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METABOLIC ALTERATIONS CAUSED BY USNIC ACID IN THE LIVER

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Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos

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BIOGRAFIA

Caroline Tessaro Moreira, nasceu no dia 30 de outubro de 1983, na cidade de Ariquemes – RO. Iniciou sua graduação em Nutrição em Fevereiro de 2003 e concluiu em dezembro 2006, pela Centro Universitário de Maringá – CESUMAR. Durante a graduação participou de dois projetos de iniciação científica junto ao CESUMAR, participou também de projetos que não foram vinculados a iniciação científica. Estes projetos foram publicados em painéis e apresentações orais de congressos conceituados nacionais e internacionais. O trabalho de conclusão de curso teve como tema "Relação da Razão Cintura/Quadril nas Doenças Cardiovasculares".

Após terminar a faculdade ingressou na especialização em Nutrição Clínica pela Universidade Federal do Paraná – UFPR, conluindo a especialização em abril de 2009. Com a monografia intitulada "Ação dos Flavonóides nas Doenças Cardiovasculares".

Em 2010 ingressou na primeira turma de mestrado do Programa de Pós-Graduação em Ciência de Alimentos - UEM. Assim iniciou sua pesquisa no laboratório de metabolismo hepático, sob a orientação do professor Dr. Adelar Bracht. No decorrer do projeto experimental ocorreram alguns imprevistos que culminaram na prorrogação desta defesa de dissertação.

Tem experiência profissional na área de nutrição clínica atuando principalmente nos seguintes temas: Atendimento Personal Diet (individual, grupos e família), clínica particular (obesidade, nutrição na estética, pré e pós operatório na cirugia plástica).

Dedico

Aos meus pais: Edson e Maria do Carmo; que nunca mediram esforços para a minha educação.

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico.

ARTIGO

O artigo **"Metabolic alterations caused by usnic acid in the liver",** será submetido a revista Internacional Toxicology and Applied Pharmacology – estrato Qualis A1 e com fator de impacto 4.447, atualizado em 2011.

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GENERAL ABSTRACT

Aims

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 dosis. The most obvious consequence of the uncoupling action of usnic acid is ATP depletion, as indeed demonstrated in two studies using hepatocytes. However, this phenomenon is probably linked directly or indirectly to a variety of modifications in metabolic fluxes. Although the toxic manifestations at the cellular level have been more extensively investigated, a precise quantification of individual metabolic pathways under the influence of usnic acid is still not available. A meaningful evaluation of individual metabolic pathways is best made in the isolated perfused rat liver, a system in which true metabolic steady-state conditions can be established under conditions that are much closer to the physiological ones than isolated and cultured cells. The present study, thus, takes advantage of the perfused liver in quantifying the metabolic effects of usnic acid on both catabolic and anabolic pathways under various conditions. The results should enhance understanding of the metabolic effects of usnic acid and also allow to compare the action of this compound with that of other drugs.

Experimental approach

Male Wistar rats (180-220 g), fed *ad libitum* with a standard laboratory diet were used. The main experimental system was the isolated perfused rat liver. The liver was perfused in the non-recirculation mode. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with O_2 and CO_2 (95:5) by means of a membrane oxygenator and simultaneously heated to 37 °C. The oxygen concentration in the perfusate was monitored continuously by means of polarography. The tissue content of adenine mononucleotides and the perfusate contents of other metabolites (glucose, lactate, pyruvate, β-hydroxybutyrate and acetoacetate) were determined by enzymatic methods. The $^{14}CO_2$ production from [1-^{14}C]octanoate or [1-^{14}C]oleate was quantified by liquid scintillation counting after trapping $^{14}CO_2$ with phenylethylamine. Fluxes were calculated from the portal-venous differences and the total flow through the liver and referred to the wet liver weight.

Results

1) Usnic acid caused stable increases in oxygen consumption at concentrations up to 2.5 μM during substrate-free perfusion and in the presence of gluconeogenic substrates; at higher concentrations oxygen uptake stimulation was generally smaller and transient in nature.
2) Glycolysis and glycogenolysis from endogenous glycogen were linearly stimulated by usnic acid in the range up to 10 μM. Usnic acid also increased the

lactate to pyruvate ratio, which closely reflects the cytosolic $NADH/NAD^+$ ratio.

3) Usnic acid also stimulated fructolysis (fructose degradation to lactate and pyruvate); in this case the effect was a saturable function of the concentration.

4) Gluconeogenesis was inhibited, the IC_{50} value depending on the substrate: lactate, 1.33 μM; fructose, 1.72 μM; alanine 3.61 μM. For all substrates, however, inhibition of gluconeogenesis was virtually complete at 10 μM usnic acid.

5) Usnic acid was also strongly inhibitory for urea production, with an IC_{50} value of 2.69 μM; ammonia production, on the other hand, was stimulated, with half-maximal stimulation occurring at 1.92 μM. The L-glutamate production was also diminished by usnic acid.

6) 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. Usnic acid did not increase oxygen upake in the presence of either octanoate or oleate. It decreased, however, the β-hydroxy-butyrate/acetoacetate ratio, an indicator for the mitochondrial $NADH/NAD^+$ ratio. This decrease was much more pronounced in the presence of octanoate.

7) Concerning the hepatic contents of adenine mononucleotides, the general tendency as the usnic acid concentration was raised from 2.5 to 10 μM, was an increase in the hepatic ADP and AMP contents and a decrease in the ATP contents. With 10 μM usnic acid the ATP content was dropped to only 31% of the control value. At this usnic acid concentration both the ADP and AMP contents were also significantly higher than those in the control condition.

Conclusions

The conclusion is allowed that the effects of usnic acid up to 2.5 μM reflect predominantly its activity as an uncoupler. The observations corroborating uncoupling, which have also been traditionally reported for other uncouplers of oxidative phosphorylation, are: a) stimulation of oxygen consumption at low concentrations; b) diminution of the ATP content combined with diminutions in the ATP/ADP and ATP/AMP ratios; c) increase in the cytosolic $NADH/NAD^+$ ratio; d) inhibition of gluconeogenesis 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; f) stimulation of glycogenolysis as a means of providing glucose 6-phosphate for the increased glycolytic flux; (g) increase in ammonia production.

Additional observations suggest that usnic acid is able to exert other effects besides uncoupling. One of these effects is inhibition of electron flow in the respiratory chain. Another effect is a direct inhibitory action on ketogenesis.

The numerous metabolic effects of usnic acid tend, in principle at least, to be harmful to the organism. It is true that there are well fundamented 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 and it has also been claimed that it increases longevity in mice associated with the improvement of several serological markers. 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 injested doses in such a way as to avoid toxic and harmful manifestations. Especially noteworthy are the combined inhibitory effects on gluconeogenesis and ureagenesis, which occur both at similar low concentrations, unlike what happens with other uncouplers.

RESUMO GERAL

Introdução

O ácido úsnico é um composto de ocorrência natural derivado do dibenzofurano, encontrado em várias espécies de liquens. O composto tem sido vendido como ingrediente de suplemento para redução de peso. Existe evidência de que este composto tem ação desacopladora da fosforilação oxidativa mitocondrial e está também claro que o seu consumo pode causar severa injúria hepática dependendo da dose. A conseqüência mais óbvia da ação desacopladora do ácido úsnico é a depleção do ATP, como de fato foi demonstrado em dois estudos utilizando hepatócitos isolados. No entanto, este fenômeno está em geral ligado, direta ou indiretamente, a uma variedade de modificações em fluxos metabólicos. Embora as manifestações estritamente tóxicas a nível celular tenham sido investigadas mais extensamente, uma quantificação precisa das vias metabólicas individuais sob a influência do ácido úsnico ainda não está disponível. Uma avaliação mais significativa das vias metabólicas pode ser feita no fígado de rato em perfusão isolada, um sistema no qual estados estacionários podem ser estabelecidos sob condições mais próximas das fisiológicas comparada com células isoladas e/ou cultivadas. Dessa maneira, o presente estudo, utiliza a vantagem da perfusão isolada do fígado para quantificar os efeitos do ácido úsnico sobre vias catabólicas e anabólicas sob várias condições. Os resultados devem aumentar o entendimento dos efeitos metabólicos do ácido úsnico e também permitir comparar a ação deste composto com a de outras drogas.

Abordagem experimental

Ratos machos Wistar, foram alimentados com a dieta padrão utilizada no laboratório. O principal sistema experimental foi o fígado de rato em perfusão isolada. O fígado foi perfundido no modo não-recirculante. O fluído de perfusão foi o tampão bicarbonato Krebs/Henseleit (pH 7,4), saturado com O_2 e CO_2 (95:5) por meio de um oxigenador de membrana e simultaneamente aquecido a 37 °C. A concentração venosa de oxigênio foi monitorada constantemente através de um polarógrafo. O teor do tecido hepático em adenina mononu-cleotídeos e a concentração de metabólitos no perfusado efluente (glicose, lactato, piruvato, β-hidroxibutirato e acetoacetato) foram determinados por mé-todos enzimáticos. A produção de $^{14}CO_2$ a partir de [1-^{14}C]octanoato ou [1-^{14}C]oleato, foi quantificada por cintilação líquida, após a captação do $^{14}CO_2$ com feniletilamina. Os fluxos metabólicos foram calculados a partir da diferença veno-portal, do fluxo de perfusado e do peso do fígado.

Resultados

1) O ácido úsnico até 2,5 μM causou um aumento estável no consumo de oxigênio durante a perfusão livre de substratos e na presença de substratos gliconeogênicos; nas concentrações mais altas o estimulo do consumo de oxigênio foi geralmente menor e de natureza transitória.

2) A glicólise e a glicogenólise a partir do do glicogênio endógeno foram

linearmente estimuladas pelo ácido úsnico até 10 μM. O ácido úsnico também aumentou a razão lactato/piruvato, a qual reflete a razão $NADH/NAD^+$ citosólica.

3) O ácido úsnico também estimulou a frutólise (degradação de frutose a lactato e piruvato); neste caso o efeito foi saturável em função da concentração.

4) A gliconeogênese foi inibida. O valor de IC_{50} dependeu do substrato: lactato, 1,33 μM; frutose, 1,72 μM; alanina 3,61 μM. A inibição da gliconeogenese foi virtualmente completa com ácido úsnico 10 μM.

5) O ácido úsnico inibiu fortemente a produção de uréia, com valor de IC_{50} igual a 2,69 μM; a produção de amônia, ao contrário, foi estimulada, com metade do estímulo ocorrendo na concentração de 1,92 μM. A produção de L-glutamato foi também inibida pelo ácido úsnico.

6) A produção de $^{14}CO_2$ com $[1-^{14}C]$octanoato e $[1-^{14}C]$oleato foi aumentada pelo ácido úsnico, mas a cetogênese com octanoato foi diminuída e a cetogênese com oleato não foi afetada. O ácido úsnico não aumentou o consumo de oxigênio na presença de ambos, oleato ou octanoato. O ácido úsnico, no entanto, diminuiu a razão β-hidroxibutirato/acetoacetato, um indicador da razão $NADH/NAD^+$ mitocondrial. Esta diminuição foi muito mais pronunciada na presença de octanoato.

7) A respeito dos conteúdos hepáticos de adenina mononucleotídeos, a tendência geral com ácido úsnico entre 2,5 e 10 μM foi de aumento nos conteúdos hepáticos de ADP e AMP e de diminuição nos conteúdos de ATP. Com ácido úsnico 10 μM o conteúdo hepático de ATP diminuiu para apenas 31% do valor controle. Com ácido úsnico nesta concentração os conteúdos de ADP e AMP foram também significativamente maiores do que na condição controle.

Conclusão

Em conclusão pode-se dizer que os efeitos do ácido úsnico até 2,5 μM, refletem predominantemente sua atividade desacopladora. As observações que corroboram o desacoplamento, e que têm sido tradicionalmente relatadas para outros desacopladores da fosforilação oxidativa, são: a) estimulo do consumo de oxigênio em baixas concentrações; b) diminuição dos conteúdos de ATP, combinada com a diminuição das razões ATP/ADP e ATP/AMP; C) aumento da razão $NADH/NAD^+$ citosólica; d) inibição da gliconeogênese a partir de três diferentes substratos, isto é, lactato, frutose e alanina; e) estimulo da glicolise e frutólise como um fenômeno citosólico compensatório para a diminuição da produção mitocondrial de ATP; f) estímulo da glicogenólise como um meio de fornecimento de glicose 6-fosfato para aumentar o fluxo glicolítico; g) aumento na produção de amônia.

Observações adicionais sugerem que o ácido úsnico é capaz de exercer outros efeitos além do desacoplamento. Um desse efeitos seria a inibição do fluxo de elétrons na cadeia respiratória. Outro efeito seria a inibição direta da cetogênese.

Os diversos efeitos do ácido úsnico tendem, pelo menos em princípio, a serem prejudiciais ao organismo. É verdade que há propostas bem fundamenta-das de que o desacoplamento pouco acentuado pode ser benéfico à saúde. O desacoplamento pouco acentuado parece desencadear mecanismos moleculares que são capazes de aumentar a expressão de genes mitocondriais e também tem sido alegado que existe um aumento na longevidade de camundongos associada à melhoria de vários marcadores sorológicos. No entanto, no caso do ácido úsnico, o estreito intervalo de concentração da ação desta droga, faz com que seja muito difícil ajustar uma dose de tal forma a evitar manifestações nocivas tóxicas.

Metabolic alterations caused by usnic acid in the liver

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Key words: usnic acid, hepatic metabolism, liver perfusion.

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 dosis. Based on these and other notions 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. The conclusion is allowed 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.

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. Its use was discontinued, however, due to a number of toxic effects (Tainter et al., 1933; Lou et al., 2007). Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3[2H,9bH]-dibenzo-furandione) 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 (Favreau et al., 2002; Han et al., 2004). Usnic acid is a dibenzofuran derivative that occurs as a secondary metabolite in several lichen species and its chemical structure is shown in Figure 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 (Müller, 2001; Ingólfsdóttir, 2002). It can exist naturally as the (+) and (−) enantiomer, but most of its biologic 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 (Ingólfsdóttir, 2002; Cocchietto et al., 2002).

The uncoupling effect of usnic acid was firstly demonstrated in the rat liver homogenate and mitochondria (Johnson et. al., 1950; Lardy et al., 1964) and subsequently confirmed in isolated mouse liver mitochondria (Abo-Khatwa et al., 1996). The uncoupling action of usnic acid in mouse liver mitochondria presents the same features as that of 2,4-dinitrophenol: impairment of respiratory control, stimulation of oligomycin-sensitive respiration, inhibition of ATP synthesis and stimulation of the Mg^{+2}-ATPase (Abo-Khatwa et al., 1996). Also as 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 are required to reach a comparable uncoupling degree(Abo-Khatwa et al., 1996).

It has been suggested that the hepatotoxicity of usnic acid is related primarily to its uncoupling action (Han et al., 2004; Pramyothin et al., 2004; Abo-Khatwa et al., 2005). It has also been suggested that the compound might

cause inhibition of the mitochondrial electron transport chain (Abo-Khatwa et al., 1996; Han et al., 2004; Sonko et al., 2011). 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 (Sonko et al., 2011). In cultured mouse hepatocytes the inhibition of oxidative phosphorylation was also proportional to necrosis (Han et al., 2004) and extensive necrosis was also found in the liver of mice that were treated with usnic acid (Silva-Santos et al., 2006; Lu et al., 2011). 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 (Han et al., 2004; Pramyothin et al., 2004; Lu et al., 2011). In humans, hepatotoxicity of usnic acid has been the subject of several reports including cases of acute liver failure and necessity of liver transplantation (Favreau et al., 2002; Durazo et al., 2004; Guo et al., 2008).

The most obvious consequence of the uncoupling action of usnic acid is ATP depletion, as indeed demonstrated in two studies using hepatocytes (Pramyothin et al., 2004; Sonko et al., 2011). However, this phenomenon is probably linked directly or indirectly to a variety of modifications in metabolic fluxes (Suzuki-Kemmelmeier et al., 1989; Lopez et al., 1998; Acco et al., 2004; Saling et al., 2011). Although the toxic manifestations at the cellular level have been more extensively investigated (Pramyothin et al., 2004; Sonko et al., 2011), 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 (Sonko et al., 2011). 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 individual metabolic pathways under the influence of usnic acid, however, is still not available. A meaningful evaluation of individual metabolic pathways is best made in the isolated perfused rat liver, a system in which true metabolic steady-state conditions can be established under conditions that are much closer to the physiological ones than isolated and cultured cells (Scholz and Bücher, 1965). The present study, thus, takes advantage of the perfused liver in quantifying the metabolic effects of usnic acid on both catabolic and anabolic pathways under

various conditions. The results should enhance understanding of the metabolic effects of usnic acid and also allow to compare the action of this compound with that of other drugs.

Materials and methods

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.

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.

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, nonrecirculating perfusion was performed (Scholz and Bücher, 1965). 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 (dimethylformamide)/(Krebs/Henseleit-bicarbonate buffer) was very low, between 3.03×10^{-4} and 3.30×10^{-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, $[1\text{-}^{14}C]$octanoate and $[1\text{-}^{14}C]$oleate) were dissolved directly into the Krebs/Henseleit-bicarbonate buffer at the desired concentration.

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 (Bergmeyer, 1974). 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 (Bracht et al., 2003a). 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 $[1\text{-}^{14}C]$octanoate or $[1\text{-}^{14}C]$oleate were infused for $^{14}CO_2$ production the outflowing perfusate was collected in Erlenmeyer flasks in 2 minute 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 the phenylethylamine was achieved 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/liter 2,5-diphenyloxazole and 0.15 g/liter 2,2-p-phenylene-bis(5-phenyl-oxazole). 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 liver with liquid nitrogen (Bracht et al., 2003b). 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 (Bergmeyer, 1974).

Treatment of data

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

Results

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 (Scholz and Bücher, 1965). 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 Figure 2A illustrates the time course of the action of 10 μM usnic acid. Panel A of Figure 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 minutes. Glucose (125%), lactate (168%) and pyruvate (95%) productions increased progressively during the first 20 minutes 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 minutes and decreased progressively. At the end of the usnic acid infusion period (40 minutes 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 minutes perfusion time caused the reversal of all stimulations except that of oxygen uptake. In panel B of Figure 2 the stimulations caused by usnic acid are 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 Figure 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 minutes usnic acid infusion (40 minutes 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 Figure 2A). Figure 2B also 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 (Scholz and Bücher, 1965). 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 similar stimulations at the concentrations of 5 and 10 μM.

Effects of usnic acid on lactate gluconeogenesis and associated variables

Figure 3A illustrates the response of the perfused liver to usnic acid infusion at the concentration of 10 μM and it 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 both 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 Figure 3B. Here again the variables represented against the usnic acid concentration correspond to the maximal changes caused by the drug. If the effect was stable (glucose production or oxygen uptake for concentrations up to 2.5 μM) this corresponds to the values found at 70 minutes perfusion time (30 minutes usnic acid infusion). If the effect was not stable (e.g., oxygen uptake for usnic acid concentrations above 2.5 μM), the peak increment was 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 (IC$_{50}$) can be expected at an usnic acid concentration of 1.33 μM. Pyruvate production was slightly increased by usnic

acid without a clearly defined concentration dependence. Oxygen uptake was maximally stimulated at 2.5 μM usnic acid.

Effects of usnic acid on fructose metabolism

Transformation of fructose in the liver allows to monitor simultaneously 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 Figure 4. As expected, the infusion of fructose produced rapid and quite pronounced increments in glucose, lactate and pyruvate productions and oxygen uptake (Figure 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 not transitory (not shown). The concentration dependences of the effects are shown in Figure 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 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 effects were stimulation, even though they might have been transient in nature.

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 hours fasted rats were used. The time courses of the experiments that were done with 10 μM usnic acid can be seen in Figure 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 L-glutamate productions (panel B). Glucose, urea and L-glutamate productions were clearly inhibited by 10 μM usnic acid. Ammonia production, on the other hand, was clearly increased,

the same what hapenned with pyruvate production. Oxygen uptake and lactate production experienced initial increases which were subsequently reversed or even transformed into inhibition. Similar results were obtained with lower usnic acid concentrations, except that lactate production and oxygen uptake experienced stable increments at the concentrations of 1 and 2.5 μM. Figure 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 L-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.

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 (Menahan and Wieland, 1969). 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 chain and a long chain fatty acid, namely [1-^{14}C]octanoate and [1-^{14}C]oleate. Figure 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}CO$_2$-production, β-hydroxybutyrate production and aceto-acetate production. Since livers from fasted rats were used, there was a significant basal production of both acetoacetate and β-hydroxybutyrate, a fact that corroborates the notion that substrate-free perfused livers are oxidizing endogenous fatty acids (Menahan and Wieland, 1969). The introduction of [1-^{14}C]octanoate caused an immediate release of ^{14}CO$_2$, stimulation of oxygen uptake and β-hydroxybutyrate production and an increase in the β-hydroxy-butyrate to acetoacetate ratio. The latter reflects the mitochondrial NADH/NAD$^+$ ratio (Sies, 1982). 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 production. The introduction of 10 μM usnic acid at 26 minutes perfusion time lead to an increased ^{14}CO$_2$ production and a

decreased β-hydroxybutyrate production (Figure 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.

Figure 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.

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 three 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. The general tendency when the usnic acid concentration was raised from 2.5 to 10 μM, was an increase in the hepatic ADP and AMP contents and a decrease in the ATP contents. The only significant modifications at the usnic acid concentration of 2.5 μM, however, was an

increase in the ATP/ADP ratio. With 10 μM usnic acid the ATP content was dropped to only 31% of the control value. At this usnic acid concentration both the ADP and AMP contents were also significantly higher than those in the control condition. The ATP/ADP ratio (an important regulatory ratio) also dropped considerably at the usnic acid concentrations of 5 and 10 μM. A tendency toward lower total mononucleotide contents (AMP + ADP + ATP) was also observed, but they lacked statistical significance at the 5% level.

Discussion

In general, 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 (Soboll et al., 1978; Suzuki-Kemmelmeier and Bracht, 1989); b) diminution of the ATP content combined with diminutions in the ATP/ADP and ATP/AMP ratios (Soboll et al., 1978); c) increase in the cytosolic $NADH/NAD^+$ ratio (Soboll et al., 1978; Suzuki-Kemmelmeier and Bracht, 1989); d) inhibition of gluconeogenesis (Kelmer-Bracht and Bracht, 1993; Suzuki-Kemmelmeier and Bracht, 1989) 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 (Soboll et al., 1978; Suzuki-Kemmelmeier and Bracht, 1989); f) stimulation of glycogenolysis as a means of providing glucose 6-phosphate for the increased glycolytic flux (Lopez et al., 1998; Soboll et al., 1978); (g) increase in ammonia production (Saling et al., 2011).

All the phenomena listed in the former paragraph 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 (Lou et al., 2007). 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 is exerted 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. 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

uncloupler 2,4-dinitrophenol and several others, for example. In this respect, comparisons of IC_{50} values will be illustrative. The usnic acid IC_{50} values for gluconeogenesis inhibition were between 1.33 and 3.61 μM; for the classical uncoupler 2,4-dinitrophenol a value of 17.9 μM has been recently reported (Saling et al., 2011). A similar value was reported for juglone (15 μM) another natural product which has also been recommended for weight loss purposes (Saling et al., 2011). 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 IC_{50} values are ~100, 40, and 22 μM, respectively (Suzuki-Kemmelmeier and Bracht, 1989; Kelmer-Bracht and Bracht, 1993; Lopez et al., 1998).

It is worth at this point to compare the influence of usnic acid on ^{13}C incorporation into lactate by isolated hepatocytes (Sonko et al., 2011). 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 minutes infusion. In isolated hepatocytes incubations a significant incorporation of ^{13}C into lactate was found only after 24 hours, solely with 5 μM usnic acid and with an increase in incorporation of less than 15%. Similarly, the ^{13}C distribution detected inhibition of new glucose formation (gluconeogenesis) only with 10 μM usnic acid and after 6 to 24 hours. This contrasts sharply with our experiments in which inhibition of gluconeogenesis developed very fast (20 minutes) 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 (Favreau et al., 2002), 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 (Abo-Khatwa et., 1996). 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 exogenous 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 that usnic acid is incapable of increasing oxygen uptake to levels above those already attained in the presence of these fatty acids alone. On the other hand, the overall effect of usnic acid on fatty acid metabolism is quite complex as evidenced by a few observations that are difficult to explain. The first of these observations is that the absence of oxygen uptake increment when octanoate and oleate were introduced contrasts with the increased $^{14}CO_2$ production that was observed, especially when [1-^{14}C]octanoate was the substrate. 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 fattty 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 but it can also 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 (Lopes-Cardoso, 1978). This phenomenon generally occurs when the mitochondrial NADH/NAD$^+$ radio is decreased, as indicated by the β-hydroxybutyrate/acetoacete ratio (Sies, 1982). 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 (Favreau et al., 2002; Durazo et al., 2004; Guo et al., 2008) is fully corroborated by several observations of the present work. In addition to ATP depletion, which had already been reported previously (Pramyothin et al., 2004; Sonko et al., 2011), 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 (Saling et al., 2011). 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 (Saling et al., 2011). These comparisons reveal a combination of effects for usnic acid that is much more toxic 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 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 well fundamented 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 (Rohas et al., 2007) and it has also been claimed that it increases longevity in mice associated with the improvement of several serological markers (Caldeira da Silva et al., 2008). 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 injested doses in such a way as to avoid toxic and harmful manifestations.

References

Abo-Khatwa, A.N., Al-Robai, A.A., Al-Jawhari, D.A., 1996. Lichen acids as uncouplers of oxidative phosphorylation of mouse liver-mitochondria. Nat Toxins 4, 96-102.

Abo-Khatwa, A.N., Al-Robai, A.A., Al-Jawhari, D.A., 2005. The uncoupling of oxidative phosphorylation of mouse liver mitochondria *in vivo* by usnic acid. JKAU Science 17, 35-45.

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

Bergmeyer, H.U. 1974. Methods of Enzymatic Anaysis, Academic Press, New York.

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

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

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

Cocchietto, M., Skert, N., Nimis, P.L., Sava, G., 2002. A review on usnic acid, an interesting natural coumpond. Naturwissenschaften 89, 137-146.

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

Favreau, J.T., Ryu, M.L., Braunstein, G., Orshansky, G., Park, S.S., Coody, G.L., Love, L.A., Fong, T.L., 2002. Severe hepatotoxicity associated with the dietary supplement LipoKinetix. Ann Int Med 136, 590-595.

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

Han, D., Matsumaru, K., Rettori, D., Kaplowitz, N., 2004. Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress. Biochem Pharmacol 67, 439-451.

Ingólfsdóttir, K., 2002. Usnic acid. Phytochemistry 61, 729-736.

Johnson, R.B., Feldott, G., Lardy, H.A., 1950. The mode of action of the antibiotic

usnic acid. Arch Biochem 28, 317-323.

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

Lardy, H.A., Connelly, J.L., Johnson, D., 1964. Antibiotics as tolls for metabolic studies. II. Inhibition of phosphoryl transfer by oligomycin and aurovertin. Biochemistry 3, 1961-1968.

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

Lopes-Cardoso, M., 1978. Regulation of ketogenesis in isolated rat liver mitochondria. In: Söling HS, Seufert CD (Eds.). Biochemical and Clinical Aspects of Ketone Body Metabolism. Georg Thieme Publishers-Stuttgart, Germany, pp. 41-58.

Lou, P.H., Hansen, B.S., Olsen, P.H., Tullin, S., Murphy, M.P., Btand, M.D., 2007. Mitochondrial uncouplers with an extraordinary dynamic range. Biochem J 407, 129–140.

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

Menahan, L.A., Wieland, O., 1969. The role of endogenous lipid in gluconeogenesis and ketogenesis of perfused rat liver. Eur J Biochem 9, 182−188.

Müller, R., 2001. Pharmaceutically relevant metabolites from lichens. Appl Microbiol Biotechnol 56, 9-16.

Pramyothin, P., Janthasoot, W., Pongnimitprasert, N., Phrukudom, S., Ruangrungsi, N., 2004. Hepatotoxic effect of (+)usnic acid from *Usnea siamensis* Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria. J Ethnopharmacol 90, 381-387.

Rohas, L.M., St-Pierre, J., Uldry, M., Jäger, S., Handschin, C., Spiegelmen, B.M., 2007. A fundamental system of cellular energy homeostasis regulated by PGC-1α. Proc Natl Acad Sci U.S.A. 104, 7933-7938.

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

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

Sies, H., 1982. Nicotinamide Nucleotide Compartmentation. In: Sies H (Ed.).

Metabolic Compartmentation. Academic Press-London, UK, pp. 205−231.

Silva-Santos, N.P., Nascimento, S.C., Wanderley, M.S.O., Pontes-Filho, N.T., Silva, J.F., Castro, C.M.M.B., Pereira, E.U., Silva, N.H., Honda, N.K., Santos-Magalhães, N.S., 2006. Nanoencapsulation of usnic acid: an attempt to improve antitumor activity and reduce hepatotoxicity. Eur J Pharm Biopharm 64, 154-160.

Sonko, B.J., Schmitt, T.C., Guo, L., Shi, Q., Boros, L.G., Leakey, J.E.A., Beger, R.D., 2011. Assessment of usnic acid in rat primary hepatocytes using ^{13}C isotopomer distribution: analysis of lactate, glutamate and glucose. Food Chem Toxicol 49, 2968-2974.

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

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

Tainter, M.L., Stockton, A.B., Cutting, W.C., 1933. Use of dinitrophenol in obesity and related conditions. JAMA 101, 1472-1475.

Table 1

Conditions	$^{14}CO_2$ production $(\mu mol\ min^{-1}g^{-1)}$	Oxygen consumption $(\mu mol\ min^{-1}g^{-1})$	Ketone bodies production $(\mu mol\ min^{-1}g^{-1})$	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(n=4)	1.13±0.13*	2.81±0.15	0.65±0.11*	0.10±0.03*
0.2 mM Octanoate + 10 μM usnic acid(n=4)	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(n=3)	0.57±0.04	2.80±0.16	1.00±0.05	1.78±0.06
0.2 mM Oleate + 10 μM usnic acid(n=3)	0.88±0.09*	2.81±0.17	0.95±0.23	0.71±0.21*

Influence of usnic acid on the transformation of [1-^{14}C]octanoate and 1-^{14}C]oleate in the perfused rat liver. Livers from fasted rats were perfused as described in the Materials and methods section according to the experimental protocols illustrated by Figures 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 Figures 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 ($p \leq 0.05$).

Table 2

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
2.5 μM usnic acid (n=4)	2.44±0.07	0.58±0.05	0.46±0.09	3.48±0.20	4.28±0.18*
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*

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 the Materials and methods section. The experimental protocol delineated in Figure 3A was followed with various concentrations of usnic acid. The livers were freeze-clamped in liquid nitrogen at 70 minutes 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 the various usnic acid concentrations ($p \leq 0.05$).
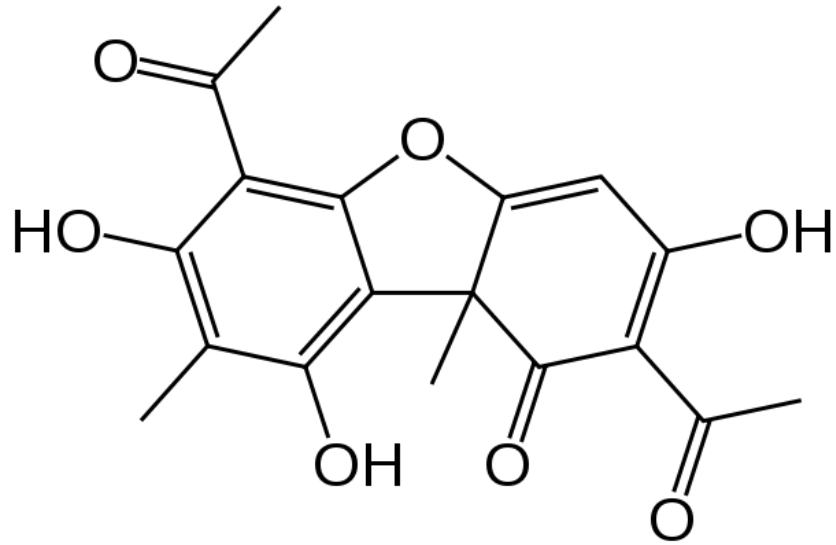
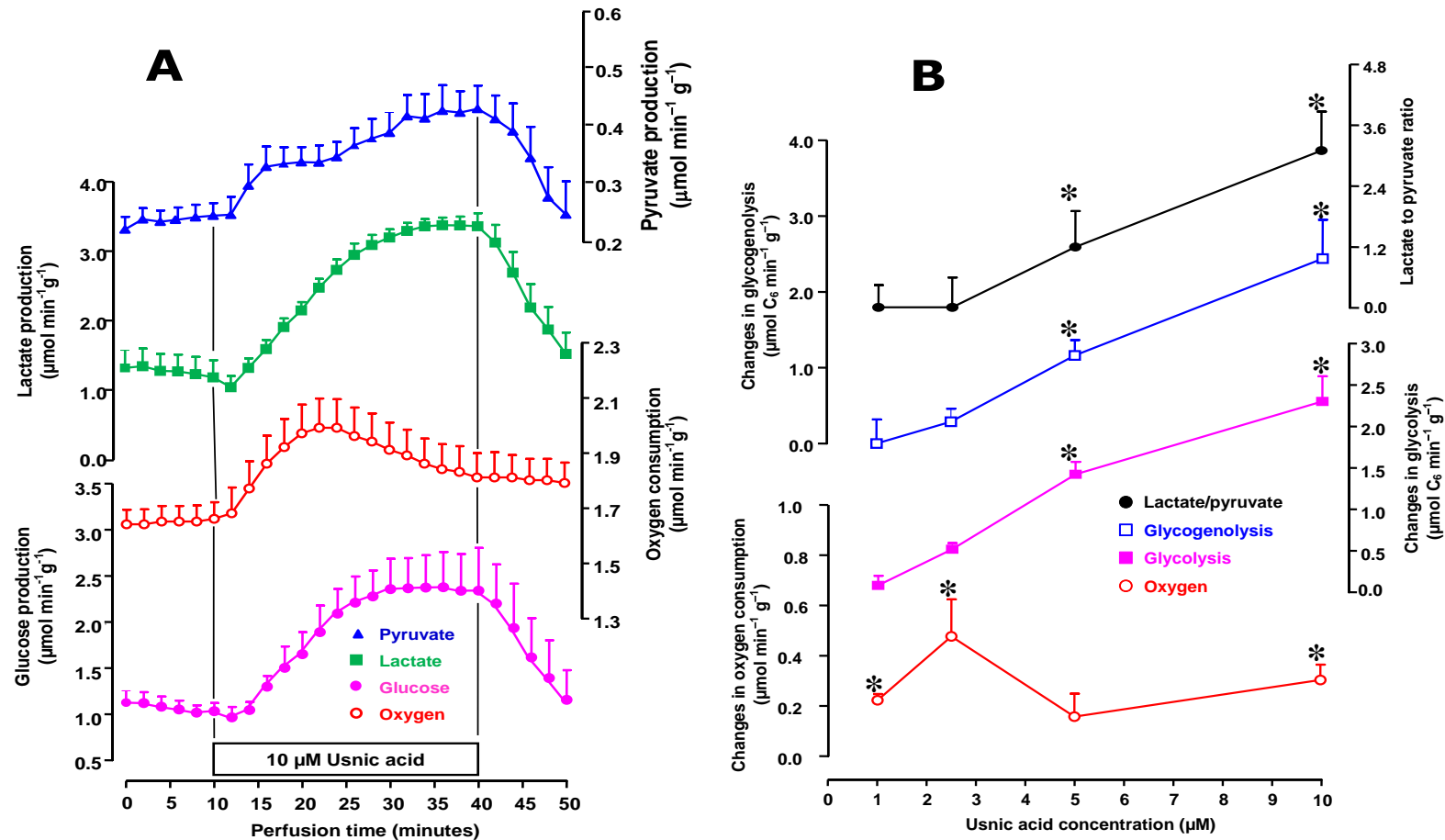Figure 1. **Chemical structure of usnic acid.**

Figure 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 Materials and methods section. 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 minutes infusion (40 minutes 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. Asterisks (*****) indicate statistical significance in comparison with the control condition($p \leq 0.05$).
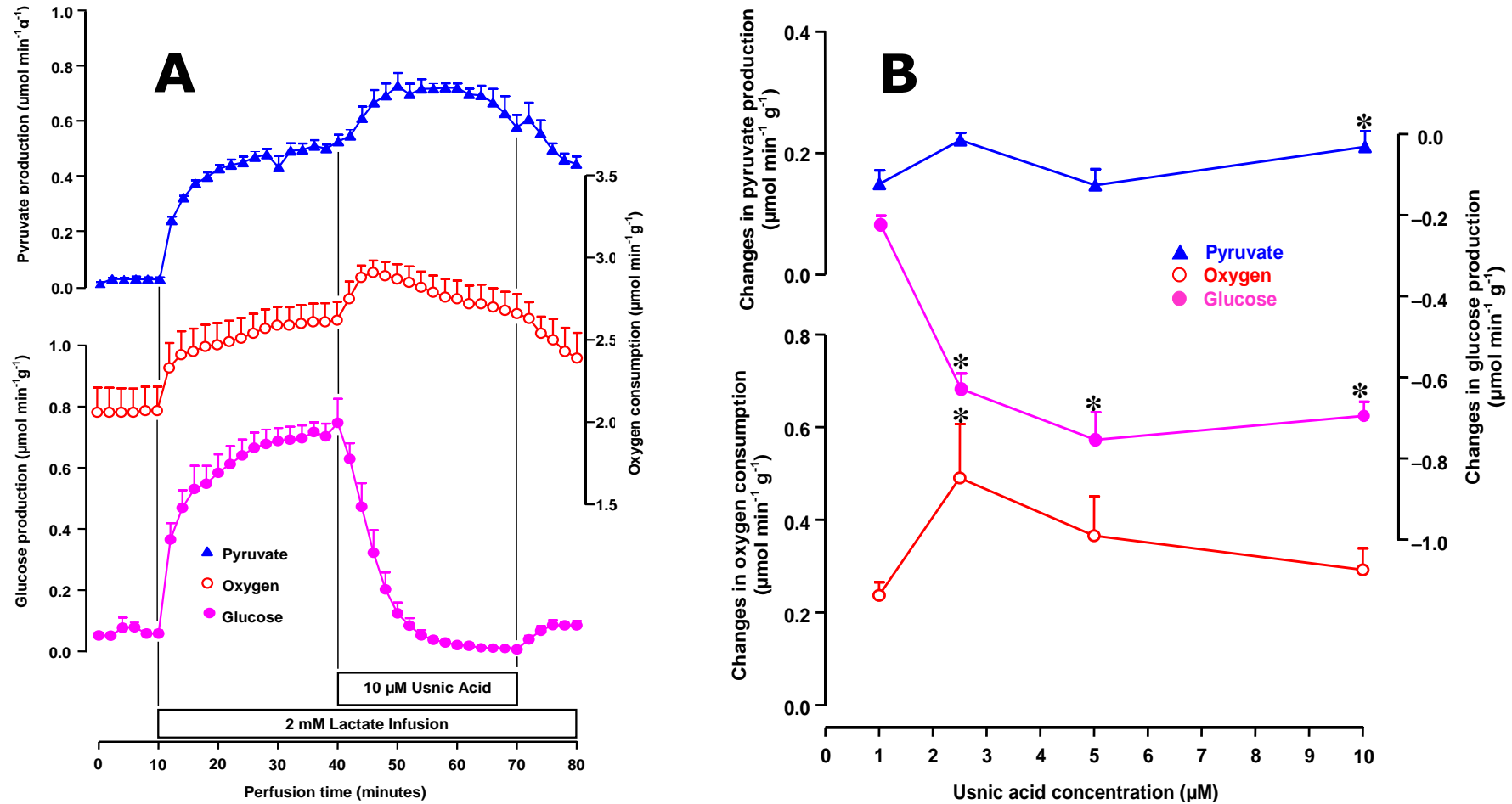
Figure 3. **Effects of usnic acid on lactate gluconeogenesis and associated variables in the perfused rat liver.** Livers from fasted rats were perfused as described in the Materials and methods section. Lactate was infused during 70 minutes (10 to 80 minutes perfusion time). Samples of the effluent 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 minutes (40 to 70 minutes perfusion time); **panel B**: concentration dependence of the changes promoted by usnic acid after 30 minutes infusion (70 minutes perfusion time). Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean. Asterisks (*) indicate statistical significance in comparison with the control condition ($p \leq 0.05$).
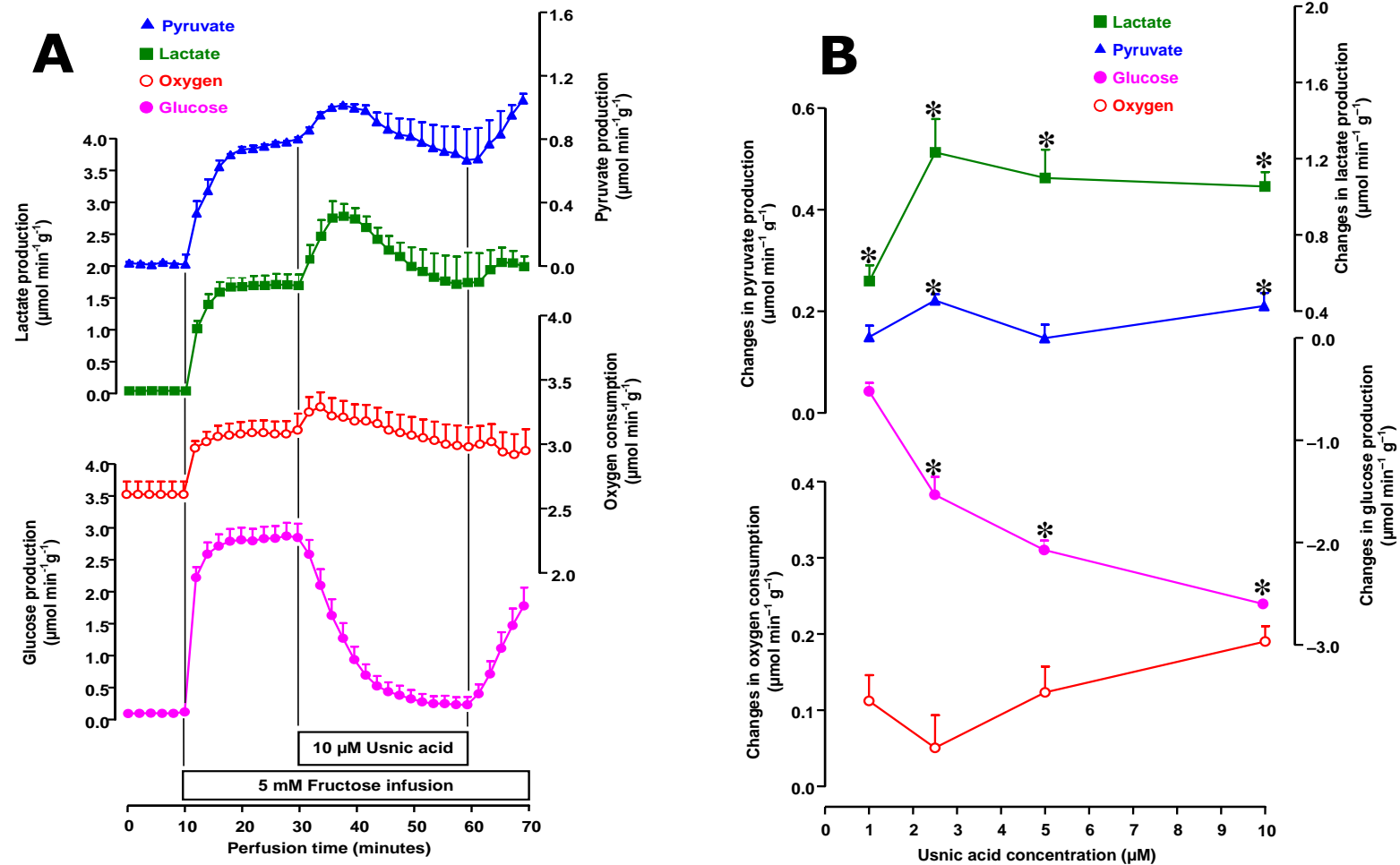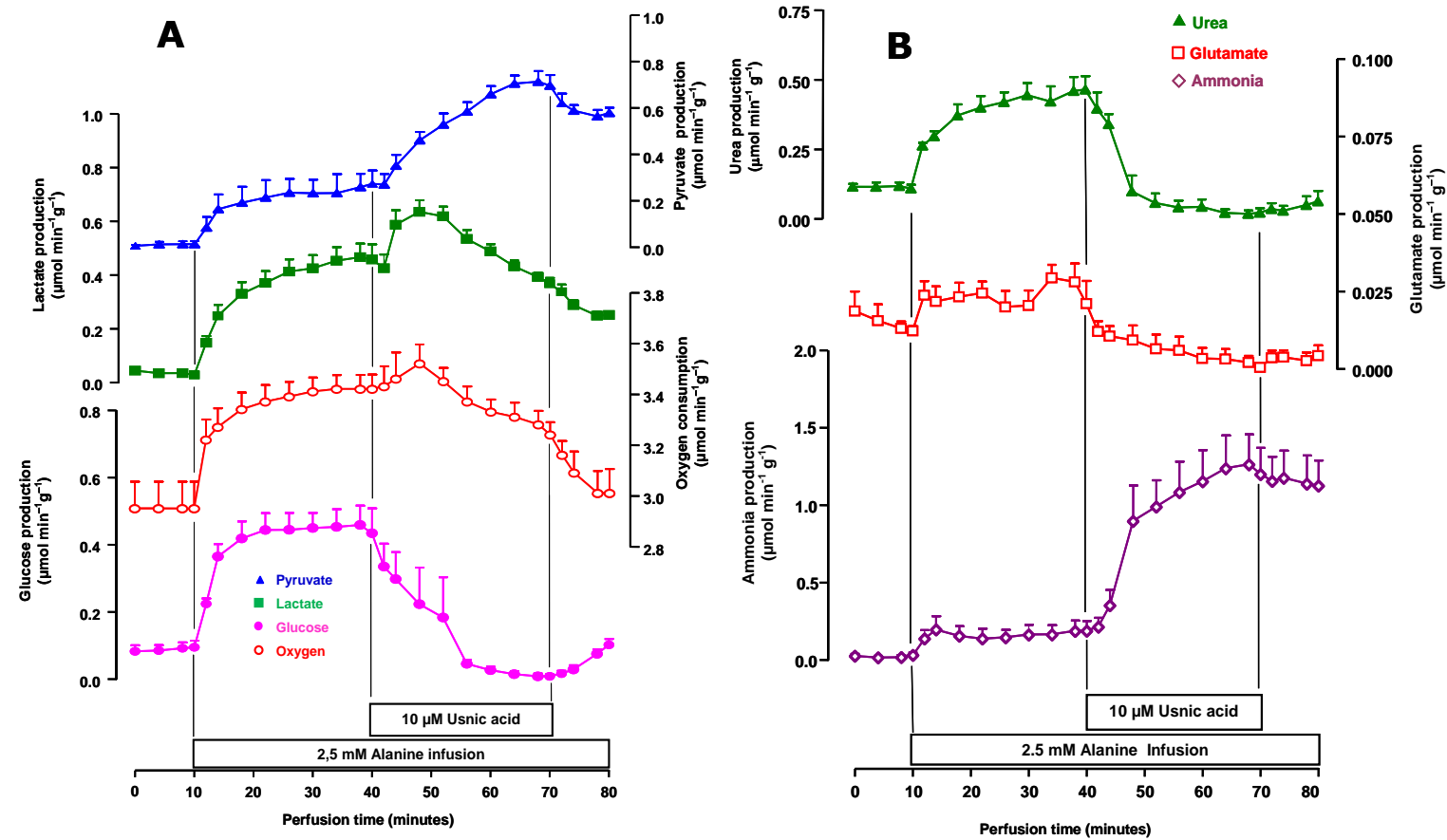
Figure 4. **Effects of usnic acid on fructose metabolism in the perfused rat liver.** Livers from fasted rats were perfused as described in the Materials and methods section. Fructose was infused during 60 minutes (10 to 70 minutes perfusion time). Samples of the effluent perfusion fluid were collected 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 minutes (30 to 60 minutes perfusion time); **panel B**: concentration dependence of the changes promoted by usnic acid after 30 minutes infusion (60 minutes perfusion time). Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean. Asterisks (∗) indicate statistical significance in comparison with the control condition(p≤0.05).

Figure 5. **Time courses of the effects of 10 μM usnic acid on alanine metabolism**. Livers from fasted rats were perfused as described in the Materials and methods section. Alanine was infused during 70 minutes (10 to 80 minutes perfusion time). Usnic acid was infused during 30 minutes (40 to 70 minutes 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; **panel B**: parameters linked to the flow of nitrogen linked from alanine. Each datum point represents the mean of four liver perfusion experiments. Bars are standard errors of the mean.
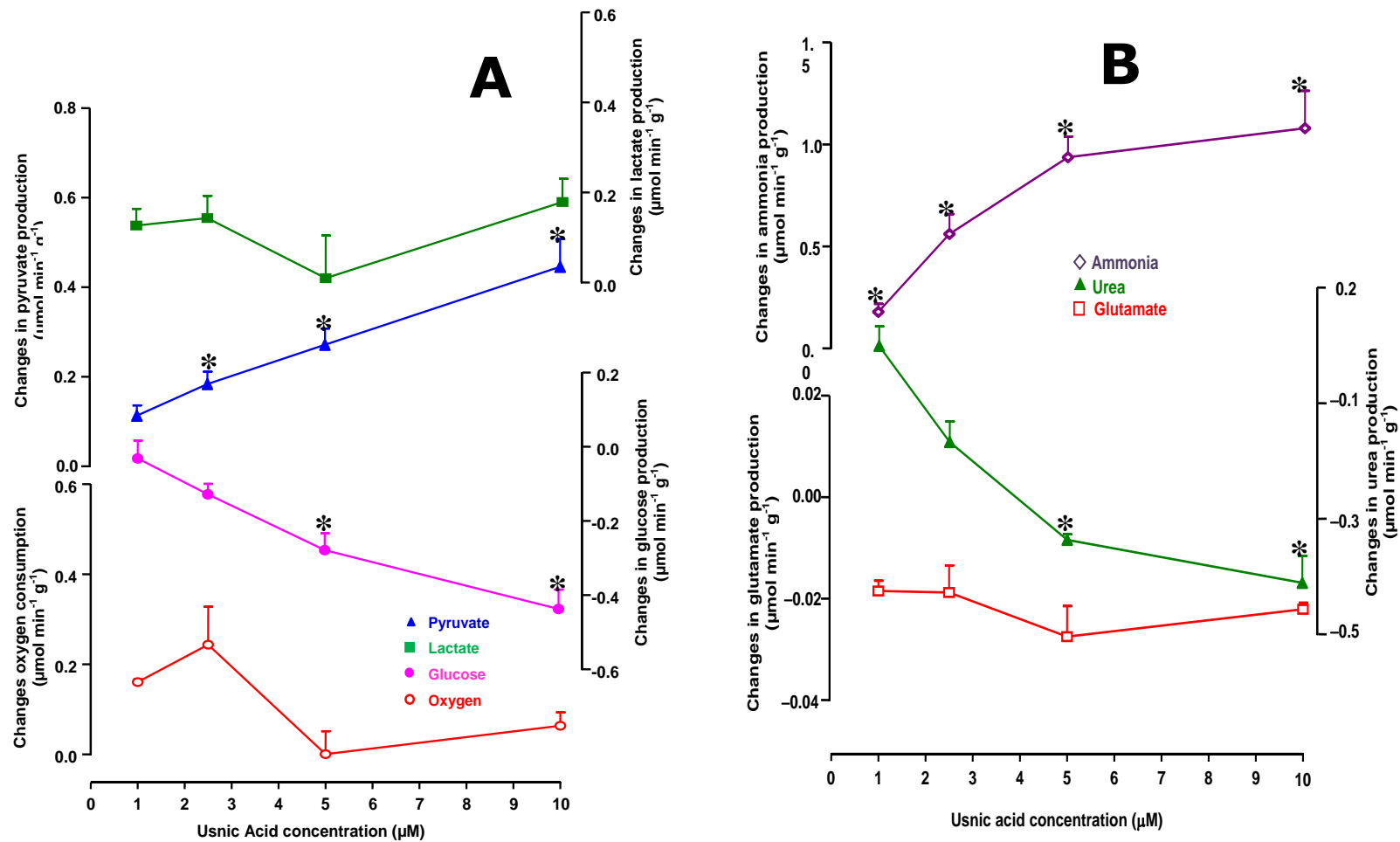
Figure 6. **Concentration dependences of the changes promoted by usnic acid on alanine metabolism.** Livers from fasted rats were perfused as described in the Material and methods section and according to the experimental protocol illustrated by Figure 5. The changes promoted by usnic acid after 30 minutes infusion (70 minutes 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; **panel B**: changes produced by usnic acid on the flow of nitrogen derived from alanine metabolism. Asterisks (*) indicate statistical significance in comparison with the control condition ($p \leq 0.05$).
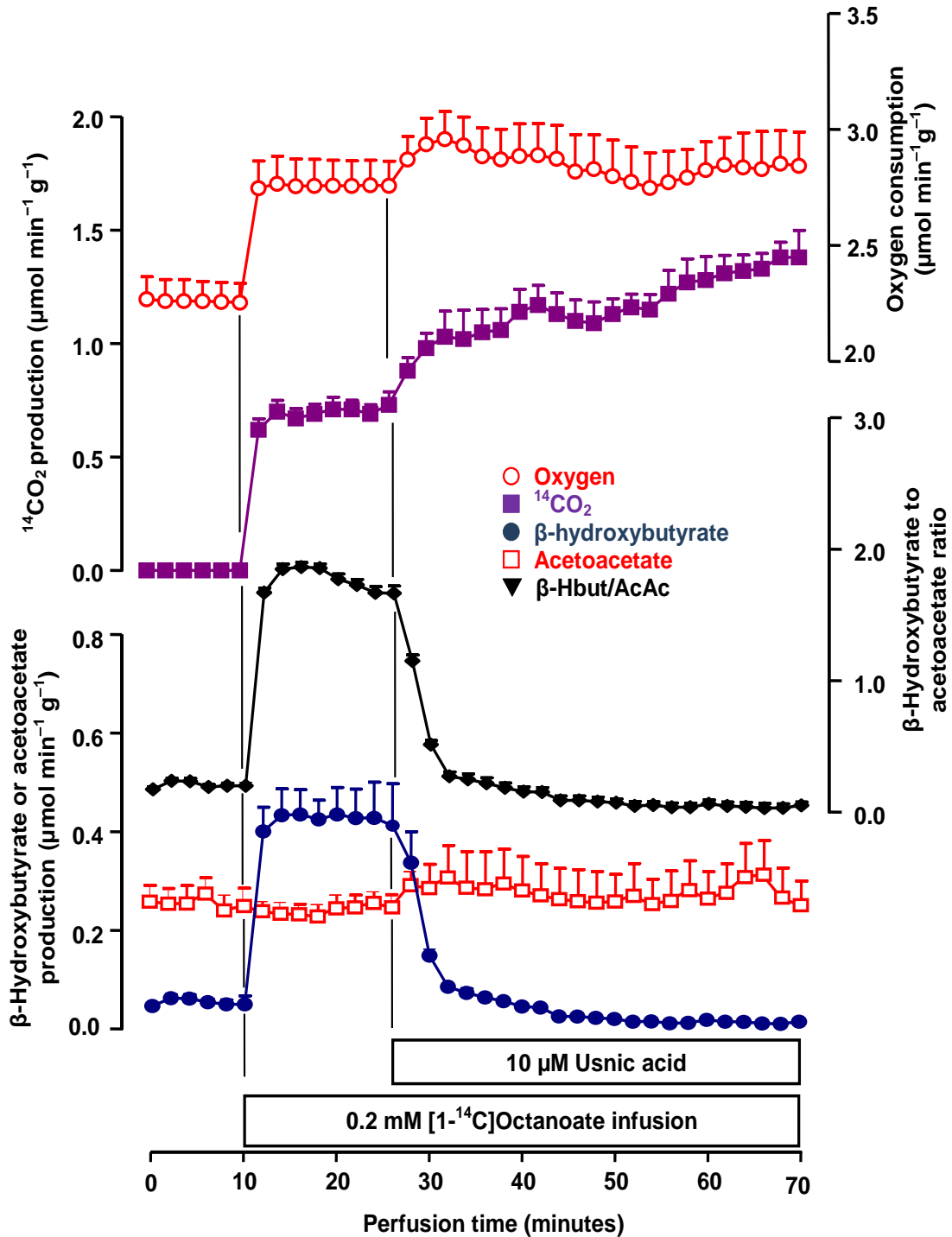
Figure 7. **Time course of the effects of 10 μM usnic acid on octanoate metabolism.** Livers from fasted rats were perfused as described in the Materials and methods section. [1-^{14}C]Octanoate (0.2 mM; 1 μCi/liter) was infused during 60 minutes (10 to 70 minutes perfusion time). Usnic acid was infused during 44 minutes (26 to 70 minutes perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic assay of β-hydroxybutyrate and aceto-acetate. 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.
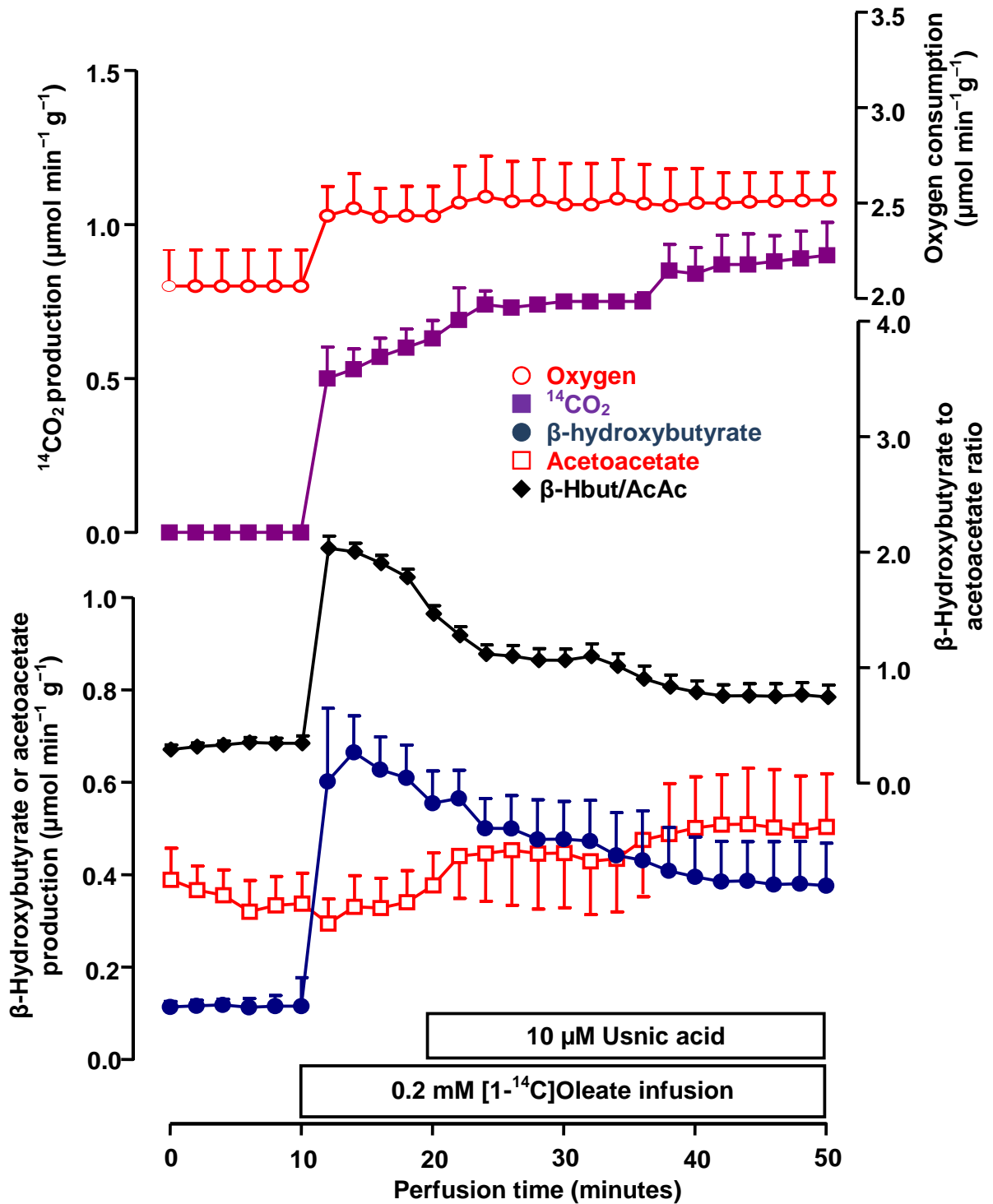
Figure 8. **Time course of the effects of 10 μM usnic acid on oleate metabolism.** Livers from fasted rats were perfused as described in the Materials and methods section. [1–^{14}C]Oleate (0.2 mM; 1 μCi/liter) was infused during 40 minutes (10 to 50 minutes perfusion time). Usnic acid was infused during 30 minutes (20 to 50 minutes perfusion time). Samples of the effluent perfusion fluid were collected for the enzymatic assay of β-hydroxybutyrate (Hbut) and acetocetate (AcAc). 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.