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The metabolic effects of diuron in the rat liver

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ABSTRACT

A systematic study on the effects of diuron on the hepatic metabolism was conducted with emphasis on parameters linked to energy metabolism. The experimental system was the isolated perfused rat liver. The results demonstrate that diuron inhibited biosynthesis (gluconeogenesis) and ammonia detoxification, which are dependent of ATP generated within the mitochondria. Conversely, it stimulated glycolysis and fructolysis, which are compensatory phenomena for an inhibited mitochondrial ATP generation. Furthermore, diuron diminished the cellular ATP content under conditions where the mitochondrial respiratory chain was the only source of this compound. 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. Some of the general signs of toxicity that were observed in diuron-treated rats can be attributed, partly at least, to the effects of the herbicide on energy metabolism.

1. Introduction

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a selective herbicide, efficient in the control of a wide range of weeds and grasses in the cultures of cotton, coffee, sugar cane and citrus. Diuron is a white and odorless powder, scarcely soluble in water (Hodge et al., 1967). Its chemical structure is shown as an inset in Fig. 1. The compound acts as an herbicide because it is an efficient inhibitor of photosynthesis. It blocks the binding site of plastoquinone to the photosystem II, thus preventing the transfer of electrons to this intermediate (Metz et al., 1986). The herbicide was introduced in the market in 1954 and is amply available in several forms and packages. Consequently, it is ubiquitous in the environment and there are frequently serious risks of human and animal contamination (Giacomazzi and Cochet, 2004).

According to Boyd and Krupa (1970), who examined the toxicology of diuron in rats, the LD_{50} is equal to 1.02 g/kg, with death occurring allegedly by respiratory collapse. The conclusion of Boyd and Krupa was based on observations in isolated mitochondria (Abo-Khatwa and Hollingworth, 1974). Diuron, as well as several herbicides, is active on the respiratory activity of rat liver mitochondria. Based on a series of observations it was concluded that diuron and several other herbicides are capable of acting as uncouplers of oxidative phosphorylation (Abo-Khatwa and Hollingworth, 1974). Later on, it was also shown that diuron blocks the electron flow at the cytochrome bc1 segment with 90% inhibition at the concentration of 50 µM (Convent and Briquet, 1978). The latter effect, however, was demonstrated in yeast mitochondria (*Saccharomyces cerevisiae)* and has, apparently, not yet been demonstrated in mammalian mitochondria.

Strong inhibition of oxidative phosphorylation may have letal consequences to animals (Boyd and Krupa, 1970), but incomplete inhibition at various degrees is known to be the cause of several effects, especially metabolic ones (Acco et al., 2004; Caldeira et al., 2008; Saling et al., 2011; Moreira et al., 2013). Investigation of the effects of several pesticides on the active transport of glucose in the intestine of mice, for example, revealed that diuron inhibited the active absorption of glucose (Guthrie et al., 1974). Actually, in general, it could be concluded that all pesticides that impair oxidative phosphorylation are equally able to inhibit the intestinal glucose absorption. With respect to the metabolism of the liver, the site where most biotransformation reactions occur, there is a single study investigating the action of diuron (Owen and Halestrap, 1993). This study had a highly specific purpose, namely, to use diuron as an inhibitor of oxidative phosphorylation with the aim of measuring the modifications in the concentrations of intermediates of the gluconeogenic pathway and of cofators such as ATP. According to this study, diuron inhibits gluconeogenesis from lactate + pyruvate (ratio 10/1) by 60% in isolated hepatocytes at the concentration of 0.5 mM (Owen and Halestrap, 1993). No concentration dependence is known and the way how diuron affects gluconeogenesis from other precursors, glycolysis, fructolysis and oxygen uptake is also unknown. As an uncoupler and a concomitant inhibitor of electron flow

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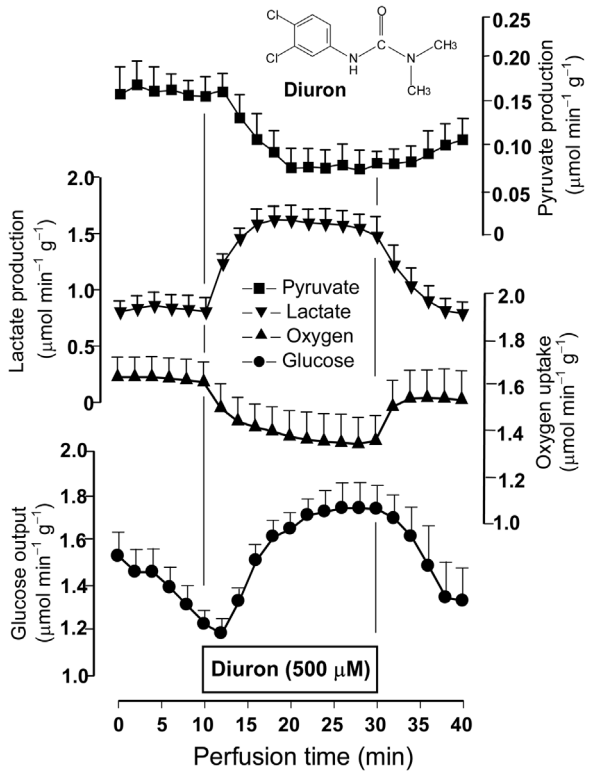

Fig. 1. Time course of the actions of diuron on glycogen catabolism and oxygen uptake. Livers from fed rats were perfused as described in the materials and methods section. Diuron was infused as indicated. The effluent perfusate was sampled in 2 min intervals and analyzed for its glucose, lactate, and pyruvate contents. Oxygen consumption was followed polarographically. Each datum point represents the mean of 3 liver perfusion experiments. Bars are standard errors of the mean.

the effect of diuron on the respiratory activity of the intact cell could be either inhibition or stimulation, depending on what effect predominates (Acco et al., 2004; Saling et al., 2011; Moreira et al., 2013). Furthermore, the hepatic transformation of diuron involves a series of demethylations (Van Boven et al., 1990; Coelho-Moreira et al., 2013), processes that require the use of reducing power in the form of NADPH and O_2 in the electron transport chain of the endoplasmic reticulum (Scholz et al., 1973).

Taking into account what was exposed above, the purpose of the present work was to conduct a systematic study on the effects of diuron on the hepatic metabolism. The experimental system was the isolated perfused rat liver in which the microcirculation and cell polarity is preserved in addition to the cell integrity. The results should bring additional information about the interactions of diuron with the liver.

2. Materials and methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringa. Diuron, 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).

2.2. 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. 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. A total of 85 rats were used in the experiments, 70 in the liver perfusion experiments and 15 in the exeriments with isolated mitochondria. 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 1219290915).

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 diuron were added to the perfusion fluid according to the experimental protocols. Due to its low water solubility, diuron was added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration. These concentrations were 50, 100 200 and 500 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 h fasted rats. Under these 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 neutralised 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-