Chemico-Biological Interactions 203 (2013) 502–511

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Harmful effects of usnic acid on hepatic metabolism

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ARTICLE INFO

Article history:
Received 1 October 2012
Received in revised form 21 December 2012
Accepted 4 February 2013
Available online 16 February 2013

Keywords:
Hepatic metabolism
Liver perfusion
Uncoupling
Toxicology

ABSTRACT

Usnic acid is a naturally occurring dibenzofuran derivative found in several lichen species. The compound has been marketed as an ingredient of food supplements for weight reduction. There is evidence that the compound acts as an uncoupler of mitochondrial oxidative phosphorylation and it is also clear that consumption of the drug can lead to severe hepatotoxicity depending on the doses. Based on these and other ideas the objective of the present work was to investigate the possible effects of usnic acid on liver metabolism. Livers of male Wistar rats were perfused in a non-recirculating system. Usnic acid stimulated oxygen consumption at low concentrations, diminished the cellular ATP levels, increased the cytosolic but diminished the mitochondrial NADH/NAD^+ ratio, strongly inhibited gluconeogenesis from three different substrates (IC_{50} between 1.33 and 3.61 μM), stimulated glycolysis, fructolysis, glycogenolysis and ammoniagenesis and inhibited ureogenesis. The $^{14}CO_2$ production from [1-^{14}C]octanoate and [1-^{14}C]oleate was increased by usnic acid, but ketogenesis from octanoate was diminished and that from oleate was not affected. It may be concluded that the effects of usnic acid up to 2.5 μM reflect predominantly its activity as an uncoupler. At higher concentrations, however, several other effects may become significant, including inhibition of mitochondrial electron flow and inhibition of medium-chain fatty acid oxidation. In metabolic terms, toxicity of usnic acid can be predicted to be especially dangerous in the fasted state due to the combination of several deleterius events such as diminished hepatic glucose and ketone bodies output to the brain and increased ammonia production.

1. Introduction

Uncouplers of mitochondrial oxidative phosphorylation have always been investigated as possible ingredients for weight-loss supplements because they are associated with increased metabolic rates and stimulated fuel oxidation. The uncoupler 2,4-dinitrophenol was indeed used for this purpose for some time [1]. Its use was discontinued, however, due to a number of toxic effects [1,2]. Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3[2H,9bH]-dibenzofurandione) is another mitochondrial uncoupler that has been investigated and marketed as an ingredient in dietary supplements for weight reduction, but it has also been associated with serious side-effects, mainly hepatotoxicity and acute liver failure [3,4]. Usnic acid is a dibenzofuran derivative that occurs as a secondary metabolite in several lichen species. Its chemical structure is shown in Fig. 1. It has been claimed that in addition to its weight reducing effects usnic acid has also antibiotic, antiviral, antiprotozoal, antiproliferative, anti-inflammatory and analgesic activities. All these activities are said to be linked to the uncoupling properties of the compound [5,6]. It can exist naturally as the (+) and (−) enantiomer, but most of its biological activity is attributed to the (+) enantiomer. The latter has been included as an ingredient of cosmetics, which are used as both active principle and preservative [5,7].

The uncoupling effect of usnic acid was first demonstrated in rat liver homogenates and mitochondria [8,9] and subsequently confirmed in isolated mouse liver mitochondria [10]. The uncoupling action of usnic acid in mouse liver mitochondria presents the same features as those of 2,4-dinitrophenol: impairment of respiratory control, stimulation of the oligomycin-sensitive respiration, inhibition of ATP synthesis and stimulation of the Mg^{+2}-ATPase [10]. Similarly to 2,4-dinitrophenol, usnic acid is considered to be a protonophoric uncoupler. However, usnic acid is 50 times more potent than 2,4-dinitrophenol, with maximal uncoupling at 1 μM, whereas 50 μM 2,4-dinitrophenol is required to reach a comparable degree of uncoupling.

It has been suggested that the hepatotoxicity of usnic acid is related primarily to its uncoupling action [4,10–12]. It has also been suggested that the compound might cause inhibition of the mitochondrial electron transport chain [4,11,13]. The viability of cultured hepatocytes, which is an indicative of hepatotoxicity, was strongly reduced when the oxidative phosphorylation was inhibited by usnic acid as indicated by the reduction in the ATP levels [13]. In cultured mouse hepatocytes the inhibition of oxidative

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http://dx.doi.org/10.1016/j.cbi.2013.02.001

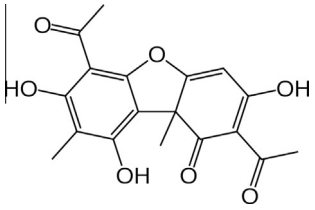

Fig. 1. Structural formula of usnic acid.

phosphorylation was proportional to necrosis [4]. Extensive necrosis was also found in the liver of mice that were treated with usnic acid [14,15]. In addition, hepatocytes and isolated mitochondria from livers of both mice and rats exposed to usnic acid showed increased levels of reactive oxygen species, diminution of reduced glutathione (GSH) in addition to necrotic and apoptotic changes, suggesting a role for oxidative stress in the hepatotoxicity [4,12,15]. In humans, hepatotoxicity of usnic acid has been the subject of several reports including cases of acute liver failure and necessity of liver transplantation [3,16,17].

The most obvious consequence of the uncoupling action of usnic acid is ATP depletion, as indeed demonstrated in two studies using rat hepatocytes [12,13]. However, this phenomenon is probably linked directly or indirectly to a variety of modifications in metabolic fluxes [18–21]. Although the toxic manifestations at the cellular level have been more extensively investigated [12,13] little information is available about the action of usnic acid on specific metabolic pathways. An attempt of approaching this question has been made using [^{13}C]glucose tracer and cultured hepatocytes [13]. Roughly speaking the data that were obtained in this study indicate what one can normally expect from an uncoupler: stimulation of catabolic pathways and inhibition of anabolic ones. A precise quantification of specific metabolic pathways under the influence of usnic acid, however, is still not available. A meaningful evaluation of specific metabolic pathways is best made in the isolated perfused rat liver, a system in which true metabolic steady states can be established under conditions that are much closer to the physiological situation than isolated and cultured cells [22]. The present study, thus, takes advantage of the perfused liver for quantifying the metabolic effects of usnic acid on both catabolic and anabolic pathways under various conditions. The results should improve understanding of the metabolic effects of usnic acid and also allow to compare the action of this compound with that of other drugs. The results should also provide an answer to the question of how adequate it is to use usnic acid for weight-losing purposes.

2. Materials and methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the enzymatic assays were purchased from Sigma–Aldrich (St. Louis, MO, USA). [1-^{14}C]Octanoic acid (O-7012, specific activity 53 mCi/mmol) was purchased from Sigma–Aldrich (St. Louis, MO, USA). [1-^{14}C]Oleic acid (NEC317050UC, specific activity 54.4 mCi/mmol) was purchased from Perkin–Elmer (Boston, MA, USA). All standard chemicals were of the best available grade.

2.2. Animals

Male albino rats (Wistar), weighing 180–220 g, were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). In several experimental protocols, the rats were starved for 18 h before the surgical removal of the liver. All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

2.3. Liver perfusion

For the surgical procedure, the rats were anesthetized by intraperitoneal injection of thiopental (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. Hemoglobin-free, non-recirculating perfusion was performed [22]. After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 °C. The flow, provided by a peristaltic pump, was between 30 and 33 mL/min. Usnic acid was infused into the perfusion fluid as a dimethylformamide solution by means of a precision pump. The rate of infusion was the same in all experiments, namely 10 μL/min. The concentration of usnic acid in the dimethylformamide solution was adjusted in such a way as to ensure the final perfusate concentrations of 1, 2.5, 5, and 10 μM. With an infusion rate of 10 μL/min the ratio (dimethyl-formamide)/(Krebs/Henseleit-bicarbonate buffer) was very low, between 3.03×10^{-4} and 3.3×10^{-4}. Control experiments have shown that this proportion of dimethylformamide does not affect the metabolic rates measured in the present work nor does it affect liver viability as can be judged from its oxygen uptake rates and perfusion fluid leakage. Substrates (lactate, fructose, alanine, [1-^{14}C]octanoate and [1-^{14}C]oleate) were dissolved directly into the Krebs/Hense-leit-bicarbonate buffer at the desired concentration.

2.4. Analytics

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate, urea, ammonia and L-glutamate [23]. The oxygen concentration in the outflowing perfusate was monitored continuously, employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [24]. Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver.

In those experiments in which [1-^{14}C]octanoate or [1-^{14}C]oleate were infused for measuring $^{14}CO_2$ production the outflowing perfusate was collected in Erlenmeyer flasks in 2-min fractions. The Erlenmeyer flasks were rapidly and tightly closed with rubber stoppers to which scintillation vials containing phenylethylamine were fastened by means of stainless steel wires. Trapping of the $^{14}CO_2$ in phenylethylamine was accomplished by acidification of the perfusate with a HCl solution which was injected into the flasks through the rubber stoppers. Radioactivity was measured by liquid scintillation spectroscopy. The scintillation solution was: toluene/ethanol (2/1) containing 5 g/l 2,5-diphenyloxazole and 0.15 g/l 2,2-p-phenylene-bis(5-phenyloxazole). The rate of $^{14}CO_2$ production was calculated from the specific activity of each labeled fatty acid and from the rate of radioactivity infusion.

The hepatic contents of adenine nucleotides were measured after freeze-clamping the perfused livers with liquid nitrogen [25]. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K_2CO_3 and AMP, ADP, and ATP were assayed by means of standard enzymatic procedures [23].