R) at 10,000 rpm at 4°C for 10 min. The supernatant was extracted either in hexane or chloroform / methanol (2:1), rotoevaporated (Buchi R-114) to dryness, and then reconstituted in 1.5 ml hexane or chloroform. Nitrogen gas was bubbled through the hexane or Folch extracts for removal of oxygen, a di-radical that interferes with the ESR spectral detection.

The hexane samples were then analysed on an ESR spectrometer (JEOL TE200), using an ESR round cell (4 mm dia), to detect lipid soluble radicals. ESR measurements were performed at room temperature using a JEOL JES-TE200 ESR spectrometer coupled with JEOL-ESPRIT computer software. The ESR parameters were set as follows: microwave power of 3.0 mW, microwave frequency of 9.425 GHz, modulation frequency of 100 kHz at 0.32 mT, time constant of 0.1 s, sweep time of 1 min, amplitude of 1×10^3, and sweep width of 5.0 mT. Multiple averages (20) were obtained for each spectrum. ESR spectral peak integrals were measured using the JEOL-ESPRIT software, and ESR simulations were performed using the JEOL Isotropic simulation version 1.11 software.

The Folch extracts were analysed on a Bruker EXM spectrometer equipped with a super high Q cavity (NIEHS, Research Triangle Park, NC, USA), using the following ESR parameters: microwave power of 20 mW, microwave frequency of 9.746 GHz, modulation frequency of 100 kHz, modulation amplitude of 1.0 G, time constant of 1.3 s, sweep time of 11.2 min, receiver gain of 1×10^5, sweep width of 70 G, and a single average. ESR simulations were performed using a program written by Duling[14] (NIEHS, Research Triangle Park, NC, USA).

In Vivo ^{1}H-NMR Spectroscopy Studies

Rats were anaesthetised with isoflurane (2.0%) via inhalation at a rate of 500 ml O_2/min. A Teflon tube (0.4 mm dia) was placed within the peritoneal cavity of the rat so that toxin could be administered after control data had been obtained without further manipulation of the animal from the bore of the magnet. Hepatotoxicity was induced in anaesthetised animals ($n = 6$) by a single i.p. injection via the Teflon tube, of a LD_{50} of stock toxin solution made up to a 200 µl aliquot with saline for both treatment groups. Control rats ($n = 6$) were administered 200 µl of saline i.p. via a Teflon tube.

^{1}H-NMR spectroscopy measurements were made using a Varian INOVA UNITY 7.0 T/18 cm horizontal bore small animal imaging spectrometer. Anaesthetised rats were placed in a ^{1}H-NMR birdcage probe with the liver localised centrally within the probe. The zyphoid process of the sternum, which can be felt externally on the ventral side of the rat, indicates the location of the central region of the liver. The probe was then placed in the centre of the bore of the magnet. A sagittal image was obtained to ensure the correct placement of the rat within the bore of the magnet, and to plan the position of the transverse slices. A respiratory gating device was used to gate the pulse sequence with the respiration of the animal to minimise motion artefacts.[15] Multiple ^{1}H-NMR T1-weighted images (spin echo sequence; repetition time (TR) of 800 ms, echo time (TE) of 23 ms) were obtained in the transverse plane to plan the position of the pixel voxel for STEAM (stimulated echo acquisition mode) localisation.

Spatial coordinates corresponding to a region of liver proximal to the hepatic portal vein in the T1-weighted images were used to form a voxel size of $6 \times 6 \times 6\ mm^3$ (216 µl). The STEAM method was used to obtain localised ^{1}H-NMR spectra from within the chosen voxel to monitor changes in lipid levels in the liver in response to cyanobacterial toxin administration. Changes in lipid hydrogen levels in response to saline administration over a period of 3 h were also monitored. A radio-frequency (RF) pulse, selective for the frequency at which water protons resonate was used to suppress the water signal. A 15 ms delay was used after the first and third 90° pulses, and a 30 ms delay was used after the second 90° pulse. The TR was 1.5 s. Localised shimming was used to obtain a spectral line width of ≤ 40 Hz. Localised spectra were obtained prior to treatment with cyanobacterial toxins, to obtain control (pretreated) data, and over a period of 3 h following administration of the toxin. Spectral analysis was performed on fitted parameters using algorithms and software on the NMR spectrometer.

Blood Serum Liver Enzyme Assays

Blood serum levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in rats used in addition to those used in the ESR and NMR studies, using Sigma reagent kits (Sigma Diagnostics, St Louis, MO). All assays were performed using a JASCO, V-530, UV–Vis spectrophotometer. Rats were anaesthetised with 2% isoflurane via inhalation at a rate of 1.0 l O_2/min. Hepatotoxicity was induced via a single i.p. injection of MCLR stock solution made up to 200 µl aliquots with saline ($n = 5$ for each time point). Control animals were injected with a single injection of 200 µl saline ($n = 5$ for each time point). Blood was collected at one and 3 h post-dosing. At each time point, the thoracic cavities of the corresponding anaesthetised animals were exposed and 1.0 ml of blood was immediately withdrawn from the left ventricle of the heart in a heparinised syringe. After the blood samples were obtained, the anaesthetised rats were immediately euthanised via cervical dislocation. The blood samples were centrifuged at 4°C and the serum kept for enzymatic analysis (Universal R 16 centrifuge, HD Scientific Supplies Pty.)

Preparation of Liver Extracts

The right- and right medial liver lobes were removed from rats used in the *in vivo* NMR experiments following the NMR assessment and freeze-clamped immediately using aluminium tongs at liquid nitrogen temperatures (−190°C). The tissue was powdered in liquid nitrogen using a mortar and pestle. The powdered tissue was poured into 50 ml disposable centrifuge tubes, the liquid nitrogen allowed to boil off, and then stored in a −80° freezer.

Powdered tissue (typically 0.1 g) was homogenised in 750 µl of ice-cold 3.6% perchloric acid (PCA) using a Biospec Mini Bead-beater. Ice-cold homogenates were centrifuged at 11,000 g for 5 min at room temperature using a Heraeus (sepatech) Biofuge 13 centrifuge. The supernatants were neutralised using 0.3 or 3 M potassium carbonate ($KHCO_3$), centrifuged at 11,000 g for 5 min and stored at −80°C.

Lactate Assay

Samples of rat liver extracts were added to cocktail (pH 9.9, 200 mM 2-amino-2-methylpropanol, 40 mM glutamate and 100 mM NAD^+) and distilled water in cuvettes. Glutamate pyruvate transaminase (GPT, pig heart, Boehringer Mannheim) was then added to each cuvette and their changes in absorbance at 340 nm was monitored using a GBC UV–Vis

spectrometer (model 918) until the reaction was complete. Lactate dehydrogenase (LDH, beef heart, Boehringer Mannheim) was subsequently added to each cuvette and their changes in absorbance at 340 nm was again monitored until the reaction was complete.

Glutamine/glutamate Assay

Glutamine and glutamate were measured in samples of liver extracts added to two sets of cuvettes. Glutaminase reagent was added to the first set and allowed to incubate for 45 min. This set of cuvettes measured glutamate only. Glutaminase reagent was not added to the second set, which measured both glutamate and glutamine. Glutamate reagent was then added to both sets of cuvettes and their absorption at 340 nm recorded. Glutamate dehydrogenase (GDH, Boehringer Mannheim) was subsequently added and the changes in absorbance at 340 nm were monitored until the reaction was complete. Glutamine was determined by subtracting the first set of cuvettes from the second set. The assay was validated using glutamine and glutamate standards.

Statistics

The ESR and NMR data were analysed using a Student's t-test with Statistica software (Statsoft, OK, USA). ESR and NMR peak integrals were expressed as mean ± SE. Significance was determined if p-values were less than 0.05.

RESULTS

ESR Spectroscopy Studies

ESR spectroscopy, coupled with spin-trapping, was used to detect and characterise the levels of free radical metabolites that arise from cyanobacterial hepatotoxin-induced liver damage in rats. Characteristic ESR signals observed in rat liver samples, pre-and post-exposure to MCLR, are shown in Fig. 1. A characteristic triplet of doublet ESR signal was obtained for the PBN radical adducts detected.

An ESR spectral simulation signal is shown in Fig. 2. The ESR spectra could only be simulated if two radical PBN adduct species were incorporated together, rather than any single species. The spin adduct coupling constants, relative amounts of each species for either the hexane or Folch extracts, and previous literature assignments[16–21] are shown in Table I.

For each hexane extract ESR spectrum, the total peak area for all trapped radicals, using a standardised sample preparation protocol, was calculated from the first peak of the triplet of doublet signal. Figure 3 illustrates the change in the ESR spectral

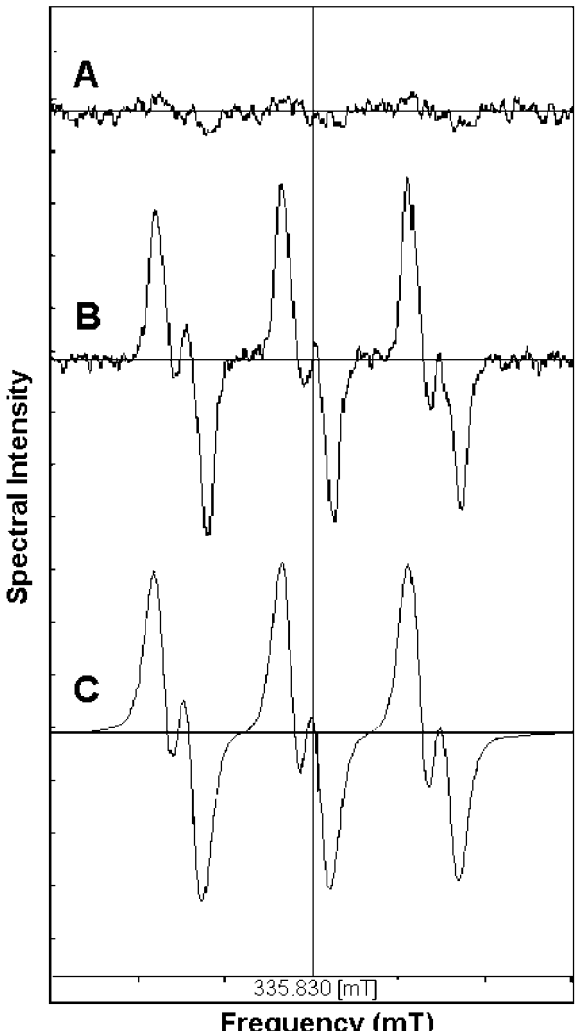

FIGURE 1 Representative electron spin resonance (ESR) PBN (α-phenyl tert-butyl nitrone) spin adduct spectra of rat liver extracts obtained from (A) control (pre-treatment), and (B) MCLR-treated (2 h post-treatment) rats. (C) Simulation of spectrum B.

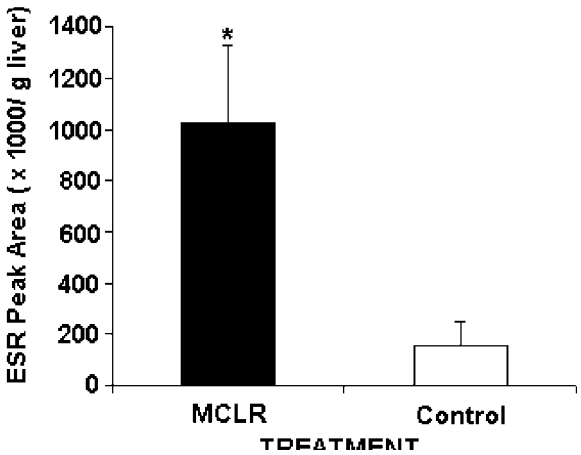

FIGURE 2 ESR spectral peak areas (mean ± SE) of PBN adducts obtained from hexane extracts of livers treated with either MCLR or PBS (control) ($n = 6$ for each treatment group). $*p < 0.05$ when comparing mean and standard deviation (SD) between the control group and the MCLR treatment group.

TABLE I ESR coupling constants for multiple PBN-adduct species detected from MCLR-treated rat liver hexane and Folch extracts, compared to assigned literature values. Solvent abbreviations: HX = hexane, FO = Folch, BE = benzene, CF = chloroform. Coupling constants: a^N = PBN nitrogen coupling constant, a^H = PBN β-hydrogen coupling constant

Solvent extract	Species	Simulated PBN adduct			PBN adduct literature assignments				
		a^N (G)	a^H (G)	%	a^N (G)	a^H (G)	Solvent	Species	References
Hexane	1	14.4	3.2	80	14.5	3.3	BE	·L	[16,17]
					14.4	3.2	HX	·C	
	2	13.7	2	20	13.7	1.9	BE	·OL	[16]
Folch	1	15	3.4	51.7	14.8	3.3	FO	·CL	[18,19]
					14.8	2.7	CF	·OCR or ·C	
	2	14.2	2.2	48.3	13.8	2.2	FO	·OL	[20,21]
					14.2	2.3	CF	$\cdot OC_2H_5$	

peak areas of PBN free radical adducts detected in hexane extracts of rat liver tissue from control or toxin-treated animals. Significant differences ($p < 0.05$) were obtained when comparing ESR spectral peak areas of control and MCLR-treated liver samples.

In Vivo Image-guided ^{1}H-NMR Spectroscopy Studies

Image-guided ^{1}H-NMR spectroscopy was used to monitor *in vivo* levels of non-adipose lipids in cyanobacterial toxin-treated rat livers over a period of 3 h. Figure 4 depicts a representation of the localised liver region where image-guided ^{1}H-NMR spectroscopy was obtained. Representative *in vivo* ^{1}H-NMR spectra from localised regions ($6\times6\times6$ mm^3, or 216 μl volumes) of MCLR-treated rat liver are shown in Fig. 5. Assignment of peaks were based on high-resolution ^{1}H-NMR analysis, and previous studies in rat liver conducted in our laboratory.[22] A decrease in the lipid methylene and glutamine (Gln)–glutamate (Glu) peak heights were observed over a period of 3 h following MCLR administration.

Peak areas (peak integrals) for the lipid methylene and Gln–Glu peaks calculated from the ^{1}H-NMR spectra of MCLR-treated rat livers are shown in Fig.

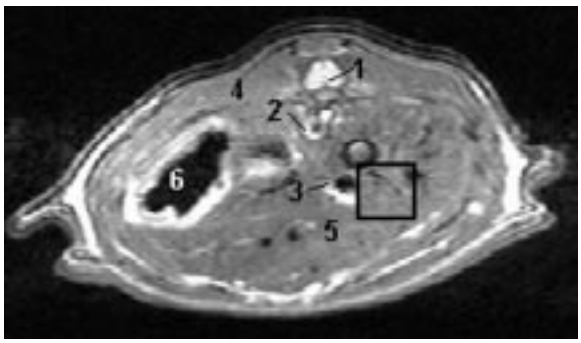

FIGURE 3 Representative *in vivo* transverse ^{1}H-NMR image (T1-weighted) of a rat liver region depicting the region of STEAM (stimulated echo acquisition mode) ^{1}H-NMR spectroscopy localisation ($6\times6\times6$ mm^3 or 216 μl volume). Anatomical features include: (1) spinal cord; (2) aorta; (3) hepatic portal vein; (4) muscle; (5) liver; and (6) stomach.

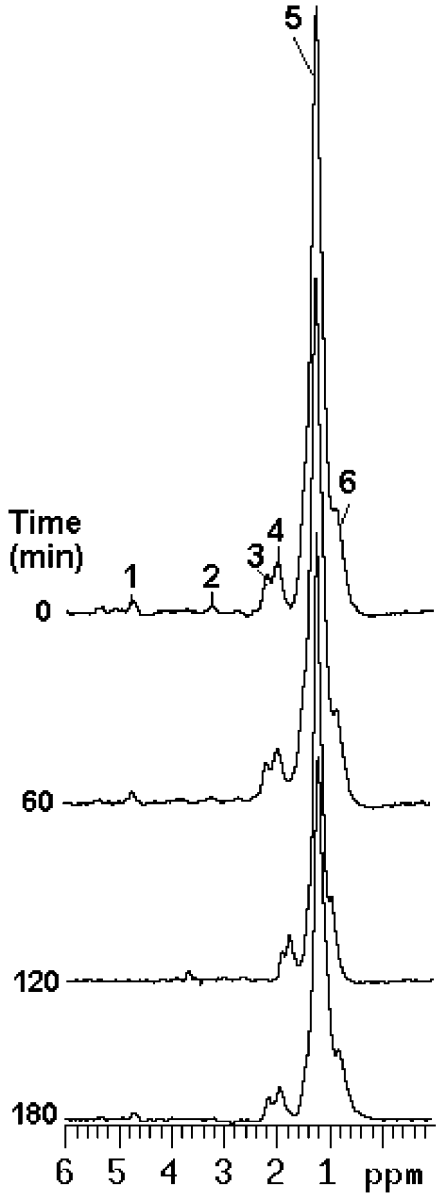

FIGURE 4 Serial representative *in vivo* STEAM ^{1}H-NMR spectra obtained from (A) control (pre-treated), and post-exposure to MCLR (B) 1 h, (C) 2 h, and (D) 3 h. Peak assignments: (1) water (4.77 ppm; suppressed), (2) choline, (3) β-methylene hydrogens of Glu–Gln (2.2 ppm), (4) triaglyceride (TAG)–CH=CH–CH_2– unsaturated methylene hydrogen peak (2.0 ppm), (5) lipid methylene hydrogen (TAG–CH_2–) peak (1.3 ppm), and (6) lipid methyl (TAG–CH_3–) peak (0.9 ppm).

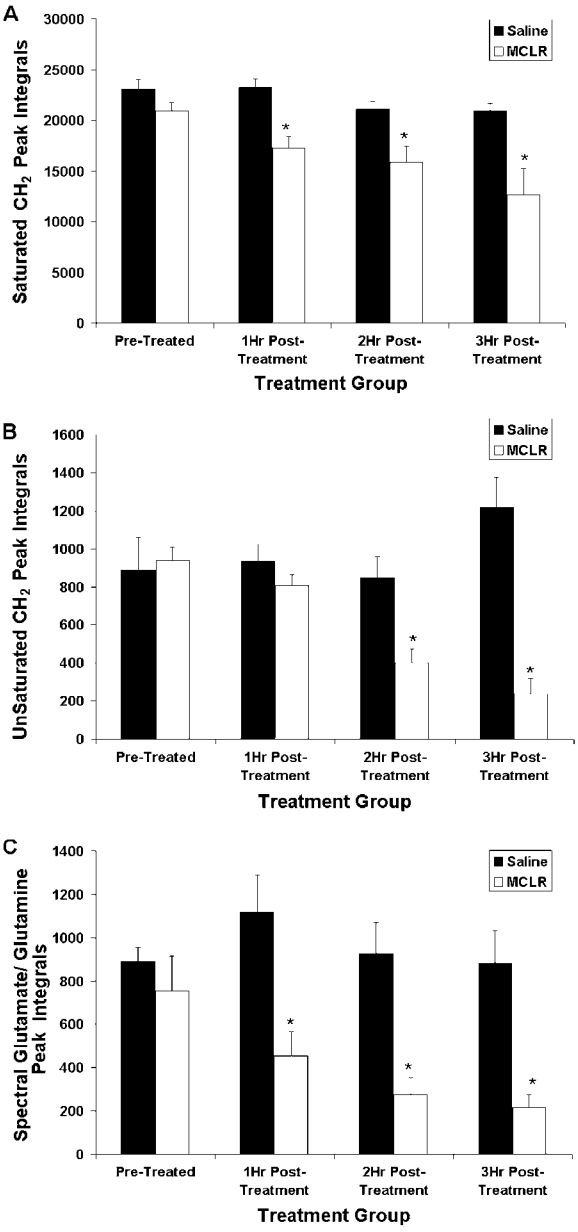

FIGURE 5 STEAM ^{1}H-NMR spectral peak areas (mean±SE) following MCLR exposure. (A) Lipid methylene hydrogens (–CH_2–; 1.2 ppm; peak 4 in Fig. 4), (B) unsaturated lipid methylene hydrogens (–CH=CH–CH_2–; 2.0 ppm, peak 3 in Fig. 4), and (C) β-methylene hydrogens from Glu–Gln (2.2 ppm, peak 2 in Fig. 4). Statistical significance obtained between control and MCLR-treatment groups when $*p < 0.05$ or $**p < 0.01$.

6(a). A statistically significant decrease ($p < 0.05$) was observed in the levels of both lipid methylene peak areas (2.1 and 1.5 ppm), as well as in the Gln–Glu peak areas in rat livers, after 1, 2 and 3 h post-exposure to MCLR, in comparison to control (pre-treated) data. Saline-treated controls, over a period of 3 h, did not show a significant difference in the levels of methylene lipids, as shown in Fig. 6(b).

Biochemical assessment of rat liver Glu, Gln and lactate obtained from perchloric acid extracts from saline- and MCLR-treated rat livers is shown in Fig. 6. Liver function was also assessed by monitoring

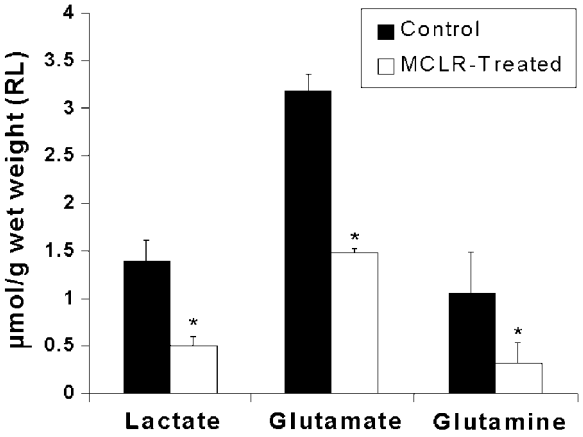

FIGURE 6 Biochemical assessment of glutamine (Glu), glutamate (Gln) and lactate concentrations from perchloric acid extracts of rat livers treated with saline or MCLR.

serum enzyme (ALT and AST) levels, as shown in Table II.

DISCUSSION

Previous evidence of lipid peroxidation following exposure to cyanobacterial extracts (measured as MDA production) has been observed *in vitro* (rat liver microsomes and hepatocytes).[8,23] In addition Ding and co-investigators also measured ROS production in microcystin-treated hepatocytes with a fluorescence probe, 2′,7′-dichloro fluorescein diacetate (DCFH-DA).[23] However, the direct detection of free radicals resulting from oxidative damage, particularly *in vivo*, remains to be further investigated. Results from this study clearly demonstrate that *in vivo* MCLR exposure leads to the formation of free radical species, possibly derived from oxidative lipid alterations.

Reactive transient free radicals can be effectively observed by the ESR spin trapping technique. The spin trap reacts with reactive free radicals to form more stable radicals, which can be observed by ESR spectroscopy. The spin trapping method was developed to extend the limits of ESR spectroscopy so that

TABLE II Serum enzyme levels (alanine amino transferase (ALT), and aspartate amino transferase (AST)) from saline- and MCLR-treated rats. MCLR (50 µg/kg) was administered i.p. to rats. Blood samples were taken via cardiac puncture from five rats per treatment group at each given time. Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase.

	1 h		3 h	
	Control	Treated	Control	Treated
ALT† (U/l)	32.1± 3.0	36.5±5.6	42.0±7.0	210.5±74.4*
AST† (U/l)	93.6±9.6	132.4±27.8	69.6±6.7	386.8±129.2*

*Significantly different from the respective control values, $p < 0.05$, by ANOVA.
†Enzyme activities (units/litre) are mean ± SE.

lower concentrations of free radicals could be detected. This analytical technique involves the trapping of a reactive short-lived free radical by a diamagnetic nitroxide spin trap compound via an addition reaction to produce relatively persistent nitroxide radical products or spin adducts. One of the most commonly used biological nitrone spin traps is PBN. Nitrones have become the most popular spin traps in biological systems due to their relative hydrophilicity, light insensitivity, low toxicity, solubility and stability, leading to reproducible results.[24]

The type of spin trap used is an important factor in determining how informative and sensitive the spin trapping technique may be for a given free radical. Careful selection of spin traps with particular solubilities, steric properties, adduct stability, and reaction specificity should be considered. As a result of its lipophilicity, PBN was used in this study to trap lipid-derived radicals. PBN is able to form relatively long-lived radical adducts and can cross cell membranes to trap free radicals generated from lipid peroxidation.[25,26]

ESR spin trapping spectroscopy was used to detect and identify two possible lipid-derived free radical metabolites formed in rat liver following MCLR exposure. The detected ESR signals in rat liver hexane and Folch extracts were simulated by including two PBN spin adduct species, which have been potentially assigned as the PBN-carbon-centred lipid alkyl adduct and the PBN-oxygen-centred lipid alkoxyl adduct, based on previous literature. The levels of PBN-trapped free radicals in hexane extracts from MCLR-treated rat livers were found to increase significantly in comparison to controls (see Fig. 3; $p < 0.05$). These results suggest that lipid-derived free radicals are generated in rat liver due to cyanobacterial toxin-induced hepatotoxicity, which clearly demonstrates that oxidative damage is occuring.

To determine whether the detection of free radicals correlated with alterations in lipid metabolism, non-adipose methylene lipid levels were monitored in cyanobacterial toxin-treated rat livers *in vivo*, utilising image-guided ^{1}H-NMR spectroscopy. Rat liver exposure to MCLR resulted in a significant ($p < 0.05$) decrease in the levels of the saturated and unsaturated methylene lipid resonance peak areas two and 3 h post-exposure, when compared to control (pre-treated) data. A possible explanation for the decrease in lipid methylene hydrogen levels could be due to a decrease in lipid mobility from lipid acyl chain cross-linking as a result of lipid peroxidation. Alternatively, or in addition to decreased lipid mobility, the decrease in lipid signal may be due to a release in free iron during necrosis, having an effect on T1 (spin–lattice relaxation) and T2 (spin–spin relaxation), which modulate signal intensity. A study by Baysal and colleagues (1989) found that T1 and T2 relaxation times decreased with increasing Fe^{3+} concentration.[27]

Both the detection of free radicals and the observed decrease in lipid levels, following MCLR exposure, occur within a relatively short time frame, a period of ~2 h. In addition, further evidence of extensive acute hepatic damage is supported by the significant increase in serum enzyme levels detected 3 h following MCLR exposure. This time frame also correlates well with histological[5,8,28] and serum enzyme[5,8] data previously published.

Of additional interest, we recently used NMR imaging to detect acute localised microcystin-induced hepatic damage within a period of 3 h.[28] Image analysis indicated that there was a significant ($p < 0.01$; $n = 6$) increase in oedema (observed as an increase in NMR image signal intensity) within 2–3 h in regions surrounding the hepatic portal vein (HPV) in MCLR-treated rats compared to non-treated rats.[28] These results were confirmed with histological and electron microscopy data obtained from biopsy samples within the observed NMR image regions of apparent "tissue damage", using spatial co-ordinates from the images.[28] The extension of the NMR imaging technique by obtaining image-guided NMR spectra provides additional information on biochemical alterations that occur as a result of toxin-induced tissue injury.

Other biochemical information obtained by NMR spectroscopy included a significant decrease in Glu and Gln levels. The β-methylene hydrogens from both of these amino acids are indistinguishable by *in vivo* NMR spectroscopy. Biochemical analysis of perchloric acid extracts from MCLR- and saline-treated rat livers clearly showed that both Glu and Gln levels decrease significantly following MCLR exposure (in comparison to saline treatment). The decrease in hepatic Glu and Gln levels may reflect membrane damage and enzyme oxidation as a result of lipid peroxidation. A study by Sundari and Ramakrishna previously found that oxidative damage, from acute alcohol- or cabon tetrachloride-induced liver injury, resulted in a significant decrease in hepatic glutamine synthase (GS) levels within 1 h.[29]

During tissue injury lactate levels are often reported to increase as a metabolic response to tissue injury.[30–32] In contrast, MCLR exposure seems to result in a decrease in measured hepatic lactate levels. The decrease in lactate levels is supported by a study which observed that *in vivo* MCLR-induced DNA damage in mouse liver was accompanied by damage to enzymes of hepatic origin, including lactate dehydrogenase.[33] The lactate data, from *in vitro* biochemical analysis, also aids in supporting the saturated lipid methylene decrease observed by

in vivo NMR spectroscopy. If there was an increase in lactate, the lactate resonance peaks at 1.33 and 1.25 ppm would interfere with the lipid peak at 1.3–1.4 ppm, which would complicate the assignment of the lipid peak. A decrease in lactate, as determined by measurement of levels in perchloric acid extracts, indicates that there is no possible interference with lipid levels in the NMR spectra, therefore, the peak assigned at 1.3–1.4 ppm is only of lipid origin.

The results in this study provide convincing evidence that oxidative processes, leading to alterations in lipid metabolism, occur as a result of in vivo cyanobacterial toxin-induced hepatotoxicity in mammals. The in vivo studies reported in this paper also support the in vitro studies conducted by other investigators that previously demonstrated indirectly that lipid peroxidation occurred in both hepatocytes and hepatic microsomes following MCLR exposure.[8,10,23]

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