Toxicology and Applied Pharmacology 329 (2017) 259–271

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Distribution, lipid-bilayer affinity and kinetics of the metabolic effects of dinoseb in the liver

Gabriela Bueno Franco Salla, Lívia Bracht, Anacharis Babeto de Sá-Nakanishi, Angela Valderrama Parizotto, Fabrício Bracht, Rosane Marina Peralta, Adelar Bracht *

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

ARTICLE INFO

Article history:
Received 13 April 2017
Revised 5 June 2017
Accepted 13 June 2017
Available online 15 June 2017

Keywords:
Herbicides
Hepatic metabolism
Hepatic perfusion
Isolated mitochondria

ABSTRACT

Dinoseb is a highly toxic pesticide of the dinitrophenol group. Its use has been restricted, but it can still be found in soils and waters in addition to being a component of related pesticides that, after ingestion by humans or animals, can originate the compound by enzymatic hydrolysis. As most dinitrophenols, dinoseb uncouples oxidative phosphorylation. In this study, distribution, lipid bilayer affinity and kinetics of the metabolic effects of dinoseb were investigated, using mainly the isolated perfused rat liver, but also isolated mitochondria and molecular dynamics simulations. Dinoseb presented high affinity for the hydrophobic region of the lipid bilayers, with a partition coefficient of 3.75×10^4 between the hydrophobic and hydrophilic phases. Due to this high affinity for the cellular membranes dinoseb underwent flow-limited distribution in the liver. Transformation was slow but uptake into the liver space was very pronounced. For an extracellular concentration of 10 μM, the equilibrium intracellular concentration was equal to 438.7 μM. In general dinoseb stimulated catabolism and inhibited anabolism. Half-maximal stimulation of oxygen uptake in the whole liver occurred at concentrations (2.8–5.8 μM) at least ten times above those in isolated mitochondria (0.28 μM). Gluconeogenesis and ureagenesis were half-maximally inhibited at concentrations between 3.04 and 5.97 μM. The ATP levels were diminished, but differently in livers from fed and fasted rats. Dinoseb disrupts metabolism in a complex way at concentrations well above its uncoupling action in isolated mitochondria, but still at concentrations that are low enough to be dangerous to animals and humans even at sub-lethal doses.

1. Introduction

Dinitrophenols are a class of compounds that exert several biological and toxic effects. The main action is impairment of energy metabolism, an effect that can be extremely harmful depending on its degree and intensity (Heusinkveld et al., 2016; Zaharia et al., 2016). Dinitrophenols can be and has been used in agriculture as herbicides and pesticides that persist in contaminated soils and waters. Although several of them have been officialy banned in many countries, their use as pharmaceutical agents has also been proposed, especially for weight loss purposes and at least one of them, 2,4-dinitrophenol, can be purchased in the internet. Many death cases have been reported as accidents in agriculture or as overdoses in weight-loss diets (Zaharia et al., 2016).

The potency by which these compounds exert their activity varies considerably. The perhaps most toxic of them seems to be 2-(butan-2-yl)-4,6-dinitrophenol, more commonly known as dinoseb (McBean, 2012). Dinoseb has been used mainly as an herbicide for weed-control in the production of crops like soybeans, vegetables, fruits and nuts, or citrus. It was also used as an insecticide to protect grapes. In the present, dinoseb is banned in the European Union and United States due to its high toxicity. However, it is still used in other countries and routinely found in rain- and drinking water (Heusinkveld et al., 2016; Zaharia et al., 2016; Guan et al., 2013). Furthermore, especially in China, large quantities of the acetate ester of dinoseb (dinoseb acetate) are manufactured annually, a compound that is equally used as pesticide (ProfResearch Reports, 2014). Dinoseb acetate can be easily transformed into dinoseb by simple hydrolysis of the ester bond, a phenomenon that is catalyzed by non-specific esterases (Preiss et al., 1988). In animals highly active esterases exist (Eler et al., 2013), leading to rapid hydrolysis of dinoseb acetate into dinoseb (McBean, 2012). Another dinitrophenol that is rapidly metabolized in animals into dinoseb is binapacryl (the 3,3-dimethylacrylic acid ester of dinoseb), a compound used as acaricide and fungicide (McBean, 2012).

The LD_{50} values for orally administrated dinoseb are relatively low and vary between 14 and 114 mg/Kg, depending on the species (McBean, 2012; Zaharia et al., 2016). Preceding death the poisoning symptoms include, prostration and convulsions, hyperventilation and hyperthermia. It is generally assumed that the main mechanism behind

* Corresponding author at: Department of Biochemistry, University of Maringá, 5790 Avenida Colombo, 87020900 Maringá, Brazil.
E-mail addresses: adebracht@uol.com.br, abracht@uem.br (A. Bracht).

http://dx.doi.org/10.1016/j.taap.2017.06.013
0041-008X/

the deleterius effects of dinoseb is uncoupling of mitochondrial oxidative phosphorylation (McBean, 2012). In plants dinoseb is also an uncoupler of photophosphorylation (Oettmeier and Masson, 1980). Dinoseb is a potent uncoupler, considerably superior to the classical uncoupler 2,4-dinitrophenol. Half-maximal stimulation of oxygen uptake driven by succinate in isolated rat liver mitochondria (state IV respiration), for example, has been reported to occur at a concentration of 0.12 μM dinoseb (Palmeira et al., 1994a). For 2,4-dinitrophenol the corresponding concentration is 15–20 μM (Chappell, 1964; Hanstein and Hatefi, 1974). In mechanistic terms dinoseb seems to be a classical inhibitor, i.e., a compound that acts as a protonophore, thus dissipating the proton gradient across the mitochondrial membrane as well as the membrane potential (Palmeira et al., 1994a). Such an action, when potent enough, inevitably leads to decreased levels of cellular ATP (Palmeira et al., 1994b), leading ultimately to energetic failure of the whole organism.

ATP depletion by dinoseb has already been demonstrated in isolated hepatocytes (Palmeira et al., 1994b). However, impairment of energy metabolism has several consequences for basic metabolic pathways, such as glycolysis and gluconeogenesis (Saling et al., 2011; Moreira et al., 2013), which in the case of dinoseb have not yet been characterized. The distribution space of dinoseb in the liver has also not yet been measured. Based on these premises we decided to undertake a systematic study on the distribution (transport), lipid bilayer affinity and kinetics of the metabolic effects of dinoseb in the liver. The experimental system was the isolated perfused rat liver in which microcirculation and cell polarity are both preserved in addition to the cell integrity. Preservation of the microcirculation may be important for characterizing the cellular uptake of dinoseb, especially if intense interactions with the cellular lipidic environment occur. In addition to obtaining experimental data, molecular dynamics simulations were also done with the purpose of broadening the theoretical basis for interpretation. The results should bring additional information about the interactions of dinoseb with the liver which could be important if one considers that dinitrophenols in general still trigger both health and legal issues worldwide (McBean, 2012; Zaharia et al., 2016).

2. Material and methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. A schematic representation of this apparatus can be seen Fig. S1 (Supplementary material). Dinoseb (2-(butan-2-yl)-4,6-dinitrophenol) was purchased from Sigma Chemical Co. (St Louis, USA). All enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, USA). All standard chemicals were from the best available grade (98–99.8% purity).

2.2. Animals

Male Wistar rats, weighing 240–260 g, were used in the experiments. The rats were housed in individual cages, maintained on a regulated light–dark cycle and fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). When required the rats were starved for 18 h prior to the experiments. Preparation of the liver for perfusion was done under sodium thiopental anesthesia, which was injected intraperitoneally (50 mg/kg). Lack of body or limb movement in response to a standardized tail clamping stimulus was the criterion of anesthesia. 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 8172271015).

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 transferred to a plexiglass chamber. See Fig. S1 (Supplementary material) for a schematic representation of the perfusion apparatus. Constant flow perfusion was done using a peristaltic pump (Minipuls 3, Gilson, France). The flow rate was adjusted between 30 and 32 mL/min, depending on the liver weight (Scholz and Bücher, 1965; Kelmer-Bracht et al., 1984). The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg/100 mL 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: 116 mM NaCl, 25 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.24 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 dinoseb were added to the perfusion fluid according to the experimental protocols. Due to its low water solubility, dinoseb was added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration. It is already amply documented that dimethyl-sulfoxide 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.

2.4. Metabolites assays

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 were referred to the wet weight of the liver.

The hepatic contents of the adenine nucleotides were measured by HPLC after freeze-clamping the perfused liver with liquid nitrogen (Mito et al., 2014). Two gram of the freeze-clamped liver were macerated in liquid nitrogen until homogeneity. To the powdered liver 6 mL of 0.6 M perchloric acid was addded and the suspension was homogenenized in a van Potter-Elvejhem homogenizer. The resulting homogenate was centrifuged at 4000*g* for 10 min and the resulting homogenate neutralized with fixed volumes of potassium carbonate (0.5 M). Extraction with perchloric acid has the advantage that the resulting insoluble potassium perchlorate salt can be eliminated by precipitation (Bergmeyer, 1974). The precipitated potassium perchlorate was eliminated by filtration and the fltrate used for the adenine nucleotides determinations. 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, 30%. 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