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VANESA DE OLIVEIRA PATEIS

Efeitos metabólicos do aditivo alimentar BHA no fígado de rato em perfusão

Maringá
Junho de 2017

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Dissertação apresentada à
Universidade Estadual de Maringá,
como requisito parcial para a obtenção
do título de mestre.

Orientador: Dr. Adelar Bracht
Coorientadora: Dra. Lívia Bracht

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Aprovado em:

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Prof. Orientador
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APRESENTAÇÃO

Vanesa de Oliveira Pateis nasceu em Maringá/PR em 03/09/1990. Possui graduação em Bioquímica pela Universidade Estadual de Maringá (2014).

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RESUMO GERAL

INTRODUÇÃO

O hidroxianisol butilado (BHA) é amplamente utilizado como antioxidante em alimentos processados, cosméticos, produtos farmacêuticos e petroquímicos. Em mitocôndrias isoladas, estudos demonstram que o BHA diminui o controle respiratório estimulando a respiração do estado 4, dissipa o potencial da membrana e induz a perda de cálcio. Portanto, estes estudos mostram que o BHA age provavelmente como um desacoplador e dois mecanismos possíveis poderiam explicar tal condição: o BHA poderia estar atuando como um transportador de prótons através da membrana interna da mitocôndria, ou então estar causando desintegração desta membrana. A hipótese de que BHA pode modificar os fluxos metabólicos em virtude de sua ação inibitória sobre o metabolismo energético ainda não foi testada. É interessante saber se BHA é capaz de interagir com as mitocôndrias na célula intacta da mesma maneira que interage com mitocôndrias isoladas. Este foi o propósito do presente trabalho e para atingir esse objetivo foi utilizado o sistema experimental de fígado de rato em perfusão. Nesse sistema a microcirculação e a polaridade celular são preservados, além da integridade celular.

MATERIAIS E MÉTODOS

Ratos machos Wistar pesando 220-240 g foram utilizados em todos os experimentos. O sistema de perfusão de fígado não-recirculante, com meio isento isento de hemoglobina, foi utilizado e o fluído de perfusão foi o tampão Krebs/Henseleit-bicarbonato (pH 7,4), contendo 25 mg% de albumina de soro bovino e saturado com uma mistura carbogênica (95% de O_2 e 5% de CO_2). Foram avaliados os efeitos de diferentes concentrações de BHA sobre as seguintes vias metabólicas: a) glicogenólise, b) glicólise c) consumo de oxigênio e d) gliconeogênese a partir de três substratos diferentes (L-lactato, Frutose e L-alanina). Com L-alanine como substrato foram avaliados também a ureogênese e a amoniogênese. Os substratos e o BHA foram adicionados diretamente no líquido de perfusão. Por ser uma substância lipofílica, o BHA foi previamente dissolvido em dimetilsulfóxido antes de ser adicionado ao líquido de perfusão. As concentrações utilizadas de BHA variaram entre 50 e 750 μM. O perfusado efluente foi coletado para análises enzimáticas dos metabólitos produzidos

(produções de glicose, lactato, piruvato, amônia e ureia) e o consumo de oxigênio foi monitorado continuamente com um eletrodo de platina. Os conteúdos de nucleotídeos de adenina também foram medidos. Para tanto, após a perfusão com BHA, o fígado foi clampeado em nitrogênio líquido, extraído com ácido perclórico e o extrato foi neutralizado com K_2CO_3. As quantidades de AMP, ADP e ATP foram determinadas por HPLC.

RESULTADOS

Os primeiros experimentos foram realizados com fígados de ratos alimentados, o que permite avaliar o catabolismo do glicogênio e a glicólise. Determinou-se, portanto, os efeitos do BHA sobre a liberação de glicose, lactato, piruvato e sobre o consumo de oxigênio. Em altas concentrações de BHA, houve uma tendência em direção à diminuição no consumo de oxigênio, embora os resultados não tenham sido estatisticamente significativos. Todavia, o BHA provocou um nítido aumento da liberação de glicose e da produção de lactato, nas concentrações de 500 e 750 μM. Avaliamos ainda, o efeito do BHA sobre a gliconeogênese e, de maneira geral, este aditivo foi capaz de inibir a produção de glicose a partir dos três substratos utilizados (L-lactato, frutose e L-alanina). Particularmente em relação à gliconeogênese a partir de L-lactato, o efeito mais forte do BHA foi sobre a produção de glicose, uma vez que a inibição foi evidenciada a partir da concentração de 100 μM e cálculos de interpolação numérica demonstram que 50% de inibição deste parâmetro pode ser esperada com uma concentração de BHA igual a 279,6 μM A inibição do consumo de oxigênio também foi evidente, mas teve início a partir da concentração de 200 μM. A produção de piruvato a partir de L-lactato foi levemente estimulada pelo BHA, também a partir da concentração de 200 μM. Quando a frutose foi o substrato, o BHA provocou uma inibição da produção de glicose a partir de 100 μM e 50% de inibição pode ser esperada com 460,5 μM. Houve também inibição do consumo de oxigênio, que foi mais mais acentuada em altas concentrações (500 e 750 μM). A produção de piruvato e de lactato foi estimulada pelo BHA, sendo que o estímulo da produção de lactato foi claramente dependente da concentração de BHA. A infusão de alanina como substrato permite a avaliação tanto do metabolismo de carbono quanto do metabolismo de nitrogênio. O BHA inibiu a produção de glicose a partir de alanina de maneira dependente da concentração, iniciando em 50 μM. Uma

inibição de 50% sobre este parâmetro pode ser esperada com uma concentração de BHA igual a 233,3 μM. A produção de lactato e piruvato foi apenas levemente estimulada pelo BHA. O consumo de oxigênio foi inibido, com uma cinética de inibição similar àquela causada pelo BHA com os substratos L-lactato e frutose. Além disso, o BHA estimulou a produção de amônia e inibiu a produção de ureia nas concentrações de 500 e 750 μM. Vale ressaltar que após a remoção do BHA do líquido de perfusão, os efeitos metabólicos deste aditivo foram apenas parcialmente revertidos, em algumas situações, ou não foram revertidos, na maioria das situações. Finalmente, analisaram-se os níveis de mononucleotídeos de adenina (ATP, ADP e AMP) em fígados de ratos alimentados e em jejum. Para a primeira situação, o BHA na concentração de 500 μM não causou diminuição no conteúdo de ATP e um discreto, mas significativo, aumento nos níveis de ADP. Quanto ao fígado de rato em jejum, o BHA na concentração de 750 μM causou uma diminuição de 48% no conteúdo de ATP e diminuição de 40% no de ADP.

DISCUSSÃO

Os resultados mostram que o BHA atua no metabolismo do fígado e é capaz de afetar várias vias metabólicas que são dependentes ou ligadas ao metabolismo energético. As observações mais importantes foram: a) inibição do consumo de oxigênio; b) diminuição do conteúdo de ATP, combinado com modificações da razão ATP/ADP e ATP/AMP; c) aumento da razão NADH/NAD^+ citosólica; d) inibição da gliconeogênese a partir de três substratos diferentes; e) estimulação da glicólise e da frutólise; f) estimulação da glicogenólise; g) dano à detoxificação da amônia. As modificações nos fluxos metabólicos podem ser, de maneira geral, atribuíveis aos efeitos do BHA sobre a cadeia respiratória mitocondrial. Em estudos com mitocôndrias isoladas, o BHA foi capaz de bloquear o transporte de elétrons na cadeia respiratória, mas também apresentou um efeito de desacoplador. O primeiro fenômeno levaria a inibição do consumo de oxigênio no fígado, enquanto o segundo estimularia o consumo de oxigênio. No fígado em perfusão, todavia, o estímulo no consumo de oxigênio não foi observado em nenhuma das concentrações de BHA utilizadas no presente trabalho. Ao contrário, no fígado perfundido de ratos em jejum, foi observado apenas inibição no consumo de oxigênio. Em ratos alimentados, todavia, na ausência de substratos exógenos, o BHA não foi capaz de inibir o consumo de

oxigênio. Uma possível explicação para esta diferença no consumo de oxigênio na condição de jejum (com substrato) e alimentada (sem substrato) deve levar em conta dois fatores: (a) as condições particulares do fígado perfundido sem substrato, nas quais as condições mitocondriais estejam mais próximas daquelas encontradas no estado 4 da respiração, onde há predomínio de desacoplamento; (b) a contribuição de oxidases de função mista microsomais responsáveis pela biotransformação de BHA, que utilizam oxigênio e NADPH. O BHA pode ser considerado com um agente metabólico brando que se torna tóxico apenas em altas doses. Um fator agravante, no entanto, é a observação de que presumivelmente um tempo considerável é necessário para a reversão da maior parte dos efeitos após a remoção do composto da circulação. Além da falta de glicose circulante devido à inibição da gliconeoigênese, pode-se esperar também acidose metabólica devido à excessiva produção de ácido lático, danos à detoxificação da amônia e prejuízos às células devido a uma deficiente manutanção da homeostasia celular.

Keywords: BHA, metabolismo, fígado perfundido, mitocôndria, gliconeogênese.

GENERAL ABSTRACT

INTRODUCTION

Butylated hydroxyanisole (BHA) is widely used as an antioxidant in processed foods, cosmetics, pharmaceuticals and petrochemicals. In isolated mitochondria, BHA decreases respiratory control by stimulating state 4 respiration, dissipates membrane potential and induces calcium loss. Studies have shown that BHA acts as a uncoupler and two possible mechanisms could explain such an action: BHA could be acting as a proton carrier or as a membrane disrupter. The hypothesis that BHA can modify metabolic fluxes by virtue of its inhibitory action on energy metabolism has not yet been tested. It is of interest to know if BHA is capable of interacting with the mitochondria in the intact cell in the same way as it interacts with isolated mitochondria. This was the purpose of the present study and to achieve this goal the isolated perfused rat liver was used as the experimental system. In this system microcirculation and cellular polarity are preserved, in addition to cellular integrity.

MATERIALS AND METHODS

Male Wistar rats weighing 220-240 g were used in all experiments. The non-recirculating, hemoglobin-free perfusion system was used, and the perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine serum albumin and saturated with a carbogenic mixture. The substrates and BHA were added directly to the perfusion liquid. BHA was added as a dimethylsulfoxide solution. The perfusate was collected for enzymatic analyses of the produced metabolites (productions of glucose, lactate, pyruvate, ammonia and urea). Oxygen consumption was monitored polarograhically. The nucleotide contents of adenine mononucleotides were also mesaured. After perfusion, the liver was clamped in liquid nitrogen at appropriate times, extracted with perchloric acid and the extract was neutralized with K_2CO_3. The amount of AMP, ADP and ATP was measured by HPLC.

RESULTS

As a first approach experiments were done with livers from fed rats, which allow assessment of glycogenolysis and glycolysis. The effects of BHA on the release of glucose, lactate, pyruvate and on oxygen uptake was determined. It was found

that, at high concentrations, BHA revealed a tendency to decrease oxygen consumption, although no statistical significance was found. However, the productions of glucose and lactate were clearly increased in a concentration-dependent manner. We also evaluated the effect of BHA on gluconeogenesis from three substrates (L-lactate, fructose and L-alanine). For lactate gluconeogenesis the strongest effect of BHA was on glucose production, inhibition being already evident at the concentration of 100 μM. Numerical interpolation revealed that 50% inhibition can be expected at a concentration of 279.6 μM. There was also inhibition of oxygen consumption, which started at the concentration of 200 μM. Pyruvate production from L-lactate was slightly stimulated by BHA, also beginning at 200 μM. When fructose was the substrate, BHA caused an inhibition of glucose production from 100 μM on and 50% inhibition can be expected at a concentration of 460.5 μM. Oxygen uptake inhibition was significant only at the concentrations of 500 and 750 μM. Stimulation of lactate production was concentration-dependent and more prominent than stimulation of pyruvate production, which did not show a well defined concentration dependence. When used as a substrate alanine allows to measure both carbon and nitrogen fluxes in the liver. BHA caused inhibition of glucose production that started at the concentration of 50 μM; 50% inhibition can be expected at the concentration of 233.3 μM. The production of lactate and pyruvate was slightly stimulated. Oxygen uptake was also inhibited with a kinetics similar to that found when lactate and fructose were the substrates. In addition, BHA stimulated the production of ammonia and inhibitied the production of urea at 500 and 750 μM. It is worth noting that, after cessation of BHA infusion, the metabolic effects of this additive were only partially reversed, in some cases, or there was no reversion at all, in most cases. Finally, the levels of adenine mononucleotides (ATP, ADP and AMP) were analyzed. Livers from fed and fasted rats were used. For the first situation, 500 μM BHA did not cause a decrease in the ATP content and there was a discrete, but significant, ADP content increase. In the fasted state, on the other hand, the ATP and the ADP contents were both diminished by 750 μM BHA, -48% and -30%, respectively.

DISCUSSION

The results show that BHA acts on the metabolism of the liver and is capable of affecting several metabolic pathways that are dependent or linked to energy

metabolism. The most important observations, which have also been reported for other inhibitors of mitochondrial respiration, are: a) inhibition of oxygen consumption; b) diminution of the ATP content combined with modifications in the ATP/ADP and ATP/AMP ratios; c) increase in the cytosolic NADH/NAD^+ ratio, as indicated by the lactate/pyruvate ratio; d) inhibition of gluconeogenesis from three different substrates, namely lactate, fructose and alanine; e) stimulation of glycolysis and fructolysis as cytosolic compensatory phenomena for the diminished mitochondrial ATP production; f) stimulation of glycogenolysis as a means of providing glucose 6-phosphate for the increased glycolytic flux; g) impairment of ammonia detoxification. Modifications in metabolic fluxes are generally attributable to their reported effects on the mitochondrial respiratory chain. In isolated mitochondria BHA blocks the electron flow, but it is also an uncoupler. The first phenomenon would inhibit oxygen uptake, and the second would cause oxygen uptake stimulation. In contrast, in the perfused liver of the fasted rat, BHA only inhibited oxygen consumption. In substrate-free perfused livers (fed condition) no inhibition was found. One possible explanation for the observed difference in the fast (infusion of a gluconeogenic substrate) and fed (substrate-free) conditions must take into account two factors: (a) the particular conditions of the perfused liver without substrate, in which the conditions surrounding the mitochondria were more close to state 4, leading to a certain compensation between the inhibitory and stimulatory tendencies; (b) the contribution of microsomal mixed function oxidases responsible for BHA biotransformation, which consume oxygen and NADPH. BHA can be considered a mild metabolic agent that becomes toxic only at high doses. An aggravating factor could be the observation that considerable time is presumably required for the reversion of most effects after removal of the compound from the circulation. Besides the lack of circulating glucose due to gluconeogenesis inhibition, one can expect metabolic acidosis due to excess lactate production, impairment of ammonia detoxification and cell damage due to a deficient maintenance of its homeostasis.

Keywords: BHA, metabolism, perfused liver, mitochondria, gluconeogenesis.

Sumário

The metabolic effects of the food additive BHA in the perfused rat liver

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Abstract

A systematic study on the effects of butylated hydroxyanisole (BHA) on the hepatic metabolism was conducted with emphasis on parameters linked to energy metabolism. The experimental system was the isolated perfused rat liver. It was found that BHA inhibits biosynthetic pathways (gluconeogenesis) and ammonia detoxification, which are dependent on ATP generated within the mitochondria. Conversely, the compound stimulated glycolysis and fructolysis, which are compensatory phenomena for an inhibited mitochondrial ATP generation. Furthermore, BHA diminished the cellular ATP content under conditions where the mitochondrial respiratory chain was the only source of this compound. Gluconeogenesis from various substrates (lactate, alanine and fructose) was the most strongly affected parameter. Inhibition of gluconeogenesis was generally pronounced at concentrations under 200 μM. Several effects, however, were prominent only at the concentrations of 500 and 750 μM. BHA can be considered, thus, a mild metabolic agent that becomes toxic only at high doses. An aggravating factor could be the observation that considerable time is required for the reversion of most effects after removal of the compound from the circulation. Besides the lack of circulating glucose due to gluconeogenesis inhibition, one can expect metabolic acidosis due to excess lactate production, impairment of ammonia detoxification and cell damage due to a deficient maintenance of its homeostasis.

Key-words: gluconeogenesis, glycolysis, respiration, energy metabolism, toxicity

1. INTRODUCTION

Butylated hydroxyanisole (BHA) and its analogue butylated hydroxytoluene (BHT) are amply utilized as antioxidants in processed foods, cosmetics, pharmaceutical products and petrochemicals (Chen e Chaw, 1974). The commercial form of BHA is usually a mixture of two isomers in which the predominating form is 3-terc-butyl-4-hydroxyanisol (3-BHA) whereas the secondary component is 2-terc-butyl-4-hydroxyanisol (2-BHA). Figure 1 shows the chemical structure. Both forms are highly hydrophobic and are capable, thus, of interacting with biological membranes and enzymes linked to membranes. In agreement to this it was shown that BHA, as also BHT, can labilize the lisosomal membranes. Besides, BHA is also able to disorganize the mitochondrial structure (Sgaragli and Rizotti-onti, 1971).

There are several reports about the interactions of BHA with rat liver mitochondria. In isolated mitochondria BHA diminishes the respiratory control by stimulating state 4 respiration, dissipates the membrane potential and induces calcium loss (Thompson and Moldéus, 1987). Consistently, death of hepatocytes was preceded by a pronounced drop in the ATP levels. These toxic actions were proposed to be independent of the formation of reactive intermediates because they were not influenced by inhibitors of cytochrome P-450 (metyrapone, SKF 525-A and piperonyl butoxide). In another study (Ferreira, 1990) it was found that BHA inhibits mitochondrial respiration stimulated by ADP or uncouplers and driven by NAD^+- or FAD-dependent substrates. Proton ejection and ATPase activity were mildly affected, suggesting an action predominantly on the electron transport chain (Ferreira, 1990). Actually, inhibition of NADH oxidase and of electron flow through the ubiquinone-cytochrome b-cytochrome c_1 complex was found, suggesting that BHA inhibits electron transport at the NADH-ubiquinone and ubiquinone-cytochrome b level. This would be possible because the structure of BHA resembles that of ubiquinol (Aldunate el. al, 1986). For the action of BHA as a mild uncoupler two possible mechanisms were proposed: BHA could be acting either as a proton carrier (protonophore) or as a membrane disruptor (Fusi et al., 1991). The first mechanism is possible due to the phenolic hydroxyl group and the second one would be caused by the lipophilic nature of BHA as it is well known that monocyclic compounds can modify membrane structures (Sgaragli et al., 1977). It should be remarked that reversion of the latter effect

(after removal of BHA) would tend to require much more time than the former one.

With respect to the effects of BHA on isolated mitochondria it should be mentioned that similar effects have been reported for the chemically related compound BHT (Thompson and Moldéus, 1987; Festje et al., 2006). In intact cells the impairment of mitochondrial energy metabolism has several consequences for basic metabolic pathways, such as glycolysis stimulation and gluconeogenesis inhibition (Saling et al., 2011; Moreira et al., 2013). When this hypothesis was tested for BHT in the isolated perfused rat liver, however, the results were negative. The compound was unable to modify gluconeogenesis and oxygen uptake in the range up to 500 μM (Castro et al., 2017). Apparently, for reasons that did not became clear, BHT is unable to inhibit mitochondrial energy transduction in the perfused liver. For BHA the hypothesis that it might modify metabolic fluxes by virtue of its inhibitory action on energy metabolism has not yet been tested. It is of interest to know, however, if BHA is able to interact with the mitochondria in the intact cell in the same manner as it interacts with isolated mitochondria or if it behaves like BHT (Castro et al., 2017). This was precisely the purpose of the present work. The experimental system was the isolated perfused rat liver in which microcirculation and cell polarity are both preserved in addition to the cell integrity. Both catabolic and anabolic pathways were measured and the results should bring additional information about the interactions of BHA with the intact liver cells.

2. MATERIALS AND METHODS

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Butylated hydroxyanisole (BHA; 2(3)-*t*-butyl-4-hydroxyanisole) was the product number B1253 from Sigma-Aldrich Co (St. Louis, USA). Enzymes and coenzymes used in the enzymatic assays were also purchased from Sigma-Aldrich Co (St. Louis, USA). All other chemicals were from the best available grade (98–99.8% purity).

2.2. Animals

Male Wistar rats weighing 220–240 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. For preparing the liver for perfusion 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 and were previously approved by the Ethics Committee of Animal Experimentation of the University of Maringá (protocol number 4507290915).

2.3. Liver perfusion

Hemoglobin-free, non-recirculating perfusion was performed (Scholz and Bücher, 1965; Kelmer-Bracht et al., 1984). 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 (Scholz and Bücher, 1965; Kelmer-Bracht et al., 1984): 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. Samples of the effluent perfusion fluid were collected and analyzed for their metabolite contents. Substrates and BHA were added to the perfusion fluid according to the experimental protocols. Due to its low solubility in water, BHA was added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration. These concentrations were 50, 100, 200, 500 and 750 µM. 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.

In accordance with the protocol, rats were used fed or starved for 18 h prior to the experiments. 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. Gluconeogenesis was measured in livers from 18 hours fasted rats. Under this condition the livers possess low glycogen levels and the rate of glucose output reflects mainly the rate of gluconeogenesis (Comar et al., 2016).

2.4. Metabolite assay

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, ammonia and urea (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 (Kelmer-Bracht et al, 1984). Metabolic rates were calculated from input–output differences and the total flow rates and referred to the wet weight of the liver.

The hepatic contents of the adenine nucleotides were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The pH of the extract was neutralized with K_2CO_3

and AMP, ADP, and ATP were assayed by means of high-performance liquid chromatography (HPLC) (Mito et al., 2014). The HPLC system (Shimadzu, Japan) consisted of a system controller (SCL-10AVP), two pumps (model LC10ADVP), a column oven (model CTO-10AVP) and an UV–Vis detector (model SPD-10AV). A reversed-phase C18 CLC-ODS column (5 µm, 250 × 4.6 mm i.d., Shimadzu) protected with a CLC-ODS precolumn (5 µm, 4 × 3 mm i.d., Phenomenex) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1.1), pH 7.0. In percent methanol, the gradient was the following: at 0 min, 0%; at 2.5 min, 0.5%; at 5 min, 3%; at 7 min, 5%; at 8 min, 12%; at 10 min, 15%; at 12 min, 20%; at 20 min, 28%; and at 30 min, 0%. The temperature was kept at 35 °C, and the injection volume was 20 µL with a flow rate of 0.8 mL/min. Monitoring was performed spectrophotometrically at 254 nm. Identification of the peaks of the investigated compounds was carried out by a comparison of their retention times with those obtained by injecting standards under the same conditions. The concentrations of the compounds were calculated by means of the regression parameters obtained from the calibration curves. The calibration curves were constructed by separating chromatographically standard solutions of the compounds. Linear relationships were obtained between the concentrations and the areas under the elution curves.

2.7. Data handling

The error parameters presented in the graphs are standard errors of the mean. Statistical analysis was done by means of the GraphPadPrism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA) Variance analysis was done with post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$). Student's t test was applied when comparing two means. Computation of the IC_{50} values was done by numerical interpolation using Stineman's formula (Wagon, 1999). The software used was the *Scientist* program from MicroMath Scientific Software (Salt Lake City, UT, USA).

3. RESULTS

3.1. Effects of BHA on glycogen catabolism and glycolysis

As a first approach experiments were planned in order to test possible effects of BHA 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; Kelmer-Bracht et al., 1984). Under these conditions the livers release glucose, lactate and pyruvate as a result of glycogen catabolism. Figure 2 illustrates the time course of the action of 750 μM BHA. This is the maximal concentration allowed by the BHA solubility in the perfusion fluid. Figure 2 also illustrates the experimental protocol that was used for all other BHA concentrations. After a pre-perfusion period of 10 min, BHA was infused during 20 min, followed by additional 10 min of drug-free perfusion. Four parameters were measured: glucose release, lactate and pyruvate productions and oxygen consumption. The introduction of BHA at 10 minutes perfusion time caused a progressive increase in lactate release which tended to stabilize at the end of the infusion period. Glucose output slightly increased, but oxygen uptake and pyruvate production were minimally affected. Cessation of the infusion did not reverse the stimulation of lactate production, at least not during the 10 minutes following the interruption of BHA infusion. The experiment shown in Figure 2 was repeated with four other BHA concentrations in order to establish effect versus concentration relationships. Figure 3 summarizes the results that were obtained with the concentrations of 50, 100 200, 500 and 750 μM. The metabolic rates were evaluated in the absence of BHA (zero BHA concentration) and at the end of the infusion period of BHA for each concentration (30 minutes perfusion time). BHA concentrations up to 200 μM produced only non-significant fluctuations in most parameters. Oxygen uptake tended to decrease at high BHA concentrations, but statistical significance was absent. Pyruvate production was not affected at all over the entire concentration range. Lactate production and glucose output, on the other hand, were clearly increased at the BHA concentrations of 500 and 750 μM. The increase in lactate production at 500 and 750 μM BHA was equal to 66 and 117%, respectively.

3.2. Effects of BHA on lactate gluconeogenesis and associated variables

Fig. 4 shows results of experiments in which the action of 500 µM BHA on lactate gluconeogenesis and associated parameters was measured. Livers from 18 h fasted rats were used in order to minimize interference by glycogen catabolism. Under these conditions glucose output reflects very closely the gluconeogenic activity of the liver (Comar et al., 2016). As expected, the infusion of 2 mM lactate produced gradual and substantial increases in glucose production, oxygen uptake and pyruvate production. The increases had almost stabilized at the time where the BHA infusion was started (34 minutes perfusion time). The introduction of BHA caused progressive decreases in both oxygen uptake and glucose production. At 56 minutes perfusion time the infusion of BHA was interrupted. At this time the increment in oxygen uptake caused by lactate had already been completely abolished by BHA, similarly to what happened with the increment in glucose production. Upon cessation of the BHA infusion the effects on oxygen uptake and glucose production were partly reversed. Pyruvate production, on the other hand, experienced a small increase upon BHA infusion. This increase was not stable, as it presented a maximum at 44 minutes perfusion time. The experiment shown in Figure 4 was repeated with four other BHA concentrations in order to establish effect versus concentration relationships. Figure 5 summarizes the results that were obtained. Oxygen uptake and glucose production were evaluated before starting BHA infusion and at the end of the infusion. For pyruvate production the values plotted in Figure 5 correspond to the peak changes experienced after starting the BHA infusion. The most evident effect of BHA is that one on glucose production, which started to be inhibited at 100 µM BHA. Numerical interpolation reveals that 50% can be expected at a concentration of 279.6 µM. The effect of BHA on oxygen uptake is less pronounced: the increment caused by lactate can be expected to be 50% inhibited at a concentration of 365.1 µM. Pyruvate production, finally, started to be stimulated at the BHA concentration of 200 µM.

3.3. Effects of BHA on fructose metabolism

Transformation of fructose in the liver allows the simultaneous monitoring of anabolic and catabolic reactions, as this substrate can be transformed into glucose via an energy-requiring pathway and into lactate or pyruvate via an energy-yielding route. The results of the experiments that were done with fructose as the substrate are shown in Figures 6 and 7. Figure 6 shows the time courses of the effects of 500 µM BHA. As expected, the introduction of 2 mM fructose increased oxygen uptake and the productions of glucose, lactate and pyruvate. The introduction of 500 µM BHA at 36 minutes perfusion time modified all these parameters: glucose production and oxygen uptake were inhibited and lactate and pyruvate production were stimulated. Cessation of the infusion of BHA did not reverse oxygen uptake inhibition. Glucose production inhibition was only partially reversed, but the stimulations of lactate and pyruvate productions were almost completely reversed. Figure 7 shows the concentration dependences of the effects. Inhibition of glucose production started to be significant at 100 µM BHA; 50% inhibition can be expected at the concentration of 460.5 µM. Oxygen uptake inhibition was significant only at the concentrations of 500 and 750 µM. Stimulation of lactate production was concentration-dependent and more prominent than stimulation of pyruvate production which did not show a well defined concentration dependence.

3.4. Effects of BHA 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 BHA affects variables linked to carbon and nitrogen metabolism. Livers from 18 h fasted rats were used. Figure 8 shows the time courses of the effects of 500 µM BHA. Alanine gluconeogenesis was also progressively inhibited by BHA, without reversion after cessation of the infusion (Figure 8A). Lactate and pyruvate productions tended to be stimulated by BHA, but the changes were not stable. Oxygen uptake was also inhibited with a kinetics similar to that found when lactate and fructose were the substrates (Figure 8B). Reversibility was also absent. Ammonia production was stimulated, an effect that was not reversed during the 10 minutes period that followed cessation of the infusion. Urea

production, finally, tended to be inhibited by BHA. The concentration dependences in Figure 9 show a clear concentration-dependent inhibition of glucose production that started at the concentration of 50 μM; 50% inhibition can be expected at the concentration of 233.3 μM. Ammonia production stimulation also showed a clear concentration dependence, but significance was evident only at the highest concentrations. Oxygen uptake and urea production inhibitions were significant only at the highest concentrations. Lactate and pyruvate productions, finally, were stimulated to relatively small degrees over the whole concentration range employed.

3.5. Effects of BHA 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 BHA. Livers from fed and fasted animals were examined. Livers from fed animals were perfused with a substrate-free perfusion medium and livers from fasted animals were perfused with a 2 mM lactate containing medium. The latter were, thus, actively synthesizing glucose (see Figures 3 and 4). The results are shown in Table 1. In livers from fed rats 500 μM BHA did not decrease the ATP content. Actually there was even a tendency toward higher levels and the ADP content was significantly increased. The AMP content was not modified. The ATP/AMP ratio, however, was increased. In the fasted state, on the other hand, the ATP and the ADP contents were both diminished by 750 μM BHA, −48% and −30%, respectively. In consequence, the total content of adenine mono-nucleotides was also decreased (−40%). Furthermore, the ATP/ADP and ATP/AMP ratios were both decreased by BHA.

4. DISCUSSION

4.1. Markers of the effect of BHA on the mitochondrial energy metabolism

The bulk of data presented above shows that BHA acts on liver metabolism and is able to affect several metabolic pathways that are dependent or linked to energy metabolism. The modifications in the metabolic fluxes are, in general, attributable to its reported effects on the mitochondrial respiratory chain (Thompson and Moldéus, 1987; Ferreira, 1990; Fusi et al., 1991). The most important observations, which have also been reported for other inhibitors of mitochondrial respiration, are (Soboll et al., 1978; Kelmer-Bracht et al., 1984; Itinose et al., 1989; Constantin et al., 1995; Simões et al., 2017): a) inhibition of oxygen consumption; b) diminution of the ATP content combined with modifications in the ATP/ADP and ATP/AMP ratios; c) increase in the cytosolic NADH/NAD^+ ratio, as indicated by the lactate/pyruvate ratio; d) inhibition of gluconeogenesis from three different substrates, namely lactate, fructose and alanine; e) stimulation of glycolysis and fructolysis as cytosolic compensatory phenomena for the diminished mitochondrial ATP production; f) stimulation of glycogenolysis as a means of providing glucose 6-phosphate for the increased glycolytic flux; g) impairment of ammonia detoxification.

In spite of these general characteristics that are common to compounds that interfere with energy metabolism, BHA also presents peculiarities when compared to other inhibitors. One of these particularities is the action of BHA on oxygen uptake in the perfused liver. In isolated mitochondria BHA blocks the electron flow, but it is also an uncoupler (Thompson and Moldéus, 1987; Ferreira, 1990; Fusi et al., 1991). Uncoupling causes oxygen uptake stimulation (Franco-Salla et al., 2017), which was indeed observed in isolated mitochondria under specific conditions. The maximal stimulation of state 4 respiration by BHA was approximately 300-350% (Ferrreira, 1990). In the perfused liver, however, oxygen uptake stimulation was not observed under any of the conditions that were employed. Inhibition, on the other hand, was found solely when respiration had been stimulated by the infusion of a gluconeogenic substrate. In substrate-free perfused livers no inhibition was found (See Figures 2 and 3). In spite of

this, however, glycolysis was increased, as revealed by the increased rates of lactate production. Glycolysis was sufficiently intense so has to avoid a significant drop in the ATP content of the overall hepatic tissue from fed rats although it is likely that oxidative phosphorylation within the mitochondria might have been impaired (Soboll et al., 1978). An explanation for these apparent contradictions must take into account two factors: (a) the particular conditions of the substrate-free perfused liver; (b) the contribution of the microsomal mixed-function oxidases responsible for the BHA biotransformation. With reference to point (a) it is possible that when the mitochondrial respiration was driven solely by endogenous substrates, the conditions surrounding the mitochondria were more close to state 4. Under this state oxygen uptake stimulation is the predominant effect of BHA in isolated mitochondria (Thompson and Moldéus, 1987) leading to a certain degree of compensation between both inhibition and stimulation tendencies within the intact cell. With reference to point (b) it is appropriate to emphasize that BHA increases the oxidation of NADPH in isolated microsomes (Rahimtula, 1983) by virtue of its biotransformation reactions (Rahimtula, 1983). This process requires molecular oxygen that is consumed stoichiometrically. This extra oxygen consumption can minimize further the difference between inhibition and stimulation of oxygen consumption in the whole liver. Oxidation of NADPH in consequence of biotransformation reactions has been demonstrated to affect biosynthetic pathways that depend on reducing power in the form of NADPH or NADH, such as gluconeogenesis (Scholz et al., 1973). Consequently, the inhibitory action of BHA on gluconeogenesis is not necessarily the sole consequence of its action on the mitochondrial energy metabolism, but could be partly due to its influence on the mixed function oxidases as it was demonstrated to occur with aminopyrine (Scholz et al., 1973).

Besides conjugated and demethylated derivatives, formaldehyde and a dimer di-BHA, biotransformation of BHA also produces reactive intermediates that can lead to irreversible formation of protein adducts (Astill et al., 1960; Rahimtula, 1983). Hypothetically at least, one cannot exclude the possibility that the irreversible formation of protein adducts could be partly responsible for the poor or at least slow reversion of the metabolic effects after cessation of the infusion. In this respect it should be remarked that even in the case of compounds such as dinoseb, that are bound with high affinity but in a reversible manner by the lipid bilayers, the metabolic effects are reversed in a period not much longer than 10

minutes after stopping the infusion (Franco-Salla et al., 2017). The poor reversiblility is an aspect of the effects of BHA deserving more detailed investigation.

4.2. Actions in the perfused liver, isolated mitochondria and cell systems

There is a clear contrast between observations that were done in isolated hepatocytes and the relatively high LD_{50} of BHA. When administered orally, the LD_{50} of BHA was found to be equal to 1690 mg/kg (Della Corte and Sgaragli, 1984). In isolated hepatocytes, on the other hand, BHA depleted 100% of the cellular ATP content in 15 minutes and 35% of the cells were already unviable as judged from their permability to trypan blue (Thompson and Moldéus, 1987). It is true that this occurred at the high concentration of 750 μM, but data obtained with the perfused liver in the present work are much more compatible with the relatively high LD_{50} doses. In fact, in livers from fasted rats 750 μM BHA diminished the ATP content by 48% after 20 minutes infusion and the cells were still respiring even though at a reduced rate. Furthermore, in livers from fed rats the equally high concentration of 500 μM did not affect the cellular ATP content. It is clear from these comparisons that experiments with isolated cells may, in some instances, produce toxicologic data that are much less realistic than those obtained in the intact organ. A possible reason is that in the perfused organ the ratio cell space/extracelular space is much higher than that is a suspension of isolated cells what means a more intense exposure to the drug. Furthermore, isolated hepatocytes expose their whole surface and all membrane proteins to the medium, whereas the same cells in the intact tissue expose only a fraction of their surface membrane proteins to the vascular space. In this respect it would be interesting to verify if BHA is able to induce release of cytchrome c in the perfused liver, a phenomenon that was described to occur in isolated hepatocytes. Observation of this phenomenon lead to the proposition that the cytotocixity of BHA results, party at least, from apoptosis induction mediated by the direct release of cytochrome c and the subsequent activation of caspases (Yu et al., 2000).

Another aspect worth to be considered is the different behaviour of BHA with respect to its analog BHT. Both compounds affect energy metabolism in isolated mitochondria and isolated hepatocytes in a similar way and at similar concentrations. BHT, however, was without significant effects on energy metabolism in the perfused liver (Castro et al., 2017). Gluconeogenesis, for example, was totally insensitive to BHT at concentrations up to 500 μM. This is a surprising difference inasmuch as BHT is more hydrophobic than BHA and should, thus, have better access to the cellular and mitochondrial membranes. The contrast between these compounds, thus, warns against straighforward assumptions that greater hydrophobicity immediately implies in greater capacity of interacting with mitochondria inside the cell.

4.3. Significance and concluding remarks

The concentration range of the effects of BHA on liver metabolism is somewhat under those of other effects proposed for the compound. For example, apoptosis triggered by cytochrome c release in hepatocytes occurs at concentrations above 250 μM (Yu et al., 2000), although there are signs of the phenomenon in isolated mitochondria at concentrations under 100 μM (Festjens et al., 2006). At these concentrations BHA already inhibits gluconeogenesis to a considerable degree. Actually, alanine gluconeogenesis is already significantly inhibited at the concentration of 50 μM. On the other hand, the endocrine disrupting effects attributed to BHA begin, apparently, at the concentration of 50 μM, requiring doses of up to 500 mg/kg in experimental rats (Pop et al., 2013). Doses of 200 mg/kg in experimental rats lead to peak concentrations of BHA in the systemic circulation of up to 2.2 μM (Verhagen et al., 1989). The latter is evidently too low a concentration to justify the great amount that was administered and also to justify the observed toxic effects attributed to BHA. Interestingly, when the dose was elevated to 2,000 mg/kg the disproportionally high peak concentration of 178 μM was observed (Guarna et al., 1983). It is possible that, especially at the lower doses, most of the absorbed drug is retained by the body tissues, a hypothesis justified by the lipophilic nature of BHA. Apparently, tissue retention in humans is much less pronounced than in rats, because in the former a dose of only 0.5 mg/kg resulted in plasma peak

concentrations of 0.7 μM. The latter corresponds to only one third of the peak concentration found in rats that received a 400-fold higher dose (Verhagen et al., 1989). Biotransformation in rats is also remarkably faster than in humans, actually by a factor of 4 as can be the deduced from the corresponding half-life times in the plasma (Verhagen et al., 1989). The liver is a site in which the drug can be retained in considerable amounts if one takes into account that it is the first organ that receives compounds absorbed from the intestinal tract. It is also highly probable that the concentration of BHA in the portal vein is considerably higher than that in the systemic circulation. For some drugs the ratio of portal vein to systemic circulation concentration reaches factors of 4.0 or above (Yamano et al., 2001). It is, thus, of interest to investigate how BHA interacts with the liver tissue, how it permeates the liver cell membrane, how fast is its biotransformation and how strongly it binds to the hepatic cellular lipids. Quantification of these phenomena is important for clarifying if the various effects attributed to BHA can really occur *in vivo* or not.

5. ACKNOWLEDGEMENTS

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7. ATTACHMENTS

Table 1. **Adenine mononucleotide contents of livers from fasted and fed rats and the effect of BHA.** Livers from fed and fasted rats were perfused in an open system as described in the Materials and methods section. Livers from fed rats were pre-perfused with Krebs/Henseleit-bicarbonate buffer for 10 min and, after that, BHA (500 μM) was infused for 30 min. For the experiments with livers from fasted rats, L-lactate (2 mM) was infused at 10 min and BHA (750 μM) was infused 26 min after L-lactate infusion for 30 min. The livers were freeze-clamped in liquid nitrogen and the adenine nucleotides were extracted with cold perchloric acid. Control determinations were done with livers that were freeze-clamped at the same perfusion time in the abscence of substrate (fed condition) or in the presence of L-lactate but without BHA infusion. Values are means ± SEM. Asterisks indicate statistical significance in comparison with the fasted control condition and crosses indicate statistical significance in comparison with the fed control condition as revealed by unpaired t-test. *p<0.05, **p<0.001 and $^{\#}$p<0.05, $^{\#\#}$p<0.001.

Parameter	**Fed**		**Fasted**	
	Control (n=6)	500 μM BHA (n=4)	Control (n=4)	750 μM BHA (n=4)
ATP (μmol/g)	2.27±0.17	2.74±0.17	2.19±0.05	1.14±0.11**
ADP (μmol/g)	0.56 ± 0.02	0.72±0.02$^{\#\#}$	1.04±0.07	0.73±0.06*
AMP (μmol/g)	0.30±0.02	0.26±0,01	0.48±0.03	0.39±0.03
Total	3.13±0.21	3.72±0.17	3.77±0.04	2.26±0.17**
ATP/ADP	3.99±0.18	3.81±0.21	2.22±0.13	1.57±0.11*
ATP/AMP	7.70±0.48	10.66±1.17$^{\#}$	4.77±0.60	2.96±0.22*

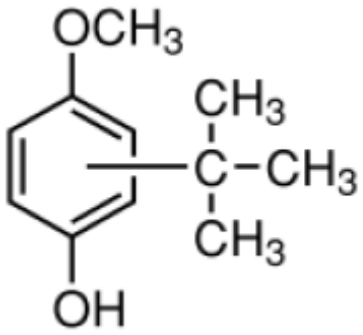

Figure 1. **Structural formula of butylated hydroxyanisole (BHA).** The sample used in the present study consisted in ≥ 90% of 3-butylated hydroxyanisole and ≥ 98.5% of 3-butylated hydroxyanisole + 2-butylated hydroxyanisole.

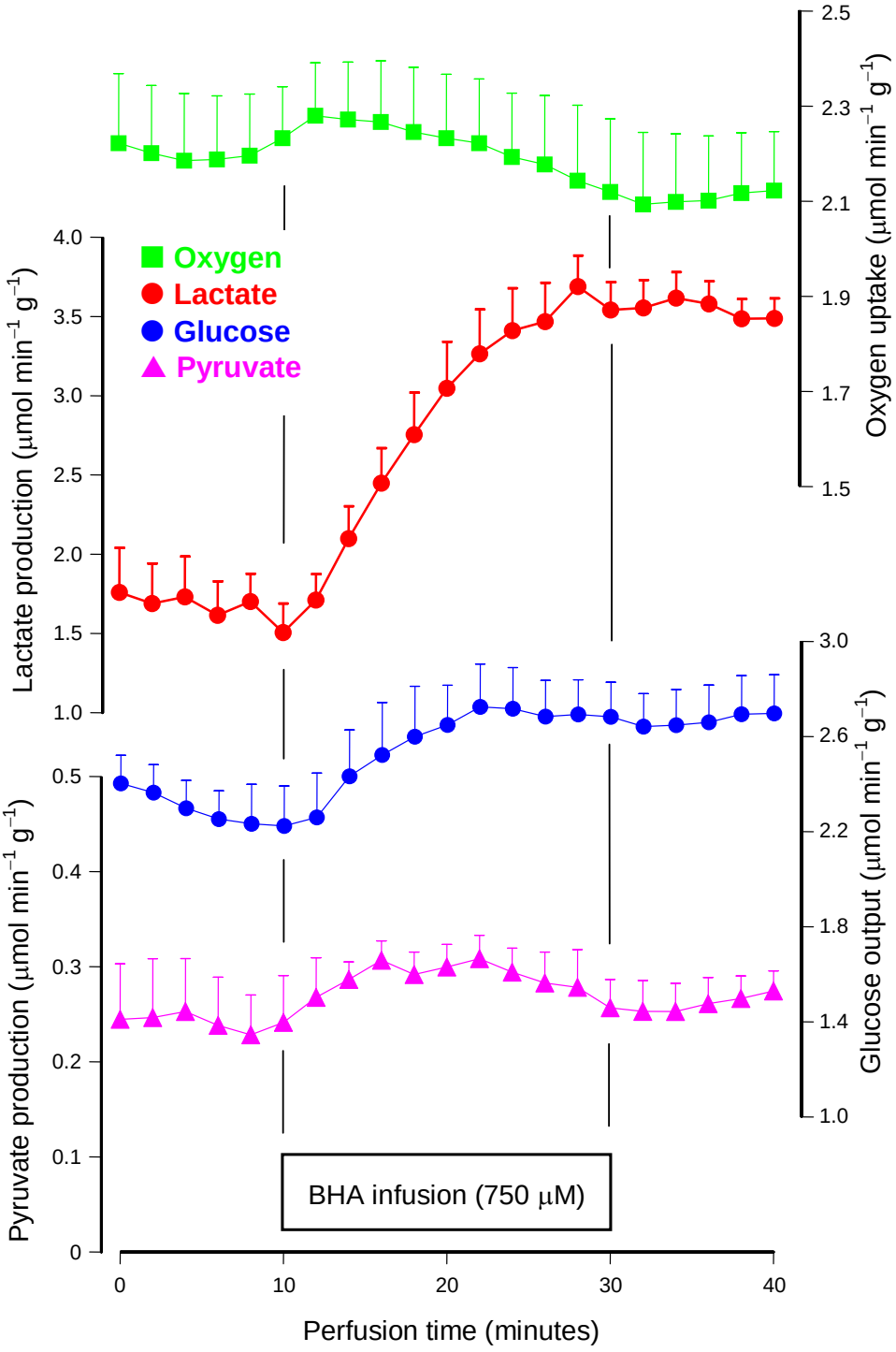

Figure 2. **Time course of the changes caused by 750 µM BHA in glycogen catabolism and related parameters in the perfused liver from fed rats.** Livers from fed rats were perfused as described in the Materials and methods section. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate, and pyruvate. Oxygen consumption was followed polarographically. Each datum point represents the mean of 3 liver perfusion experiments. Bars are standard errors of the mean. Time period of BHA infusion is indicated by the box near to the time scale.

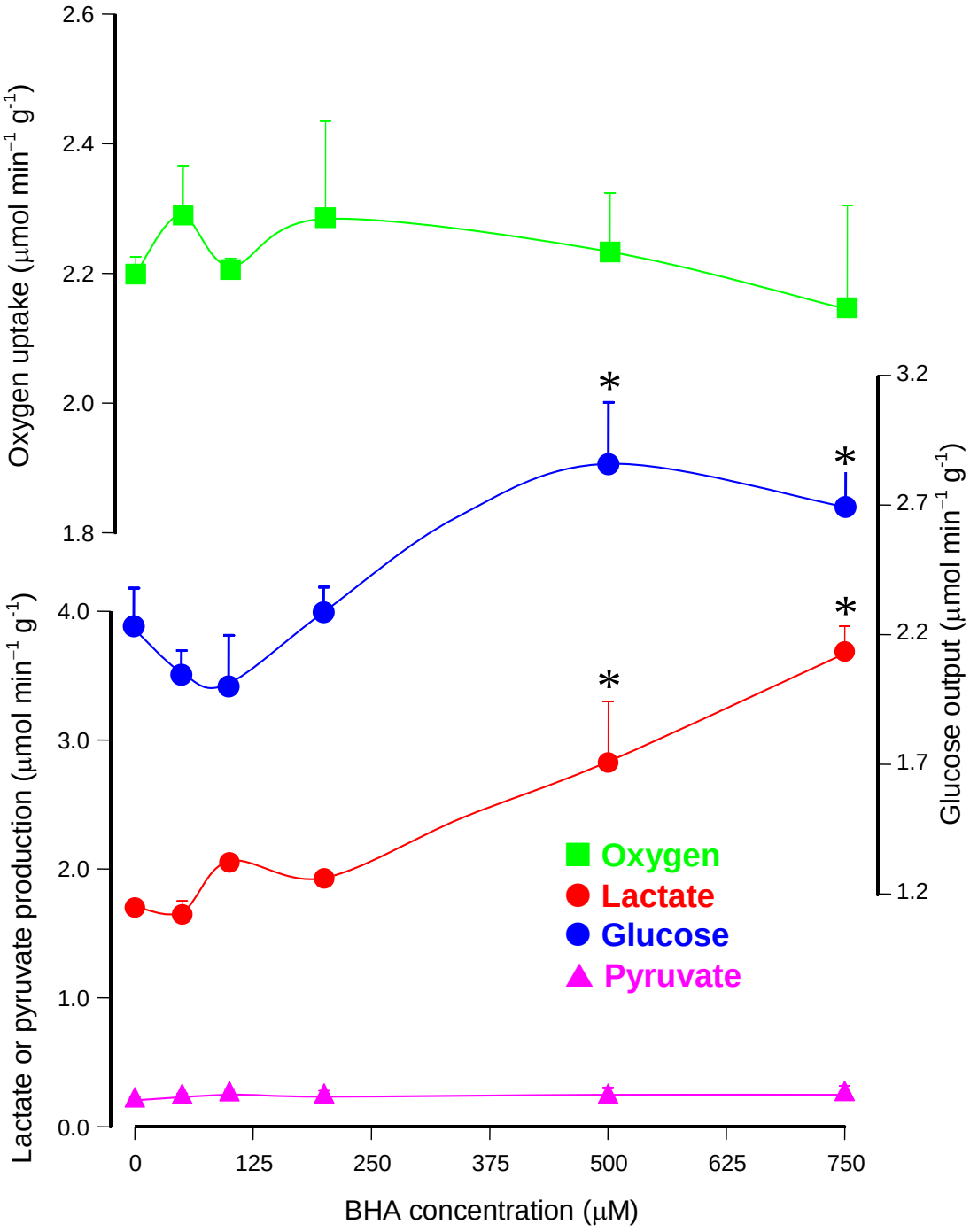

Figure 3. **Concentration dependence of the effects of BHA on glycogen catabolism and related parameters.** Perfusion experiments were done following the protocol illustrated by Figure 1 with several BHA concentrations. The rates of oxygen uptake, lactate and pyruvate productions and glucose output were evaluated before and at 20 minutes after starting BHA infusion. The control values (absence of BHA) are the means of 15 perfusion experiments; data in the presence of BHA represent the means of 3 liver perfusion experiments. Bars are standard errors of the mean. Asterisks indicate those rates that differ from the control condition (absence of BHA), as indicated by post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$).

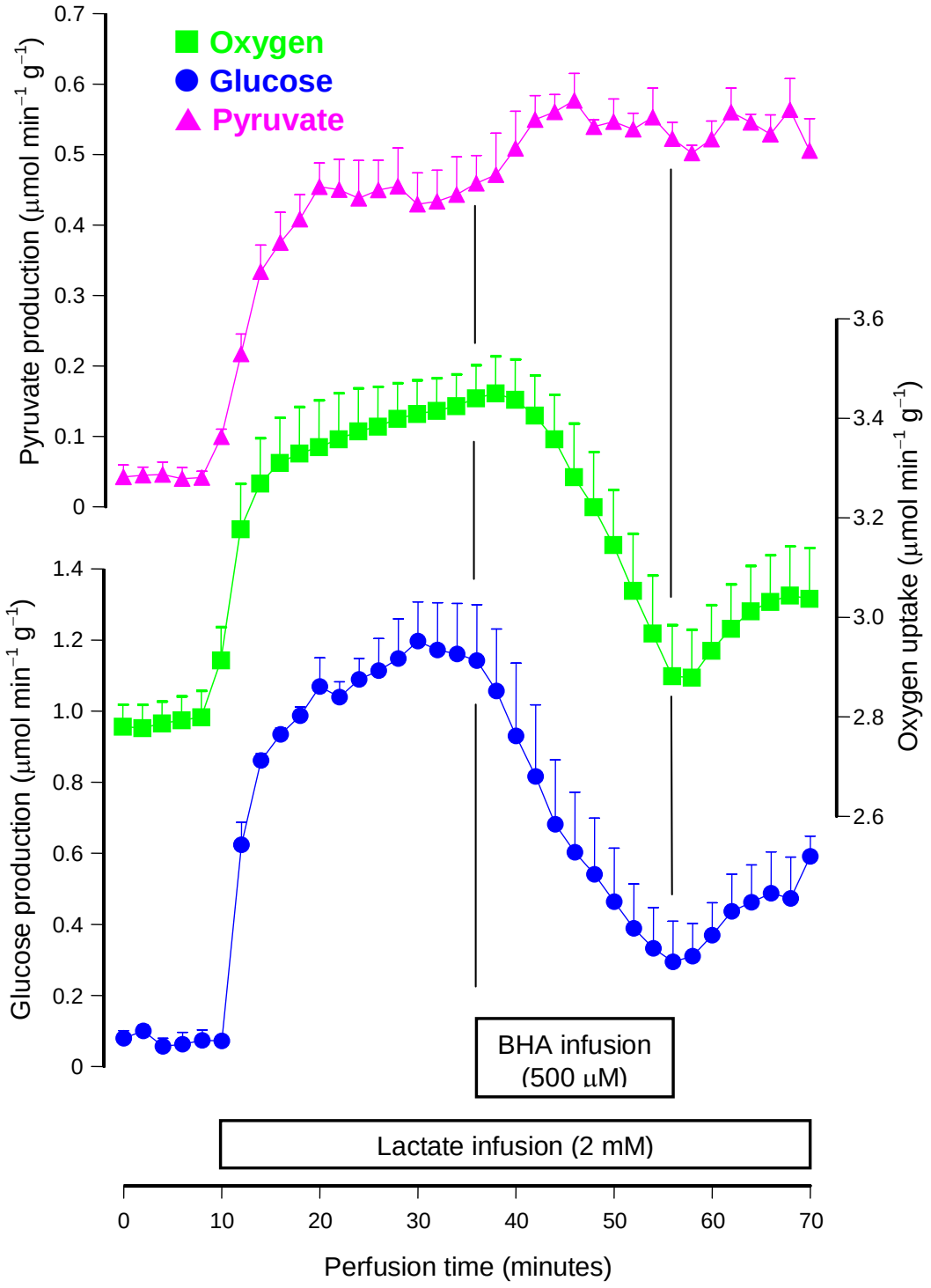

Figure 4. **Time course of the effects of 500 µM BHA on lactate gluconeogenesis and related parameters.** Livers from 18 hours fasted rats were perfused as described in the Materials and methods section. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose and pyruvate. Oxygen consumption was followed polarographically. Each datum point represents the mean of 3 liver perfusion experiments. Bars are standard errors of the mean. The time periods of BHA and lactate infusions are indicated by the boxes near to the time scale.

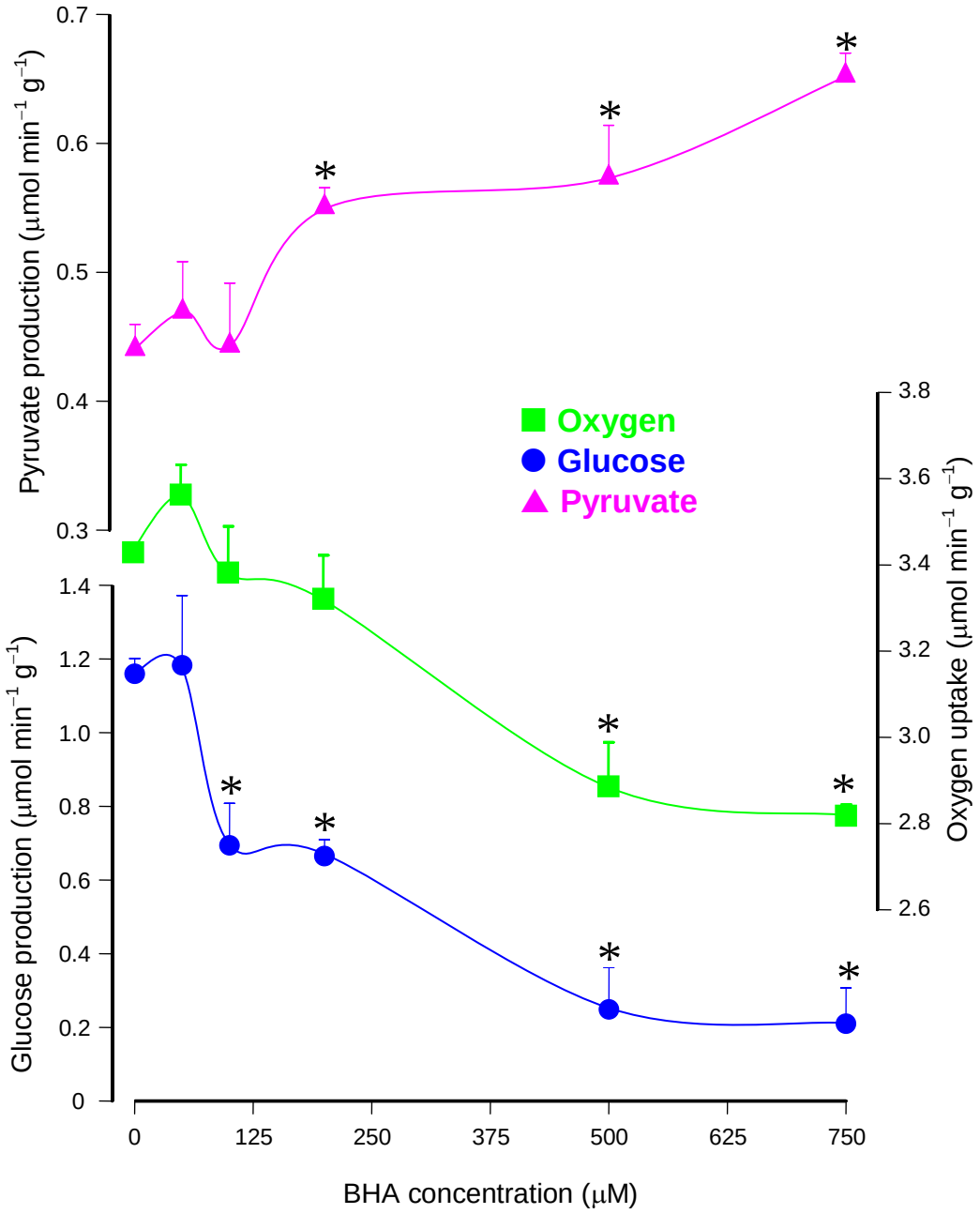

Figure 5. **Concentration dependences of the effects of BHA on lactate gluconeogenesis and related parameters.** Perfusion experiments were done following the protocol illustrated by Figure 3 with several BHA concentrations. The rates pyruvate and glucose productions and of oxygen uptake were evaluated before and at 20 minutes after starting BHA infusion (56 minutes perfusion time). The control values (absence of BHA) are the means of 15 perfusion experiments; data in the presence of BHA represent the means of 3 liver perfusion experiments. Bars are standard errors of the mean. Asterisks indicate those rates that differ from the control condition (absence of BHA), as indicated by post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$).

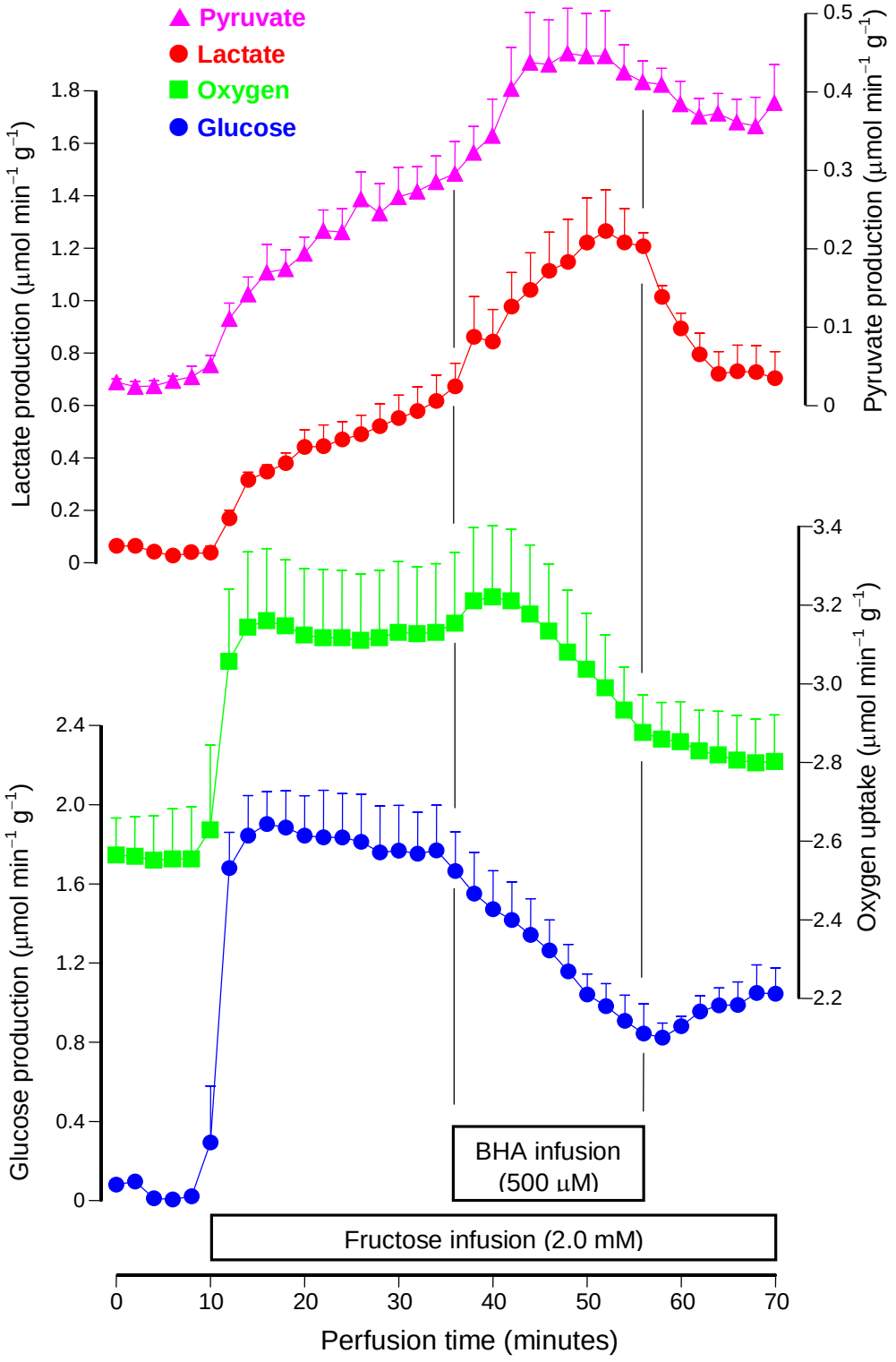

Figure 6. **Time course of the effects of 500 µM BHA on fructose metabolism.** Livers from 18 hours fasted rats were perfused as described in the Materials and methods section. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate and pyruvate. Oxygen consumption was followed polarographically. Each datum point represents the mean of 3 liver perfusion experiments. Bars are standard errors of the mean. The time periods of BHA and lactate infusions are indicated by the boxes near to the time scale.

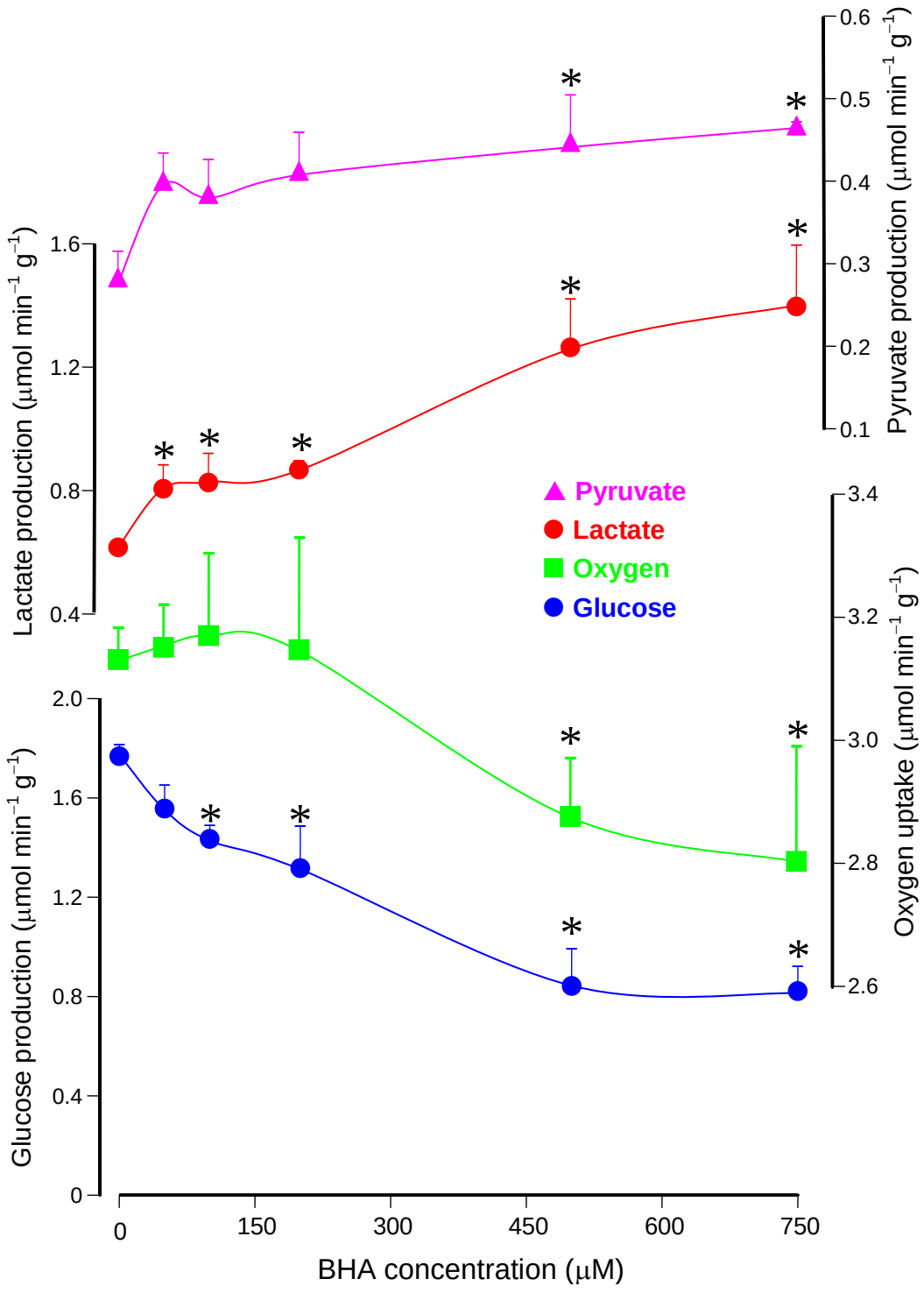

Figure 7. **Concentration dependences of the effects of BHA on fructose metabolism.** Perfusion experiments were done following the protocol illustrated by Figure 5 with several BHA concentrations. The rates pyruvate, lactate and glucose productions and oxygen uptake were evaluated before and at 20 minutes after starting BHA infusion (56 minutes perfusion time). The control values (absence of BHA) are the means of 15 perfusion experiments; data in the presence of BHA represent the means of 3 liver perfusion experiments. Bars are standard errors of the mean. Asterisks indicate those rates that differ from the control condition (absence of BHA), as indicated by post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$).

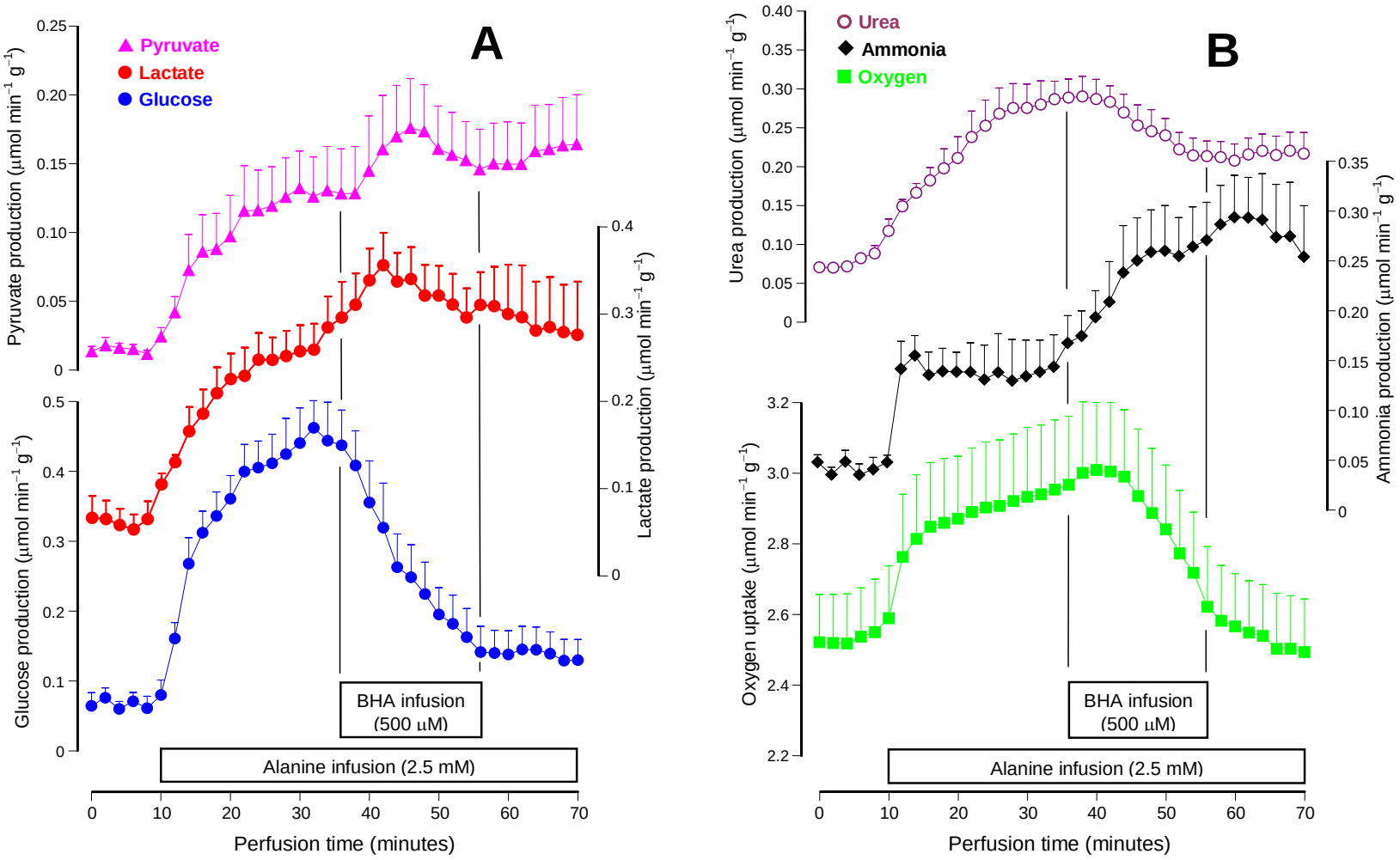

Figure 8. **Time course of the effects of 500 μM BHA on alanine metabolism.** Livers from 18 hours fasted rats were perfused as described in the Materials and methods section. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate, pyruvate, urea ad ammonia. Oxygen consumption was followed polarographically. Each datum point

represents the mean of 3 liver perfusion experiments. Bars are standard errors of the mean. The time periods of BHA and lactate infusions are indicated by the boxes near to the time scale.

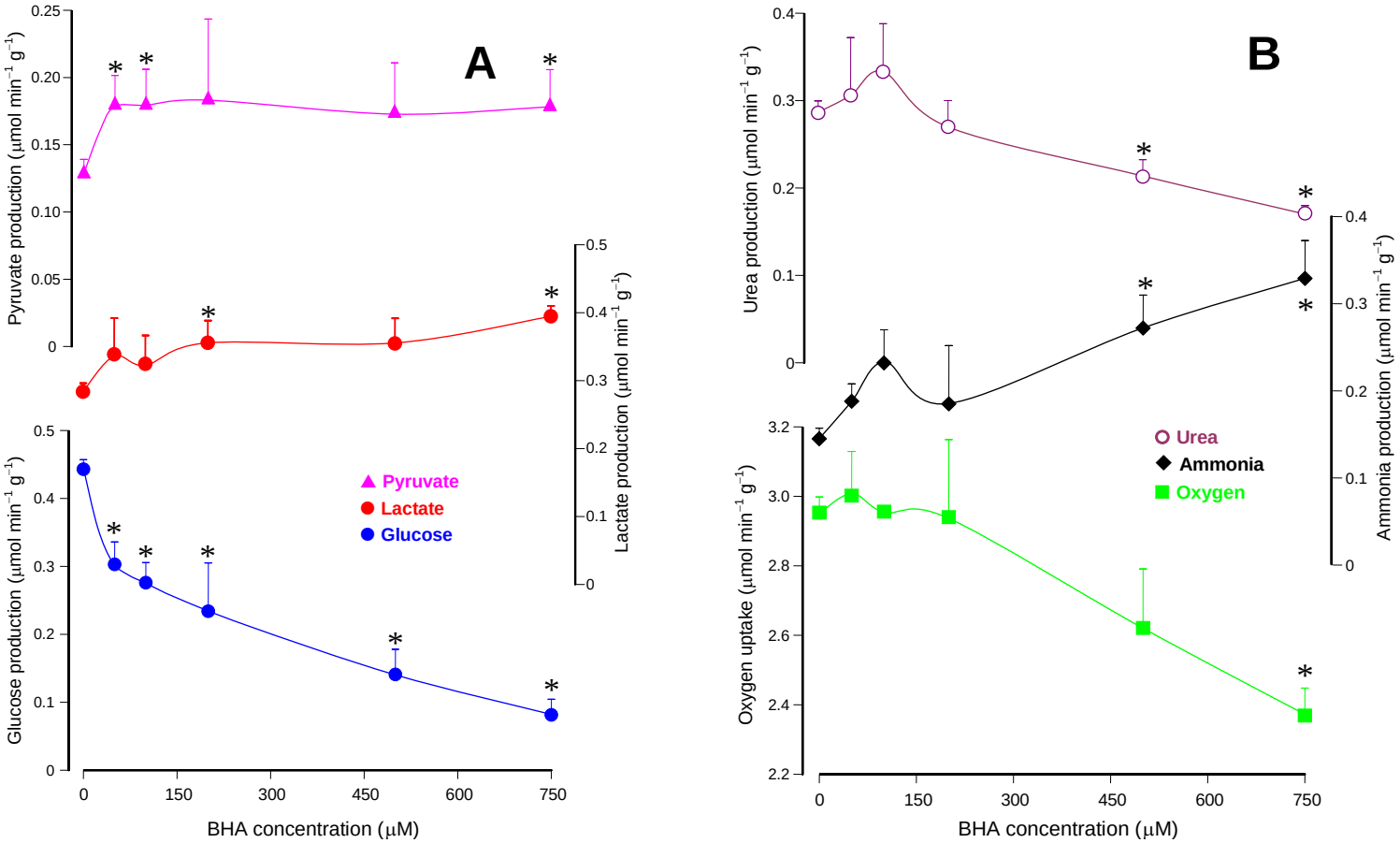

Figure 9. **Concentration dependences of the effects of BHA on alanine metabolism.** Perfusion experiments were done following the protocol illustrated by Figure 7 with several BHA concentrations. The rates pyruvate, lactate, glucose, ammonia and urea productions and oxygen uptake were evaluated before and at 20 minutes after starting BHA infusion (56 minutes perfusion time). The control values (absence of BHA) are the means of 15 perfusion experiments; data in the presence of BHA represent the means of 3 liver perfusion experiments. Bars are standard errors of the mean. Asterisks indicate those rates that differ from the control condition (absence of BHA), as indicated by post-hoc testing according to Student-Newman-Keuls ($p \leq 0.05$).