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Actions of juglone on energy metabolism in the rat liver

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ABSTRACT

Juglone is a phenolic compound used in popular medicine as a phytotherapic to treat inflammatory and infectious diseases. However, it also acts as an uncoupler of oxidative phosphorylation in isolated liver mitochondria and, thus, may interfere with the hepatic energy metabolism. The purpose of this work was to evaluate the effect of juglone on several metabolic parameters in the isolated perfused rat liver. Juglone, in the concentration range of 5 to 50 μM, stimulated glycogenolysis, glycolysis and oxygen uptake. Gluconeogenesis from both lactate and alanine was inhibited with half-maximal effects at the concentrations of 14.9 and 15.7 μM, respectively. The overall alanine transformation was increased by juglone, as indicated by the stimulated release of ammonia, urea, L-glutamate, lactate and pyruvate. A great increase (9-fold) in the tissue content of α-ketoglutarate was found, without a similar change in the L-glutamate content. The tissue contents of ATP were decreased, but those of ADP and AMP were increased. Experiments with isolated mitochondria fully confirmed previous notions about the uncoupling action of juglone. It can be concluded that juglone is active on metabolism at relatively low concentrations. In this particular it resembles more closely the classical uncoupler 2,4-dinitrophenol. Ingestion of high doses of juglone, thus, presents the same risks as the ingestion of 2,4-dinitrophenol which comprise excessive compromising of ATP production, hyperthermia and even death. Low doses, i.e., moderate consumption of natural products containing juglone, however, could be beneficial to health if one considers recent reports about the consequences of chronic mild uncoupling.

Introduction

Juglone (5-hydroxy-1,4-naphthoquinone) is a phenolic compound with allelopathic properties belonging to the class of naphthoquinones. Its chemical structure is shown in Fig. 1. This quinone is found in roots, leaves, bark and nuts of several species of walnut from the plant family Juglandaceae (Lee and Campbell, 1969). The α-hydrojuglone is the reduced form of juglone and is related to developmental processes and defense mechanisms of the nuts. When exposed to the air, the α-hydrojuglone is readily oxidized to juglone (Duroux et al., 1998; Rietveld, 1983).

The extract of walnut is widely used in popular medicine as a phytotherapic to treat inflammatory diseases, eczema, acne, herpes, psoriasis, and bacterial, fungal, viral and parasitic diseases (Bell, 1981; Jin, 2010; Mahoney et al., 2000). On the other hand, juglone has been investigated by the National Toxicology Program (USA) as a potentially toxic natural product (Mahoney et al., 2000). The naphthoquinones can cause a variety of hazardous effects in vivo, including acute cytotoxicity and immunotoxicity (Bolton et al., 2000). The mechanisms by which juglone causes cell toxicity are complex. This is partly caused by the fact that juglone can assume three structures which are in equilibrium: in addition to the oxidized and fully reduced forms shown in Fig. 1, the partially reduced semiquinone is also usually present. The mechanisms of action of juglone comprise mixed actions which include the reactivity of the electrophilic quinoidal group and the ability to undergo oxidation–reduction cycles with concomitant formation of free radicals (Duroux et al., 1998; O'Brien, 1991; Rath et al., 1996). Juglone can also interact with nucleophilic biomolecules such as glutathione and thiol groups of proteins which lead to the oxidation of nucleophilic sites. This, in turn, causes inactivation of enzymes or cellular signaling proteins (Klaus et al., 2010).

The toxicity of juglone on bacteria is attributed to changes in the plasma membrane (Zhang et al., 1994). In human lymphocytes, 50 μM juglone inhibits cell proliferation by blocking potassium channels. In consequence it induces polarization of the plasma membrane (Varga et al., 1996). The juglone also appears to inhibit enzymes such as protein kinase C (Frew et al., 1995) and cytochrome P_{450} aromatase in human placental microsomes in a dose-dependent manner (Muto

Abbreviations: Ala, alanine; Pyr, pyruvate; Lac, lactate; $\alpha Keto_C$, cytosolic α-ketoglutarate; Glu_C, cytosolic L-glutamate; $\alpha\text{-}Keto_M$, mitochondrial α-ketoglutarate; Glu_M, mitochondrial L-glutamate; Asp, aspartate; OXAL, oxaloacetate; TCA, tricarboxylic acid cycle; ALT, alanine aminotransferase; AST, aspartate aminotransferase; $Icitr_C$, cytosolic isocitrate; LDH, lactate dehydrogenase; IDH_C, cytosolic isocitrate dehydrogenase; IDH_M, mitochondrial isocitrate dehydrogenase; GluDH, glutamate dehydrogenase.

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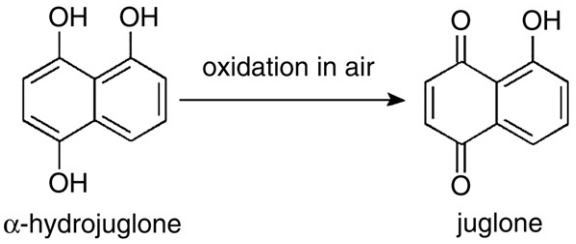

Fig. 1. Chemical formulae of α-hydrojuglone and juglone.

et al., 1987). It also blocks transcription, induces DNA damage, reduces protein levels and induces cell death (Paulsen and Ljungman, 2005). In addition, juglone interferes with cellular energy metabolism due its uncoupling of mitochondrial oxidative phosphorylation. On this respect, Makawiti et al. (1990) reported preliminary experiments which strongly suggest that juglone is able to uncouple rat liver mitochondria.

As it occurs with most xenobiotics, juglone also undergoes hepatic biotransformation, primarily reduction reactions and conjugation with sulfate and glucuronic acid to form various metabolites which are excreted mainly into urine (Chen et al., 2005). The compound has thus clearly full access to the liver cells and several metabolites are generated, some of which are also potentially toxic. Due to its uncoupling action detected with isolated mitochondria (Makawiti et al., 1990), it is highly probable that this activity will manifest itself when the compound is used for medicinal purposes. For this reason it is also of great interest to evaluate the possible effects that juglone might have on the liver functions that are energy-dependent or that are linked in some way to energy metabolism. To investigate these effects was exactly the purpose of the present study. The isolated perfused rat liver was used, because this system allows to measure several related processes such as oxygen consumption, glycolysis, gluconeogenesis and ureogenesis, which are linked to energy metabolism. Experiments with isolated mitochondria were also done in order to present confirmative evidence and to complement previous reports (Makawiti et al., 1990). The results should improve our understanding of the action mode of juglone on mammalian cells and to help in the decision of using or not the compound as a therapeutic agent.

Materials and methods

Materials. The liver perfusion apparatus was built in the workshops of the University of Maringa. Juglone, enzymes and coenzymes used in the enzymatic assays were purchased from Sigma-Aldrich Co (St. Louis, USA). All other chemicals were from the best available grade (98–99.8% purity) and were purchased from Sigma-Aldrich, Merck (Darmstad, FRG) and Reagen (Rio de Janeiro, Brazil).

Animals. Male Wistar rats weighing 200–280 g were used in all experiments. Animals were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil) and maintained on a regulated light–dark cycle. In accordance with protocol, rats were used fed or starved for 18 h prior to the experiments. For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. All experiments were done in accordance with the worldwide accepted ethical guidelines for animal experimentation.

Liver perfusion. Hemoglobin-free, non-recirculating perfusion was performed according to the technique described by Scholz and Bücher (1965). After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The constant flow was provided by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 32 mL/min, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37 °C). The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$. The perfusion fluid enters the liver via a cannula inserted into the portal vein and leaves the organ via a cannula inserted into the cava vein (Scholz and Bücher, 1965). Samples of the effluent perfusion fluid were collected and analyzed for their metabolite contents. Substrates and drugs were added to the perfusion fluid according to the experimental protocols. Due to its low water solubility, juglone was added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration. It is already amply documented that dimethylsulfoxide does not significantly affect liver metabolism, at least not when infused at rates up to 32 μL/min (Acco et al., 2004), a limit that was never surpassed in the present work.

Analytical. In the effluent perfusion fluid the following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate, ammonia, urea and 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 (Scholz and Bücher, 1965). Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver.

For measuring the hepatic contents of glutamate, α-ketoglutarate and adenine nucleotides (AMP, ADP, ATP, NAD^+ and NADH) the perfused livers were frozen in liquid nitrogen and extracted. The acid-stable adenine nucleotides (AMP, ADP, ATP and NAD^+), glutamate and α-ketoglutarate were extracted with a 0.6 M perchloric acid solution. After mixing the liver powder with 3 volumes of the perchloric acid solution the suspension was homogenized in a Van-Potter homogenizer. The homogenate was centrifuged for 10 min at 3000 g (2 °C) and the supernatant was neutralized with potassium carbonate. Alpha-ketoglutarate and glutamate in the neutralized extract were determined by enzymatic procedures (Bergmeyer, 1974) and the adenine nucleotides by high-performance liquid chromatography (HPLC) analysis.

The acid-labile NADH was extracted with alkali. Two grams of the frozen tissue were suspended in a water–ethanol mixture (1:1) containing 0.5 M KOH in a centrifuge tube previously cooled in ice. The tubes were closed and maintained in bath at 90 °C for 5 min. After more 5 min, triethanolamine-phosphate buffer (0.5 M triethanolamine + 0.4 M KH_2PO_4 + 0.1 M K_2HPO_4) was added and the suspension was neutralized with HCl (5 M). After 10 min at room temperature the neutralized suspension was centrifuged for 5 min at 30,000 g (2 °C) and the supernatant was used for NADH assay by HPLC.

The HPLC system (Shimadzu, Japan) consisted in a system controller SCL-10AVP, two pumps model LC10AVDP, a column oven model CTO-10AVP, and a UV–VIS detector model LC10AVP. A reversed-phase column C18 HRC-ODS (5 lm; 150 · 6 mm I.D.; Shimadzu, Japan), protected with a pre-column GHRC-ODS (5 μm; 10 · 4 mm I.D.; Shimadzu, Japan), was used with a gradient from reversed-phase 0.044 M phosphate buffer solution pH 6.0 to 0.044 M phosphate buffer solution plus methanol (1.1) pH 7.0 at 0.8 mL min^{-1}. The gradient was (in% of methanol): 0 min, 0%; 2.5 min, 0.5%; 5 min, 3%; 7 min, 5%; 8 min, 12%; 10 min, 15%; 12 min, 20%; 20 min, 30%. Temperature was kept at 35 °C and the injection volume was always 20 μL. The UV-absorbance detector was auto-zeroed at the start of each chromatogram and the absorbance was measured at 254 nm for the perchloric acid extract and 340 nm for the KOH extract.

Identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained injecting standards in the same conditions, as well as by spiking liver samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. The calibration curves were constructed by separating chromato-graphically standard solutions of the compounds. Linear relationships were obtained between the concentrations and the areas under the absorbance curves.

Mitochondria isolation and measurement of respiratory activity. Fed rats were decapitated and their livers removed immediately and placed in ice-cold buffer containing 200 mM mannitol, 75 mM sucrose, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM tris (hydroxymethyl)amino-methane (Tris–HCl), pH 7.4 and 50 mg% bovine serum albumin. The tissue was minced, washed with the buffer and homogenized in the same medium by means of a Dounce homogenizer for lysing the cells. After homogenization, the mitochondria were isolated by differential centrifugation (Bracht et al., 2003; Voss et al., 1961) and suspended in the same medium, which was kept at 0–4 °C.

Oxygen uptake by isolated mitochondria was measured polarographically using a teflon-shielded platinum electrode (Clark, 1956; Voss et al., 1961). Mitochondria (0.90 ± 0.20 mg protein/mL) were incubated in the closed oxygraph chamber in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 25 mg% fatty acid-free bovine serum albumin, 10 mM Tris–HCl (pH 7.4) and two different substrates in addition to various juglone concentrations in the range between 1 and 10 μM. The substrates were succinate and β-hydroxybutyrate, both at a concentration of 10 mM. ADP, for a final concentration of 0.125 mM, was added at appropriate times. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol min^{-1} $(mg\ protein)^{-1}$. The respiratory control ratio (RC) and the ADP/O ratio were calculated according to Chance and Williams (1955).

Protein content of the mitochondrial suspensions was measured by the method described by Lowry et al. (1951), using the folin-phenol reagent and bovine serum albumin as standard.

Membrane-bound enzyme activities. The mitochondrial ATPase activity was measured in intact (coupled and uncoupled) and in freeze-thawing disrupted mitochondria according to the protocol of Bracht et al. (2003) with modifications. Intact mitochondria (1.0 mg protein/mL) were incubated in a medium containing 0.2 M sucrose, 50 mM KCl, and 10 mM Tris–HCl (pH 7.4) plus 0.2 mM EGTA and 5.0 mM ATP for 20 min, at 37 °C, in the absence (coupled) and presence (uncoupled) of 0.2 mM 2,4-dinitrophenol (DNP), in a final volume of 0.5 mL. When disrupted mitochondria were used as enzyme source, the medium contained 20 mM Tris–HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP and stopped by the addition of ice-cold 5% trichloroacetic acid. ATPase activity was evaluated by measuring the released inorganic phosphate as described by Fiske and Subbarow (1925) at 700 nm.

Freeze-thawing-disrupted mitochondria were used as enzyme source for assaying NADH and succinate oxidases. The activity of the enzymes was measured polarographically using a 20 mM Tris–HCl (pH 7.4) medium. The reaction was started by the addition of substrates, 1 mM NADH and 1 mM succinate, for NADH oxidase and succinate oxidase, respectively.

Transaminase activities in the liver homogenate. For the determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) the livers were surgically removed from anesthetized rats and homogenized in a Dounce type homogenizer. The resulting homogenate was centrifuged at 105,000 g for 30 min. The supernatant of this centrifugation was used as enzyme source. Standard commercial Kits (Gold Analisa Diagnóstica Ltda®, Belo Horizonte, Brazil) were used for AST and ALT determination. Juglone was added directly to the reaction medium at the desired concentrations.

Statistical analysis. The error parameters presented in graphs and tables are standard errors of the means. Statistical analysis was performed by means of the GraphPad Prism Software (version 5.0). Variance analysis was done with post-hoc Student-Newman–Keuls testing. The 5% level ($p<0.05$) was adopted as a criterion of significance.

Results

Effects of juglone on glycogen catabolism and glycolysis

The first experiments were planned for testing possible effects of juglone 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. Fig. 2A illustrates the responses of perfused livers to juglone infusion at the concentration of 50 μM. It also illustrates a typical experimental protocol, which was used for all other juglone concentrations. After a pre-perfusion period of 10 min, juglone 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. As revealed by Fig. 2A all parameters were stable before the initiation of juglone infusion. Upon juglone infusion, oxygen uptake increased and remained so during the entire infusion period. Glucose release was increased with a peak value 50% above the basal values. Lactate production was also increased with peak values 60% above the basal rates. Pyruvate production increased slowly and at the end of the juglone infusion (40 min perfusion time) it was 90% above the basal value. After removing the drug from the perfusion liquid, stimulations of oxygen consumption and pyruvate production were maintained for at least 20 min, but glucose release and lactate production returned to their basal levels.

Experiments like those illustrated in Fig. 2A were repeated with 10 and 20 μM juglone in order to establish concentration dependences for the effects. The mean values for each parameter at the end of the juglone infusion period (40 min perfusion time) were evaluated. Oxygen consumption, glycogenolysis [glucose release plus ½(pyruvate plus lactate productions)] and glycolysis (pyruvate plus lactate productions) were represented against the juglone concentration in Fig. 2B. All stimulations present saturation, with little changes after 20 μM juglone. In Fig. 2C, the lactate to pyruvate ratio, an indicator for the cytosolic NADH/NAD^+ ratio (Scholz and Bücher, 1965), was plotted against the drug concentration. Juglone up to 20 μM increased the NADH/NAD^+ ratio, but with 50 μM it returned to the value in the absence of juglone.

Effects of juglone on gluconeogenesis and ureogenesis

Since juglone affects mitochondrial energy metabolism (Makawiti et al., 1990) it should also affect ATP-dependent pathways, such as gluconeogenesis and ureogenesis. Figs. 3A and B show results of experiments in which the action of juglone on lactate gluconeogenesis 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 juglone infusion at the concentration of 50 μM and it also represents a typical experimental protocol. After a pre-perfusion period of 10 min in the absence of substrate, 2 mM lactate was infused during 20 min, followed by additional 30 min of juglone plus lactate infusion. In the absence of juglone the infusion

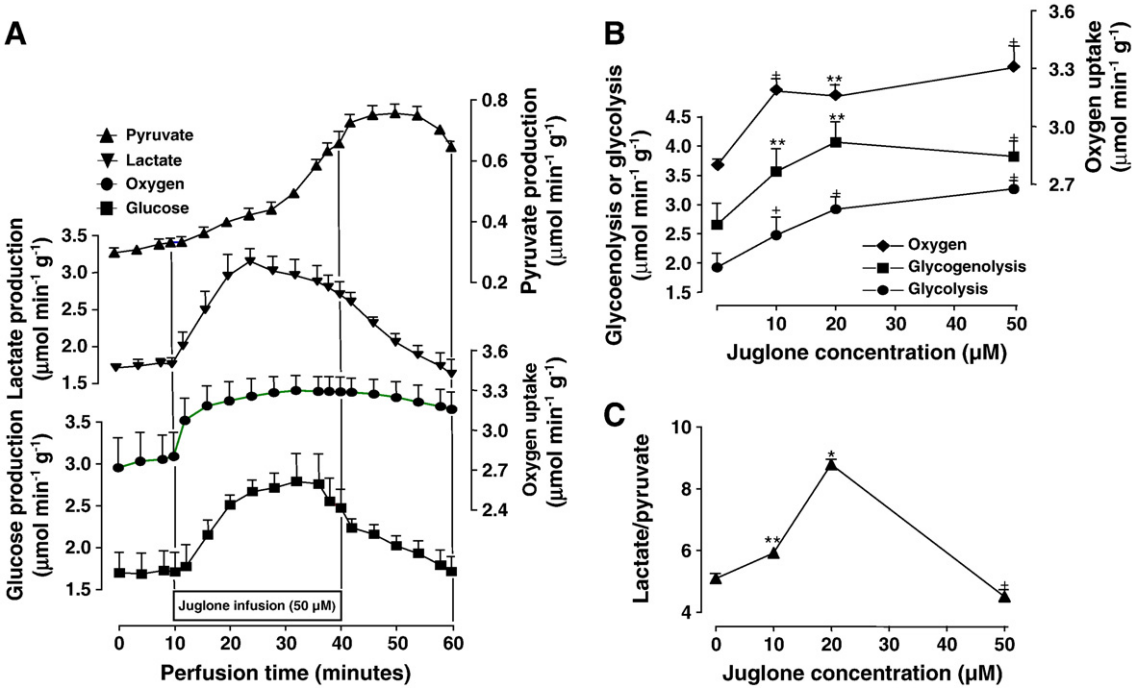

Fig. 2. Actions of juglone in glycogen catabolism and oxygen uptake. Livers from fed rats were perfused as described in the Materials and methods section. The effluent perfusate was sampled in 4-min intervals and analyzed for glucose, lactate, and pyruvate. Oxygen consumption was followed polarographically. Panel A shows the time course of experiments in which 50 μM juglone was infused as indicated. In the graphs of panels B and C the concentration dependences of the effects of juglone on oxygen uptake, glycogenolysis, glycolysis (panel B) and the lactate to pyruvate ratio (panel C) were represented. Values of the parameters at the end of juglone infusion (40 min perfusion time) were represented. Glycogenolysis and glycolysis are expressed as glucosyl units and were calculated as: glucose production + 1/2(lactate + pyruvate production) and lactate + pyruvate production, respectively. Each datum point represents the means of 3–4 liver perfusion experiments. Bars are standard errors of the mean. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($^{+}$ $p<0.05$, ** $p<0.01$, * $p<0.001$).

of 2 mM lactate produced rapid and sustained increases in both glucose production and oxygen uptake. The infusion of 50 μM juglone caused a progressive and, at the end, very strong decrease in glucose production. No recovery occurred during the 20 min following cessation of the drug infusion. Initially no changes in oxygen consumption were apparent when the juglone infusion was started. Inhibition, however, begun to occur at 54 min perfusion time. This inhibitory trend was maintained after cessation of juglone infusion. Fig. 3B allows an evaluation of the effects of several juglone concentrations on oxygen uptake and glucose production from lactate in the range of 5.0–50 μM. The final values observed at the end of the juglone infusion period (60 min perfusion time) were represented against the juglone concentrations. Glucose production was inhibited over the whole range of the juglone concentrations. Numerical interpolation revealed 50% inhibition at the juglone concentration of 14.9 μM. Oxygen uptake, on the other hand, was stimulated by juglone up to 20 μM, with maximal stimulation at 5 μM. Inhibition occurred at 50 μM, as also shown in Fig. 3A.

Alanine gluconeogenesis was also investigated. This substrate induces a more oxidized state when compared to lactate and the transfer of the amine group also influences the urea cycle and several related pathways. Fig. 4A shows the effects of 50 μM juglone on the carbon fluxes and oxygen uptake due to alanine infusion whereas Fig. 4B illustrates the changes in the nitrogen fluxes. The infusion of 2.5 mM alanine caused a rapid increase in glucose production and oxygen uptake (Fig. 4A). The subsequent infusion of 50 μM juglone was strongly inhibitory for glucose production. Oxygen consumption underwent an initial transitory increase that was reversed to inhibition at 60 min perfusion time (Fig. 4A). Finally, 50 μM juglone strongly stimulated lactate and pyruvate production. The nitrogen fluxes were also affected (Fig. 4B). Ammonia and glutamate production were both clearly stimulated by the drug. Urea production underwent an initial transitory increase, which was followed by inhibition. The concentration dependences of the juglone effects on alanine metabolism are shown in Fig. 5. Inhibition of glucose production presents a clear concentration dependence, with 50% inhibition at the concentration of 15.7 μM. Stimulations of ammonia and glutamate productions were saturable functions of the juglone concentration in the range up to 50 μM, with half-maximal stimulations at 4.15 and 5.1 μM, respectively. Lactate and urea production were stimulated in the range up to 20 μM, with a declining tendency at 50 μM. Oxygen uptake was also stimulated by juglone up to 20 μM, but diminished to values below the basal ones at the concentration of 50 μM. Pyruvate production, finally, was stimulated over the whole concentration range with a parabolic dependence.

For the sake of comparison the experiments with alanine as the substrate were repeated using the classical uncoupler 2,4-dinitrophenol (experiments not shown). The effects of this compound were similar to the actions of juglone. Gluconeogenesis was 50% inhibited at a concentration of 17.9 μM. Ammonia release and urea production were also stimulated by 2,4-dinitrophenol, with half-maximal effects at 4.55 and 4.76 μM, respectively.

Effects of juglone on transaminases and tissue contents of α-ketoglutarate and glutamate

Although it inhibits gluconeogenesis from alanine, juglone increases all other parameters, notably glutamate and ammonia production. This indicates an overall increase in alanine transformation. Increased alanine transformation necessarily requires increased alanine aminotransferase (ALT) activities in the cytosol. For this reason the action of juglone on this enzyme from liver homogenates was measured. No effects, however, were detected in the range up to 50 μM after four determinations (control, 0.19 ± 0.01 and 50 μM juglone,

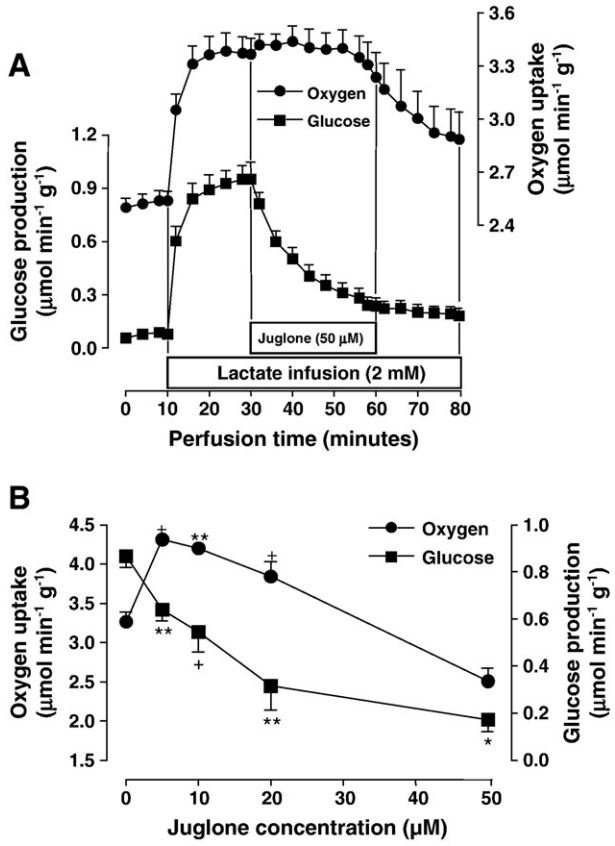

Fig. 3. Effects of juglone on gluconeogenesis from lactate in perfused livers isolated from fasted rats. Livers from fasted rats were perfused as described in the Materials and methods section. Lactate (2 mM) and juglone were infused as indicated by the horizontal bars in panel A, which shows the time course of the changes caused by 50 µM juglone in glucose production and oxygen consumption. Panel B shows the concentration dependence of the effects of juglone on oxygen consumption and gluconeogenesis. The experimental protocol was the same for all juglone concentrations. Values of the parameters at the end of juglone infusion (60 min perfusion time) were represented. Each datum point is the mean of 4 experiments. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($^{+}$ $p<0.05$, ** $p<0.01$, * $p<0.001$).

0.18 ± 0.01 µmol min^{-1} mg $protein^{-1}$). Juglone was also without effect on the activity of aspartate aminotransferase (AST; control, 0.29 ± 0.01 and 50 µM juglone, 0.28 ± 0.06 µmol min^{-1} mg $protein^{-1}$).

In the absence of direct effects on alanine aminotransferase, an increased flux through this enzyme in the cell can be caused by increased concentrations of α-ketoglutarate, the second substrate of the enzyme. Fig. 6 shows the results of experiments in which the tissue contents of α-ketoglutarate and L-glutamate were measured in the presence of alanine alone and in the simultaneous presence of alanine and juglone at two different concentrations, 20 and 50 µM. The graph in Fig. 6 reveals a very pronounced increase in the hepatic α-ketoglutarate content in the presence of both 20 and 50 µM juglone. The glutamate content, however, was not significantly increased by 20 µM juglone and even diminished by 50 µM juglone.

Effects of juglone on the hepatic contents of adenine mono- and dinucleotides

Measurement of the adenine mono- and dinucleotide levels under the gluconeogenic conditions induced by alanine can perhaps be helpful in the interpretation of the effects of juglone. Table 1 lists the results found using livers from fasted rats in the presence of 2.5 mM alanine alone and in the simultaneous presence of 20 µM juglone. It is apparent that 20 µM juglone reduced the levels of ATP and increased those of ADP and AMP. Consequently, the ATP/ADP and ATP/AMP ratios were also reduced by 37% and 60%, respectively. Concerning the NAD^{+}–NADH couple, 20 µM juglone significantly diminished the level of the oxidized form, but increased that of the reduced form. In consequence, the NADH/NAD^{+} ratio was elevated six-fold by juglone.

Effects of juglone on respiration and on membrane-bound enzymatic activities of isolated rat liver mitochondria

The effects of juglone on the respiratory activity of isolated mitochondria were investigated in the concentration range between 1 and 10 µM. Succinate and β-hydroxybutyrate were used as substrates in the presence or absence of ADP. The respiration rates were measured under three conditions: a) before the addition of ADP (substrate respiration), b) just after ADP addition (state III respiration) and c) after cessation of the ADP stimulation (state IV respiration). With succinate as the substrate (Fig. 7A) juglone increased gradually in a concentration dependent manner both substrate and state IV respiration but diminished state III respiration. When β-hydroxybutyrate was the substrate (Fig. 7B), state III respiration was also diminished, but to a higher degree. Substrate respiration and state IV respiration, however, tended to increase at low concentrations and to decrease at higher concentrations.

Table 2 shows the effects of juglone on the ADP/O and respiratory control ratios (RC). As noted, juglone reduced significantly the ADP/O ratio already at the concentration of 1 µM when β-hydroxybutyrate was the substrate. At the concentration of 5 µM the ADP/O ratio could no longer be determined. The respiratory control ratio was also reduced and eventually abolished, depending on the concentration. Similar results were obtained when succinate was the substrate, but at somewhat higher concentrations.

The uncoupling action of juglone was further investigated by measuring the ATPase, NADH-oxidase and succinate-oxidase activities of rat liver mitochondria. The ATPase activity was measured using mitochondria under three different conditions: intact (coupled), freeze-thawing disrupted and 2,4-dinitrophenol uncoupled. Fig. 8A shows that the ATPase activity was stimulated by juglone in the range between 1 and 10 µM, but with a maximum at 2.5 µM. The ATPase activity of disrupted and uncoupled mitochondria, however, was relatively insensitive to juglone in the range up to 2.5 or 5 µM, and inhibited at higher concentrations. The actions of juglone on the NADH- and succinate-oxidase activities are shown in Fig. 8B. The NADH-oxidase activity was stimulated at concentrations between 5 and 10 µM; the succinate-oxidase activity, however, was not significantly affected.

Discussion

The main conclusion that can be drawn from the bulk of the data obtained in the present work is that juglone is active on liver metabolism and able to affect several metabolic routes which are linked in some way to energy metabolism. In general, most observations in the perfused liver are compatible with its reported uncoupling action. The most important 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 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 two different substrates, namely lactate and alanine; e) stimulation of glycolysis as a cytosolic compensatory phenomenon for the diminished mitochondrial ATP production (Soboll et al., 1978; Suzuki-Kemmelmeier and

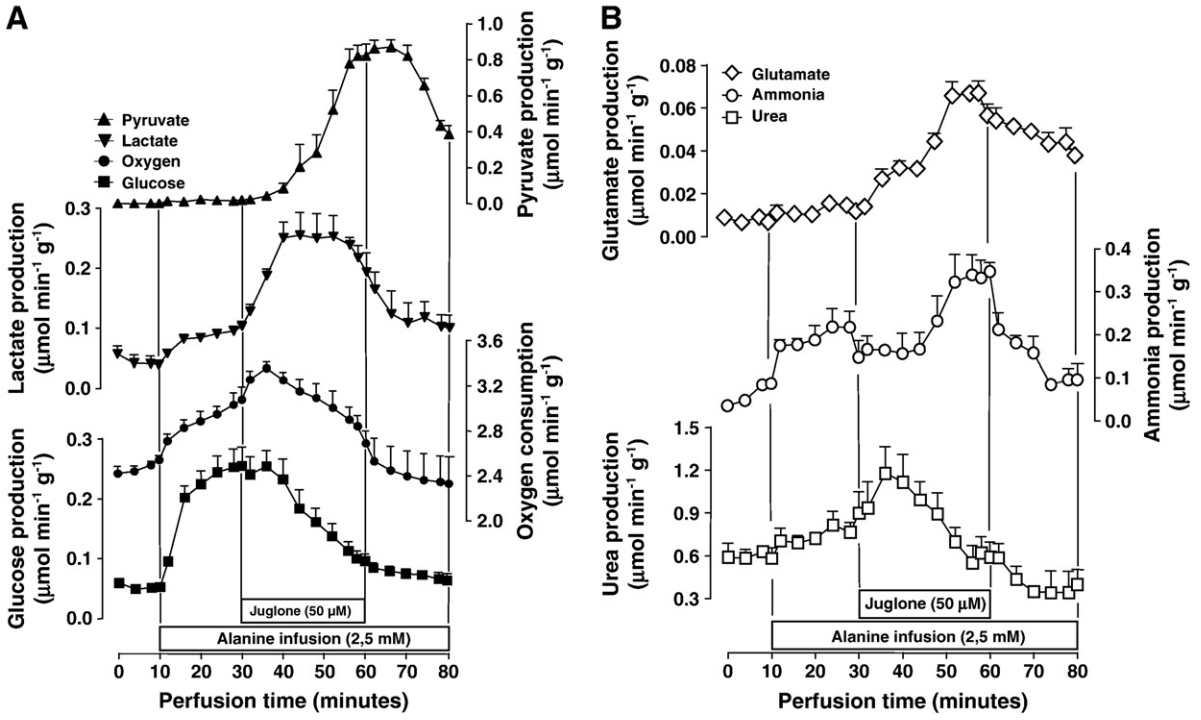

Fig. 4. Time courses of the effects of 50 μM juglone on alanine metabolism. Livers from fasted rats were perfused as described in the Materials and methods section. Alanine (2.5 mM) and juglone were infused as indicated. Oxygen uptake (panel A) was measured polarographically in the effluent perfusate. Samples of the effluent perfusate were collected for the enzymatic assay of pyruvate, lactate, glucose (panel A), glutamate, ammonia and urea (panel B). Each datum point is the mean ± SEM of 4 liver perfusion experiments.

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).

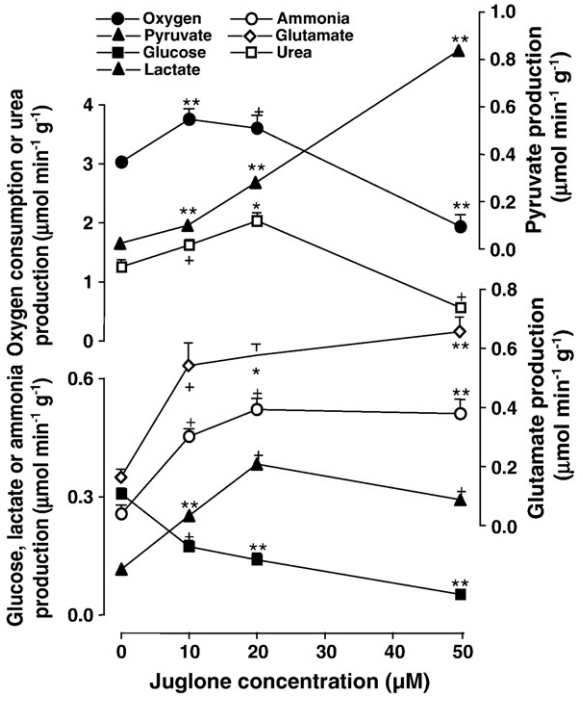

Fig. 5. Concentration dependence of the effects of juglone on parameters dependent on alanine metabolism. The experimental protocol was the same illustrated by Fig. 4. Each datum point is the mean of 4 experiments. Values of the parameters at the end of juglone infusion (60 min perfusion time in Fig. 4) were represented. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($^{+}$ $p<0.05$, ** $p<0.01$, * $p<0.001$).

Experiments with isolated mitochondria, based on the original observations of Makawiti et al. (1990), allowed to characterize further the actions of juglone on the organelle. The uncoupling action of juglone was fully corroborated by the diminished ADP/O and respiratory control ratios as well as by the stimulated ATPase activity in intact mitochondria. It must be remarked, however, that in disrupted mitochondria and in mitochondria that were previously uncoupled by the addition of 2,4-dinitrophenol, juglone promoted inhibition of the ATPase activity at concentrations above 5 μM. This suggests an additional effect for the compound, namely inhibition of the ATP-synthase. This conclusion is further corroborated by the observation that state III respiration was inhibited even at low concentrations. Furthermore, in intact mitochondria, stimulation of the ATPase

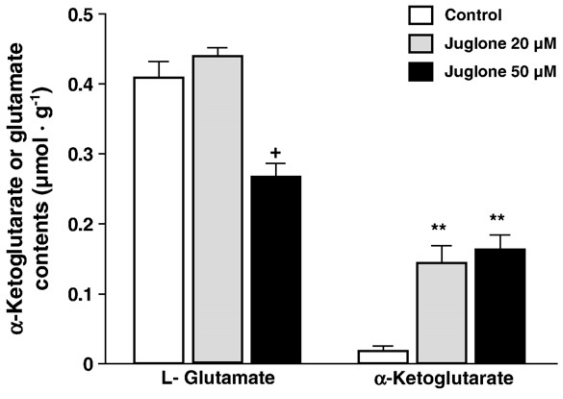

Fig. 6. Influence of juglone on the hepatic content of glutamate and α-ketoglutarate. Livers from fasted rats were perfused with 2.5 mM alanine and juglone as described in Material and methods. At 60 min perfusion time (see Fig. 4) the perfused livers were clamped in liquid nitrogen and extracted with perchloric acid. The neutralized extracts were used for glutamate and α-ketoglutarate enzymatic assay. Values are the means ± SEM for 4 perfusion experiments. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing (** $p<0.01$, $^{+}$ $p<0.05$).

Table 1
Influence of juglone on hepatic contents of adenine mono- and dinucleotides in the presence of 2.5 mM alanine.

Parameter	Control (n = 5)	20 μM juglone (n = 5)
	μmol × (g liver wet weight)$^{-1}$	
ATP	1.704 ± 0.095	1.389 ± 0.076*
ADP	0.622 ± 0.042	0.794 ± 0.053*
AMP	0.246 ± 0.046	0.442 ± 0.066*
ATP + ADP + AMP	2.571 ± 0.170	2.626 ± 0.165
NAD^+	0.457 ± 0.042	0.332 ± 0.016*
NADH	0.007 ± 0.001	0.040 ± 0.010*
	Ratios	
ATP/ADP	2.766 ± 0.151	1.766 ± 0.102*
ATP/AMP	7.923 ± 1.292	3.354 ± 0.392*
NADH/NAD^+	0.017 ± 0.001	0.119 ± 0.029*

Livers from fasted rats were perfused in an open system as described in the Materials and methods section. Alanine (2.5 mM) was infused at 10 min and juglone (20 μM) was infused 20 min after alanine infusion for 30 min. The livers were freeze-clamped in liquid nitrogen and the adenine nucleotides were extracted with cold perchloric acid (with alkali for NADH determination). Control determinations were done with livers that were freeze-clamped at the same perfusion time in the presence of alanine but without juglone infusion. Values are means ± SEM.
* $p<0.05$.

Table 2
Actions of juglone on the mitochondrial respiration driven by succinate or β-hydroxybutyrate in the presence and absence of exogenously added ADP.

Juglone (μM)	β-Hydroxybutyrate (n = 8)		Succinate (n = 8)	
	ADP/O ratio	Respiratory control ratio	ADP/O ratio	Respiratory control ratio
0	3.73 ± 0.20	3.51 ± 0.17	2.55 ± 0.15	3.96 ± 0.22
1	2.85 ± 0.16*	2.83 ± 0.18*	2.28 ± 0.10*	3.67 ± 0.27
2.5	2.77 ± 0.13*	1.80 ± 0.26*	1.98 ± 0.12*	2.31 ± 0.27*
5	–[a]	1.31 ± 0.14*	1.71 ± 0.15*	2.31 ± 0.29*
7.5	–[a]	1.82 ± 0.07*	–[a]	1.50 ± 0.15*
10	–[a]	1.00 ± 0.00*	–[a]	1.30 ± 0.12*

Mitochondria were isolated and assayed as described in the Materials and methods section. Incubations were done in the presence of substrate (10 mM) as indicated. Values are means ± SEM.
* Statistically significant relative to the controls (variance analysis with post hoc Newman–Keuls esting; $p<0.05$).
[a] Very low value, no accurate determination possible.

activity was maximal at 2 μM and declined at higher concentrations. This kind of response is normally observed when two opposite effects are present (Kelmer-Bracht et al., 1985). It should be noted that the inhibitory effect at higher concentrations was also found in the intact cells: under several experimental conditions inhibition of oxygen consumption by the liver was found at the highest juglone concentrations, especially under gluconeogenic conditions. Concerning the enzymatic systems responsible for electron flow in the respiratory chain, juglone did not inhibit succinate-oxidase but, surprisingly,

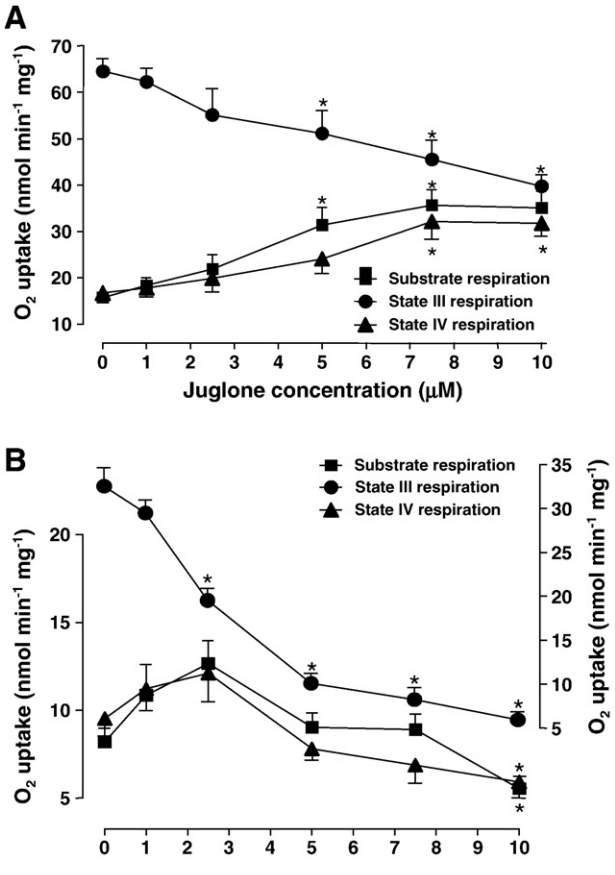

Fig. 7. Effects of juglone on the respiratory activity of isolated rat liver mitochondria. Mitochondria (0.25–2.5 mg/mL) were added to the reaction medium in the closed vessel of the oxygraph. The reaction was initiated by the addition of succinate (panel A) or β-hydroxybutyrate (panel B) and the oxygen consumption was followed polarographically for 5 min. After this time 0.25–0.5 nmol of ADP was added. Rates of oxygen consumption were computed from the slopes of the polarographic records. Each datum point is the mean ± SEM of 6 independent experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($p<0.05$).

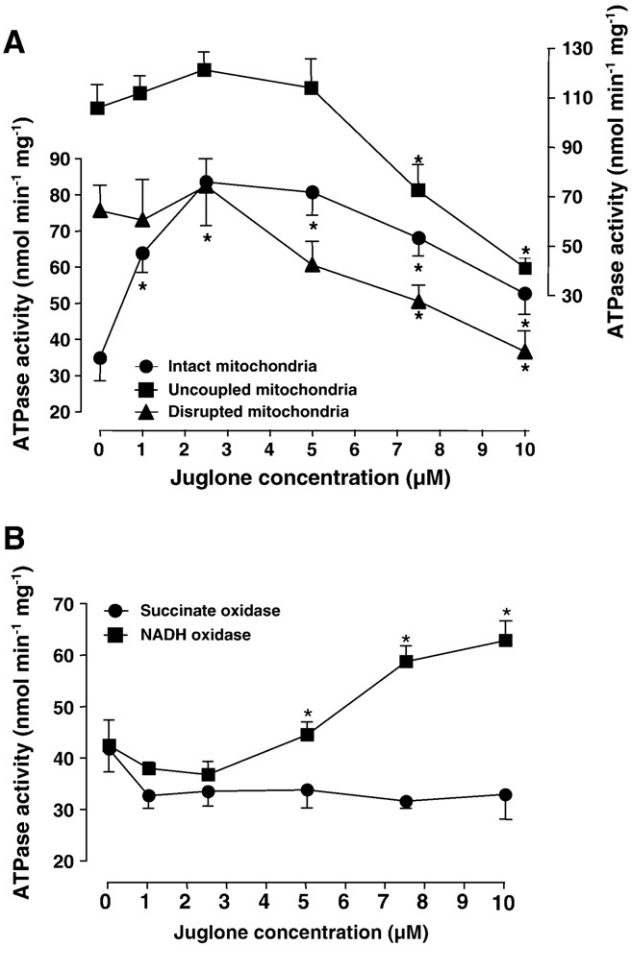

Fig. 8. Effects of juglone on mitochondrial membrane-bound enzymatic activities. The experiments in panel A (ATPase activity) were done with intact mitochondria and those in panel B (succinate and NADH oxidases) with freeze-thawing disrupted mitochondria. The mitochondria were incubated at 37 °C in reaction media described in the Materials and methods section. Each datum point represents the mean ± SEM of 6 experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Student-Newman–Keuls testing ($p<0.05$).

stimulated the NADH-oxidase activity of disrupted mitochondria. The latter phenomenon could be contributing for the stimulation of oxygen uptake in the intact liver. No mechanistic explanation for this effect can be drawn from our experimental data.

The experiments with alanine as the substrate allowed us to examine in more detail the action of uncouplers on nitrogen metabolism. To our knowledge, there are no reports in which this aspect has been analyzed more extensively in the intact liver cell although some indications can be drawn from experiments with isolated mitochondria. The scheme in Fig. 9 summarizes some of the events related to alanine metabolism that will be discussed here. The scheme shows several enzyme catalyzed transformations but it also emphasizes the compartmentation of both α-ketoglutarate and L-glutamate (Soboll et al., 1980). In isolated mitochondria it has been found that uncouplers increase L-glutamate deamination which leads to α-ketoglutarate production (Nilova, 1977; Quagliariello et al., 1965). Uncouplers also increase the tricarboxylic acid cycle where the mitochondrial isocitrate dehydrogenases (NAD^+ and $NADP^+$-dependent) can transform isocitrate into α-ketoglutarate. Consistent with these notions about the mitochondrial metabolism, we found increased cellular levels of α-ketoglutarate during juglone infusion. Alpha-ketoglutarate can also be produced in the cytosol by the cytosolic isocitrate dehydrogenase as shown in Fig. 9. There is a recent suggestion that this might even be the most important route for α-ketoglutarate production (Rokhmanova and Popova, 2006) but there are not data about the action of uncouplers on this event. The increased levels of α-ketoglutarate are the most probable cause for the increased rates of alanine transformation caused by juglone. The latter phenomenon is indicated by the increased release of ammonia and urea caused by the drug, in spite of the reduced rates of glucose production. In the absence of any direct effect of juglone on the alanine aminotransferase (ALT), the only possibility for enhancing alanine deamination in the presence of a constant concentration of this amino acid is to raise the concentration of the second substrate of this enzyme, which is α-ketoglutarate. It should be added that no short-term regulation mechanism for ALT is known.

The increase in L-glutamate release caused by juglone must be examined in terms of the characteristics of the pertinent transport system. Transport of L-glutamate into the cell is of the concentrative type. The cellular concentration of L-glutamate is generally much higher than the extracellular concentration. In our experiments, for example, a L-glutamate production rate of 0.39 μmol min^{-1} g^{-1} corresponds to a mean portal-venous concentration of 0.05 mM, whereas the cellular content reaches 0.5 mM. The high-affinity glutamate transporters mediate transport of L-glutamate by the cotransport of 3 Na^+ and 1 H^+, and the countertransport of 1 K^+ (Kanai and Hediger, 2004; Mann et al., 2003). It is this coupling that allows uphill transport of glutamate into cells against a concentration gradient. Consequently, it would not be surprising if uncoupling, which changes the membrane permeability to H^+, causes an increased leakage of L-glutamate because the inward directed concentration gradient cannot be maintained. Furthermore, the coupling is ultimately energy-dependent, which under energy deficient conditions can also be impaired. This would explain the increased rates of L-glutamate release in the presence of juglone even in the absence of increased cellular concentrations. On the other hand, compartmentation of L-glutamate could equally play some role. Soboll et al. (1980) have shown that L-glutamate is present at different concentrations in the cytosol and in the mitochondria. In the liver of fasted rats under substrate-free perfusion, for example, the cytosolic and mitochondrial concentration of L-glutamate are 2.65 and 0.65 mM, respectively. It could be that in our experiments, the cytosolic concentration of L-glutamate was raised by juglone whereas the mitochondrial one was decreased in such a way that the total content of the liver cells remained more or less the same. This is a real possibility if one takes into account the fact that uncoupling stimulates L-glutamate deamination in the mitochondria (Quagliariello et al., 1965; Nilova, 1977; see Fig. 9) a phenomenon that tends to decrease the mitochondrial concentration. The opposite occurs in the cytosol where the L-glutamate concentration can be expected to increase by virtue of the increased α-ketoglutarate concentration which increases the rate of the ALT reaction.

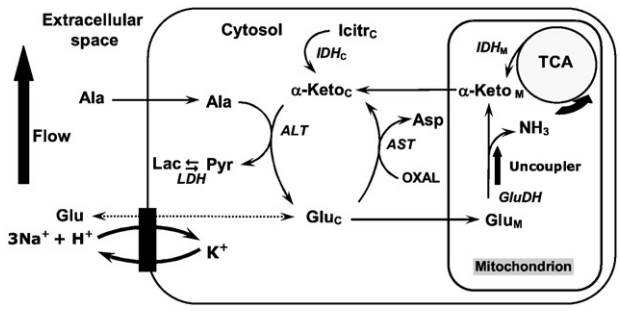

Fig. 9. Schematic representation of important events leading to the formation of ammonia, aspartate and glutamate in consequence of alanine infusion. Symbols: Ala, alanine; Pyr, pyruvate; Lac, lactate; α-Keto$_C$, cytosolic α-ketoglutarate; Glu$_C$, cytosolic L-glutamate; α-Keto$_M$, mitochondrial α-ketoglutarate; Glu$_M$, mitochondrial L-glutamate; Asp, aspartate; OXAL, oxaloacetate; TCA, tricarboxylic acid cycle; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Icitr$_C$, cytosolic isocitrate; LDH, lactate dehydrogenase; IDH$_C$, cytosolic isocitrate dehydrogenase; IDH$_M$, mitochondrial isocitrate dehydrogenase; GluDH, glutamate dehydrogenase. The thick arrows indicate the steps that can be directly stimulated by uncouplers.

A final point to be discussed is one concerned with the significance of the effects that were observed in the present work which are potentially toxic to animals. In nature it is known that juglone retards the growth of competing plants under walnut trees (Jose and Gillispie, 1998). Since uncouplers usually break down the proton electrochemical gradient in chloroplasts in the same way as in mitochondria, this could be the likely reason why juglone is also toxic to plants. Juglone is unavoidably ingested by humans when walnut extracts are used in popular medicine and it is worth to examine how this could affect the general physiology (Bell, 1981; Jin, 2010; Mahoney et al., 2000). Uncouplers were used in the past as weight loss agents, especially 2,4-dinitrophenol. Since uncouplers reduce the efficiency of energy transduction in the mitochondrial electron transport chain, more fuel has to be oxidized in order to produce the same amount of ATP. This fuel comprises largely fatty acids, weight loss is thus an understandable effect of uncoupling agents. Most of them are quite dangerous due to their narrow therapeutic window, i.e., the small concentration range between mild and nearly full uncoupling. The latter is a highly toxic condition. It has been proposed that uncouplers with a wide therapeutic window would be more appropriate and less dangerous as therapeutic agents for weight loss (Lou et al., 2007). One such compound is 2,6-bis(1,1-dimethylethyl)-4-methylphenol, more commonly known as BHT. This compound already uncouples at extremely low concentrations, 2×10^{-12} M, but it produces only modest increases in uncoupling as its concentration is raised to 2 μM (Lou et al., 2007). Most other uncouplers, including 2,4-dinitrophenol show a much narrower range of activity, generally comprising not much than one order of magnitude. From the results obtained in the present work it is evident that juglone must be classified as a narrow range uncoupler. In isolated mitochondria its action is exerted in the 10^{-6} to 10^{-5} M range. In the perfused liver, the consequences of this action are detectable in the 10^{-6} to 2×10^{-5} M range. In this particular, thus, it resembles more closely the classical uncoupler 2,4-dinitrophenol. Ingestion of high doses of juglone, consequently, presents the same risks as the ingestion of high doses of 2,4-dinitrophenol which comprise excessive compromising of ATP production, hyperthermia and even death. It should also be noted that blocking of transcription, induction of DNA damage, reduction of protein levels and induction of cell death are all effects that occur within the same concentration range as the effects observed in the present work (Paulsen and Ljungman, 2005). The use of juglone as an anticancer agent, thus, is not deprived of considerable risk if one takes into account the doses that are

necessary for this action. On the other hand, there are recent reports showing that mild uncoupling could have beneficial effects because in medium term the increased energy expenditure and oxygen consumption causes little or no changes in the ATP levels. Mild uncoupling apparently trigers molecular mechanisms that are able to increase both mitochondrial gene expression and mitochondrial volume (Rohas et al., 2007). In agreement with these observations it has also been shown that mild uncoupling increases longevity in mice a phenomenon that was associated with the improvement of several serological markers such as glucose, triglycerate and insulin levels (Caldeira da Silva et al., 2008). These observations suggest that moderate consumption of natural products containing juglone can be beneficial to health especially during aging.

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Conflict of interest

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

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