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Inhibition of serine palmitoyltransferase by myriocin, a natural mycotoxin, causes induction of c-*myc* in mouse liver

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Abstract

Myriocin, a fungal metabolite isolated from *Myriococcum albomyces*, *Isaria sinclairi*, and *Mycelia sterilia*, is a potent inhibitor of serine palmitoyltransferase (SPT), a key enzyme in *de novo* synthesis of sphingolipids. To evaluate the biological effects of myriocin *in vivo*, we investigated the levels of free sphingoid bases and expression of selected genes regulating cell growth in mouse liver. Male Balb/c mice, weighing 22 g were injected intraperitoneally with myriocin at 0, 0.1, 0.3, and 1.0 mg kg^{-1} body weight daily for 5 days. Animals were euthanized 24 hours after the last treatment. Levels of plasma alanine aminotransferase and aspartate aminotransferase were not significantly altered by the treatment. A dose-dependent decrease in free sphinganine but not sphingosine was detected by high performance liquid chromatography in both liver and kidney. The decrease of free sphinganine paralleled the decrease in SPT activity. Reverse transcriptase polymerase chain reaction analysis on liver mRNA revealed an increase in expression of c-*myc*, but no changes in tumor necrosis factor α, transforming growth factor β, and hepatocyte growth factor. Results showed that myriocin blocked *de novo* synthesis of sphingolipids *in vivo* by SPT inhibition and induced c-*myc* expression in liver.

Introduction

Myriocin (ISP-1, thermozymocidin, Figure 1a) was initially isolated as an antibiotic and immunosuppressant from *Myriococcum albomyces*, *Isaria sinclairi*, and *Mycelia sterilia*. This natural product potently inhibits proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction (MLR), T-cell-dependent antibody production, and the generation of allo-reactive cytotoxic T lymphocytes in mice with a potency of 10- to 100-fold greater than that of cyclosporin, a clinically prescribed immunosuppressant [8]. The growth inhibition of myriocin on mouse cytotoxic T cell line, CTLL-2, was due to its induction of apoptosis, and the apoptotic effect of myriocin on these cells could be reversed by addition of sphingosine to the cultures [22]. The structure of myriocin resembles that of sphingosine (Figure 1a), it has been revealed *in vitro* that myriocin inhibited the activity of serine palmitoyltransferase (SPT), a rate-limiting enzyme in *de novo* biosynthesis of sphingolipids, in a mouse cytotoxic T cell line, CTLL-2 (Figure 1b) [21].

Sphingolipids form specialized membrane structures, mediate cell-cell interactions, regulate the behaviour of cellular proteins and receptors, and signal transduction [17]. Bioactive intermediates of sphingolipid metabolism, such as ceramide, sphingosine, sphinganine, and sphingosine-1-phosphate, modulate cellular signaling functions and regulate cell growth, differentiation, apoptosis and proliferation [18, 19]. The discovery of myriocin and fumonisin as specific inhibitors of SPT and ceramide synthase, respectively, the two important enzymes in sphingolipid biosynthesis pathway, has helped in identifying key roles for the *de novo* biosynthesis in sphingolipid-mediated cellular signaling functions [22, 3, 4]. Fumonisin B_1, a mycotoxin from *Fusarium verticillioides*, increases accumulation of free sphinganine and sphingosine but decreases *de novo* synthesis of ceramide as a result of ceramide synthase inhibition (Figure 1b), [31].

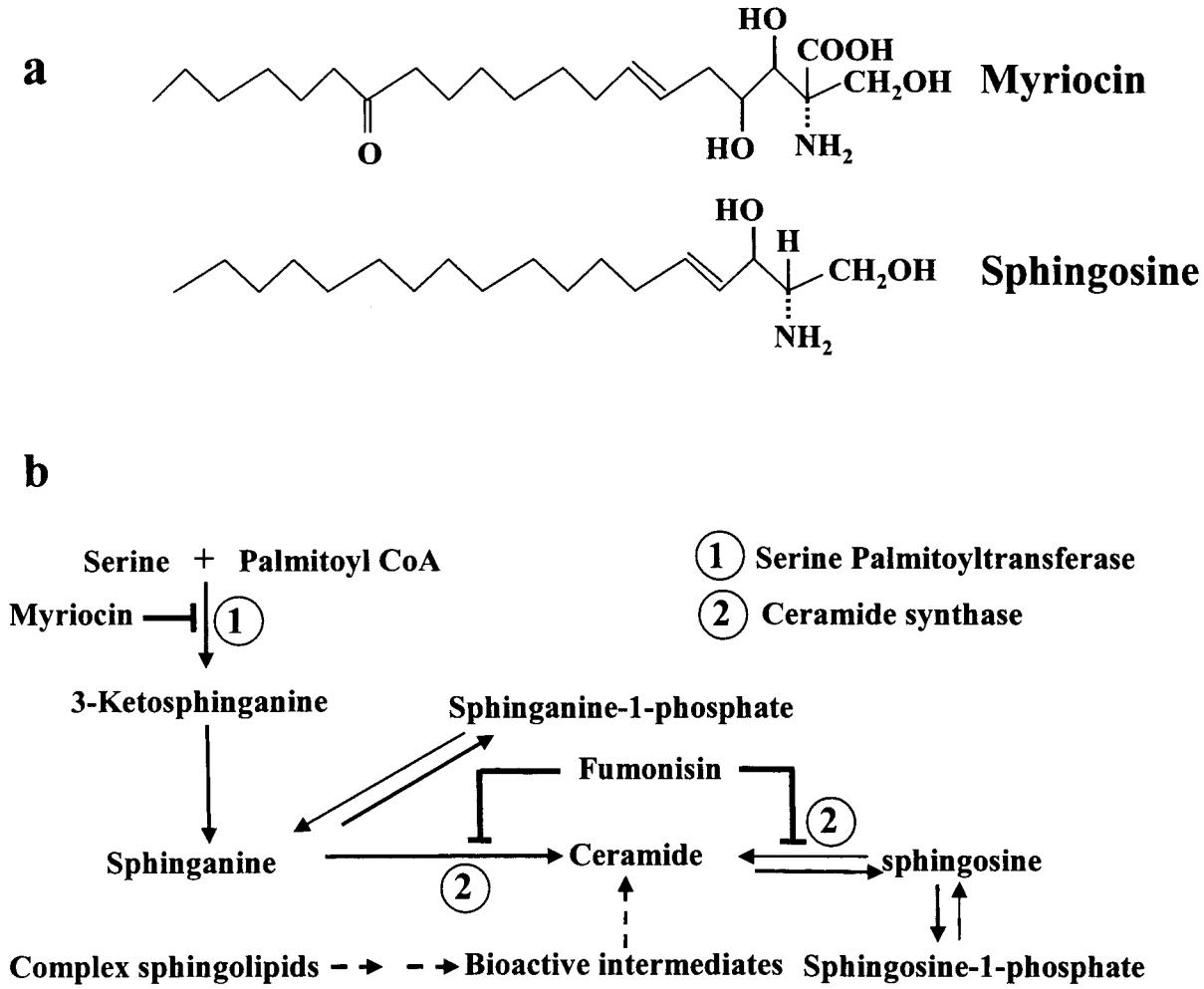

Figure 1. Structures of myriocin and sphingosine (a), and the inhibition sites of myriocin or fumonisins in the pathway of sphingolipids biosynthesis (b).

Fumonisins are known to induce hepatotoxicity and nephrotoxicity, as well as carcinogenesis in rodents [13, 23, 30], and have been linked to human esophageal cancer [16]. Fumonisins induce apoptotic cell death and/or cell proliferation in different types of cells [29, 35]. These toxicities of fumonisins are mediated by disruption of sphingolipid metabolism resulting from fumonisins-induced ceramide synthase inhibition [19, 23, 35]. However, very little is known about myriocin toxicity *in vivo*.

Myriocin has been shown to suppress the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction (MLR). It potently inhibits interleukin-2 (IL-2)-induced T cell proliferation but not IL-2 production from alloantigen-stimulated T cells [8], it also inhibits generation of T-cell-dependent antibody and allo-reactive cytotoxic T lymphocytes after intraperitoneal or oral administration in Balb/c mice [8]. Myriocin-induced SPT inhibition results in decrease of *de novo* synthesized free sphingoid bases and ceramide [21]. The property of myriocin has been used to elucidate the cellular mechanisms of fumonisin toxicity [12, 25], and shown to inhibit fumonisin B_1-induced accumulation of free sphinganine in mouse kidney [24]. It has been suggested that myriocin could be used as a therapeutic agent to treat fumonisin-related diseases based on its blocking fumonisin B_1-induced accumulation of free sphingoid bases [24]. However, decrease of sphingolipid by myriocin induced cellular apoptotic death in CTLL-2 [22], and rat Purkinje cells [9]. *De novo* sphingolipid biosynthesis is probably required for survival

in vivo because the sphingoid bases from food are largely degraded in mammalian intestine [17]. In the current study, we report that myriocin treatment significantly decreased free sphinganine content and SPT activity, but increased c-*myc* expression in mouse liver. The decrease of free sphinganine was correlated with myriocin-induced inhibition of SPT activity. Results suggest that myriocin may have adverse effects on human health by SPT inhibition and oncogenes induction.

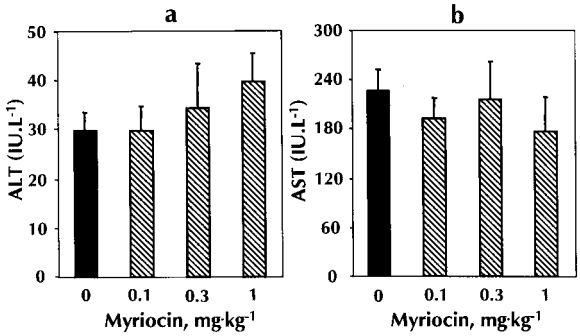

Figure 2. Effects of myriocin on plasma ALT (a) and AST (b) in mice. The mice were treated with the indicated dose of myriocin for 5 days. Data are presented as mean ± SE ($n = 5$).

Materials and methods

Animals and treatment

Male Balb/c mice, 22 g body weight and 7–8 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for one week at 23 °C and 50% relative humidity, with a 12-h light/dark cycle. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Myriocin, (2S, 3R, 4R, 6E-2- amino-3,4-dihydroxy-2-hydroxymethyl-14-oxo-6-eicosenoic acid, Figure 1a) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Four groups of 5 animals each were treated with 5 daily intraperitoneal injections of physiologically buffered saline (PBS, vehicle), or 0.1, 0.3, or 1.0 mg kg^{-1} day^{-1} of myriocin as a suspension in PBS. Blood was collected in heparinized tubes, and plasma was subsequently isolated for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Tissues were dissected into several pieces, quickly frozen on dry ice and stored at −85 °C until analysis.

Analysis of liver enzymes in plasma

Levels of plasma ALT and AST were determined using Spectrum Series II autoanalyzer (Abbott Laboratories, Abbott Park, IL, USA) as reported previously [27].

Sphingolipid analysis

Free sphingosine and sphinganine of liver and kidney in base-treated lipid extracts were determined by high performance liquid chromatography (HPLC) utilizing a modification of the extraction methods described earlier [20]. Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, Matreya Inc. Pleasant Gap, PA, USA). The HPLC apparatus and derivatization procedure was similar to the one described before (20) except the fluorescence detector used in this study was Luminescence Spectrometer LS30 (Perkin-Elmer Inc., Norwalk, CT, USA).

Assay of serine palmitoyl transferase (SPT) activity

The activity of SPT in liver was analysed using the method described by Williams et al. [32] with minor modification. Briefly, the frozen tissues were homogenized in homogenization buffer (50 mM HEPES, 5 mM DTT, 10 mM EDTA, 0.25 M sucrose, pH 7.4), and the homogenate was centrifuged at 30,000 g for 30 min, then the supernatant was used for analysis of SPT activity. The reaction volume of 0.1 ml contains 100 mM HEPES (pH 8.3), 5 mM dithiothreitol (DTT), 2.5 mM EDTA (pH 7.0), 50 μM pyridoxal phosphate, 200 μM palmitoyl CoA (Sigma Chemical Inc., St. Louis, MO, USA), 1 mM ^{3}H-labeled L-serine (20 mCi/mmol, Moravek, Brea, CA, USA), and supernatant containing 50–150 μg protein. The ^{3}H-labeled 3-ketosphinganine product was extracted in chloroform-methanol, and radioactivity counted in Rack Beta liquid scintillation counter (Pharmacia, Wallac OY, Turku, Finland). The concentration of protein was determined by Bio-Rad Bradford reagent according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

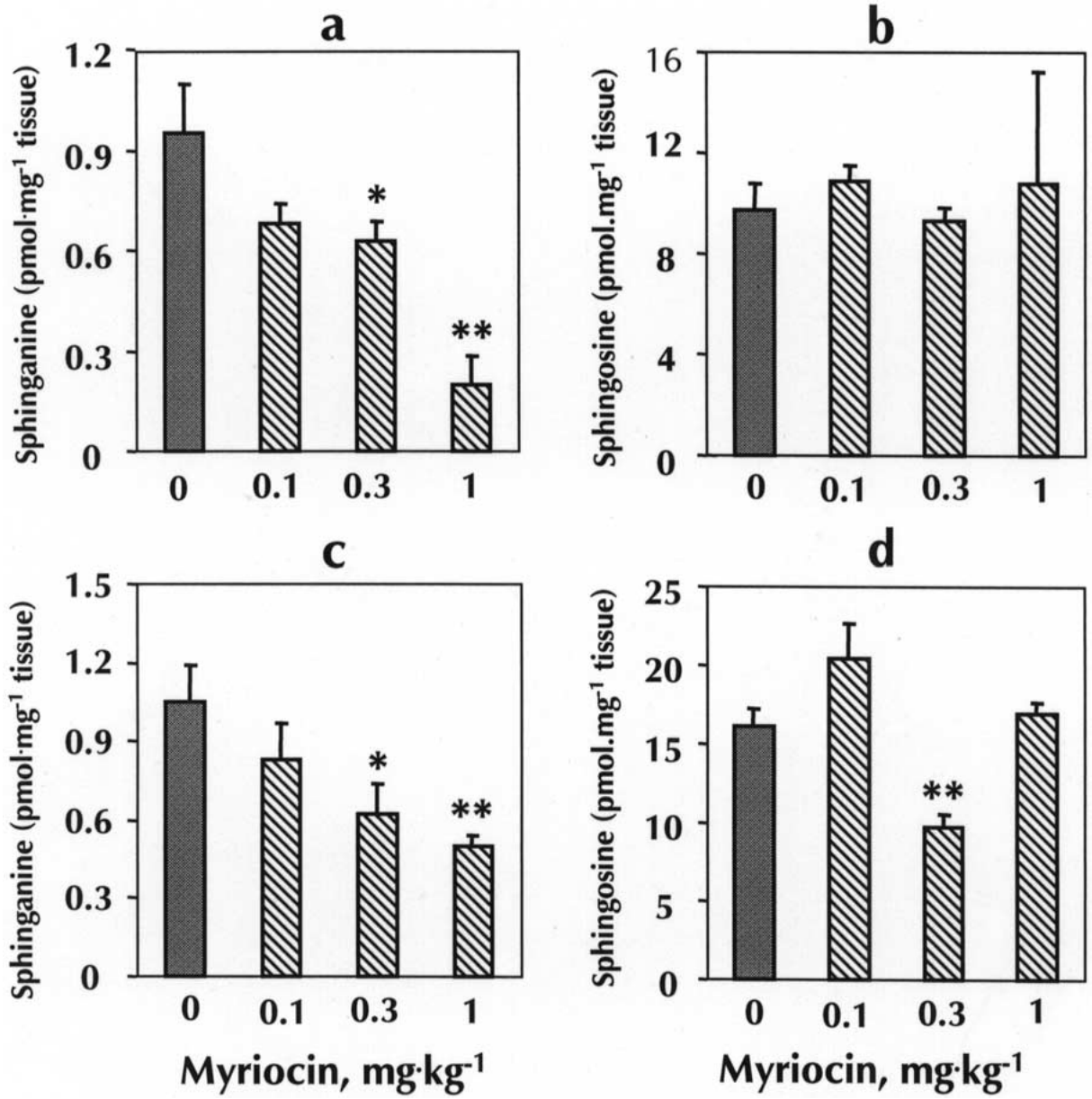

Figure 3. Concentrations of free sphinganine and sphingosine in mouse liver (a, b) and kidney (c, d) treated with myriocin with the indicated doses daily for 5 days. Data are presented as mean ± SE ($n = 5$). * $p < 0.05$, ** $p < 0.01$ compared to control (0 mg kg^{-1}).

Semiquantitative estimation of gene expression by cDNA amplification

Total RNA was isolated from the tissue powdered in liquid nitrogen as described by Sharma et al. [27]. RNA (2.5 μg) was transcribed to cDNA using oligo $(dT)_{12-18}$ and Superscript® II (Life technologies, Grand Island, NY, USA) at 42 °C for 50 min. The abundance of selected genes in liver tissues was analysed by polymerase chain reaction (PCR) using Taq DNA polymerase and 0.2 μM of each primer in 1X PCR buffer containing 2 mM $MgCl_2$. The PCR reactions were performed in an Eppendorf Mastercycler® gradient (Eppendorf Scientific Inc., Westbury, NY, USA). The respective primers (chosen by Primer3 program, Whitehead Institute, Cambridge, MA, USA) are shown in Table 1. The annealing temperatures for each reaction were optimized, and the number of cycles was

optimized to yield the product in exponential range to avoid saturation. The PCR products were separated on 2% agarose gel containing ethidium bromide and detected by UV transilluminator (Ultra Lum Inc., Carson, CA, USA). Images were captured using a Kodak DC290 camera followed by digitization using UNSCAN-IT software (Silk Scientific Inc., Orem, UT, USA). Density of β-actin in the same sample was used to normalize the expression of each gene. The quantitative validity of RT-PCR was confirmed by either northern blot in lipopolysaccharide-treated J774. A macrophages [12] or RNase protection assay in mouse liver [1]

Statistical analysis

All data are expressed as mean $\pm$ standard error (SE). Data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The level of $p < 0.05$ was considered significant.

Results

No apparent behaviour or gross pathological effects of myriocin treatment were evident after 5 days of exposure. No difference was observed in either absolute or relative liver weights among these groups (data not shown). The increases in plasma ALT and AST levels after myriocin treatment were not significant (Figure 2).

There was a dose-dependent decrease in the content of free sphinganine in both liver and kidney (Figure 3a, c). The decrease of free sphinganine in liver was significantly correlated with the doses of myriocin ($r = -0.78$, $p < 0.0001$). No significant changes were detected in the concentration of free sphingosine in both liver and kidney except a decrease in 0.3 mg kg^{-1} of myriocin treated group in kidney (Figure 3b, d).

A dose-dependent and significant decrease in the activity of liver SPT was observed after myriocin treatment with a correlation coefficient of -0.76 ($p < 0.0001$, Figure 4). The SPT activity in liver was correlated with the free sphinganine content ($r = 0.6$, $n = 18$, $p = 0.004$).

After myriocin treatment, the expression of liver c-*myc* was significantly increased in a dose-dependent fashion (Figure 5a); no change was observed in the expression of hepatocyte growth factor (HGF, Figure 5b); dose-related increases in liver mRNA for transforming growth factor (TGF)β1 and tumour necrosis factor (TNF)α were not significant statistically (Figure 5c, d).

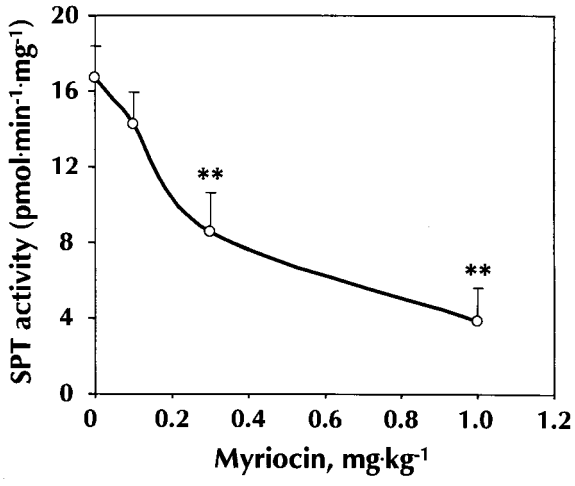

Figure 4. Dose-dependent decrease in the activity of serine palmitoyltransferase (SPT) in livers from mice treated with the indicated doses of myriocin daily for 5 days. * $p < 0.05$, ** $p < 0.01$ compared to control (0 mg kg^{-1}).

Discussion

The current study showed that myriocin treatment in mice did not produce overt liver damage indicated by the levels of plasma enzymes, ALT and AST. It suggested that the doses employed here were not clinically hepatotoxic. Fumonisin B_1, the ceramide synthase inhibitor, caused hepatotoxicity in the same strain mouse model after a 5-day treatment [28]. A lack of hepatotoxic effect by myriocin has been reported earlier. In fact, a single intraperitoneal dose of 0.1 mg kg^{-1} myriocin protected mice from the carbon tetrachloride- induced hepatotoxicity [7]. This protective effect of myriocin was attributed to the modulation of adenosine 3′-monophosphate (3′-AMP) forming enzyme activity.

The first step involved in sphingolipid biosynthesis is the condensation of serine and palmitoyl CoA, a reaction catalysed by SPT to produce 3-ketosphinganine, the precursor of free sphinganine [17]. It has been reported that myriocin potently inhibits the activity of SPT with an apparent inhibition constant of 0.28 nM in CTLL-2 cells [22]. Two subunits, LCB1 and LCB2, are directly responsible for SPT activity [10]; both LCB1 and LCB2 are specific myriocin-binding proteins [5]. Analysis in the present

Table 1. Primers and PCR conditions for genes analyzed in the liver following exposure to myriocin*

	Primers	Annealing temperature (°C)	PCR cycles
c-*myc*	Sense 5′ ATC TGC GAC GAG GAA GAG AA 3′ Anti-sense 5′ ATC GCA GAT GAA GCT CTG GT 3′	54	32
HGF	Sense 5′ CAC AGT GCT GTG AAT GAG AC 3′ Anti-sense 5′ ATG TTT GGG TCA GTG GTA AA 3′	53	29
TGFβ1	Sense 5′ CCA GAT CCT GTC CAA ACT AA 3′ Anti-sense 5′ TTT CTC ATA GAT GGC GTT G 3′	53	40
TNFα	Sense 5′ GTT CTA TGG CCC AGA CCC TCA CA 3′ Anti-sense 5′ TCC CAG GTA TAT GGG TTC ATA CC 3′	56	35
β-actin	Sense 5′ ATG GAT GAC GAT ATC GCA 3′ Anti-sense 5′ ATG AGG TAG TCT GTC AGG T 3′	55	22

*Other PCR conditions include: hot start: 95 °C 5 min, 1 cycle, followed by: denaturation, 94 °C 30 sec, annealing, indicated temperature 30 sec; elongation, 72 °C 1 min for indicated cycles, and finally elongation at 72 °C 1 min for 1 cycle. For all experiments, the conditions were optimized to keep the number of cycles within the range of exponential product increase.

study showed that liver free sphinganine is decreased significantly in response to myriocin treatment, and the decrease of sphinganine was correlated to the activity of SPT. These data suggests that myriocin-induced inhibition of SPT activity was responsible for the reduction of sphinganine.

The apoptotic effects of myriocin could be blocked by addition of sphingosine or ceramide in the cultures [9, 22]. It has been implied that the biological effects of myriocin are primarily due to the depletion of sphinganine, the product of SPT, and downstream sphingoid metabolites. A Chinese hamster ovary (CHO) cell mutant, LY-B strain, which expresses no appreciable activity of SPT, grew very poorly in sphingolipid-deficient medium, whereas LY-B/cLCB1 cells that acquired SPT activity by stable transfection of LY-B strain with the wild-type hamster *LCB1* cDNA, could grow with a high yield in the same culture conditions as LY-B strain [10]. The growth of LY-B cells was restored to the level of LY-B/cLCB1 cells by addition of sphingosine to the growth medium [10]. Myriocin inhibited cell growth via apoptotic cell death in CTLL-2 [22], and rat Purkinje cells [9]. These studies suggested that inhibition of SPT activity leading to depletion of sphingosine and/or ceramide is a plausible mechanism for myriocin-induced effects on cell growth.

In the present study, the observation that the sphingosine content in liver did not alter significantly in the five-day myriocin treatment might account for the absence of evident liver damage. We further investigated the expression of selected genes that regulate cell growth and survival. Results showed that only c-*myc* significantly increased with minor alteration of other selected genes, including HGF, TGFβ, and TNFα, in liver. It has been reported that ceramide down-regulated c-myc mRNA in HL-60 cells [33]. Myriocin treatment results in a marked inhibition of intracellular ceramide biosynthesis [4, 21]. It is not known whether a decrease of ceramide in response to myriocin could potentiate c-*myc* expression in mouse liver.

It is interesting that inhibition of ceramide synthase by fumonisin B_1 also caused a marked induction of c-*myc* in mouse liver [2]. The c-*myc* expression was increased more than 50-fold in mouse liver after a 5-day treatment with 2.25 mg kg^{-1} of fumonisin B_1, using a quantitative RNase protection assay. Together these findings indicate that depletion of the same downstream signaling intermediates, such as ceramide, glucosylceramide, may be responsible for the c-Myc induction. It has been reported that both myriocin and fumonisin induce apoptosis [9, 22, 28]. Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-Myc depending on different cellular conditions; and the release of mitochondrial cytochrome c is involved in c-Myc-accelerated apoptosis [6, 14]. The loss of ceramide and/or sphingosine resulting from exposure to myriocin induced apoptotic cell death [9, 22]. It has been shown that myriocin-induced apoptosis in CTLL-2 cells is caspase-3-independent, but related to other caspases activation and mitochondrial depolarization [34]. The ability of myriocin to increase c-*myc* expression might account for myriocin-induced mito-

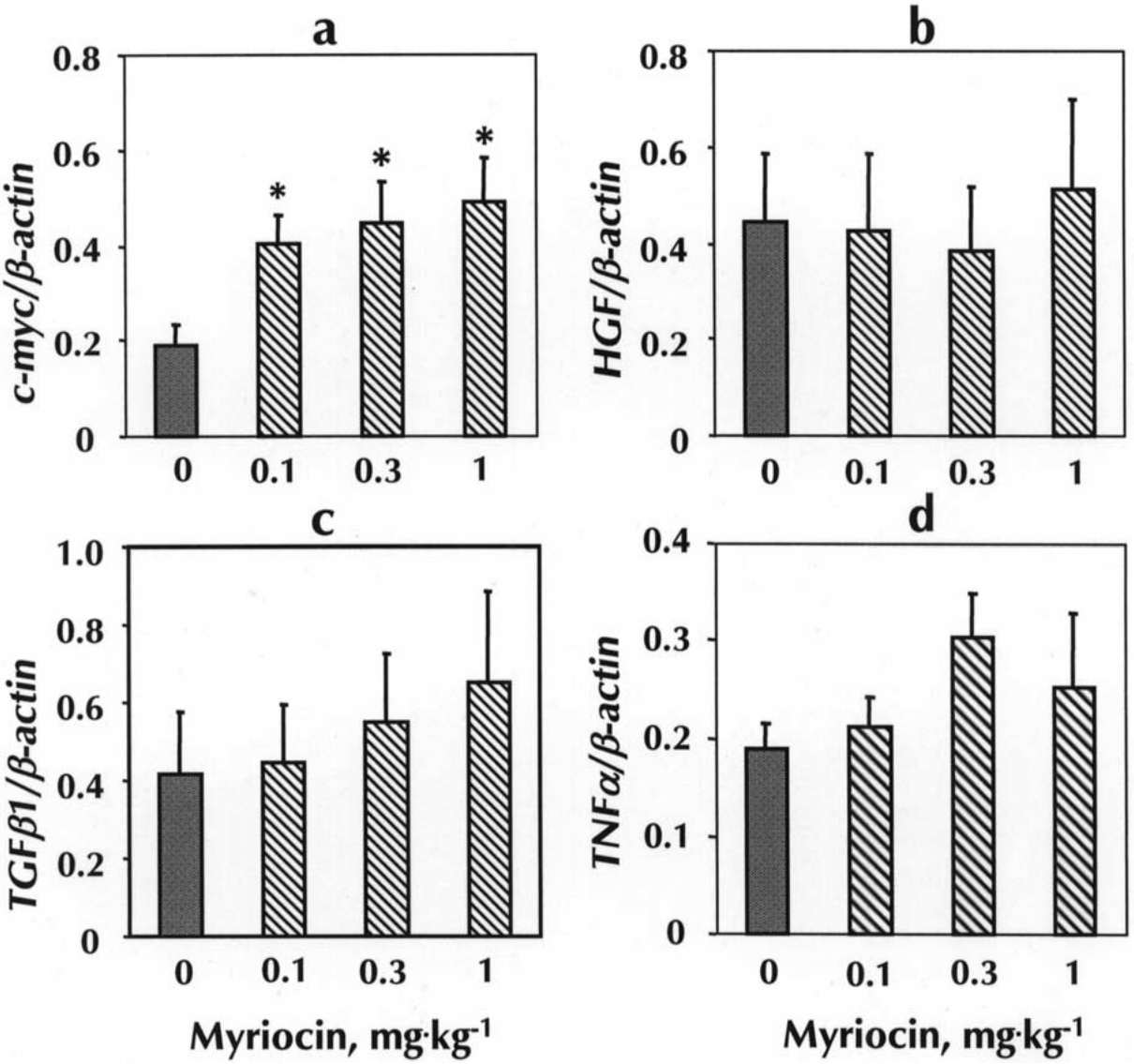

Figure 5. Expression of selected genes in liver of mice treated with myriocin at the indicated doses daily for 5 days. Data are presented as mean ± SE ($n = 5$). * $p < 0.05$ compared to the control (0 mg kg^{-1}).

chondrial damage and apoptosis observed in CTLL-2 cells [34]. However, there are very many compounds with a diverse spectrum of biological functions and toxicities in sphingolipid metabolism pathways. The perturbation of sphingolipid metabolism allows alteration of the levels of various bioactive lipids simultaneously. As each of these bioactive metabolites has its own direct actions, one would then predict a variety of responses mediated by the integration of these distinct pathways [11, 17]. Myriocin treatment would alter the levels of many bioactive intermediates resulting from SPT inhibition. It is very difficult to define the exact relationships between myriocin-induced biological effects and alteration in respective bioactive metabolites; these effects of myriocin are likely the result of many bioactive lipid interactions.

Myc regulates cellular growth, proliferation, and apoptosis under specific conditions where survival growth factors are limited. Myc overexpression generally increases cellular proliferation and growth, but in some circumstances, increased Myc stimulates cells to undergo apoptosis [6, 14, 15, 26]. Myc-induced sensitization of cells to apoptotic stimuli is mediated by release of cytochrome c into the cytoplasm, and this

process can be blocked by survival growth factors such as insulin-like growth factor 1 [14]. The observation in the present study suggests that the relative normal expression of selected hepatocyte growth factors such as HGF might maintain the liver functions within normal range by interaction between these growth factors and c-Myc in myriocin exposure. Myriocin protects against carbon tetrachloride-induced acute hepatotoxicity in mice [7]. These studies suggest that myriocin may not be detrimental under low-dose short-term exposures; however, long-term use of myriocin may produce adverse effects on health by modulating biological functions of cells as a result of persistent disruption of sphingolipid metabolism.

Acknowledgements

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