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Toxicogenomic evaluation of microcystin-LR treated with ultrasonic irradiation

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Abstract

Microcystins are a family of toxins produced by cyanobacteria found throughout the world in marine and freshwater environments. The most commonly encountered form of microcystin is microcystin-LR (MC-LR). Humans are exposed to MC-LR by drinking contaminated water. The toxin accumulates rapidly in the liver where it exerts most of its damage. Treatment of water containing MC-LR by ultrasonic irradiation leads to the breakdown of the toxin. Both the parent toxin and the treated toxin reaction products (TTRP) were evaluated for toxic effects in mice. Animals were exposed to purified MC-LR or an equivalent dose of the TTRP and sacrificed after 4h or 24h. Serum was collected and assayed for lactate dehydrogenase (LDH) activity as an indicator of hepatotoxicity. LDH activity was detected in the serum of MC-LR exposed mice indicative of liver damage, but not in control mice. Only a fraction of that activity was detectable in mice exposed to TTRP. Liver RNA was used for microarray analysis and real-time PCR. Individual animals varied in their overall genomic response to the toxin, however, only 20 genes showed consistent changes in expression. These include: chaperones which may be part of a generalized stress response; cytochrome P450 which may be involved in metabolizing the toxin; and lipid dystrophy genes such as lipin-2, uridine phosphorylase, and a homolog to tribbles, a stress-inducible gene involved in cell death. Of the genes that responded to the MC-LR, none showed significant changes in expression profile in response to TTRP. Taken together, the data indicate that ultrasonic irradiation of MC-LR effectively reduces hepatotoxicity in mice and therefore may be a useful method for detoxification of drinking water.

Keywords

toxicogenomics; cyanobacterial toxins; microcystin; hepatotoxicology; microarrays; xenobiotics

Introduction/Background

Most of the world relies on surface freshwaters as its primary source for drinking water and the drinking water industry is constantly searching for treatment technologies to deal with contaminants that must be removed to protect public health. Recent reports demonstrate that toxic cyanobacteria blooms are an emerging issue in the United States. The presence of toxic

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cyanobacteria blooms in surface water has led to livestock deaths and a variety of human health disorders (Duy et al., 2000). Primary public exposure to algal biotoxins can come from drinking water (Carmichael, 1994;Ressom et al., 1994), water used in medical treatments, recreational water and dietary supplements (Saker et al., 2007). For example, in 1996, 116 patients who underwent hemodialysis at a clinic in Brazil developed acute toxic symptoms due to microcystin exposure, 26 of whom died of liver failure (Jochimsen et al., 1998). Secondary human exposure may come from algal biotoxin residue on fruits and vegetables from contaminated irrigation water and consumption of animal tissue. The most commonly encountered cyanobacterial toxins are the microcystins. Microcystins are a family of cyclic heptapeptide hepatotoxins produced by several different species of cyanobacteria including *Microcystis*, *Anabaena*, *Oscillatoria*, *Planktothrix*, *Nostoc*, *Anabaenopsis*, and *Hapalosipon* (Carmichael, 1992). Variants of microcystin are designated by the two variable amino acids and vary in their toxicity. Microcystin-LR is the most common and the most toxic variant (Gupta et al., 2003). Epidemiological studies have indicated that one of the risk factors for the high occurrence of primary hepatocellular carcinoma is consumption of microcystins in drinking water (Fleming et al., 2000;Falconer and Humpage, 1996). As with most drinking water contaminants, the limited availability of markers for the toxins has hindered the understanding of algal toxin effects on human health. The World Health Organization, however, has recommended a value of 1 μg l^{-1} as a guideline for safe drinking water (WHO, 1998).

Conventional water treatment methods, such as chemical coagulation, flocculation, sand filtration and chlorination, have been proved to be ineffective in removing MCs from potable water; hence specialized treatment is usually recommended for treatment of contaminated water (Lawton et al., 1999a). Advanced oxidation technologies (AOTs) are attractive alternatives to traditional water treatments and have received considerable attention recently. AOTs involve the generation of •OH as the predominant species responsible for the degradation of pollutants. Several reports indicated that TiO_2 photocatalysis and UV/H_2O_2 effectively remove cyanotoxins from drinking water and •OH is the predominant species responsible for the degradation of pollutants (Lawton et al., 1999b;Liu et al., 2003;Qiao et al., 2005). Ultrasonic irradiation, an AOT, has been studied for the oxidation of organics, destruction of pathogenic organisms, and treatment of water and wastewater either as a sole means of treatment or in combination with other oxidation processes such as ozone, UV irradiation and photocatalysis (Adewuyi, 2005a,b). Unlike other AOTs, ultrasound does not require addition of chemicals and can be used for treatment of turbid solutions. Ultrasonic irradiation promotes the growth and collapse of gas bubbles (cavitation), leading to extreme conditions (5000 K, 1000 atm) under which the pyrolysis of water produces •H and •OH. Our previous studies (Song et al., 2005;2006) have demonstrated that ultrasonic irradiation leads to rapid degradation of MC-LR and that •OH is responsible for a significant fraction of the observed degradation. Primary toxicity studies indicate that byproducts of ultrasonic irradiation do not exhibit appreciable inhibition of protein phosphatase in vitro.

Studies of the uptake of radiolabeled microcystin have demonstrated that after a single sublethal injection, most of the toxin is localized to the liver within one hour (Robinson et al., 1989). The efficient accumulation of the toxin is due to an active transport system in hepatocytes (Runnegar et al., 1995) and makes the liver the target organ for damage from microcystin in mammals. The toxin binds to and irreversibly inhibits protein phosphatases PP1 and PP2A (Runnegar et al., 1993) which leads to collapse of actin filaments and subsequent structural damage to the liver (Batista et al., 2003). High doses of MC-LR generate oxidative stress in hepatocytes (Jayaraj et al., 2006) followed by death. Less is known about the effects of lower exposures to the toxin. The study of changes in gene expression in response to MC-LR may elucidate more subtle effects of exposure and lead to a better understanding of chronic toxic exposure.

Toxicogenomics is the application of microarray analysis to the field of toxicology to facilitate the identification of genes and pathways involved in mechanisms of toxicity. Microarrays have been utilized in an attempt to establish toxic profile signatures and to look for specific biomarkers of toxic exposure. The specific genes responding vary due to differences in the treatment, the purity of the toxin, the animals or the tissue or cell type used in the study (Gehringer, 2004). In the present study we used microarrays as a tool for gene discovery rather than extensive profiling. The main purpose of our study is to evaluate a novel method used to break down microcystins in drinking water supplies. The overall genomic response of the liver to toxin was compared to that of the treated toxin in mice. Quantitative PCR was performed on a subset of genes to validate the array data and give a more detailed picture of the genetic response to the compounds.

Materials and Methods

Purification of microcystin-LR.

Microcystis aeruginosa was obtained from the University of Toronto Culture Collection, (UTCC299). Microcystin LR was purified from cultures by established methods (Lawton and Edwards, 2001) involving a combination of methanol extraction, solid phase (C18) extraction and a final purification by HPLC (70:30 MeOH: 20mM NH_4OAc, Agilent Technologies, hypersil-ODS column), at the NIEHS Toxic Algae Culture Center at Florida International University, Miami FL. Identity and purity were confirmed by mass spectrometry, ^1H NMR (Bruker Avance, 400 MHz, in MeOH-*d4*), (Harada et al, 1990) and reverse phase HPLC comparison to an authentic sample (Sigma-Aldrich).

Ultrasonic irradiation.

An ultrasonic transducer UES 1.5−660 Pulsar (Ultrasonic Energy Systems, Inc.) operating at 640 kHz under operational power of 500 W equipped with a 2.0 ml reaction vial was fitted with a polyethylene window facing the transducer. The reaction vessel was centered at a distance of 4.50 cm from the face of the horn. Since modest heating is observed during ultrasonic irradiation, the entire assembly was submerged in an ice bath to maintain a constant temperature of 4 °C throughout the reaction process. The ultrasonic irradiation experiments were conducted using pure MC-LR at a starting concentration of 500 mg/L. After 90 min irradiation, more than 99 % MC-LR was decomposed. Several byproducts of ultrasonic irradiation have been identified by LC-MS and the degradation pathways elucidated (Song et al., 2006).

Animals and animal maintenance.

All animal work was done according to University of Miami (UM) Institute Animal Care and Use Committee approved protocols. Adult male BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and were maintained by UM Division of Veterinary Resources DVR for one week to allow the animals to acclimate after shipment. Animals were housed in polycarbonate cages in an approved facility at 20−22°C with 12h dark/light cycle. Food and water were provided ad libitum. Mice were exposed as follows (n=4 for each): 35, 45, or 65 μg kg^{-1} of MC-LR (aqueous) or equal volume of TTRP administered by intraperitoneal injection. or water (control) by a single intraperitoneal injection. The animals were observed for signs of distress post-injection. The mice were handled and sacrificed according to UM SOP at two different intervals: 4h and 24h post-injection. Animals were euthanized by carbon dioxide inhalation. Tissues were collected immediately. Representative samples of all major organs were collected and preserved in 10% neutral buffered formalin. Tissues were processed by routine histological technique which included paraffin embedding, sectioned at 5 micrometers thickness, and stained with hematoxylin and eosin. Routine light microscopic evaluation was performed.

Enzyme assays.

A terminal blood sample was collected from the heart. After coagulation, serum was separated from the cells by centrifugation. LDH activity was determined by using the TOX-7 assay kit (Sigma, St. Louis, MO). All samples were assayed in triplicate in 96-well plates. Absorbance was read at 490 nm on a microplate reader.

RNA purification.

A portion of the liver was homogenized and total RNA was isolated using the QIAzol reagent protocol (Qiagen, Valencia, CA) and further purified using the RNeasy total RNA kit (Qiagen, Valencia, CA). RNA concentration was determined by spectrophotometry on a NanoDrop instrument and purity was evaluated on an RNA 6000 Nano LabChip in a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA).

DNA Microarrays.

Microarray work was performed at the UM Microarray Facility. Microarray studies utilized a reference design with the control animals used as the reference, similar to experiments done with brevetoxin (Walsh et al., 2003). RNA (10 μg of each) for hybridization was prepared by amplification using a modified Eberwine protocol (van Gelder et al., 1990) using the Amino Allyl MessageAmp aRNA Kit (Ambion). The Cy3/Cy5 labeled target was combined with control targets and applied to the Mouse 1A Oligo 22K Microarray (Agilent Technologies). Dye swaps were performed to avoid dye bias in the results. Cy3 and Cy5 labeled aRNAs were hybridized to slides and incubated 17 hours at 60°C. Following hybridization, slides were scanned using a calibrated GenePix 4000a Microarray Scanner (Axon Instruments) at wavelengths 532 nanometers and 635 nanometers with PMT settings of 650 and 700 respectively. The 16-bit two channel TIFF array image was analyzed by GenePix Pro 4.1 (Axon Instruments) software. Data from non-control features were used to derive log expression ratios between the Cy-3 and Cy-5 signal values for each feature. Features that exhibit differential expression of greater than 1.7-fold were deemed significant. Gene annotation and ontological data were derived from Onto-Express (Open Channel Foundation). LOWESS normalization and extensive annotation were performed using Acuity 3.1 (Axon Instruments). Each array was normalized for signal intensities across the whole array and locally, using Lowess normalization. Features for further analysis were selected according to the following quality criteria: (1) at least 90% of the pixels in the spot had intensity higher than background plus two standard deviations; (2), there were less than 2% saturated pixels in the spot; (3) signal to noise ratio (defined as ratio of the background subtracted mean pixel intensity to standard deviation of background) was 3 or above for each channel; (4) the spot diameter was between 110 and 150 micron; (5) the regression coefficient of ratios of pixel intensity was 0.6 or above. To identify significantly expressed genes across all replicate arrays one-class SAM (Significant Analysis of Microarray, http://www-stat.stanford.edu/~tibs/SAM) analysis was used with the smallest FDR (False Discovery Rate). Genes that were identified as responsive to the toxins in the microarray work were further evaluated by real-time (quantitative) PCR.

Quantitative RT-PCR.

Microarray data was validated by one-step quantitative PCR, normalized to two different housekeeping genes, histone (HIS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time reverse transcriptase PCR was performed using 100 ng of total RNA in a Stratagene MX4000 real-time instrument. Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and MultiScribe reverse transcriptase enzyme mix (Applied Biosystems, Foster City, CA) were used in a one-step RT-PCR reaction. Optimization and standard curves were run for each primer set initially and melting curves were run in each experiment. In addition, negative control reactions were run without reverse transcriptase for

each assay. PCR amplified products were visualized by agarose gel electrophoresis to verify size and purity. Each product was sequenced to validate correct gene amplification. The quantitative PCR results are presented as the fold change in expression (the ratio of the change in the threshold of fluorescence in the experimental samples to that of the controls).

Primers were designed using the Primer3 program (online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3), except for cytochrome P450 1A2 and GAPDH (Uno et al., 2005) and histone H2A.Z (Jeong et al., 2005) which are qPCR primer pairs previously used by others. Sequences of oligonucleotide primer (Operon Biotechnologies, Germantown, MD) pairs for each gene of interest (F = 5' and R= 3'):

CYP1A2F AAGACAATGGCGGTCTCATC; CYP1A2R GACGGTCAGAAAGCCGTGGT;

GAPDHF TGCACCACCAACTGCTTAG; GAPDHR GATGCAGGGATGATGTTC;

HISF CGTCAGAGAGACGCTTACCG; HISR AAGCCTCCAACTTGCTCAAA;

LIPIN2F CCCTCCTGGGATTCTGTCTC; LIPIN2R TACGTGAAAAGGCGAGCACT;

TRIBFGTGCTGGTTCATGGGACAGT; TRIBR TTTTGGCTTGAATCCCACAG;

UPP2F AAATCCCCAATGTTCCAACC; UPP2R GCGAACACAGTCGATTCCAT;

ASS1F CTGGAGGATGCCTGAGTTTTAC; ASS1R CTGATGTGCATGAGGTTTTCAT

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Statistical analysis. Data from quantitative PCR experiments were analyzed and graphed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Significant differences in means (P-value < 0.05) were determined by one-way ANOVA analysis and Tukey multiple comparison tests and nonparametric t-tests.

Results

In order to evaluate whether ultrasonic irradiation reduced toxicity of MC-LR, we needed to compare the effects of purified toxin with that of TTRP in a sublethal toxic exposure. The median lethal dose (LD_{50}) of microcystins by intraperitoneal injection in mice and rats has been shown to vary over a considerable range, anywhere from 36−122 μg kg^{-1} body weight depending on animal, strain and purity of the compound (Dawson, 1998). In studies in BALB/c mice the median lethal dose was determined to be 82 μg kg^{-1} (Gehringer, 2003). In a separate study in mice only a minor transient effect was observed after a dose of 35 μg kg^{-1} (Robinson, 1991). Changes in gene expression can occur in a transient, delayed or prolonged manner. In our studies mice received a single sublethal dose of 35, 45, or 65 μg kg^{-1} of MC-LR (aqueous) or equal volume of TTRP administered by intraperitoneal injection. To look for both immediate and delayed or prolonged changes, we sacrificed animals at two time points, 4h or 24h post injection. Control animals received an equal volume of carrier alone. The mice were observed for signs of distress after injection of toxin. No observable ill effects were seen at any of the doses given during the 24h post injection interval.

Histopathology

Tissues representing all major organs were evaluated from 8 animals: control, 35, 45, and 65 μg kg^{-1} experimental for two time periods, 4 and 24 hours post-intraperitoneal injections. There were no significant light microscopic findings in any of the tissues from the animals that were examined. This result suggests that the doses of toxin used in the study were relatively low

since there was no morphological evidence of cytotoxic effects. And further suggests that the threshold for morphological damage may be fairly steep since no gross damage was observed even though the highest dose used was 80% of the reported LD_{50}.

LDH assays

MC-LR has been shown to disrupt the integrity of the cell membrane in the liver (Gupta et al., 2003), allowing some cytosolic enzymes to leak into the circulatory system. Lactate dehydrogenase (LDH) is a very abundant cellular enzyme that readily leaks from the cell upon damage to the membrane (Hudder et al., 2003). The presence of LDH activity in the serum is a good indicator of hepatotoxicity. Serum samples were assayed for the presence of LDH activity (fig. 1). The assay used is very sensitive and a background level of activity is detectable in control animals. Even the lowest dose of toxin used caused leakage of LDH significantly above background levels at both the 4h and 24h time points. In contrast, animals that received TTRP had little or no LDH activity present in their serum. This is a good indication that ultrasonic treatment of MC-LR reduces toxicity in mice. The amount of LDH detected in the serum of animals receiving the highest dose of TTRP may be due to a remainder of unmodified toxin in the reaction solutions.

DNA microarray analysis

DNA microarray analysis is a powerful tool in evaluating patterns of gene expression. We utilized mouse DNA oligonucleotide microarrays to look at overall changes in gene expression in response to toxin and to identify candidate toxin-responding genes. Samples from animals at the 24h time points that received the highest doses of toxin or irradiated products were used for microarray analysis (n = 4). A reference design was employed and dye swaps were performed on each sample to eliminate dye bias in the results (8 microarray analyses in total). The reference was composed of a pool of the control samples. As a further precaution, samples from control animals (n = 4) were individually compared against pooled control samples to look for natural variation in gene expression among control animals. There were very few genes exhibiting changes in expression among the control animals. For the experimental samples, less than 5% of genes on the array showed an altered pattern of expression with a magnitude of greater than 1.7-fold in response to toxin and there was considerable variability among individual microarray analyses due to dye bias The small sample size and the variability in individual array data make statistical analyses difficult. A short list of genes that had changes in expression profile of greater than 1.7 fold on more than one array are shown in Table 1. These data were used to identify genes of interest for further studies based on the criteria that the expression profile changed in response to toxin on two or more microarray analyses and did not change in the controls. Animals that were injected with TTRP did not exhibit many changes in gene expression profiles significantly different from controls and none were consistent on more than one microarray. Of special note is that none of the genes that responded to microcystin exposure had the same patterns of expression in animals exposed to the TTRP, suggesting that ultrasonic irradiation reduces toxicity of the compound.

Quantitative PCR

A subset of toxin responding genes were selected for validation and further analysis by quantitative PCR. Quantitative PCR was performed on all samples, experimental and control, from all doses and time points (total number of animals is 28). Assays were performed in triplicate and repeated with two different reference (housekeeping) genes. The reference genes were chosen on the basis that neither of them showed changes in expression in the microarray analysis. In addition, GAPDH has been used by others as a stable reference gene to study relative changes in gene expression in response to MC-LR exposure (Jayaraj et al, 2006). Genes representing different pathways and genes known to be involved in human disease processes

were of particular interest. Three of the selected genes, uridine phosphorylase, tribbles and cytochrome P450 showed enhanced expression in response to toxin while two of the selected genes, arginosuccinate synthetase and lipin, were reduced in expression compared to the control animals by microarray analysis. The same RNA samples were analyzed in quantitative PCR assays and confirmed the microarray data. In addition RNA from the other doses and time points was assayed by quantitative PCR and the analysis provided additional information about the changes in expression of the subset of genes in response to MC-LR and TTRP. There was no correlation of dose and magnitude of change in genetic profile, however there were differences in gene expression at 4h and 24h post injection in some cases.

Arginosuccinate synthetase (Ass1, figure 2, panel A) is a key enzyme involved in nitrogen metabolism (urea synthesis) and also functions in stress response through nitric oxide production. Ass1 expression was reduced in response to MC-LR in both microarray analysis and Q-PCR at the 24h timepoint. The TTRP did not elicit any changes greater than the 1.7 fold cutoff as evaluated by microarray analysis. Q-PCR confirmed that MC-LR reduces expression of Ass1 at the 24h time point and extends that to include the 4h timepoint. The TTRP, however, elicited a small but significant ($P<0.0001$) increase in expression of this gene at both 4h and 24h post exposure.

Lipin-2 (Lpin2, figure 2, panel B) is involved in lipid metabolism. Expression of this gene is also reduced by toxin both in microarray analysis and quantitative PCR. While microarray analysis showed no change in expression of this gene in response to TTRP, a small reduction was detectable by quantitative PCR at 4h, but is nearly back to control levels by 24h.

Uridine phosphorylase (Upp2, figure 2, panel C) participates in pyrimidine metabolism and its expression is enhanced by the toxin. Of the five genes chosen for further analysis, it is the only one in which the Q-PCR results show some discrepancy when compared to the microarray analysis at the 24h time point. This is due to wide variation in the response of individual animals at this time point ($n = 6$). The animals used for the microarrays also showed a greater than 1.6 fold increase in the Q-PCR assay; however, other members of the cohort had little or no increase in expression of this gene as reflected by the error bar on the graph, yielding no significant difference between the effects of MC-LR and TTRP at the 24h time point ($p>0.05$). Since the expression of this gene is clearly induced at 4h, the results at the later time point may reflect differences in recovery time in response to toxin. TTRP, however, did not cause enhanced expression of this gene ($p< 0.01$).

Tribbles (Trb3, figure 2, panel D) functions as an inhibitor of insulin signaling and also plays a role in apoptosis. Toxin induces expression of this gene at both 4h and 24h time points. The TTRP showed significantly less induction of this gene at 4h ($P<0.015$) and by 24h post-injection is expressed at the same level as control animals.

It was anticipated that genes involved in xenobiotic metabolism may be induced by exposure to cyanobacterial toxins. While there were 60 cytochrome P450 (Cyps) genes represented on the DNA microarrays, only one of these, Cyp 1a2, showed significant changes in expression level. Q-PCR analysis of Cyp1a2 indicated that the expression of this gene is enhanced by MC-LR at both the 4h and 24h time points. Exposure to TTRP, however, results in a small decrease in gene expression that was not detected by microarray analysis.

Each of the genes examined by quantitative PCR was consistently up or down regulated in response to toxin, confirming what had been observed in the microarray analysis. Furthermore, animals exposed to TTRP had different expression patterns for these genes than the toxin exposed animals. In most cases, the gene expression in response to TTRP was close to the level of expression in control animals indicating an overall reduction in toxicity of microcystin.

Discussion

The current study was undertaken in order to evaluate a novel method for detoxification of MC-LR in freshwater supplies. In addition, we sought to elucidate changes in gene expression in response to toxin at sublethal doses. Death as an endpoint or other measures of cytotoxicity could be used to determine the toxicity of the TTRP versus the parent toxin in mice and is perhaps still the preferred method for routine screening of compounds. The application of genomics techniques, however, provides insight into the environment-gene interaction. This type of applied genomic research will likely play an increasing role in clinical and public health research (Khoury et al., 2005).

The effects of injection of MC-LR or TTRP were evaluated in mice, specifically in the liver. In the absence of morphological damage to the liver, even small doses of MC-LR elicit minute structural perturbations of the cell membrane and allow leakage of LDH. LDH activity in serum was still at an elevated level even 24h post-exposure to toxin. These data suggest that the damage caused by MC-LR is not readily reversed as it persists for at least 24h after exposure to toxin. Thus, even low dose exposures can potentially cause harm. This type of exposure would be underreported by epidemiological studies because of the absence of acute illness or obvious symptoms. This result suggests that more vigilant efforts may be needed to assess the epidemiological implications of MC-LR exposure and underscores the need to find accurate biomarkers of exposure to this toxin. Animals injected with TTRP had very little LDH activity in their serum. The residual amount of LDH activity detected by the assay may be due to unmodified toxin remaining in sample. Perhaps this residual toxin could be further detoxified by increasing the duration of the ultrasonic irradiation.

The microarray analysis showed that small but significant changes in gene expression occurred at low dose exposure. The genes affected are representative of many diverse metabolic pathways. While it is not certain what effect the observed changes in gene expression could have on overall health, there is evidence that even small fold changes in gene expression can have a significant physiological effect (Oleksiak et al., 2005).

Of the genes that show changes in expression profile compared to controls in response to toxic challenge, none respond in the same manner when exposed to TTRP. Some toxin responsive genes, such as transcription factors and factors involved in protein synthesis, may be part of a generalized toxic response. Others may be specifically responding to MC-LR, such as Cyp 1a2. Some of these toxin responding genes will be the subject of further investigation.

Exposure to environmental agents can cause fundamental changes in basic physiology through alterations in patterns of gene expression, which can lead to increased risk of disease. Each of the genes chosen for analysis by quantitative PCR in the current work is representative of different cellular pathways. However, it is interesting to note that changes in expression levels of several of these genes have been linked to disease phenotypes. The fatty liver dystrophy (*fld*) mutant mouse strain arose from a spontaneous mutation in the lipin gene. The *fld* mice exhibit hypertriglyceridiemia and insulin resistance (Peterfy et al., 2001). Alteration in the level of expression of the tribbles gene was identified as contributing to the *fld* mouse phenotype (Klingenspor et al., 1999). It has been shown that increased levels of tribbles block Akt activation, blunting insulin stimulation and leading to hyperglycemia (Du et al., 2003). In fact it has been suggested that increased expression of the Tribbles gene plays a key role in insulin resistance in the diabetic liver and that it may be a suitable therapeutic target for treatment of Type II Diabetes (Koo et al., 2004).

Similar to the *fld* mice, microcystin exposed mice have increased levels of expression of tribbles and decreased levels of lipin. It is tempting to speculate that chronic exposure to MC-LR may exacerbate certain disease conditions e.g., exposure to low levels of toxin results in changes

in gene expression that could contribute to insulin resistance in diabetes. Therefore, a better understanding of the effects of altered gene expression on key pathways is necessary for evaluating the impact of environmental toxins on human health.

In summary, we defined MC-LR toxicity by leakage of LDH, changes in gene expression profiles on microarrays and by quantitative PCR analysis of a few selected genes of interest. Treatment of MC-LR by ultrasonic irradiation greatly reduced or eliminated the toxic response in the animals by all three methods of evaluation. We conclude that our results provide evidence that ultrasonic irradiation of the cyanobacterial toxin, MC-LR, in an aqueous environment reduces the toxicity of the compound on mouse liver.

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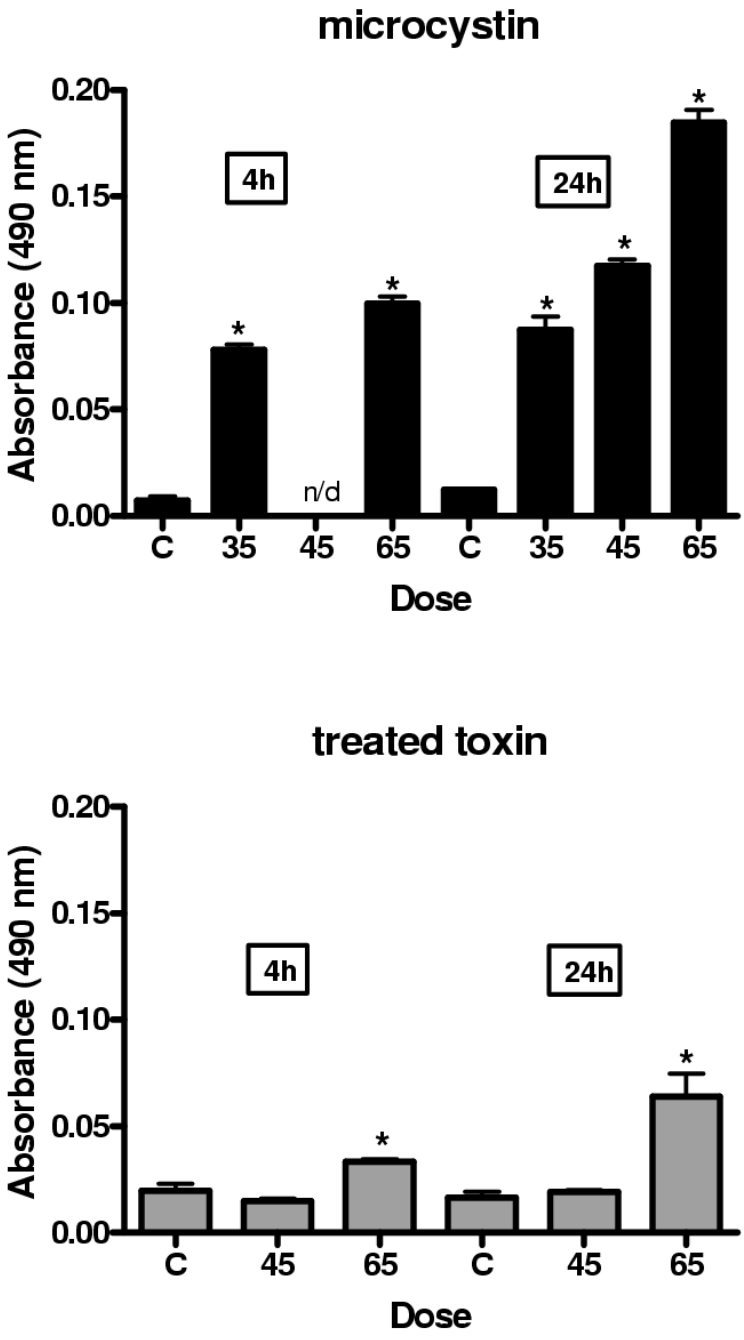

Figure 1.
LDH Assay. Serum from a terminal blood draw was assayed for LDH using the TOX-7 system. Error bars represent +/− SEM. Asterisk denote values differ significantly from controls ($P<0.001$).

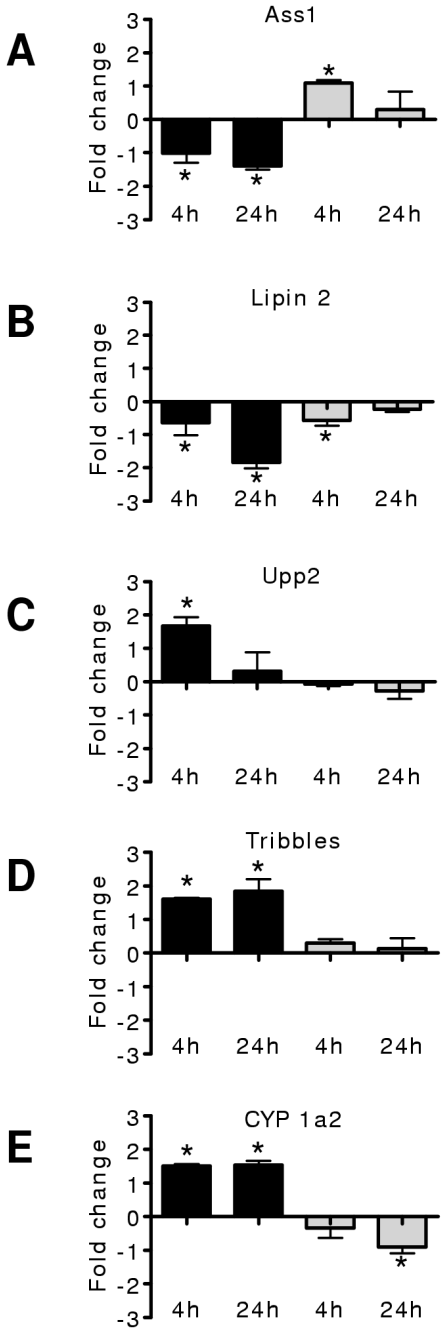

Figure 2.
Real-time PCR of selected genes of interest. RNA from animals exposed to microcystin (black bars) or treated reaction products (gray bars) was isolated 4h or 24h post-injection. The y-axis is fold change as determined by the ΔΔCt method. Error bars represent +/− SEM. Asterisks denote values differ significantly from controls (P<0.001).

Table 1

Genes with greater than 1.7 fold change in expression profile in DNA microarrays. Mice (n = 2) were injected with 65 μg kg^{-1} body weight MC-LR and sacrificed after 24h. Dye swaps were performed for each sample. The data shown are the expression levels relative to controls on one microarray experiment.

GenBank Accession No	UniGene Name	UniGene Symbol	Fold Change
NM_009787	calcium binding protein, intestinal	Cai	−1.82
NM_010497	isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	−2.03
NM_011072	profilin 1	Pfn1	−1.73
NM_010012	cytochrome P450, family 8, subfamily b, polypeptide 1	Cyp8b1	−1.72
NM_013485	complement component 9	C9	−2.04
AK045176	succinate-Coenzyme A ligase, GDP-forming, beta subunit	Suclg2	−2.00
NM_009993	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	2.25
NM_007494	argininosuccinate synthetase 1	Ass1	−1.70
NM_011082	polymeric immunoglobulin receptor	Pigr	−1.95
NM_007393	actin, beta, cytoplasmic	Actb	−1.83
NM_144554	Mus musculus TRB-3 (TRB-3), tribbles	Ifld2	3.14
NM_009804	catalase	Cat	−2.03
BI106083	basigin	Bsg	−1.89
NM_010240	ferritin light chain 1	Ftl1	−2.09
NM_008200	histocompatibility 2, D region locus 4	H2-D4	−2.14
NM_029692	RIKEN cDNA 1700124F02 sim to uridine phosphorylase	Upp2	2.63
NM_011933	2−4-dienoyl-Coenzyme A reductase 2, peroxisomal	Decr2	−1.59
NM_008184	glutathione S-transferase, mu 6	Gstm6	−2.12
AK090130	fibronectin 1	Fn1	−1.81
NM_026931	RIKEN cDNA 1810011O10 gene	1810011O10Rik	−2.03
NM_153526	insulin induced gene 1	Insig1	2.39
NM_013602	metallothionein 1	Mt1	−2.04
NM_011817	growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	−2.25
NM_010358	glutathione S-transferase, mu 1	Gstm1	−2.02
NM_007679	CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	−2.49
NM_029239	protein kinase C, nu	Prkcn	1.97
NM_007811	cytochrome P450, family 26, subfamily a, polypeptide 1	Cyp26a1	−2.45
NM_007913	early growth response 1	Egr1	2.46
NM_022882	lipin 2	Lpin2	−2.37