Harmful effects of usnic acid on hepatic metabolism

CrossMark

Caroline T. Moreira, Andrea L. Oliveira, Jurandir F. Comar, Rosane M. Peralta, A. Bracht *

Department of Biochemistry, University of Maringá, 87020900 Maringá, Brazil

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.

© 2013 Elsevier Ireland Ltd. Open access under the Elsevier OA license.

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
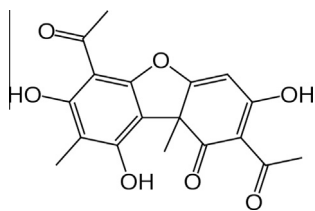
Fig. 1. Structural formula of usnic acid.

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].

2.5. Treatment of data

The metabolic rates were expressed as μmol per minute per gram liver wet weight ($\mu mol\ min^{-1}\ g^{-1}$). Statistical analysis of the data was done by means of the Statistica™ program (Statsoft®, 1998). The results are mentioned in the tables as the *p* values.

3. Results

3.1. Effects of usnic acid on glycogen catabolism and glycolysis

As a first approach experiments were planned in order to test possible effects of usnic acid on glycogen catabolism and glycolysis. Livers from fed rats when perfused with substrate-free medium survive at the expense of glycogen degradation via glycolysis and oxidation of endogenous fatty acids [22]. Under these conditions the livers release glucose, lactate and pyruvate as a result of glycogen catabolism. The results of experiments in which the actions of four usnic acid concentrations (1, 2.5, 5, and 10 μM) were investigated are summarized in Fig. 2. Panel A illustrates the time course of the action of 10 μM usnic acid. It also illustrates the experimental protocol that was used for all other usnic acid concentrations. After a pre-perfusion period of 10 min, usnic acid was infused during 30 min, followed by additional 20 min of drug-free perfusion. Four parameters were measured: glucose release, lactate and pyruvate productions and oxygen consumption. Panel A of Fig. 2 shows that all parameters were relatively stable before usnic acid

infusion. The introduction of the drug changed all parameters after a short period of approximately 4 min. Glucose, lactate and pyruvate productions increased progressively during the first 20 min and remained elevated up to the point at which the usnic acid infusion was discontinued. Oxygen uptake also increased, reached a maximum at 20–24 min and decreased progressively. At the end of the usnic acid infusion period (40 min perfusion time), however, the rate of oxygen uptake was still above the rate observed before the onset of the infusion. Cessation of the infusion of usnic acid at 40 min perfusion time caused the reversal of all stimulations except that of oxygen uptake. In panel B of Fig. 2 the stimulations caused by usnic acid were represented against the concentrations of the drug. For this analysis the data were recalculated in terms of glycolysis (lactate + pyruvate production) and glycogenolysis (glucose release + ½[lactate + pyruvate production]). These calculations provide good approximations for the metabolic pathways due to the low flux through the pyruvate dehydrogenase under the specific perfusion conditions. It should be remarked that Fig. 2B shows the increments in glycolysis and glycogenolysis, i.e., the differences between the rates found in the presence of usnic acid and the rates found before the onset of usnic acid infusion. The points of maximal increment by usnic acid were taken for the calculations. For all parameters except oxygen uptake the maximal increment was always found at 30 min usnic acid infusion (40 min perfusion time). In the case of oxygen uptake the maximal increment was found at this time only for concentrations up to 2.5 μM; for the concentrations of 5 and 10 μM the maximal increment occurred earlier in time (see Fig. 2A). Panel B of Fig. 2 also
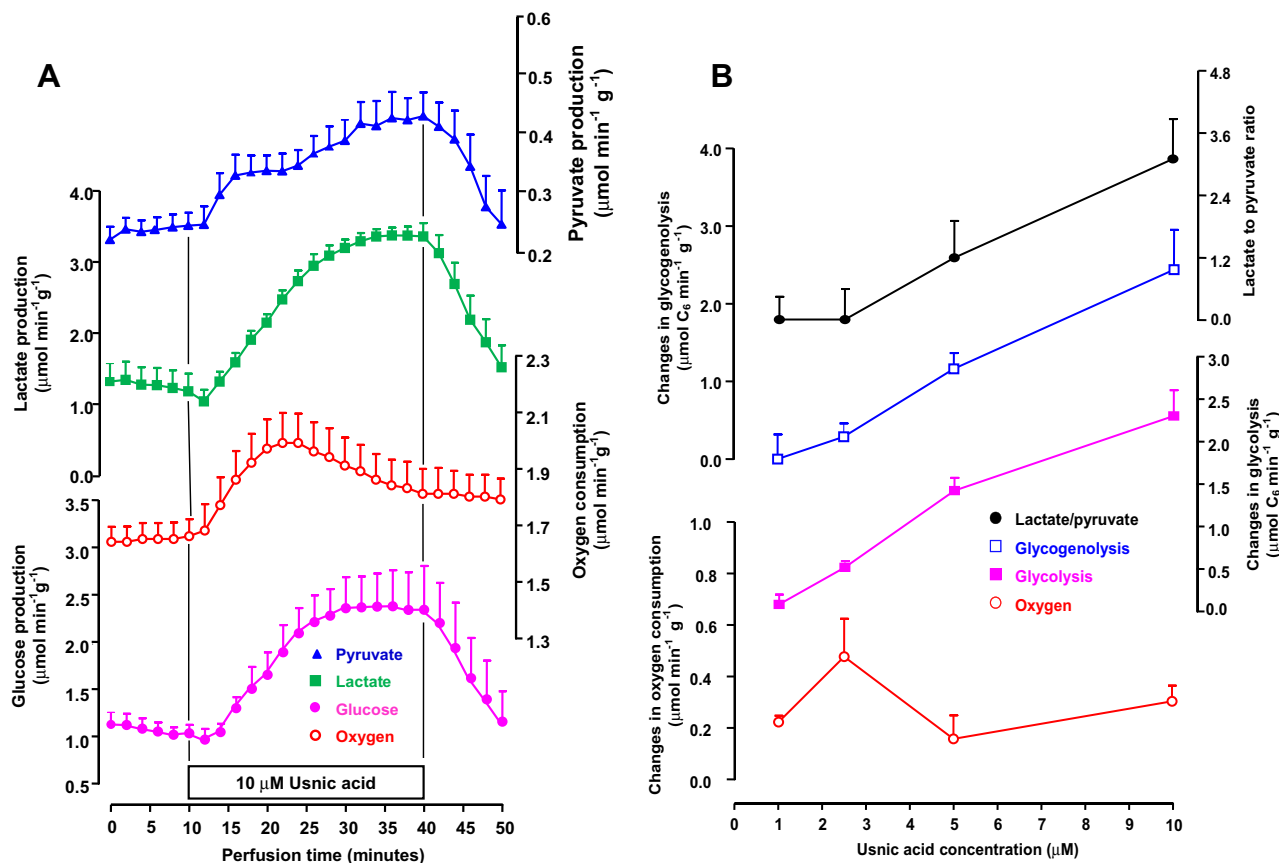

Fig. 2. Effects of usnic acid on glycogen catabolism and endogenous fatty acids-driven oxygen uptake in the perfused rat liver. Livers from fed rats were perfused with substrate-free perfusion fluid as described in the Section 2. Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: time course of the effects of 10 μM usnic acid; panel B: concentration dependence of the changes (increases) promoted by usnic acid after 30 min infusion (40 min perfusion time). Glycogenolysis means glucose production + ½(lactate + pyruvate production); glycolysis means lactate + pyruvate production. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.

shows the lactate to pyruvate ratio as a function of the usnic acid concentration. This parameter is an indicator for the cytosolic $NADH/NAD^+$ ratio [22]. Excepting oxygen uptake, all calculated parameters increased almost linearly with the usnic acid concentration, i.e., no saturation was apparent for usnic acid up to 10 μM. For oxygen uptake a clear maximum at 2.5 μM usnic acid was found, with less pronounced stimulations at the concentrations of 5 and 10 μM.

3.2. Effects of usnic acid on lactate gluconeogenesis and associated variables

Fig. 3 shows results of experiments in which the action of usnic acid on lactate gluconeogenesis and associated parameters was measured. Livers from 18 h fasted rats were used in order to minimize interference by glycogen catabolism. Fig. 3A illustrates the response of the perfused liver to usnic acid infusion at the concentration of 10 μM and also illustrates the experimental protocol. After a pre-perfusion period of 10 min in the absence of substrate, 2 mM lactate was infused during 30 min, followed by additional 30 min of usnic acid plus lactate infusion. In the absence of usnic acid the infusion of 2 mM lactate produced rapid and sustained increases in glucose production, oxygen uptake and pyruvate production. The infusion of 10 μM usnic acid caused a progressive and, at the end, almost complete inhibition of glucose production. Only a slight recovery occurred during the 10 min following cessation of the drug infusion. Oxygen consumption was stimulated initially by usnic acid. This stimulation was not stable, however, as it declined progressively and was minimal at the end of the infusion. Pyruvate production was also stimulated by usnic acid, but stimulation was not stable. Analysis of the concentration dependence is allowed by Fig. 3B. Here again the variables represented against the usnic acid concentration correspond to the maximal changes caused by the drug. If the changes were stable (glucose production

or oxygen uptake for concentrations up to 2.5 μM) they correspond to the values found at 70 min perfusion time (30 min usnic acid infusion). If the changes were not stable (e.g., oxygen uptake for usnic acid concentrations above 2.5 μM), the peak increments were represented. Inhibition is evidenced by the negative values on the scale. Inhibition of glucose production was already evident at 1 μM usnic acid and practically maximal at concentrations above 2.5 μM. Numerical interpolation revealed that 50% of maximal inhibition can be expected at an usnic acid concentration of 1.33 μM. Pyruvate production was slightly increased by usnic acid without a defined concentration dependence. Oxygen uptake was maximally stimulated at 2.5 μM usnic acid.

3.3. Effects of usnic acid on fructose metabolism

Transformation of fructose in the liver allows the simultaneous monitoring of anabolic and catabolic reactions, as this substrate can be transformed into glucose via an energy-requiring pathway and into lactate or pyruvate via an energy-yielding route. The results of the experiments that were done in the present study are summarized in Fig. 4. Here again livers from 18 h fasted rats were used in order to minimize interference by glycogen catabolism. As expected, the infusion of fructose produced rapid and quite pronounced increments in glucose, lactate and pyruvate productions and oxygen uptake (Fig. 4A). The subsequent introduction of 10 μM usnic acid caused a marked decrease in glucose production, transient stimulations in lactate and pyruvate productions and a relatively small transient increase in oxygen uptake. At the usnic acid concentrations of 1 and 2.5 μM, the increments in lactate and pyruvate productions and oxygen uptake were stable (not shown). The concentration dependences of the effects are shown in Fig. 4B. The inhibition of glucose production is a clear function of the usnic acid concentration. Numerical interpolation predicts 50% inhibition at an usnic acid concentration of 1.72 μM. Lactate
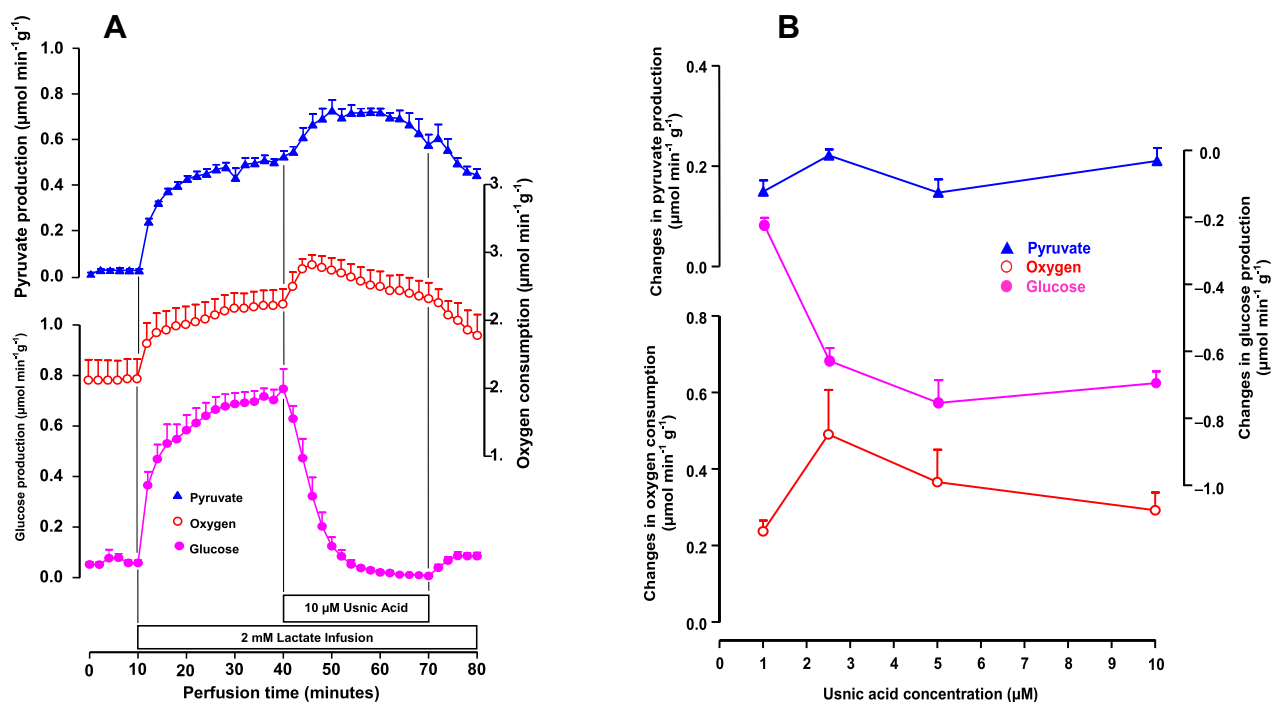

Fig. 3. Effects of usnic acid on lactate gluconeogenesis and associated variables in the perfused rat liver. Livers from fasted rats were perfused as described in the Section 2. Lactate was infused during 70 min (10–80 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: time course of the effects of 10 μM usnic acid, which was infused during 30 min (40–70 min perfusion time) and panel B: concentration dependence of the changes promoted by usnic acid after 30 min infusion (70 min perfusion time). Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.
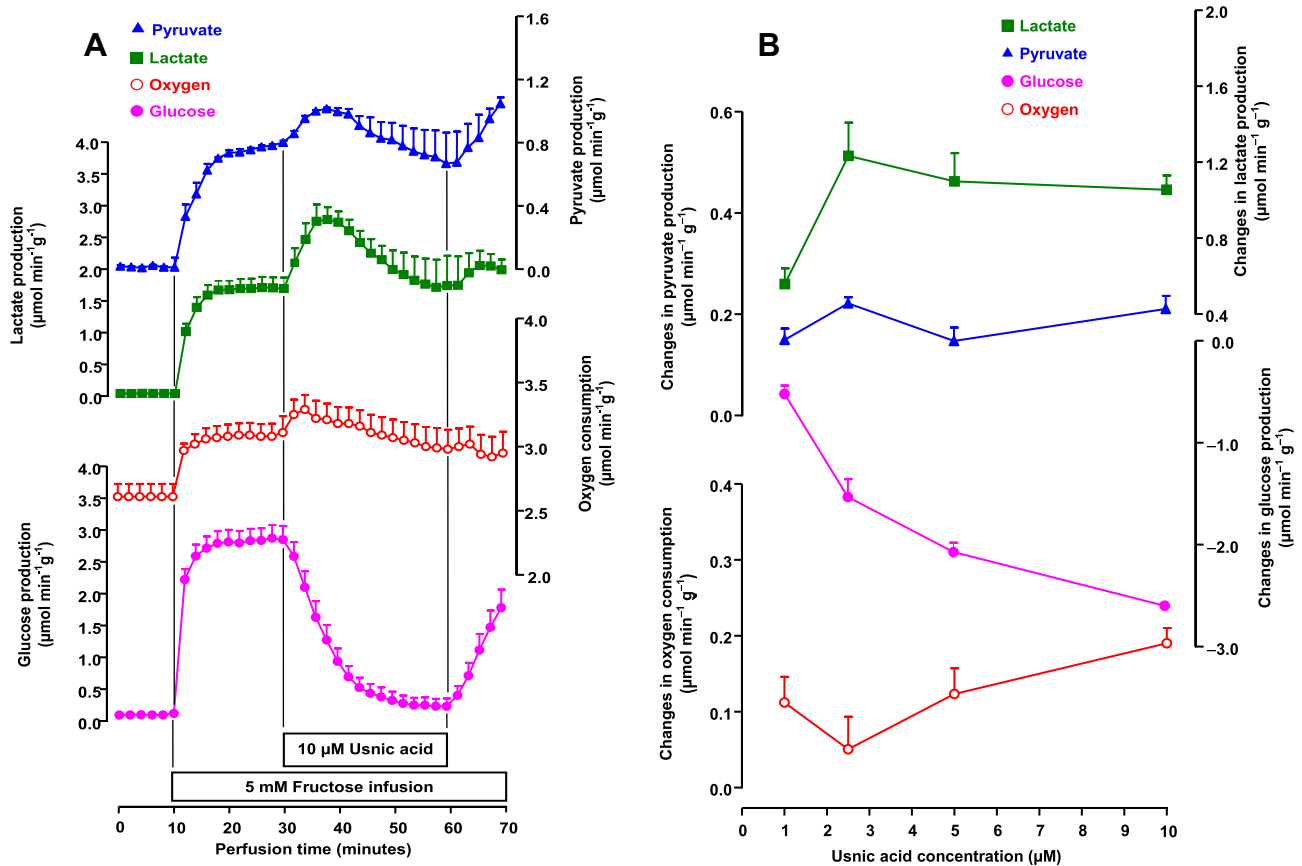
Fig. 4. Effects of usnic acid on fructose metabolism in the perfused rat liver. Livers from fasted rats were perfused as described in the Section 2. Fructose was infused during 60 min (10–70 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: time course of the effects of 10 μM usnic acid, which was infused during 30 min (30–60 min perfusion time) and panel B: concentration dependence of the changes promoted by usnic acid after 30 min infusion (60 min perfusion time). Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.

production ceased to increase at usnic acid concentrations higher than 2.5 μM. Pyruvate production and oxygen uptake stimulations showed poorly defined concentration dependences, but the overall effect was stimulation, even though it might have been transient in nature.

3.4. Effects of usnic acid on alanine metabolism

When used as a substrate alanine allows to measure both carbon and nitrogen fluxes in the liver. Experiments were thus planned with alanine in order to see how usnic acid affects variables linked to carbon and nitrogen metabolism. Livers from 18 h fasted rats were used. The time courses of the experiments that were done with 10 μM usnic acid can be seen in Fig. 5: panel A shows carbon fluxes e panel B the simultaneous nitrogen fluxes. The introduction of alanine produced stable increments in oxygen uptake, glucose, lactate and pyruvate productions (panel A) and urea, ammonia and ʟ-glutamate productions (panel B). Glucose, urea and ʟ-glutamate productions were clearly inhibited by 10 μM usnic acid. Ammonia production, on the other hand, was clearly increased, the same what occurred with pyruvate production. Oxygen uptake and lactate production experienced initial increases which were subsequently reversed or even turned into inhibition. Similar results were obtained with lower usnic acid concentrations, except that lactate production and oxygen uptake experienced stable rather than transitory increments at the concentrations of 1 and 2.5 μM. Fig. 6 shows the concentration dependences of the changes caused by usnic acid. Clearly defined

concentration dependences were found for pyruvate production (stimulation, no saturation), glucose production (50% inhibition at 3.61 μM), ammonia production (stimulation; 50% of maximal effect at 1.92 μM) and urea production (50% inhibition at 2.69 μM). The inhibition of ʟ-glutamate production was similar for all concentrations; the same can be said about the stimulation of lactate production. Oxygen uptake was stimulated by usnic acid concentrations up to 2.5 μM; at higher concentrations stimulation was practically absent.

3.5. Effects of usnic acid on fatty acid metabolism

Oxygen uptake in substrate-free perfused livers is driven essentially by the oxidation of endogenous fatty acids [26]. Since usnic acid stimulates oxygen uptake under these conditions, it is of interest to know whether it also stimulates oxidation of exogenous fatty acids. In the present work this question was investigated using the ^{14}C-labeled forms of a medium- and a long-chain fatty acid, namely [1-^{14}C]octanoate and [1-^{14}C]oleate. Fig. 7 shows the time courses of the experiments that were done with 0.2 mM [1-^{14}C]octanoate and 10 μM usnic acid. Four parameters were measured: oxygen uptake, ^{14}C-production, β-hydroxybutyrate production and acetoacetate production. Since livers from fasted rats were used, there was a significant basal production of acetoacetate and β-hydroxybutyrate, a fact that corroborates the notion that substrate-free perfused livers are oxidizing endogenous fatty acids [26]. The introduction of [1-^{14}C]octanoate caused an immediate release of ^{14}CO_2, stimulations of oxygen uptake and β-hydroxybutyrate pro-
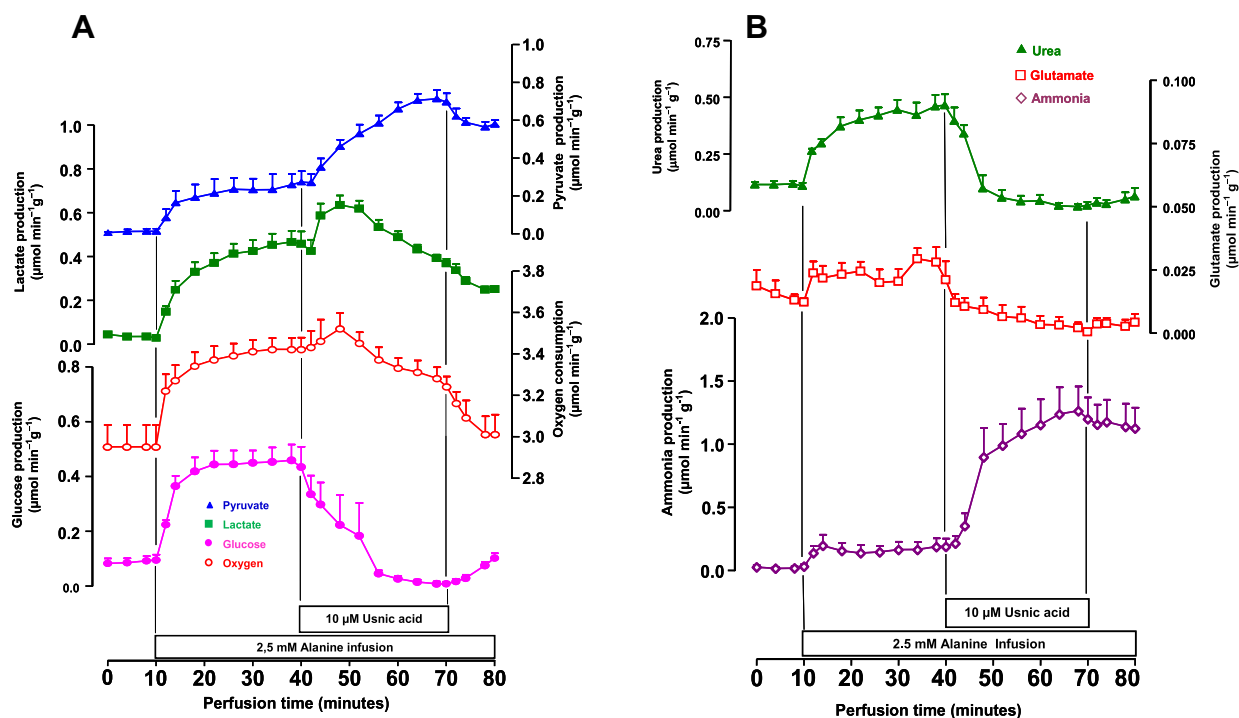
Fig. 5. Time courses of the effects of 10 μM usnic acid on alanine metabolism. Livers from fasted rats were perfused as described in Section 2. Alanine was infused during 70 min (10–80 min perfusion time). Usnic acid was infused during 30 min (40–70 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic metabolite assay. Oxygen consumption was monitored polarographically. Panel A: parameters linked to the flow of carbon derived from alanine and oxygen uptake and panel B: parameters linked to the flow of nitrogen derived from alanine. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.
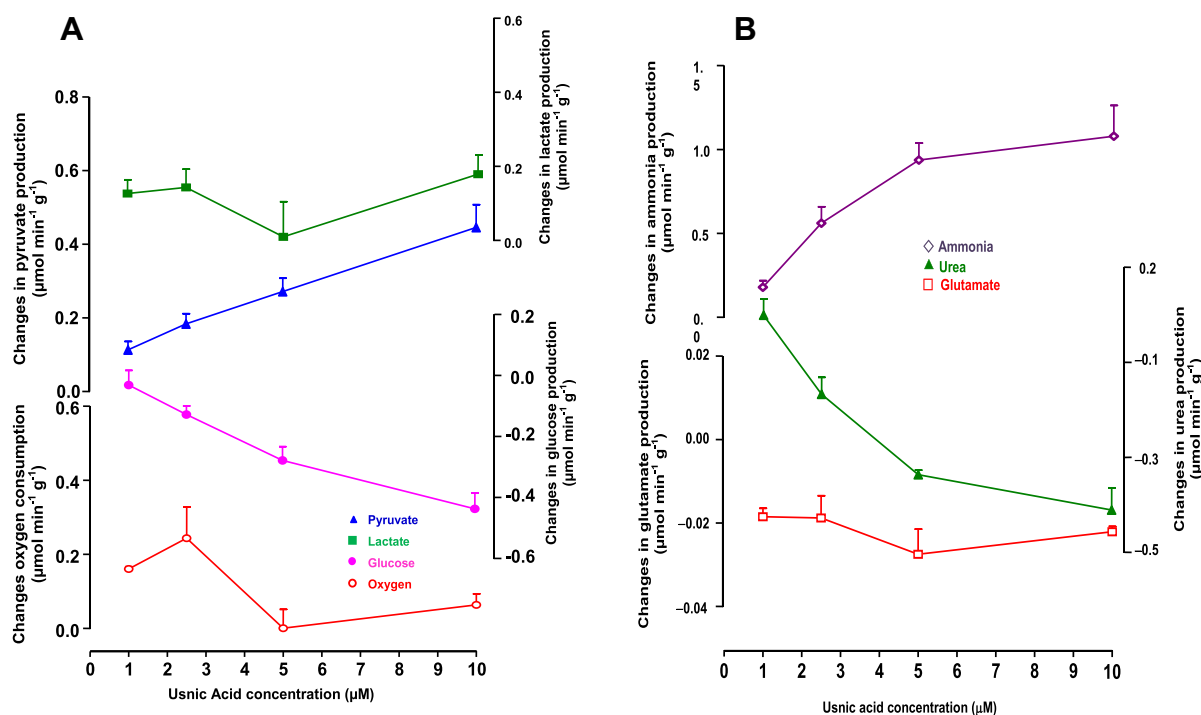

Fig. 6. Concentration dependences of the changes promoted by usnic acid in alanine metabolism. Livers from fasted rats were perfused as described in the Section 2 and according to the experimental protocol illustrated by Fig. 5. The changes promoted by usnic acid after 30 min infusion (70 min perfusion time) were computed. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean. Panel A: changes produced by usnic acid on the flow of carbon derived from alanine metabolism and panel B: changes produced by usnic acid on the flow of nitrogen derived from alanine metabolism.

duction and an increase in the β-hydroxybutyrate to acetoacetate ratio. The latter reflects the mitochondrial NADH/NAD⁺ ratio due to the near equilibrium of the mitochondrial β-hydroxybutyrate

dehydrogenase [27]. The exact values of the metabolic fluxes are listed in Table 1. In this table the productions of β-hydroxybutyrate and acetoacetate were added up and labeled as ketone bodies pro-
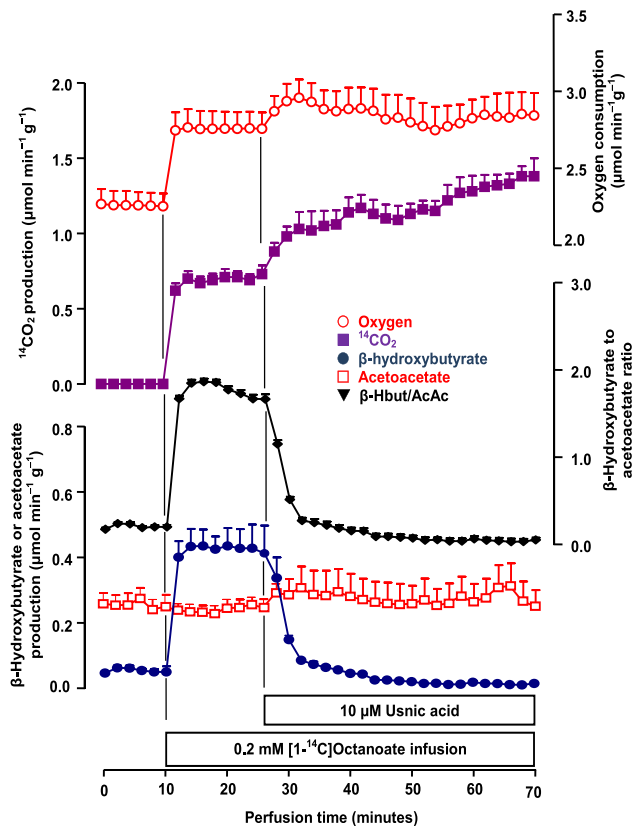
Fig. 7. Time course of the effects of 10 μM usnic acid on octanoate metabolism. Livers from fasted rats were perfused as described in the Section 2. [1-^{14}C]Octanoate (0.2 mM; 1 μCi/l) was infused during 60 min (10–70 min perfusion time). Usnic acid was infused during 44 min (26–70 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic assay of β-hydroxybutyrate and acetoacetate. Oxygen consumption was monitored polarographically. ^{14}CO$_2$ was trapped in phenylethylamine and measured in a liquid scintillation counter. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.

duction. The introduction of 10 μM usnic acid at 26 min perfusion time led to an increased ^{14}CO$_2$ production and a decreased β-hydroxybutyrate production (Fig. 7). The pronounced decrease in the β-hydroxybutyrate production also caused a marked decrease in the β-hydroxybutyrate/acetoacetate ratio, a phenomenon that was further enhanced by the slight increase in the acetoacetate production. Table 1 shows that the actions of usnic acid on octanoate metabolism were concentration-dependent, as the effects of 2.5 μM usnic acid were less pronounced than those of 10 μM usnic acid but always in the same direction for each parameter. It should be remarked that usnic acid caused a pronounced inhibition of ketone bodies production. With 10 μM usnic acid the ketone bodies production with octanoate was even 35% smaller than that found during substrate-free perfusion. It should equally be remarked that usnic acid was without effect on oxygen uptake in the presence of 0.2 mM octanoate.

Fig. 8 shows the time courses of the effects of 10 μM usnic acid on [1-^{14}C]oleate metabolism. Table 1 lists the values of the various parameters in the absence and presence of 2.5 and 10 μM usnic acid. The infusion of [1-^{14}C]oleate produced changes in all the measured parameters that were similar to those found with octanoate: stimulation of oxygen uptake and ketone bodies production and enhancement of the β-hydroxybutyrate/acetoacetate ratio. Usnic acid did not affect the stimulated oxygen uptake. Also, the ketone bodies production was not significantly affected by either 2.5 or 10 μM usnic acid (Table 1). The β-hydroxybutyrate/acetoacetate

ratio, however, was diminished by usnic acid, though by a far lesser extent when compared to the similar action when octanoate was the substrate. The ^{14}CO$_2$ production from [1-^{14}C]oleate, on the other hand, was not affected by 2.5 μM usnic acid and only slightly stimulated at the concentration of 10 μM.

3.6. Effects of usnic acid on adenine mononucleotide contents

The levels of the adenine mononucleotides (AMP, ADP and ATP) were measured in order to obtain information about the energy status of the liver in the presence of two different usnic acid concentrations. The measurements were carried out in livers from fasted rats in the presence of 2 mM lactate. The results are shown in Table 2. Usnic acid decreased the ATP content. This decrease reached 61% at the concentration of 10 μM. At this usnic acid concentration both the ADP and AMP contents were significantly higher than those in the control condition. The ATP/ADP ratio (an important regulatory ratio) also dropped considerably. A tendency toward lower total mononucleotide contents (AMP + ADP + ATP) was also observed, but it lacked statistical significance at the 5% level.

4. Discussion

Several observations of the present work about the action of usnic acid in the perfused liver are compatible with its reported uncoupling action. The most important of these observations, which have also been traditionally reported for other uncouplers of oxidative phosphorylation, are: (a) stimulation of oxygen consumption at low concentrations [18,28]; (b) diminution of the ATP content combined with diminutions in the ATP/ADP and ATP/AMP ratios [28]; (c) increase in the cytosolic NADH/NAD$^+$ ratio [18,28]; (d) inhibition of gluconeogenesis [18,29] from three different substrates, namely lactate, fructose and alanine; (e) stimulation of glycolysis and fructolysis as a cytosolic compensatory phenomenon for the diminished mitochondrial ATP production [18,21,28]; (f) stimulation of glycogenolysis as a means of providing glucose 6-phosphate for the increased glycolytic flux [19,28]; (g) increase in ammonia production combined with impaired urea production [21].

All the phenomena listed above are more or less common to most uncouplers, a fact that does not exclude the possibility that each individual compound with uncoupling action presents its own peculiarities. One such peculiarity is the concentration range between mild and full uncoupling, the latter being a highly toxic condition. Concerning the concentration range between mild and full uncoupling, the uncouplers have been recently classified into two different groups [2]. The first group comprises those uncouplers which have a wide uncoupling range, as for example, 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT), whose uncoupling action occurs between 2×10^{-12} M and 10^{-6} M. Uncouplers of the second group, by contrast, have a relatively narrow uncoupling range which hardly extends over more than one order of magnitude. The classical uncoupler 2,4-dinitrophenol belongs to this group. Uncouplers with a wide uncoupling range are said to be less dangerous when used for purposes of weight loss because it is much more difficult to attain per accident highly toxic plasma concentrations [2]. In the case of usnic acid, it is clear that it must be classified as a narrow range uncoupler because all the effects that are characteristic of uncoupling were found in the concentration range between 1 and 10 μM. Within this concentration range usnic acid is in fact a very potent metabolic effector, considerably more potent than the classical uncoupler 2,4-dinitrophenol and several others. In this respect, comparisons of IC$_{50}$ values will be illustrative. The usnic acid IC$_{50}$ values for gluconeogenesis inhibition were

Table 1

Influence of usnic acid on the transformation of [1-14C]octanoate and 1-14C]oleate in the perfused rat liver. Livers from fasted rats were perfused as described in the Section 2 according to the experimental protocols illustrated by Figs. 7 and 8. Basal rates refer to the metabolic rates measured just before the onset of octanoate or oleate infusion. Rates in the presence of the fatty acids but absence of usnic acid refer to the metabolic rates measured just before the onset of usnic acid infusion. Rates referring to the simultaneous presence of the fatty acid and usnic acid were evaluated at the end of the perfusion experiment, as ilustrated by Figs. 7 (octanoate) and 8 (oleate). Ketone bodies production refers to the sum of β-hydroxybutyrate (β-Hbut) and acetoacetate (AcAc) productions. Values are means ± SEM. Asterisks (*) indicate statistical differences between the values observed in the absence of usnic acid and the values found in the presence of the various usnic acid concentrations ($p \leqslant 0.05$).

| Conditions	14CO2 production (µmol min⁻¹g⁻¹)	Oxygen consumption (µmol min⁻¹g⁻¹)	Ketone bodies production (µmol min⁻¹g⁻¹)	β-Hbut/AcAc
Basal rates (substrate-free perfusion)	–	2.30 ± 0.09	0.43 ± 0.13	0.28 ± 0.03
0.2 mM Octanoate	0.67 ± 0.02	2.95 ± 0.13	1.27 ± 0.15	2.34 ± 0.40
0.2 mM Octanoate + 2.5 µM usnic acid	1.13 ± 0.13*	2.81 ± 0.15	0.65 ± 0.11*	0.10 ± 0.03*
0.2 mM Octanoate + 10 µM usnic acid	1.34 ± 0.08*	2.90 ± 0.14	0.28 ± 0.06*	0.12 ± 0.03*
0.2 mM Oleate	0.58 ± 0.02	2.81 ± 0.20	1.11 ± 0.29	2.13 ± 0.14
0.2 mM Oleate + 2.5 µM usnic acid	0.57 ± 0.04	2.80 ± 0.16	1.00 ± 0.05	1.78 ± 0.06
0.2 mM Oleate + 10 µM usnic acid	0.88 ± 0.09*	2.81 ± 0.17	0.95 ± 0.23	0.71 ± 0.21*
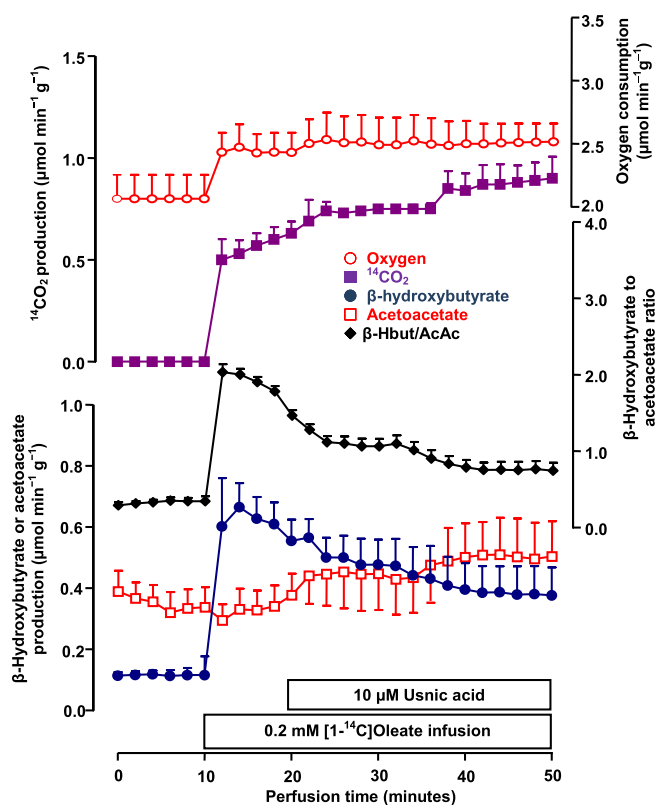
Fig. 8. Time course of the effects of 10 µM usnic acid on oleate metabolism. Livers from fasted rats were perfused as described in the Section 2. [1-14C]Oleate (0.2 mM; 1 µCi/l) was infused during 40 min (10–50 min perfusion time). Usnic acid was infused during 30 min (20–50 min perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic assay of β-hydroxybutyrate (Hbut) and acetoacetate (AcAc). Oxygen consumption was monitored polarographically. 14CO2 was trapped in phenylethylamine and measured in a liquid scintillation counter. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.

between 1.33 and 3.61 µM; for the classical uncoupler 2,4-dinitrophenol a value of 17.9 µM has been recently reported [21]. A similar value was reported for juglone (15 µM) another natural product which has also been recommended for weight loss purposes [21]. Usnic acid is also much more potent as an inhibitor of gluconeogenesis than several antiinflammatories with uncoupling action, as for example, mefenamic, flufenamic and niflumic acid whose IC50 values are ∼100, 40 and 22 µM, respectively [18,19,29].

It is worth at this point to compare the influence of usnic acid on 13C incorporation into lactate by isolated hepatocytes [13] with the results found in the present work. Lactate production from endogenous glycogen in our experiments increased linearly with the concentration of usnic acid and stimulation reached 180% at the concentration of 10 µM. Steady-state conditions were reached after 20 min infusion. In isolated hepatocytes a significant incorporation of 13C into lactate was found only after 24 h, solely with 5 µM usnic acid and with an increase in incorporation of less than 15%. Similarly, an inhibition of 13C incorporation into new glucose (gluconeogenesis) occurred only with 10 µM usnic acid and after 6–24 h. This contrasts sharply with our experiments in which inhibition of gluconeogenesis developed rapidly (20 min) and reached 50% inhibition at concentrations between 1.33 and 3.61 µM. These comparisons emphasize the importance of direct measurements of metabolic fluxes in a system that is as close as possible to the physiological conditions.

Although usnic acid has been used for weight loss purposes [3], the data obtained in the present work indicate that the drug might not be as effective as can be expected from its strong uncoupling action. For an uncoupler to be a highly effective promoter of weight loss, stimulation of fatty acid oxidation is important. Stimulation of fatty acid oxidation requires stimulation of oxygen uptake in the mitochondrial respiratory chain. Although usnic acid in fact stimulates oxygen consumption in the perfused liver, this effect is not very pronounced and even absent under some conditions. Our observations suggest that there is a narrow limit between the concentrations that stimulate oxygen uptake by means of uncoupling and those that inhibit electron flow [10]. A stable increment in oxygen uptake was found only for usnic acid concentrations up to 2.5 µM. At higher concentrations an initial increment was rapidly followed by a progressive decrease. This initial increment was more pronounced during substrate-free perfusion of the liver, i.e., when the organ was respiring mainly at the expense of endogenous fatty acids. In the presence of substrates this initial increment was generally smaller or even absent. This phenomenon was especially pronounced when the perfusion was carried out in the presence of fatty acids. The results of our experiments with the fatty acids octanoate and oleate suggest very strongly that usnic acid is incapable of increasing oxygen uptake to levels above those already attained in the presence of these fatty acids alone. Possibly the mitochondrial capacity of shuttling reducing equivalents into the respiratory chain is already at its limits when exogenous fatty acids are introduced so that the abolition of the respiratory control by an uncoupler has little influence. This is indeed indicated by the very high mitochondrial NADH/NAD+ ratio induced by fatty acids, especially octanoate. On the other hand, the overall effect of usnic acid on fatty acid metabolism is quite complex as evidenced by a few observations. The first of these observations is that the absence of oxygen uptake increments when octanoate and oleate were introduced contrasts with the increased 14CO2 productions that were observed, especially when [1-14C]octanoate was the sub-

Table 2
Influence of usnic acid on the hepatic contents of adenine mononucleotides in the presence of 2 mM lactate. Livers from fasted rats were perfused in an open system as described in Section 2. The experimental protocol delineated in Fig. 3A was followed with two different usnic acid concentrations. The livers were freeze-clamped in liquid nitrogen at 70 min perfusion time and the adenine mononucleotides were extracted with cold perchloric acid. Control determinations were done with livers that were freeze-clamped at the same perfusion time in the presence of lactate but without usnic acid infusion. Values are means ± SEM. Asterisks (*) indicate statistical differences between the control experiments and the experiments that were done in the presence of usnic acid ($p \leqslant 0.05$).

Conditions	ATP (μmol/g)	ADP (μmol/g)	AMP (μmol/g)	(AMP + ADP + ATP) (μmol/g)	ATP/ADP
Control ($n = 4$)	2.40 ± 0.22	0.74 ± 0.04	0.41 ± 0.10	3.55 ± 0.34	3.24 ± 0.22
5 μM usnic acid ($n = 4$)	1.56 ± 0.15*	0.88 ± 0.01	0.50 ± 0.03	2.94 ± 0.13	1.78 ± 0.18*
10 μM usnic acid ($n = 4$)	0.74 ± 0.13*	1.23 ± 0.07*	0.82 ± 0.13*	2.79 ± 0.25	0.62 ± 0.10*

strate. The most simple explanation for this combination of events, increased $^{14}CO_2$ production/no oxygen uptake increment, is that usnic acid could be further enhancing the preference of the liver cells for oxidizing exogenous fatty acids in detriment to endogenous ones. This explanation works very well for oleate, but less well for octanoate, because in the latter case stimulation of $^{14}CO_2$ production was more pronounced and coupled to a clear inhibition of ketone bodies production. This observation suggests that usnic acid affects differently the transformation of medium- and long-chain fatty acids. It can even be suggested that usnic acid causes a specific inhibition of octanoate transformation because no inhibition of ketone bodies production was found with oleate. The fact that the $^{14}CO_2$ production from octanoate was stimulated does not disprove this possibility. An increased $^{14}CO_2$ production does not necessarily imply in an increased rate of fatty acid oxidation because the endogenous fatty acids are not ^{14}C-labeled. Furthermore, the increased $^{14}CO_2$ production can also partly reflect an increased activity of the tricarboxylic acid cycle which is the preferred route for the transformation of the carbon units of acetyl-CoA when compared to ketogenesis [30]. The latter phenomenon generally occurs when the mitochondrial NADH/NAD$^+$ radio is decreased, as indicated by the β-hydroxybutyrate/acetoacetate ratio [27]. In this respect it is worth to emphasize that usnic acid decreases the β-hydroxybutyrate/acetoacete ratio to a much greater extent in the presence of octanoate than in the presence of oleate, again strongly suggesting specific effects on octanoate metabolism that go beyond the simple uncoupling action.

General toxicity and hepatotoxicity of usnic acid [3,16,17] is fully corroborated by several observations of the present work. In addition to ATP depletion, which had already been reported previously [12,13], there are other aspects which have not yet been described. One of these points is the strong inhibition of ureagenesis (IC_{50} = 2.69 μM), combined with the comparably strong inhibition of gluconeogenesis (IC_{50} between 1.33 and 3.61 μM). The classical uncoupler 2,4-dinitrophenol, while inhibiting gluconeogenesis (IC_{50} = 17.9 μM), actually stimulates urea production at concentrations up to 50 μM with half-maximal stimulation at 4.76 μM [21]. Juglone, the natural uncoupler found in the walnut, begins to inhibit urea production at concentrations higher than 20 μM. At concentrations up to 20 μM it is stimulatory [21]. These comparisons reveal a combination of effects for usnic acid that is much more harmful when compared to the classical uncoupler 2,4-dinitrophenol, namely a strong inhibition of both gluconeogenesis and ammonia detoxification within a very short concentration range. Particularly in the fasted state one can predict that the ingestion of usnic can be especially harmful. Under this metabolic condition the glucose supply to the nervous tissue depends basically on hepatic gluconeogenesis, which will be strongly inhibited by usnic acid. This limitation may occur in combination with a shortage in the supply of ketone bodies, an alternative carbon source for the brain, since usnic acid has been found to limit or even to inhibit ketogenesis depending on the conditions.

In conclusion, usnic acid exerts numerous metabolic effects that tend, in principle at least, to be harmful to the organism. It is true that there are serious claims that mild uncoupling can be beneficial for health. Mild uncoupling seems to trigger molecular mechanisms that are able to increase the expression of mitochondrial genes [31] and it has also been claimed that it increases longevity in mice associated with the improvement of several serological markers [32]. In the case of usnic acid, however, it seems that the very narrow concentration range of its action makes it very difficult to adjust the ingested doses in such a way as to avoid toxic and harmful manifestations.

Conflict of interest statement

The authors state that they have no conflict of interest concerning the present article.

Acknowledgements

References

[1] M.L. Tainter, A.B. Stockton, W.C. Cutting, Use of dinitrophenol in obesity and related conditions, JAMA 101 (1933) 1472–1475.
[2] P.H. Lou, B.S. Hansen, P.H. Olsen, S. Tullin, M.P. Murphy, M.D. Btand, Mitochondrial uncouplers with an extraordinary dynamic range, Biochem. J. 407 (2007) 129–140.
[3] J.T. Favreau, M.L. Ryu, G. Braunstein, G. Orshansky, S.S. Park, G.L. Coody, L.A. Love, T.L. Fong, Severe hepatotoxicity associated with the dietary supplement LipoKinetix, Ann. Int. Med. 136 (2002) 590–595.
[4] D. Han, K. Matsumaru, D. Rettori, N. Kaplowitz, Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress, Biochem. Pharmacol. 67 (2004) 439–451.
[5] R. Müller, Pharmaceutically relevant metabolites from lichens, Appl. Microbiol. Biotechnol. 56 (2001) 9–16.
[6] K. Ingólfsdóttir, Usnic acid, Phytochemistry 61 (2002) 729–736.
[7] M. Cocchietto, N. Skert, P.L. Nimis, G. Sava, A review on usnic acid, an interesting natural coumpond, Naturwissenschaften 89 (2002) 137–146.
[8] R.B. Johnson, G. Feldott, H.A. Lardy, The mode of action of the antibiotic usnic acid, Arch. Biochem. Biophys. 28 (1950) 317–323.
[9] H.A. Lardy, J.L. Connelly, D. Johnson, Antibiotics as tolls for metabolic studies II. Inhibition of phosphoryl transfer by oligomycin and aurovertin, Biochemistry 3 (1964) 1961–1968.
[10] A.N. Abo-Khatwa, A.A. Al-Robai, D.A. Al-Jawhari, Lichen acids as uncouplers of oxidative phosphorylation of mouse liver-mitochondria, Nat. Toxins 4 (1996) 96–102.
[11] A.N. Abo-Khatwa, A.A. Al-Robai, D.A. Al-Jawhari, The uncoupling of oxidative phosphorylation of mouse liver mitochondria *in vivo* by usnic acid, JKAU Sci. 17 (2005) 35–45.
[12] P. Pramyothin, W. Janthasoot, N. Pongnimitprasert, S. Phrukudom, N. Ruangrungsi, Hepatotoxic effect of (+) usnic acid from *Usnea siamensis* Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria, J. Ethnopharmacol. 90 (2004) 381–387.
[13] B.J. Sonko, T.C. Schmitt, L. Guo, Q. Shi, L.G. Boros, J.E.A. Leakey, R.D. Beger, Assessment of usnic acid in rat primary hepatocytes using ^{13}C isotopomer distribution: analysis of lactate, glutamate and glucose, Food Chem. Toxicol. 49 (2011) 2968–2974.
[14] N.P. Silva-Santos, S.C. Nascimento, M.S.O. Wanderley, N.T. Pontes-Filho, J.F. Silva, C.M.M.B. Castro, E.U. Pereira, N.H. Silva, N.K. Honda, N.S. Santos-Magalhães, Nanoencapsulation of usnic acid: an attempt to improve antitumor activity and reduce hepatotoxicity, Eur. J. Pharm. Biopharm. 64 (2006) 154–160.

[15] X. Lu, Q. Zhao, Y. Tian, S. Xiao, T. Jin, X. Fan, A metabolomic characterization of (+)-usnic acid-induced liver injury by gas chroma-tography-mass spectrometry-based metabolic profiling of the plasma and liver, Int. J. Toxicol. 30 (2011) 478–491.

[16] F.A. Durazo, C. Lassman, S.H.B. Han, S. Saab, N.P. Lee, M. Kawano, B. Saggi, S. Gordon, D.G. Farmer, H. Yersiz, L.I. Goldstein, M. Ghobrial, R.W. Busuttil, Fulminant liver failure due to usnic acid for weight loss, Am. J. Gastroenterol. 99 (2004) 950–952.

[17] L. Guo, Q. Shi, J.L. Fang, N. Mei, A.A. Ali, S.M. Lewis, J.E. Leakey, V.H. Frankos, Review of usnic acid and Usnea barbata toxicity, J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev. 26 (2008) 317–338.

[18] F. Suzuki-Kemmelmeier, A. Bracht, The effects of the nonsteroidal antiin-flammatory mefenamic acid on energy metabolism in the perfused rat liver, Biochem. Pharmacol. 38 (1989) 823–830.

[19] C.H. Lopez, A. Bracht, N.S. Yamamoto, M.D. Santos, Metabolic effects and distribution space of flufenamic acid in the isolated perfused rat liver, Chem. Biol. Interac. 116 (1998) 105–122.

[20] A. Acco, J.F. Comar, A. Bracht, Metabolic effects of propofol in the isolated perfused rat liver, Basic Clin. Pharmacol. Toxicol. 95 (2004) 166–174.

[21] S.C. Saling, J.F. Comar, M.S. Mito, R.M. Peralta, A. Bracht, Actions of juglone on energy metabolism in the rat liver, Toxicol. Appl. Pharmacol. 257 (2011) 319–327.

[22] R. Scholz, T. Bücher, Hemoglobin-free perfusion of rat liver, in: B. Chance, R.W. Estabrook, J.R. Williamson (Eds.), Control of Energy Metabolism, Academic Press, New York, 1965, pp. 393–414.

[23] H.U. Bergmeyer (Ed.), Methods of Enzymatic Anaysis, Academic Press, New York, 1974.

[24] A. Bracht, E.L. Ishii-Iwamoto, A.M. Kelmer-Bracht, O estudo do metabolismo no fígado em perfusão, in: A. Bracht, E.L. Ishii-Iwamoto (Eds.), Métodos de Laboratório em Bioquimica, Editora Manole, São Paulo, 2003, pp. 275–289.

[25] A. Bracht, J. Constantin, M.V.C. Lonardoni, N.S. Yamamoto, Análise enzimática, in: A. Bracht, E.L. Ishii-Iwamoto (Eds.), Métodos de Laboratório em Bioquimica, Editora Manole, São Paulo, 2003, pp. 139–173.

[26] L.A. Menahan, O. Wieland, The role of endogenous lipid in gluconeogenesis and ketogenesis of perfused rat liver, Eur. J. Biochem. 9 (1969) 182–188.

[27] H. Sies, Nicotinamide nucleotide compartmentation, in: H. Sies (Ed.), Metabolic Compartmentation, Academic Press, London, UK, 1982, pp. 205–231.

[28] S. Soboll, R. Scholz, H.W. Heldt, Subcellular metabolite concentrations. Dependence of mitochondrial and cytosolic ATP systems on the metabolic state of perfused rat liver, Eur. J. Biochem. 87 (1978) 377–390.

[29] A.M. Kelmer-Bracht, A. Bracht, Inhibition of hepatic gluconeogenesis by niflumic acid correlates with the concentration of the free form, Res. Commun. Chem. Pathol. Pharmacol. 80 (1993) 241–248.

[30] M. Lopes-Cardoso, Regulation of ketogenesis in isolated rat liver mitochondria, in: H.S. Söling, C.D. Seufert (Eds.), Biochemical and Clinical Aspects of Ketone Body Metabolism, Georg Thieme Publishers, Stuttgart, Germany, 1978, pp. 41–58.

[31] L.M. Rohas, J. St-Pierre, M. Uldry, S. Jäger, C. Handschin, B.M. Spiegelmen, A fundamental system of cellular energy homeostasis regulated by PGC-1, Proc. Natl. Acad. Sci. USA 104 (2007) 7933–7938.

[32] C.C. Caldeira da Silva, F.M. Cerqueira, L.F. Barbosa, M.H.G. Medeiros, A.J. Kowaktowski, Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity, Aging Cell 7 (2008) 552–560.