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Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress

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Abstract

Usnic acid, a lichen acid, is a compound found in crude medicines and dietary supplements, including Lipokinetix®, a supplement marketed as a weight loss agent that caused hepatotoxicity and acute liver failure in patients. In this study, we examined the toxicity of usnic acid and assessed whether usnic acid may be contributing to hepatotoxicity caused by Lipokinetix. In primary cultured murine hepatocytes, usnic acid treatment (5 μM) resulted in 98% necrosis within 16 hr (no apoptosis was detected). Usnic acid treatment was associated with early inhibition and uncoupling of the electron transport chain in mitochondria of cultured hepatocytes. This inhibition of mitochondria by usnic acid corresponded with a fall in ATP levels in hepatocytes. In isolated liver mitochondria, usnic acid was observed to directly inhibit and uncouple oxidative phosphorylation. Oxidative stress appears to be central in usnic acid-induced hepatotoxicity based on the following findings: (1) pretreatment with antioxidants (butylated hydroxytoluene + Vitamin E) decreased usnic acid-induced necrosis by nearly 70%; (2) depletion of mitochondrial GSH with diethylmaleate increased susceptibility of hepatocytes to usnic acid; (3) usnic acid treatment was associated with increase free radical generation, measured using the fluorescent probe, dichlorodihydrofluorescin. The source of reactive oxygen species after usnic acid treatment include autoxidation of usnic acid and increased hydrogen peroxide generation by mitochondria caused by usnic acid inhibition of the respiratory chain, with the latter playing a more prominent role. Taken together, our results suggest that usnic acid is a strong hepatotoxic agent that triggers oxidative stress and disrupts the normal metabolic processes of cells. Usnic acid therefore may contribute to the hepatotoxic effects of Lipokinetix and its use in any supplement must come into question.

Keywords: Usnic acid; Hepatocytes; Mitochondria; Oxidative stress; Antioxidants

1. Introduction

Nutritional and herbal supplements have become a billion dollar industry in the United States. Unfortunately, many commonly sold supplements have been found to cause adverse effects and in some cases may have directly or indirectly resulted in death of some individuals [1]. Liver, as the major site of metabolism for drugs and xenobiotic compounds [2], may be highly susceptible to injury by some of these supplements. For example, hepatotoxicity has been associated with the intake of Chinese herbal medicines, such as Jin Bu Huan [3], and botanical dietary supplements, such as chaparral [4].

Lipokinetix® (Syntrax) is a dietary supplement that had been marketed as a weight loss agent. A recent study found that patients that took Lipokinetix supplements developed hepatotoxicity and in some cases developed fulminant hepatic failure [5]. This prompted the FDA to issue a warning about Lipokinetix [6] and it has subsequently been taken off the market.

Usnic acid (Fig. 1), a metabolite found in lichen [7], is a key constituent of Lipokinetix. Previous studies have shown that usnic acid can cause the uncoupling of mitochondria [8], and it may have been for this reason that Lipokinetix was advertised as a fat burner. Overall, the

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Abbreviations: BHT, butylated hydroxytoluene; DCFH, dichlorodihydrofluorescin diacetate; DCF, dichlorofluorescin; DEM, diethylmaleate.

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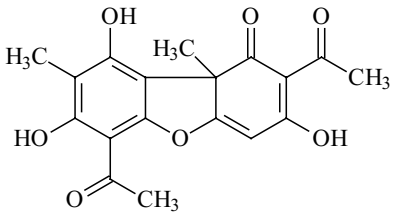

Fig. 1. Structure of usnic acid.

pathophysiological effects of usnic acid have not been well characterized either *in vivo* or *in vitro*. Initial studies have found usnic acid to inhibit cell proliferation and cause cytotoxicity in cultured keratinocytes [9]. Usnic acid treatment of mice *in vivo* was found to inhibit proliferation of polychromatic erythrocytes [10]. In addition, usnic acid has been described to have antimicrobial and antiviral effects in various systems [7].

Since usnic acid remains a component of some crude drugs and supplements sold throughout the world, such as Cladonia (used to treat pulmonary tuberculosis in Asia, Europe, and Africa) [7] and Kambala teas, the potential adverse effects of usnic acid were investigated in cultured murine hepatocytes.

2. Material and methods

2.1. Animals and reagents

The experiments were carried out using C57BL/6 mice (6–8 weeks) and male Wistar rats (4–7 months), obtained from Harlan Bioproducts for Science Inc. The animals were housed in a room at 25° under normal laboratory lighting conditions. They were maintained on a commercial pellet *ad lib*. All animals received care according to methods approved under institutional guidelines for the care and use of laboratory animals in research.

(+)-Usnic acid was purchased from Sigma. Usnic acid was dissolved in DMSO prior to treatment of hepatocytes. In some chemical studies, usnic acid was dissolved in water and pH adjusted to 11.5 at which it becomes soluble. Dichlorodihydrofluorescin diacetate (DCFH), Hoechst 33258, and Sytox Green were purchased from Molecular Probes. All other chemicals were purchased from standard commercial sources.

2.2. Cell isolation and culture

Hepatocytes were isolated as previously described [11]. The liver was perfused with collagenase and isolated hepatocytes were suspended in DMEM-F12 containing 10% heat-inactive fetal bovine serum, 1 nM bovine insulin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 nM hydrocortisone, and 0.15 mg/mL methionine. 1.2×10^6 cells in 4 mL were plated in individual 60 mm diameter LUX culture dishes coated with 0.03% rat tail collagen and cultured in a 5% CO_2 atmosphere at 37°. The viability of the isolated hepatocytes was greater than 90% as judged by trypan blue exclusion. After 3 hr, the culture medium was changed to serum-free medium containing 100 U/mL penicillin and 0.1 mg/mL streptomycin. At this time, usnic acid was added and the cells were incubated for up to 16 hr at 37°. In some experiments, hepatocytes were pretreated for 1 hr with 50 μM α-tocopheryl succinate (Vitamin E) and 20 μM butylated hydroxytoluene (BHT) and then exposed to usnic acid. Similarly, in some experiments hepatocytes were treated with 0.25 and 0.50 mM diethylmaleate (DEM) for 1 hr before addition of usnic acid, followed by continuous exposure to both for up to 16 hr.

2.3. Isolation of liver mitochondria

Liver mitochondria were isolated from adult male Wistar rats by differential centrifugation as previously described [12]. Livers from rats were excised, washed with 0.25 M sucrose, and homogenized in an H-medium (210 mM mannitol, 70 M sucrose, 2 mM HEPES, and 0.05% BSA). The homogenate was centrifuged at 800 *g* for 8 min, the pellet removed, and the centrifugation process repeated. The resulting supernatant was centrifuged at 8000 *g* for 10 min, washed with H-medium, and the centrifugation repeated.

2.4. Assay of apoptosis and necrosis

After various treatments, cells were double-stained with 8 μg/mL Hoechst 33258 and 1 μM Sytox Green. After staining, the culture dishes were placed on the ice and cells were observed under an OLYMPUS fluorescent microscope. Quantitation of total and apoptotic cells (nuclear fragmentation with Hoechst dye) were performed as previously described [13] by counting more than 500 cells per well and necrotic cells (Sytox Green positive) by counting the same field, excluding cells with segmentation of nucleus (secondary necrosis).

2.5. ATP measurement

ATP was measured as previously described [13]. The culture medium was replaced with 1 mL of cold 0.6 M/L $HClO_4$, and cells were scraped with a disposable cell scraper. After centrifugation (9000 *g*, 1 min), 0.8 mL of the supernatants were neutralized with 5 M/L KOH and 0.4 M/L imidazole. After one more centrifugation, supernatants were diluted 200-fold with deionized, distilled water. The ATP content was determined using a commercial ATP assay system kit (Promega), following the procedure indicated by the manufacture, and MGM Instruments Optocomp I luminometer. ATP standard curve (linear in the range of 10–1000 μM) was carried out in all experiments.

2.6. Measurements of mitochondrial respiration in cultured hepatocytes and isolated mitochondria

Mitochondrial respiration was measured by monitoring oxygen uptake polarographically with a Clark-type electrode (Hanstech) in a respiration buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris–HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4. For isolated mitochondria, 0.7 mg of mitochondria was added to 1 mL of respiration buffer and oxygen consumption monitored in the presence of mitochondrial substrates.

For measurement of mitochondrial respiration in cultured hepatocytes, cells were first detached by trypsin treatment and resuspended in PBS. 2.4 million hepatocytes were then placed in 1 mL of respiration buffer. Digitonin (0.025 mg) was subsequently added to cells to disrupt the cell membrane and allow ADP and mitochondrial substrates to reach mitochondria [14]. Oxygen consumption was monitored following addition of various mitochondrial substrates.

2.7. Determination of reactive oxygen species generation by hepatocytes

Reactive oxygen species levels in hepatocytes were measured using DCFH. DCFH is taken up by cells and oxidized to dichlorofluorescin (DCF), with the rate of DCFH oxidation depending on the levels of reactive oxygen species in cells [15,16]. DCF formation in hepatocytes was measured fluorometrically ($\lambda_{ex} = 490$ nm; $\lambda_{em} = 520$ nm) using a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermal-controlled and magnetic stirring sample compartment.

DCF measurements were made as follows: At various time points, the medium in control and usnic acid-treated hepatocytes was removed and cells were resuspended in PBS containing 2 μM DCFH in the presence or absence of usnic acid or uncouplers. Hepatocytes were returned to the incubator to allow DCFH uptake into cells. After 20 min, hepatocytes were washed with cold PBS, scraped, and resuspended in 3 mL PBS. DCF fluorescence was subsequently measured in hepatocytes.

2.8. Hydrogen peroxide measurements

H_2O_2 production by isolated liver mitochondria was measured by monitoring fluorescence of *p*-hydroxyphenylacetate oxidation in the presence of horseradish peroxidase. Fluorescence measurements ($\lambda_{ex} = 320$ nm; $\lambda_{em} = 400$ nm) were performed with a Perkin-Elmer LS-5 spectrofluorometer equipped with a thermal-controlled and magnetic stirring sample compartment. For all measurements mitochondria were incubated in 230 mM mannitol, 70 mM sucrose, 30 mM Tris–HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4 at 25°. The absorption spectrum of usnic acid overlaps the fluorescence spectrum of *p*-hydroxyphenylacetate, and slightly quenches *p*-hydroxyphenylacetate fluorescence. This quenching was measured and calculated in the final data analysis.

H_2O_2 production from the autoxidation of usnic acid was similarly made using *p*-hydroxyphenylacetate and horseradish peroxidase. 50–500 μM usnic acid was placed in PBS buffer and H_2O_2 production monitored for 20 min.

2.9. GSH measurements

GSH level was measured by the method of Tietze [17]. At end of each incubation period, cells were scraped from the plate with a rubber policeman and collected in 1 mL PBS. The reaction rate was monitored by measuring absorption at 412 nm and total GSH content of sample was determined by comparing the reaction rate to that obtained using a series of dilutions of GSH. GSH was normalized to the amount of cellular protein present and expressed as nanomoles per milligram of protein.

2.10. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were obtained with a Bruker ECS 106 spectrometer (operating at X-band) equipped with a cylindrical room temperature cavity operating in TM_{110} mode. Aliquots (150 μL) of the reaction mixtures were transferred to bottom-sealed Pasteur pipettes and measured at room temperature with the following instrument settings: microwave frequency, 9.77 GHz; microwave power, 20 mW; field modulation frequency, 25 kHz; field modulation amplitude, 0.1 G; receiver gain, 2×10^5; time constant, 82 ms; scan rate, 0.38 G/s; number of scans accumulated, 3.

Computer simulations of spectra were performed using the program WinSIM (EPR calculations for MS-Windows NT, 95 version: 0.96 from Public EPR Software Tools—PEST) written by Duling [18].

3. Results

3.1. Effect of usnic acid on hepatocyte viability

The cytotoxic effects of usnic acid were assessed in primary cultured murine hepatocytes. Figure 2A shows a dose-dependent effect of usnic acid treatment on hepatocyte viability after 16 hr. Hepatocytes given usnic acid doses of 2 μM or less remained completely viable, while treatment of 5 μM or greater caused almost a 100% cell death. Using dual staining, it was found that cell death was entirely necrotic, with no increase in apoptotic features being observed (data not shown). The time-dependent effects of usnic acid (5 μM) are shown in Fig. 2B.

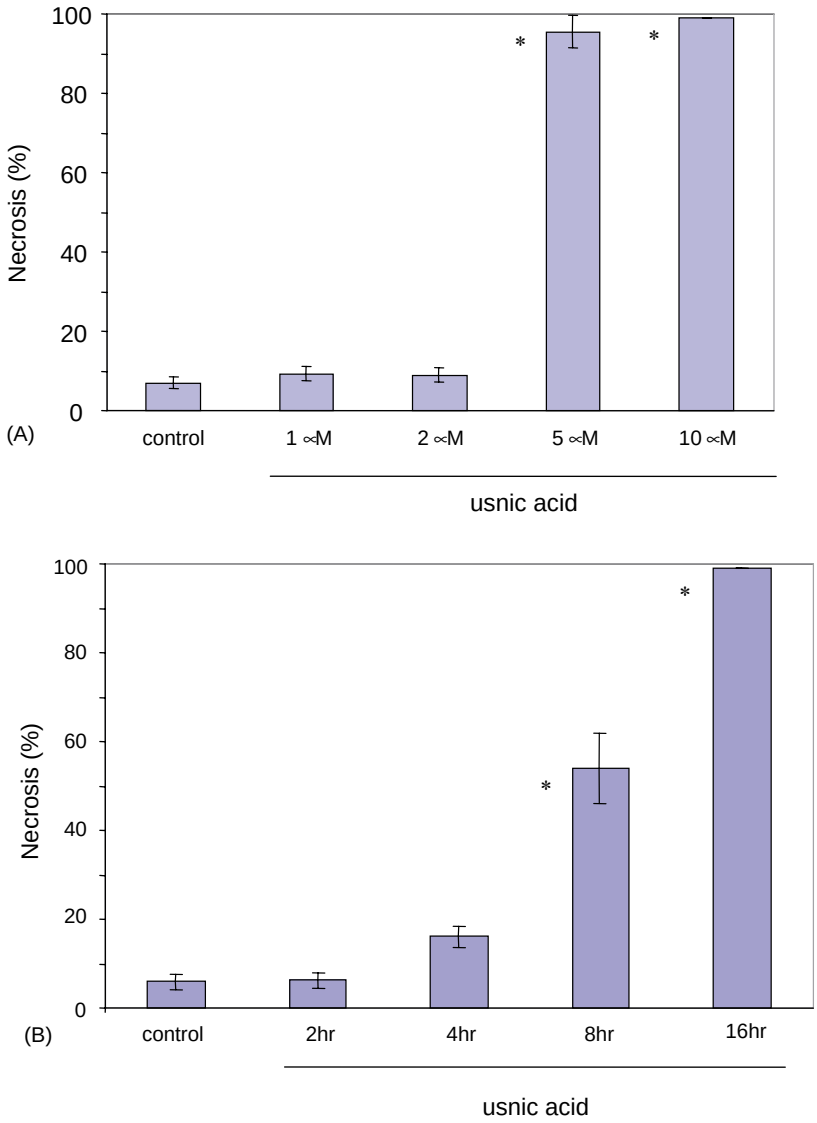

Fig. 2. Dose and time course of hepatotoxicity induced by usnic acid. (A) Dose–response of usnic acid hepatotoxicity. Primary mouse hepatocytes were treated with various doses of usnic acid for 16 hr. (B) Time course of hepatotoxicity by 5 μM usnic acid treatment. Hepatocyte viability was determined as described in Section 2. Results are mean ± SD for three independent experiments. $^{*}P < 0.05$ compared with control.

Necrotic cell death increased slightly at 4 hr and was >50% by 8 hr.

3.2. Disruption of mitochondrial respiration and energy metabolism by usnic acid

3.2.1. ATP levels following usnic acid treatment

ATP levels were measured in hepatocytes to determine if usnic acid was affecting energy metabolism of cells (Fig. 3). After only 2 hr, 5 μM usnic acid reduced ATP levels by 40% of control. At this time no increase in cell death was observed. The subsequent larger drops of ATP—70% at 8 hr and 90% at 16 hr—is likely due to cell necrosis that occurs at those times. 2 μM usnic acid treatment did not significantly reduce cellular ATP levels. Thus, the initial rapid fall in ATP preceded cell death while the later fall accompanied cell death.

3.2.2. Inhibition of mitochondrial respiration in cultured hepatocytes following usnic acid treatment

Usnic acid at low levels (1 μM) has been previously described to uncouple isolated liver mitochondria [8]. Mitochondrial-dependent oxygen uptake in cultured hepatocytes was measured polarographically to determine if usnic acid treatment was similarly affecting mitochondrial respiration in cultured hepatocytes. Two-hour treatment of usnic acid resulted in both the inhibition and uncoupling of mitochondria in cultured hepatocytes in the presence of succinate (Table 1). The treatment of 2 μM usnic acid inhibited state III respiration by 40% accompanied by a modest uncoupling of mitochondria (as seen by decline in RCR). The primary effect of 5 μM usnic acid administration to cultured hepatocytes, on the other hand, was the near complete inhibition of both states III and IV respiration. The little respiration observed in hepatocytes

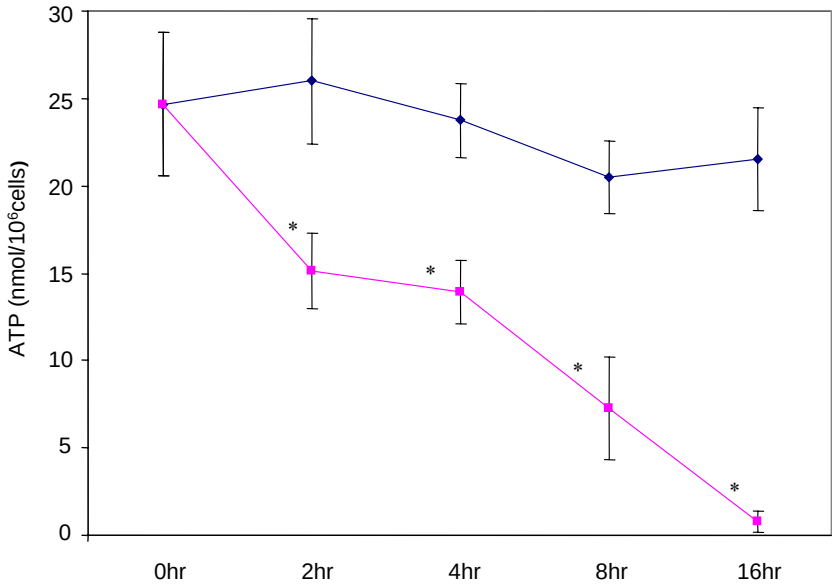

Fig. 3. ATP levels in cultured hepatocytes after usnic acid treatment. Primary mouse hepatocytes were treated with 5 μM usnic acid for various periods; control (◆); 5 μM usnic acid (■). At the indicated time points hepatocytes were harvested and ATP levels measured using luciferin/luciferase, as described in Section 2. Results are mean ± SD for three independent experiments. $^{*}P < 0.05$ compared with control.

treated with 5 μM usnic acid was almost completely uncoupled. These results suggest that the early drop in ATP after 5 μM usnic acid treatment may be due to inhibition of mitochondrial respiration and disruption of oxidative phosphorylation.

3.2.3. Uncoupling and inhibitory effects of usnic acid in isolated liver mitochondria

Oxygen uptake experiments with cultured hepatocytes demonstrated that usnic acid was acting as both an inhibitor and uncoupler of mitochondria. Previous work with isolated mitochondria only describes usnic acid as an uncoupler in isolated liver mitochondria [8]. Using isolated liver mitochondria in the presence of succinate (complex II substrate), we observed that usnic acid can act as only an uncoupler or as an uncoupler and inhibitor of mitochondria, depending on the presence of BSA in buffer (Fig. 4A and B). In the presence of BSA (0.10%), usnic acid uncoupled mitochondrial respiration (Fig. 4B), and even high doses did not inhibit respiration (Fig. 4A). When BSA was not present, usnic acid treatment resulted in both inhibition and uncoupling of isolated liver mitochondria (Fig. 4A and B). Similar results were observed with complex I substrates (glutamate/malate), suggesting that usnic acid inhibition of mitochondria was occurring at complex III or at some other component in mitochondria. When working with isolated mitochondria, BSA is commonly added to buffers for removal of fatty acids that can interfere with respiration [12]. BSA was not present in oxygen measurements with cultured hepatocytes because hepatocyte respiration and RCR was greater without BSA present in buffer (data not shown).

Table 1
Effect of usnic acid on mitochondrial respiration in cultured hepatocytes

	O_2 consumption (μM/min/million cells)		RCR
	State IV respiration	State III respiration	
Control	4.27 ± 0.25	11.5 ± 0.80	2.70
Usnic acid (2 μM)	4.37 ± 0.24	7.00 ± 0.36*	1.60*
Usnic acid (5 μM)	3.53* ± 0.27	4.14 ± 0.54*	1.17*

Cultured hepatocytes were incubated in DMEM-F12 medium in the presence and absence of usnic acid for 2 hr at 37°. Hepatocytes were subsequently detached by trypsin treatment and resuspended in PBS. 2.4 million hepatocytes were then placed in 1 mL of respiration buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris–HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4. Digitonin (0.025 mg) was added to disrupt the cell membrane and allow ADP and succinate to reach mitochondria. Oxygen consumption was measured polarographically with a Clark-type electrode. Mitochondrial respiration in hepatocytes was monitored in the presence of succinate (state IV) and succinate plus ADP (state III). Respiratory control ratio (RCR) is defined as the state III/state IV ratio. Completely uncoupled mitochondria would have an RCR value of 1. Values are expressed as mean ± SD. $^{*}P < 0.05$ compared with control.

3.3. Involvement of oxidative stress in usnic acid-mediated hepatotoxicity

3.3.1. Antioxidant pretreatment protects hepatocytes against usnic acid

The role of oxidative stress in usnic acid-induced necrosis in hepatocytes was investigated. Pretreatment of hepatocytes with antioxidants (Vitamin E and BHT) was found to protect 70% of hepatocytes from usnic acid-induced necrosis (Fig. 5). However, antioxidants did not protect mitochondria from inhibition caused by usnic acid treatment

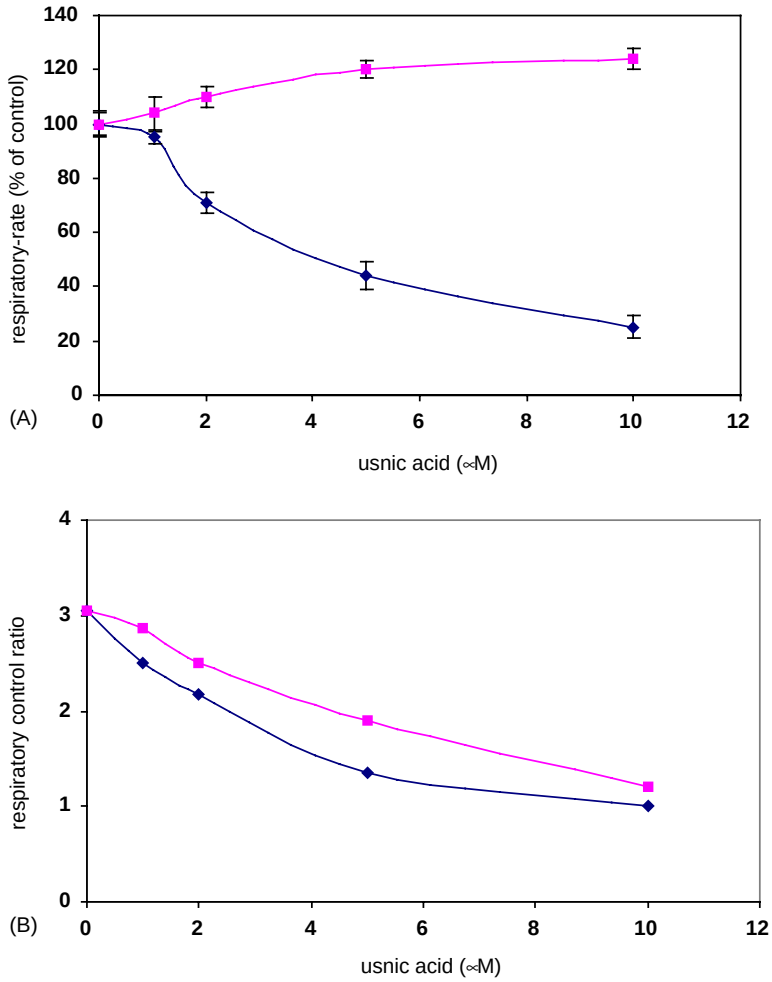

Fig. 4. Uncoupling and inhibitory effects of usnic acid in isolated liver mitochondria. Respiratory rate of isolated liver mitochondria (0.7 mg) was measured with an oxygen electrode in respiration buffer (230 mM mannitol, 70 mM sucrose, 30 mM Tris–HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4) in the presence (■) or absence (◆) of BSA (0.10%). (A) State III respiration in presence of succinate and ADP. The respiratory rate of control mitochondria is 42.3 ± 10 ng of atoms of O/min/mg protein (B) RCR values. Respiratory control ratio (RCR) is defined as the state III/state IV ratio. Completely uncoupled mitochondria would have an RCR value of 1. Values are expressed as mean ± SD.

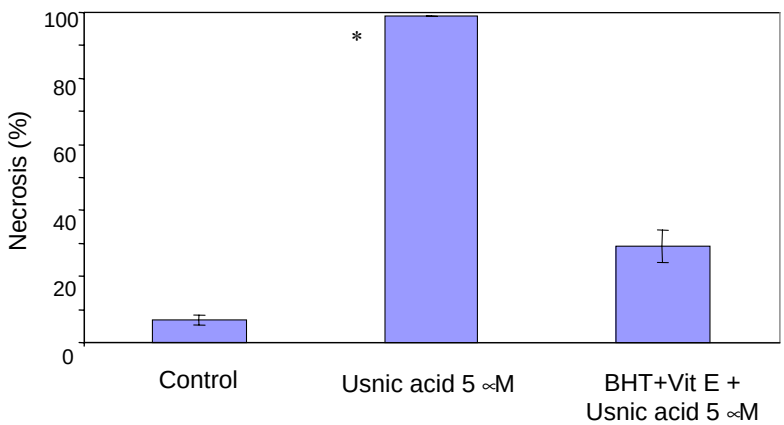

Fig. 5. Antioxidant treatment protects hepatocytes from usnic acid-induced necrosis. Following pretreatment for 1 hr with Vitamin E and butylated hydroxytoluene (BHT), cells were exposed to 5 μM usnic acid. Cell death was assessed with fluorescent dyes after 16 hr. Results are mean ± SD for three independent experiments. $^{*}P < 0.05$ compared with control.

Table 2
Effect of antioxidants on mitochondrial respiration in cultured hepatocytes

	O_2 consumption (μM/min/million cells)		RCR
	State IV respiration	State III respiration	
Control	4.01 ± 0.35	10.6 ± 0.57	2.65
Antioxidants	3.91 ± 0.44	9.81 ± 0.22	2.51
Usnic acid (5 μM)	3.42* ± 0.35	4.27 ± 0.44*	1.25*
Usnic acid (5 μM) + antioxidants	3.35* ± 0.40	3.95 ± 0.48*	1.18*

Hepatocytes were pretreated for 1 hr with antioxidants (50 μM Vitamin E and 20 μM BHT) before usnic acid addition. Cultured hepatocytes were incubated 37° for 6 hr after usnic acid administration or DMSO treatment in control cells. Oxygen consumption was measured polarographically with a Clark-type electrode in the presence of digitonin (0.025 mg), succinate, and ADP. Values are expressed as mean ± SD. *$P < 0.05$ compared with control.

(Table 2). Hepatocytes given usnic acid plus antioxidants had inhibited respiration and low RCR similar to usnic acid-treated hepatocytes in the absence of antioxidants. These data indicate that oxidative stress, not mitochondria inhibition alone, is important in mediating usnic acid-induced hepatotoxicity.

3.3.2. GSH levels following usnic acid treatment

GSH is the major low molecular weight antioxidant in cells and its levels are often decreased during oxidative stress [19]. Usnic acid treatment, however, did not alter intracellular GSH levels in cultured hepatocytes at early time points (Fig. 6A). A delayed decrease in GSH was observed at the later time points which paralleled the extent of necrosis and is likely due to membrane rupture and release of GSH during necrosis rather than being caused by

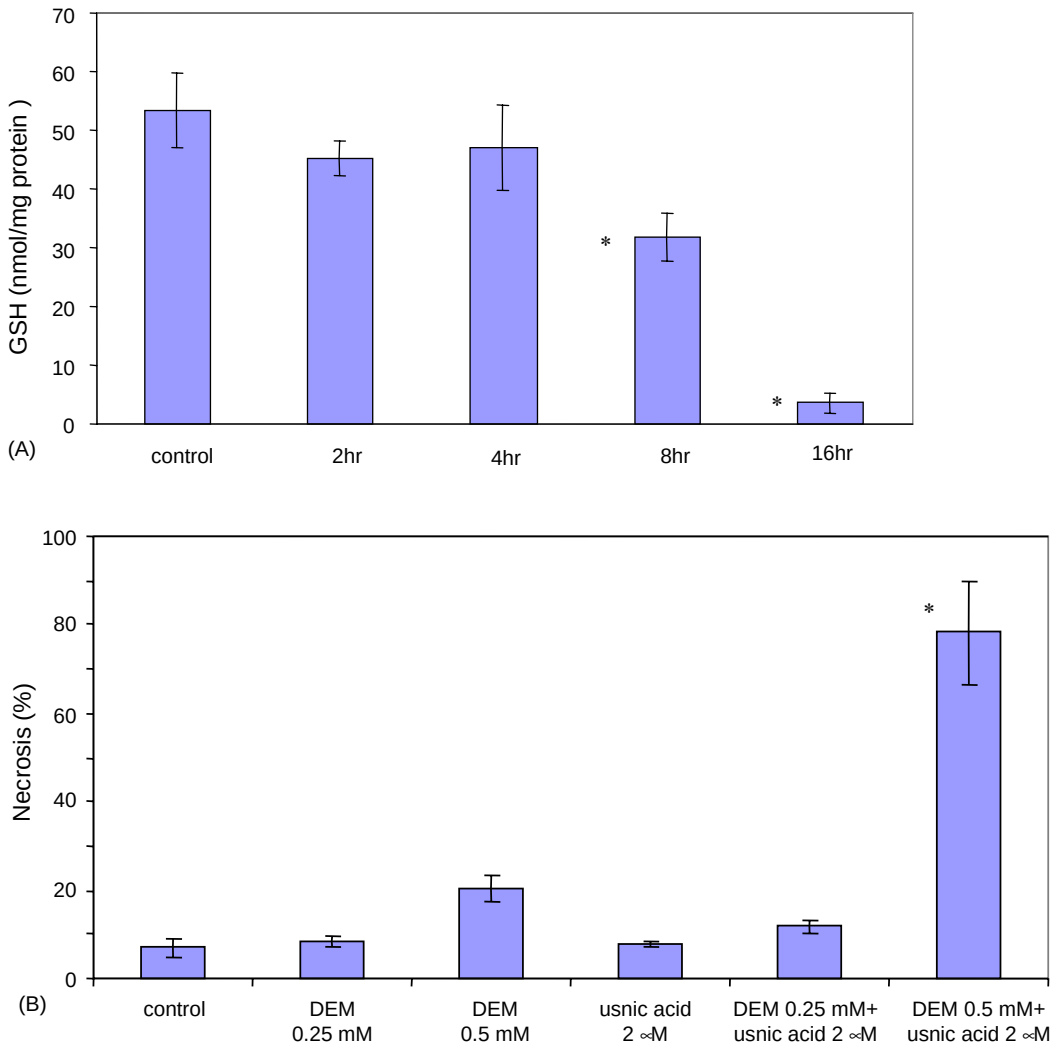

Fig. 6. Role of GSH in usnic acid-mediated hepatotoxicity. (A) GSH levels after usnic acid treatment. Primary mouse hepatocytes were treated with 5 μM usnic acid for various time periods. Hepatocytes were harvested at various time points and GSH levels were measured as described in Section 2. (B). Effect of DEM on cultured mouse hepatocytes treated with usnic acid. Primary mouse hepatocytes were pretreated for 1 hr with DEM and then exposed to 2 μM usnic acid for 16 hr. Cell death was assessed with fluorescent dyes. Results are mean ± SD for three independent experiments. *$P < 0.05$ compared with control.

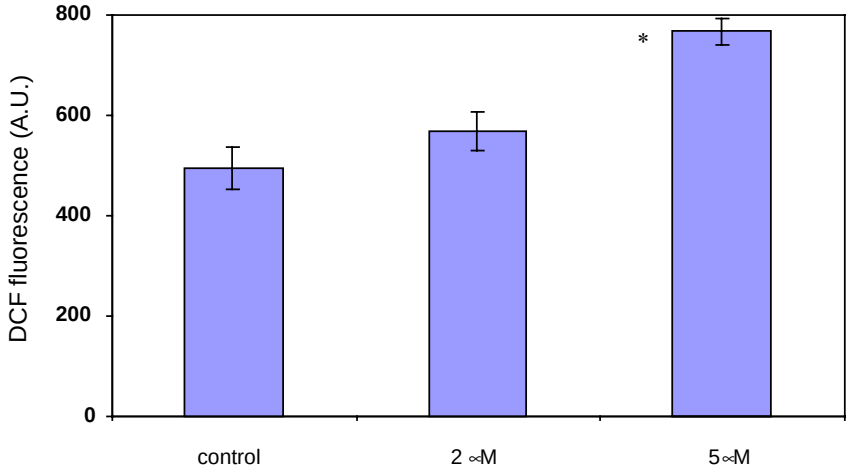

Fig. 7. Reactive oxygen species generation by usnic acid-treated hepatocytes. The media of control and usnic acid-treated hepatocytes (6 hr treatment) were removed and cells were resuspended in PBS containing 2 μM DCFH. Hepatocytes were returned to the incubator to allow DCFH uptake into cells. After 20 min, hepatocytes were washed with cold PBS, scrapped, resuspended in 3 mL PBS, and DCF fluorescence was subsequently measured. Results are mean ± SD for three independent experiments. $^{*}P < 0.05$ compared with control.

oxidative stress. Furthermore, these findings suggest that a specific interaction between usnic acid or a metabolite did not directly and rapidly deplete GSH levels.

3.3.3. GSH depletion sensitizes hepatocytes to usnic acid

Although a fall in GSH levels with usnic acid treatment did not precede necrosis, we considered that GSH might be important in protecting hepatocytes from usnic acid. Treatment of hepatocytes with 0.25 mM DEM markedly depletes cytosolic GSH (≥80%), while causing only a slight depletion of mitochondria GSH levels (<25%) [20]. Pretreatment of hepatocytes with 0.50 mM DEM comparably depletes cytosolic GSH (≥80%) while also depleting mitochondrial GSH levels (>55%) [20]. Treatment of hepatocytes with 0.5 mM DEM, but not 0.25 mM DEM, sensitized hepatocytes to 2 μM usnic acid, a normally non-toxic dose (Fig. 6B). Co-treatment of 2 μM usnic acid and DEM synergistically increased necrosis to 79% in hepatocytes. Since usnic acid appears to increase H_2O_2 production in mitochondria (see below), depletion of

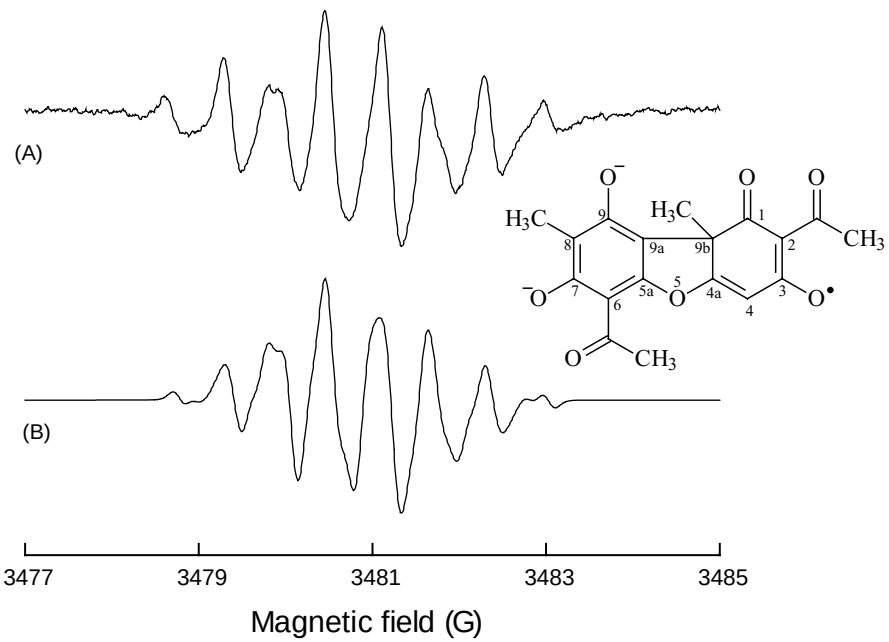

Fig. 8. EPR spectrum of usnic acid radical. (A) Usnic acid (100 mM) plus H_2O_2 (200 μM) in aqueous NaOH solution (pH 11.5). (B) Computer simulation of spectrum A ($r = 0.902$). Because a racemic mixture of usnic acid was used to obtain the experimental spectrum, simulation was performed considering the existence of two enantiomeric radicals present in a 1:1 molar ratio. For the first radical, the simulation provided hyperfine coupling constants (hfcc) of 0.41 (phenylic 4-H), 0.46 and 0.69 G; and for the second, 0.47 (phenylic 4-H), 0.58 and 0.68 G. Each of the hfcc values without attribution correspond to three equivalent hydrogens. The simulation did not permit further attributions.

mitochondrial GSH appears to be necessary for the sensitization of hepatocytes to usnic acid cytotoxicity.

3.3.4. Reactive oxygen species generation after usnic acid treatment

The addition of DCFH to cells, and monitoring its oxidation to DCF is frequently used to monitor oxidative stress in cells [15,16]. Hepatocytes were therefore treated with DCFH, and DCF fluorescence was measured to assess the involvement of reactive oxygen species in usnic acid-induced hepatotoxicity. At 6 hr, DCF fluorescence increased by 15% with 2 μM usnic acid and 54% with 5 μM usnic acid treatment, confirming increased oxidative stress after usnic acid administration (Fig. 7). The protective effect of antioxidants, the sensitization by GSH depletion, and increased DCF fluorescence taken together suggests a central role of oxidative stress in usnic acid-induced hepatotoxicity.

3.4. Sources of oxidative stress in usnic acid-mediated hepatotoxicity

3.4.1. Autoxidation of usnic acid

The source of oxidative stress as seen by increase DCF fluorescence in usnic acid-treated hepatocytes was investigated. Usnic acid appears to be a redox-active molecule; at high concentrations (>50 mM) the absorption spectrum of usnic acid changes slowly in air (data not shown). Usnic acid, which is yellowish in aqueous solutions due to absorption in violet region (the complementary color of yellow), becomes brownish-black after oxidation with increased absorption in the visible region. The same changes occur more rapidly in the presence of catalytic amounts of H_2O_2 (data not shown).

3.4.2. One electron oxidation of usnic acid

The oxidation of usnic acid, by catalytic amounts of H_2O_2, generated a stable radical species detectable by EPR under alkaline conditions (pH 11.5). Figure 8A shows the EPR spectrum of the radical. The pK_a values of the enolic 3-OH, phenolic 9-OH, and 7-OH are 4.4, 8.8, and 10.7, respectively [7]; thus, at pH 11.5, usnic acid is mainly found in its fully deprotonated form. In order to interpret the eight-line pattern spectrum (Fig. 8A), a suitable hyperfine interaction would be that which considers the coupling of the unpaired electron to seven hydrogens. This configuration is achieved by abstracting an electron from oxygen at position 3 to form the radical depicted in Fig. 8. Within this structure, the unpaired electron (due to delocalization) couples to the phenylic 4-H, the three methylic 9b-H, and the three hydrogens from the 2-acetyl group, totaling a coupling to seven hydrogens.

The usnic acid radical may act similarly to other stable radicals, like semiquinones, and react with oxygen to generate superoxide [21]. However, usnic acid autoxidation appears to occur slowly, and no significant amount of superoxide or H_2O_2 was detected from its autoxidation in buffer (50–500 μM usnic acid was tested, data not shown).

3.4.3. Increase H_2O_2 production in isolated mitochondria following usnic acid treatment

The mitochondrial electron transport chain was also explored as source of oxidative stress in usnic acid-treated cells. Inhibitors of the electron transport chain, such as rotenone and antimycin, are well known to cause increased H_2O_2 generation in mitochondria. Since usnic acid also appears to inhibit mitochondrial respiration, we explored whether usnic acid treatment of isolated liver mitochondria could similarly induce increased H_2O_2 generation. In the

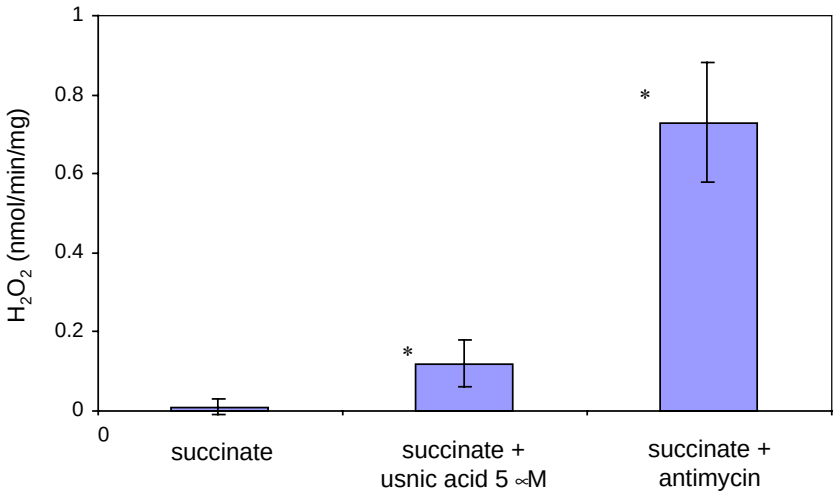

Fig. 9. Effect of usnic acid on H_2O_2 production by isolated liver mitochondria. H_2O_2 production in isolated liver mitochondria was monitored in the presence of succinate with and without usnic acid (5 μM) or antimycin (1 μg/mg protein). H_2O_2 was measured by monitoring fluorescence of *p*-hydroxyphenylacetate oxidation ($\lambda_{ex} = 320$ nm; $\lambda_{em} = 400$ nm) in the presence of horseradish peroxidase. For all experiments, isolated liver mitochondria (0.10 mg) were incubated in a buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris–HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4 at 25° with horseradish peroxidase (10 U) and *p*-hydroxyphenylacetate (1 mM). Succinate = 7.5 mM. $^*P < 0.05$ compared with control.

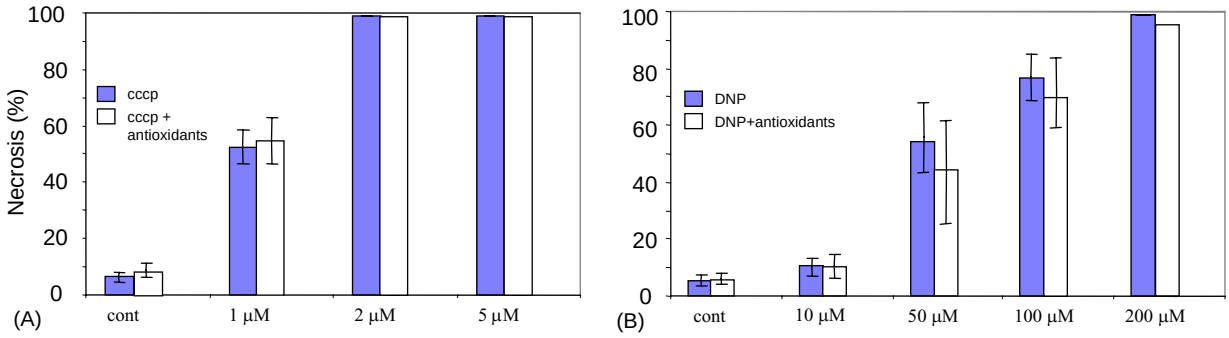

Fig. 10. Effect of various uncouplers on hepatocyte viability: (A) cccp treatment. (B) DNP treatment. Various concentrations of mitochondrial uncouplers, cccp and DNP, were added to cultured hepatocytes and viability was determined after 16 hr. In some samples, antioxidants (Vitamin E and BHT) were added to hepatocytes for 1 hr before addition of uncouplers. Cell death was assessed with fluorescent dyes as described in Section 2.

presence of succinate, little H_2O_2 is observed effluxing from isolated liver mitochondria (Fig. 9). The addition of usnic acid significantly increased H_2O_2 production in mitochondria, although not to the extent of antimycin treatment. Thus, the increased DCF fluorescence observed in hepatocytes treated with usnic acid is likely due to increased generation of H_2O_2 by mitochondria caused by usnic acid inhibition of the electron transport chain.

3.4.4. Effect of various uncouplers on hepatocyte viability

Mitochondrial inhibitors, such as rotenone, have been previously shown induce necrosis in hepatocytes [22]. Antioxidant treatment was shown to protect hepatocytes from necrosis induced by rotenone [22]. The question of whether uncouplers induce hepatocyte cell death and whether antioxidants can protect cells has not been explored. 2,4-Dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (cccp) have been characterized as mitochondria uncouplers [23,24]. The administration of cccp and DNP to hepatocytes induced necrotic cell death in a dose-dependent manner (Fig. 10A and B). However, the necrotic cell death was not inhibited by antioxidants, as seen with usnic acid. This suggests that mitochondrial uncouplers and usnic acid have different mechanisms for inducing cell necrosis in cultured hepatocytes. DCF fluorescence analysis suggests that hepatocytes treated with usnic acid experienced oxidative stress, while cccp- and DNP-treated hepatocytes actually had less oxidative stress (Table 3). This is in agreement with studies that showed uncoupling of mitochondria decreased reactive oxygen species generation in isolated mitochondria [25], unless the respiratory chain is inhibited [23] and is in agreement with a previous study that found lower DCF fluorescence in cccp-treated hepatocytes [26]. As seen with usnic acid, necrogenic doses of DNP and cccp to hepatocytes caused an uncoupling of mitochondria, inhibited mitochondrial respiration, and caused a drop in ATP levels (Table 3). The extent of ATP depletion was significantly greater in DNP- and cccp-treated heptocytes than in usnic acid-treated hepatocytes, indicating ATP depletion may be involved in necrosis caused by cccp and DNP. The fact that DNP and cccp could also inhibit mitochondrial respiration is not surprising since at high level uncouplers can disrupt the inner mitochondrial membrane.

4. Discussion

Mitochondria are considered to be the major source of reactive oxygen species in cells [27]. Components of the electron transport chain (e.g. flavoproteins, ubisemiquinone) are known to undergo autoxidation and generate reactive oxygen species in mitochondria [28,29]. Inhibition of the electron transport chain can increase the steady-state levels of these autoxidizable components and consequently increase reactive oxygen species generation by mitochondria [30,31]. Mitochondrial respiratory chain inhibitors, such as rotenone, antimycin, and myxothiazol, can all increase reactive oxygen species generation in isolated mitochondria and cells [30,32]. Consequently, mitochondria

Table 3
Effect of uncouplers on oxidative stress and energy metabolism in cultured hepatocytes

	DCF fluorescence (% of control)	RCR	Respiratory rate (% of control)	ATP (% of control)
Control (+ antioxidants)	100 (88 ± 10)	2.72 (2.57)	100 (97 ± 5)	100 (105 ± 12)
Usnic acid, 5 μM (+ antioxidants)	140 ± 11 (129 ± 12)	1.21 (1.13)	44 ± 8 (42 ± 7)	65 ± 11 (68 ± 9)
DNP, 100 μM (+ antioxidants)	79 ± 14* (68 ± 13*)	1.55 (1.61)	49 ± 7 (45 ± 9)	43 ± 8* (40 ± 9*)
cccp, 2 μM (+ antioxidants)	67 ± 13* (61 ± 14*)	1.11 (1.05)	38 ± 8 (37 ± 7)	32 ± 7* (27 ± 13*)

Hepatocytes were pretreated for 1 hr with antioxidants (50 μM Vitamin E and 20 μM BHT) before usnic acid addition. Cultured hepatocytes were incubated 37° for 4 hr after administration of uncouplers or DMSO treatment in control cells. ATP, DCF fluorescence, and RCR were measured in cells as described in Section 2. Respiratory rate represents state III respiration. Values are expressed as mean ± SD. *$P < 0.05$ compared with usnic acid-treated cells.

inhibitors can induce necrosis and apoptosis in cultured cells, an effect that in some cases is mediated by increased reactive oxygen species generation [22,33,34]. For example, rotenone treatment has been shown to induce necrosis in primary cultured hepatocytes [22]. Tocopherol succinate was shown to protect hepatocytes from rotenone-induced hepatotoxicity, indicating cell death was mediated by reactive oxygen species generation [22].

Our work suggests that the mechanism by which usnic acid induces hepatotoxicity may be similar to rotenone. Usnic acid directly inhibits mitochondria function, which causes an increase in reactive oxygen species production by the electron transport chain, and ultimately leads to cell death. Although previously reported as a mitochondria uncoupler, usnic acid appears to also inhibit the electron transport when given to cultured hepatocytes. Within 2 hr of usnic acid treatment, mitochondria of hepatocytes were effectively inhibited, with the little remaining respiration being uncoupled. A corresponding drop in cellular ATP levels was observed after usnic acid treatment, confirming a disruption in mitochondrial bioenergetics. An increase in reactive oxygen species production, as measured by DCF fluorescence, was observed in hepatocytes treated with usnic acid. The addition of usnic acid to isolated liver mitochondria caused an enhancement in H_2O_2 production, suggesting that mitochondria were a source of reactive oxygen species measured using DCF. The autoxidation of usnic acid may also generate reactive oxygen species, but due to its slow rate of oxidation and low concentrations used (low micromolar), it is not likely to contribute significantly to increase DCF fluorescence seen in usnic acid-treated cells. However, many toxins [21] are known to redox cycle and, if usnic acid can be redox cycled by various cellular systems, then autoxidation of usnic acid may also significantly contribute to the oxidative stress causing cell necrosis. The question of whether usnic acid can redox cycle in cell remains to be explored.

The central role of mitochondria in usnic acid-induced necrosis was also supported by work with DEM. Hepatocytes that had both cytoplasmic and mitochondrial GSH depleted using 0.5 mM DEM were susceptible to low doses of usnic acid, while hepatocytes that had only cytoplasmic GSH levels depleted using 0.25 mM DEM, did not exhibit increased susceptibility to low doses of usnic acid. Mitochondrial GSH is essential to detoxify H_2O_2 produced by the electron transport chain and mitochondrial GSH depletion is associated with increased reactive oxygen species generation [35]. Thus, it is likely that depletion of mitochondrial GSH levels by DEM treatment potentiates usnic acid toxicity by decreasing the capacity of mitochondria to detoxify H_2O_2 induced by usnic acid inhibition of the electron transport chain.

Usnic acid treatment to hepatocytes acted similarly to classic uncouplers in causing uncoupling of mitochondria in digitonin permabilized hepatocytes. However, surprisingly the extent of ATP depletion was significantly less for usnic acid-treated hepatocytes than for hepatocytes treated with DNP or cccp. This suggests the extent of mitochondrial uncoupling in hepatocytes caused by usnic acid in undisturbed cells may be less than in cccp- or DNP-treated cells or that some other mechanism of ATP loss is occurring in DNP- or cccp-treated cells. It also suggests that necrosis after cccp or DNP treatment may be occurring because a certain threshold of ATP loss is crossed, and merits further investigation. In addition, usnic acid differs from uncouplers, like cccp and DNP, in that the inhibition of mitochondrial respiration by usnic acid caused increased generation of reactive oxygen species while cccp and DNP decreased mitochondrial reactive oxygen species production in agreement with previous observations for uncouplers [25]. This suggests that the site of usnic acid binding to mitochondria and/or the mechanism by which usnic acid inhibits mitochondrial respiration differs from cccp and DNP.

The site and mechanism by which usnic acid affects mitochondrial respiration and oxidative phosphorylation remains to be explored. It is clear that the presence of BSA in respiration buffer strongly affected the action of usnic acid in isolated liver mitochondria, influencing whether it inhibited or uncoupled mitochondria. BSA is traditionally added in many buffers used with isolated mitochondria in part to remove fatty acids that can interfere with oxidative phosphorylation [12]. Earlier studies have found that usnic acid can bind to BSA in plasma [36]. The effect of BSA on mitochondria may be due to BSA binding of usnic acid and lowering levels of free usnic acid that can bind to mitochondria. However, BSA has also been suggested to be effective in delivering cholesterol to isolated mitochondria [37]. The possibility that BSA helps deliver and orient usnic acid in isolated mitochondria or that the BSA–usnic acid complex affects mitochondria differently than usnic acid alone cannot be ruled out.

An interesting finding of this work was the observation that addition of antioxidants was able to protect hepatocytes from usnic acid-induced necrosis without protecting mitochondria. Thus, it is likely that antioxidants prevented the consequences of increased reactive oxygen metabolites but would not be expected to affect their formation. The fact that BHT and Vitamin E are lipophilic antioxidants that can protect membranes from lipid oxidation, suggest that usnic acid-mediated necrosis may involve lipid peroxidation.

2–5 μM unbound concentration of usnic acid was acutely toxic to hepatocytes. No information on measurements in humans of blood levels of usnic acid are presently available. Based on the estimated amount of usnic acid consumed by patients and assuming pharmacokinetics in man are similar to rabbits [36,38], it has been suggested that steady-state levels of ~100 μM might be expected in 70 kg patients.[1] Since at least 99% is protein bound, free concentrations in the 1 μM range seem plausible, which is

[1] Michael Bolger, personal communication.

similar to concentrations used in our studies. Furthermore, toxicity in humans was delayed for several weeks as opposed to immediate effects in cultured cells. This difference might reflect the need to accumulate usnic acid or its effects in the liver over time to achieve the type of toxicity seen acutely in culture. Therefore, hepatotoxicity *in vivo* may similarly occur only after a threshold hepatic level of usnic acid is crossed. Of course, we can only speculate about these issues since species comparisons and chronic dosing studies in mice are not available.

Our work demonstrates that usnic acid is toxic to cultured hepatocytes, and therefore suggests that usnic acid may be mediating, at least in part, hepatotoxicity and liver failure seen in patients taking Lipokinetix. Because usnic acid is a potent hepatotoxic agent that disrupts electron transport in mitochondria and induces oxidative stress in cells, the use of usnic acid in any supplements and medicines must be questioned.

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