The Uncoupling of Oxidative Phosphorylation of Mouse-Liver Mitochondria *in vivo* by Usnic Acid

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ABSTRACT. (+) Usnic acid isolated from the local lichen species *Usnea articulata* had a median lethal dose of 180 mg/kg to mice (s.c. injection). The symptoms of the toxicity exhibited by (+) usnic acid was similar to those of classical uncouplers of oxidative phosphorylation. Mice injected with 200 mg/kg of (+) usnic acid produced significant uncoupling of isolated hepatic mitochondria. The signs of uncoupling included: release of respiratory control, inhibition of respiration (80% in the presence of ADP and 60% in its absence), hindering of ATP biosynthesis, and stimulation of Mg^{+2}-ATPase activity (two-fold increase). In this respect, (+) usnic acid, a lipophilic weak acid with two ionizable hydrogens, acts in a similar fashion to many protonophoric uncouplers which dissipate the proton motive force (delta mu H+) across the inner mitochondrial membrane, therefore disrupting the tight coupling mechanism between electron transport and ATP synthesis.

Introduction

The antibiotic activity of usnic acid (Fig. 1), a widespread lichen acid produced by many lichen species, is well documented[1- 4]. A previous study had suggested that the antibiotic activity of (+) usnic acid may be related to its capacity to disrupt energy metabolism by inhibiting selectively ATP biosynthesis[5]. This suggestion was based on the fact that (+) usnic acid exhibited characteristic uncoupling activity to oxidative phosphorylation of mouse-liver mitochondria *in vitro* similar to that produced by the classical uncoupler, 2, 4-dinitrophenol (DNP). Thus, (+) usnic acid released respiratory control and oligomycin-

inhibition of respiration, hindered ATP synthesis and stimulated ATPase activity significantly[5].
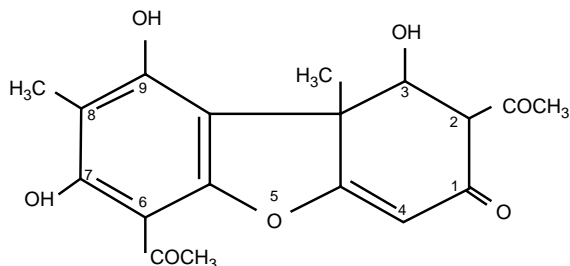

FIG. 1. Chemical structure of (+) usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-di-methyl-1,3 (2H,9bH)-dibenzofurandione).

Since the uncoupling activity of (+) usnic acid on isolated mitochondria *in vitro,* should reflect similar effects operating *in vivo,* the present study was carried out to determine its effects on some energy-linked functions of hepatic mitochondria isolated from intoxicated animals *in vivo.*

Materials and Methods

Collection of Usnea articulata

The lichen *U. articulata,* growing as long hairy festoons on juniper trees, *Juniperus procera,* in Al-Sawdah National park ("Jabal Asuda" 18º20, Nº42º 18,E) at Asir Region (South Western) of Saudi Arabia, was collected in October, 1991. Thalli of this lichen were stored in a carton box at room temperature for 5 days after its collection. The lichen sample was then spread out on newspapers and air-dried for 6 days with turning it over several times.

Preparation of Usnea articulata Extracts

The air-dried lichen sample was coarsely sheared using sharp scissor, and each 50 gm of thalli were soaked for 5 days in a dark bottle containing either one liter of 75% ethanol (Ethanol extract; EE) or one liter of chloroform (chloroform extract; CE). By filtering through several layers of clean cheesecloth, the residues were discarded and the crude EE and CE filtrates were filtered again twice through Whatman filter paper (42 ashless). The solvents of both extracts were removed by evaporation at 90ºC for EE, and 63ºC for CE, using a rotary evaporator. The semidried residues of EE and CE were kept in a warm drying oven at 37ºC to dry. The dry residues were then weighed and kept in stoppered vials at room temperature.

Extraction of (+) Usnic Acid

The chloroform extract (CE) of *U. articulata* was further purified by adding ethanol (3 vol.) to the concentrated crude extract. Long yellowish needles of (+) usnic acid were precipitated by overnight and cooling at 04ºC. The yield was purified by recrystalization in ethanol and subjected to various chemical and spectroscopic analyses for chemical identification[6].

Animals

Adult male MFI albino mice (home and out bred) aged 4-6 weeks and weighing 20-30 gm were obtained from the animal house of King Fahd Medical Research Center, Jeddah, Saudi Arabia. The mice diet consisted of water *ad libitum* and chow containing crude protein (29%), fat (3%), fiber (5.5%), ash (6.6%), calcium (0.8%), salt (0.5%), phosphorus (0.6%), vitamin A (20 IU/g), vitamin D (22.2 IU/g), vitamin E (70 IU/g), energy (2850 Kcal/kg) and trace of other elements, such as cobalt, copper, iodine, manganese, zinc (Grain Silos and Flour Mills Organization, Saudi Arabia).

Determination of LD_{50}

Pilot experiments were performed to determine the range of the most suitable median lethal dose (LD_{50}) of EE and CE of *U. articulata* and (+) usnic acid to mice. Several dilutions (20-89 mg/ml) of EE in 10% ethanol were prepared. The mice were slowly injected subcutaneously (s.c.) with 1 ml of each dilution. Control experiment was run simultaneously using 10% ethanol only. Other dilutions (1 ml each) of CE (4-8 mg/ml) in 100 % pure corn oil s.c. injected. Serial dilutions (2-7 mg/ml) of authentic sample of usnic acid in pure corn oil were prepared, and mice were injected with 1 ml of each dilution. Control mice were similarly injected with 1 ml pure corn oil each.

Counts of surviving mice were made at 24 hours after being intoxicated and LD_{50} values were calculated by statistical and graphic methods[7].

Mitochondrial Preparation and Assay

Hepatic mitochondria were isolated, as described by Katyare *et al.*[8], from mice intoxicated with a lethal dose of (+) usnic acid (65 mg in 1 ml pure corn oil) when symptoms of toxicity were evident (about 2 hrs after injection). Procedure of mitochondrial isolation and polargraphic measurements of respiration rates (state-3 and state-4; *i.e.* in the presence and absence of ADP, respectively), ADP/O ratio and respiratory control index (RCI) were carried out as already described[5]. Control hepatic mitochondrial preparation was made from mice injected with pure corn oil.

Determination of ATPase Activity

Mg^{+2}-ATPase activity of hepatic mitochondria isolated from intoxicated mice with 5 mg (+), was determined essentially as described elsewhere[5].

Determination of Mitochondrial Protein

Mitochondrial protein was determined colorimetrically by the biuret method according to Gornall *et al.*[9].

Results

The most evident symptoms of intoxicated mice, which took place 2-5 hrs, after treatment with either chloroform extract of *Usnea articulata* lichen (CE) or (+) usnic acid, were long chalasia accompanied by lucidness ponopalmosis. These symptoms started 5 min after mice were injected with lethal doses of usnic acid. Immediately before the onset of death, clear restlessness was observed followed by palms, spastic paralysis with obvious limbs thrill and acrocyanoisis. Death was accompanied with obvious *rigor mortis* symptoms.

Ethanolic extract of *U. articulata* (EE) at a high dose level of 3200 mg/kg produced no mortality or any sign of intoxication on mice for several days. On the other hand, chloroform extract (CE) was highly toxic and a linear dose-response relationship between CE concentration and % mortality was established (Fig. 2). The calculated LD_{50} was 240 mg/kg.
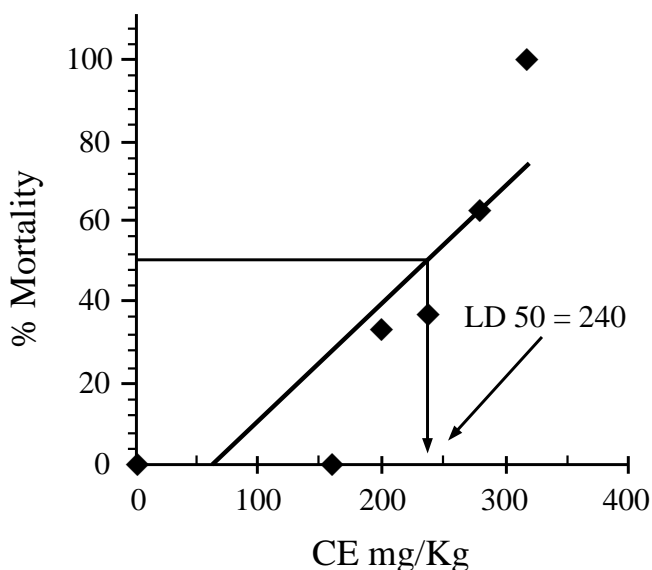

Fɪɢ. 2. Toxicity of the chloroform extract (CE) of the lichen *Usnea articulata* on mice.

Table 1 shows the range of toxicity of (+) usnic acid (80-280 mg/kg) to mice. A dose-response relationship is clearly demonstrated. The mortality was 36.7% when mice were injected with 120 mg/kg, while 100% mortality occurred when the dose was raised to 280 mg/kg. The calculated LD_{50} was 180 mg/kg.

TABLE 1. Toxicity of (+) usnic acid to mice.

(+) usnic acid[*] (mg/kg)	n[**]	% Mortality[***]
0.0	24	0.0
80	23	0.0
120	30	36.7
160	25	40.0
200	23	52.2
240	23	60.9
280	24	100.0

[*](+) Usnic acid was dissolved in corn-oil and 1 ml/mouse was injected (s.c.) to give final concentration as indicated; corn oil alone (0.0)/mouse was injected (s.c.) (1ml/mouse) as control.
[**]n = number of mice.
[***]24 h after administration.

Oxygen uptake and ADP/O ratio of isolated liver-mitochondria from mice intoxicated with (+) usnic acid, when using succinate as a respiratory substrate, is presented in Table 2. Mitochondria isolated from liver of untreated mice showed normal rates of oxygen uptake as well as normal values of both ADP/O and RCI. However, total loss of mitochondrial coupling activity resulted from treated animals with 200 mg/kg of (+) usnic acid.

TABLE 2. Oxygen uptake and oxidative phosphorylation of isolated liver-mitochondria from mice treated with (+) usnic acid.

Condition[*]	nmoles O_2 mg protein^{-1} min^{-1}		Mean ± SD	
	State 4[**]	State 3[**]	RCI[**]	ADP/O[***]
Control	70.5	144.2	2.1 (± 0.2)	2.3 (± 0.1)
Treated	31.0	32.0	1.0	n.m.
% Reduction	56	78		

[*]Control mice s.c. were injected with 1 ml pure corn-oil each; treated mice s.c. were injected with 1 ml pure corn oil containing 5 mg (+) usnic acid each .
[**]Mean ± Standard deviation (SD) of three independent experiments.
[***]$P < 0.05$ level and each value represents three independent experiments.
n. m. = not measurable .

In control experiment, the respiration rates of states 4 and 3 were 70.5 and 144.2 nmoles O_2 mg protein^{-1} min^{-1}, respectively. These rates however, were significantly reduced by 56% and 78% after mice were treated with a lethal dose of (+) usnic acid. The ADP/O ratio was 2.3 in control mitochondria, which is near the theoretical value for a FAD-linked substrate. This ratio however, was lost and not practically measurable in treated animals.

Mg^{+2}-ATPase activity of isolated liver-mitochondria from mice treated with (+) usnic acid is shown in Table 3. Significant stimulation of Mg^{+2}-ATPase activity (P < 005) was observed (about 2-fold increase) as a result of treating mice with a lethal dose of (+) usnic acid.

TABLE 3. Mg+2-ATPase activity of isolated liver-mitochondria from mice treated with (+) usnic acid.

Condition*	μg *pi* mg protein^{-1} min^{-1}	Significance	% Mg^{+2}-ATPase
	Mean (±SD)	(p)	activity
Control	5.2 (± 1.8)		100.0
Treated	9.7 (±3.6)	< 0.05**	186.5

*Conditions as described in the footnote of Table 2.
**Represents four independent experiments.

Discussion

In order to determine the toxicity of *U. articulata,* a water-soluble extract (ethanolic extract; EE) and a lipid-soluble extract (chloroform extract; CE) were injected (s.c) into mice. No mortality was observed for the EE at a dose of 3,200 mg/kg. The EE contains mostly the water-soluble fraction of lichen's thallus such as sugars, proteins, salt, etc., and these compounds should have no or very little toxicity to experimental animals. However, the CE was toxic to mice (LD_{50} = 240 mg/kg). This toxicity is undoubtedly due to the presence of (+) usnic acid. This fact is further confirmed by using pure (+) usnic acid crystals separated from this extract: The LD_{50} of the pure sample was 180 mg/kg. According to the classification of chemicals[10,11], the above values are considered to be of moderate toxicity.

The clinical signs accompanied the administration of either a lethal dose of CE or of a pure sample of (+) usnic acid, was long chalasia and lucidness ponopalmosis.

These symptoms commenced within 5 min after mice injection, and clear restlessness followed by palmus, spastic paralysis with obvious limbs thrill and acrocyanosis was observed immediately before the time of death.

(+) Usnic acid toxicity to mice is in fact similar to that produced by 2,4-dinitophenol (DNP) which is a highly toxic substance (LD_{50} of orally administration into rats is 30 mg/kg). A common feature of lethal poisoning of both compounds is that they are slow in their action and produce almost immediate onset of *rigor mortis*[12].

The uncoupling activity of (+) usnic acid on isolated hepatic mitochondria reported in previous study[5] should be interpreted as reflective of similar effects operating *in vivo*. Thus, in the present study, treating mice with a lethal dose (200 mg/kg) of (+) usnic acid showed an array of adverse effects on mitochondrial energetics similar to those obtained from the in *vitro studies*[5].

(+) Usnic acid administration to mice released respiratory control completely, inhibited both state 3 and 4 respiration rates by 68% and 56% respectively, abolish ATP synthesis in hepatic mitochondria, and stimulated Mg+2-ATPase activity by almost two-fold. These results are typical of many uncoupling agents [13-17].

It is, therefore, concluded that the toxic effects produced by (+) usnic acid on mice is likely related to its ability to interfere with mitochondrial energetics, in particular with the mechanism of ATP production. Loss of ATP from the cell may lead to an increase of cystosolic Ca^{+2} and simultaneous stoppage of Na^+/K^+-ATPase and other transporting systems[18]. These changes are usually sufficient to cause adverse biochemical and pathological damages to most animal cells, which may lead to cell death[19,20]. Concerning the mechanism of mitochondrial uncoupling by usnic acid, it is considered that usnic acid is among the so-called protonophoric uncouplers (*i.e.* proton-transporting ionophores) which constitute the most important class of potent uncouplers[21,23].

Usnic acid, a highly lipophilic substance (Fig. 1), behaves as a dibasic weak acid, *i.e.* having two ionizable hydrogen atoms[24]. However, usnic acid possesses also a third acidic hydrogen (the phenolic OH at C7), which is thought to be bound to intermolecular hydrogen bonding with the acetyl group attached to C6[24].

The concept that uncoupling is a function of lipophilicity and pKa between 3 and 9 of the uncoupler, is held by many investigators[22,25,26]. Thus, it is assumed that according to the chemiosmotic hypothesis[27] usnic acid, being a lipophilic weak acid, readily passes through the mitochondrial membranes in its neutral protonated state. In the presence of a pH gradient, it binds protons on the acidic side of the membrane, diffuses through, and releasing them on the alkaline side, thereby dissipating the proton gradient across the inner mitochondrial membrane. The fall of the proton motive force (*delta mu* H+) had been thought to disrupt the tight coupling between electron transport and ATP synthesis, thus causing the uncoupling effects[28,29].

From the present study and that of others[30,31], the (+) usnic acid is proved to behave similar to hundreds of microbial products that act as uncouplers of oxidative phosphorylation[32-35]. It also has inhibitory effect against various microorganisms such as gram positive bacteria[4,36,37]. These findings may substantiate the postulation that the antimicrobial activity of (+) usnic acid is linked to the uncoupling of microbial cell oxidative phosphorylation. Bacteria have no mitochondria, yet they possess mesosomes containing the electron transport system[28] and a mechanism of ATP synthesis similar to that of mitochondria[27]. It is possible, therefore, that the results of this study may pave the way for new antimicrobial agents; based on disruption of energy metabolism.

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منع تزامن الفسفرة التأكسدية في ميتوكوندريا كبد الفأر الحي بواسطة حمض الأوزينك

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المستخلص. حمض الأوزنيك (+) المعزول من الأشنة المحلية أوزنيا أرتكيولاتا له متوسط جرعة قاتلة (LD_{50}) للفئران تقدر بنحو ١٨٠ ملجم/ كجم حقنًا تحت الجلد. وقد أظهرت أعراض التسمم على الفئران تشابهًا كبيراً مع موانع الاقتران التقليدية التي تمنع الاقتران بين عمليتي الفسفرة والأكسدة (الفسفرة التأكسدية) في عضيات الميتوكوندريا. وقد أوضحت النتائج التجريبية « في الكائن الحي » أن الميتوكوندريا المعزولة من أكباد الفئران المعاملة بحمض الأوزنيك (٢٠٠ملجم/ كجم بالحقن تحت الجلد) قد اعترتها تغيرات ملحوظة في خصائصها الطاقية. مثلا، انعدم التحكم التنفسي الذي يسببه إضافة أدينوسين ثنائي الفوسفات (ADP) كما انخفضت معدلات تنفس الميتوكوندريا بنحو ٨٠٪ (في وجود ADP– أي في المرحلة ٣ من مراحل تنفس الميتوكوندريا) وبنحو ٦٠٪ (في غيابه – إي في المرحلة ٤). كما توقف إنتاج الطاقة كلية في العضيات، إضافة إلى زيادة ملحوظة (نحو ١٠٠٪) في نشاطية إنزيم أتبيز الماغنسيوم.

وتدل هذه النتائج على أن حمض الأوزنيك، ثنائي التأين والمحب للذوبان في الدهون، يتشابه في عمله مع طائفة كبيرة من موانع الفسفرة التأكسدية المعروفة باسم « حوامل البروتونات » التي من خصائصها منع تكوين « القوة الدافعة البروتونية » (PMF) عبر الغشاء الداخلي للميتوكوندريا، وهي المضخة اللازمة لإنتاج الطاقة.