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Comparative effects of fipronil and its metabolites sulfone and desulfinyl on the isolated rat liver mitochondria

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ABSTRACT

Fipronil is an insecticide extensively used to control pests in crops and animals. There are relates of poisoning due to exposure of fipronil in mammals and the liver has been suggested as potential target. In this study, we evaluated the effects of fipronil and its metabolites sulfone and desulfinyl on the bioenergetics, reactive oxygen species (ROS) production and calcium efflux from mitochondria isolated from rat liver. Fipronil (5–25 μM) inhibited state-3 respiration in mitochondria energized with glutamate plus malate, substrates of complex I of the respiratory chain and decreased the mitochondrial membrane potential resulting in inhibition of ATP synthesis. Fipronil also caused uncoupling in succinate-energized mitochondria and calcium efflux. The metabolites sulfone and desulfinyl also acted as mitochondrial inhibitors and uncouplers and caused calcium efflux, but with different potencies, being the sulfone the more potent one. These effects of fipronil and its metabolites on mitochondrial bioenergetics and calcium homeostasis may be related to toxic effects of the insecticide in the liver.

1. Introduction

Fipronil is an insecticide that is widely used agriculturally or in the veterinary for controlling a variety of pests, such as cockroaches, mosquitoes, grasshoppers, fleas, ticks and lice (Chanton et al., 2001; Wilde et al., 2001; Aajoud et al., 2003). It belongs to the new generation of insecticides that have been developed to reduce damage to the environment and mammals. In addition, these new insecticides serve as alternative control agents that are necessary because some insects have developed resistance to other compounds such as organophosphate insecticides, pyrethroids and carbamates; thus, these new insecticides demonstrate great effectiveness at lower doses compared with the agents previously cited (Bobe et al., 1997).

The toxic action of fipronil is due to its ability to act as a non-competitive γ-aminobutyric acid-gated chloride channel blocker, leading to insect death by neuronal hyperexcitation and paralysis (Zhao et al., 2004). Although recommended for insects control in crops or animals, fipronil has demonstrated high toxicity on insects no target as honeybee (El Hassani et al., 2005; Aliouane et al., 2009) or stingless bees (Jacob et al., 2013). In the first instance, fipronil does not represent a severe toxic agent to mammals; however, any species may be affected if the dose is greater than that recommended by the manufacturers. According to reports in the literature, there are cases of poisoning due to accidental exposure or incorrect use of the compound (Chodorowski and Anand, 2004; Lee et al., 2010). A case of acute poisoning after ingestion of a high fipronil dose caused human death (Mohamed et al., 2004).

Beyond the central nervous system, other organs may be affected by fipronil treatment, including the liver. A study by Silva (2008) evaluated the effects of prolonged exposure of rats to the insecticide; they observed swollen hepatocytes and increased liver weights in animals treated with an oral 10 mg kg^{-1} dose, which corresponds to one tenth of the LD_{50} established for rat (Tingle et al., 2003). Additionally, mice livers examined following exposure to different fipronil doses (15, 25 and 50 mg kg^{-1}) revealed cytological, morphological, and histochemical alterations in hepatocytes with autophagic processes, steatosis and necrotic cell death (Ferreira et al., 2012; Oliveira et al., 2012). Recent study using perfused rat liver showed that fipronil (10–50 μM) interferes with energy metabolism causing hepatotoxicity, as demonstrated by the increase in the activity of lactate dehydrogenase in the effluent perfusate (Medeiros et al., 2015).

Once in the bloodstream, fipronil and its metabolites are widely distributed, particularly in adipose tissues, in addition to having a

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high level of enterohepatic recirculation (Hainzl and Casida, 1996; Mohamed et al., 2004). Sulfone metabolite formation, which was derived from an oxidation reaction, corresponded to the major route of hepatic metabolism of this compound in mammals (Caboni et al., 2003; Tang et al., 2004). According to Leghait et al. (2009), this oxidation reaction is catalyzed by cytochrome P450 enzymes. Both fipronil as well as its metabolite sulfone can induce cell death in HepG2 cells and human hepatocytes (Das et al., 2006). In addition, one of its main degradation products on vegetation, soil and in water, fipronil-desulfinyl, is very persistent and may bioaccumulate, particularly in fish (Tingle et al., 2003).

The liver performs many functions relating to animal metabolism as it receives both xenobiotics as well as nutrients, which are absorbed through the digestive tract and the portal vein. In relation to xenobiotics the primary role of liver is the biotransformation mediated primarily by cytochrome P450 enzyme complex, which puts hydrophobic compounds in water-soluble compounds to be more easily eliminated by the organism (Guillouzo, 1998).

Mitochondria are responsible for synthesis of almost all of the ATP that is required for maintaining cellular structure and function. The proton motive force, whose major impetus is the membrane potential ($\Delta\psi$) that is generated by electron transport along the respiratory chain in the inner mitochondrial membrane, drives ATP synthesis via oxidative phosphorylation (Mitchell, 1961). Experimental evidences indicate that mitochondria represent a critical and preferred target for the action of drugs and toxins. The toxic effects on mitochondria can occur through direct and indirect mechanisms, leading to mitochondrial dysfunction such as changes in electron transport and oxidative phosphorylation, in inner mitochondrial membrane permeability, calcium transport, and the oxidative state as well as a series of other events that deplete ATP (Nadanaciva et al., 2007; Dykens et al., 2008; Castanha-Zanoli et al., 2012; Li et al., 2012).

Thus, in this work, we addressed the actions of fipronil and its metabolites sulfone and desulfinyl on mitochondrial bioenergetics by assessing its effect on respiration, membrane potential and ATP levels, besides reactive oxygen species (ROS) generation and calcium homeostasis in isolated rat liver mitochondria. The influences of fipronil biotransformation on mitochondrial function and liver toxicity are considered.

2. Materials and methods

2.1. Chemicals

Fipronil was kindly supplied by the company Ourofino Agribusiness (Cravinhos, São Paulo, Brazil), fipronil-sulfone was obtained from Dr. Ehrenstorfer-Schäfers (Augsburg, Germany) and fipronil-desulfinyl was purchased from Sigma–Aldrich (São Paulo, Brazil). All of the other reagents were of the highest commercially available grade. Dimethyl sulfoxide (DMSO), which was used to dissolve fipronil and its metabolites, had no effect on the assays. The volume of DMSO added never exceeded 0.1% of the total media volume. All of the stock solutions were prepared using glass-distilled deionized water.

2.2. Animals

Male Wistar rats weighing approximately 200 g were used in this study. The animals, obtained from the Central Bioterium of UNESP – Univ Estadual Paulista, Campus de Botucatu, SP, Brazil, were maintained with a maximum of 4 rats per cage under standard laboratory conditions with water and food provided ad libitum. The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the UNESP – Univ Estadual Paulista, Campus de Dracena, SP, Brazil.

2.3. Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen et al., 1978). Rats were sacrificed by decapitation, and the liver was immediately removed, sliced into 50 ml medium containing 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES–KOH, pH 7.2, and homogenized three times for 15 s at 1-min intervals with a Potter-Elvehjem homogenizer. Homogenate was centrifuged at 770 × *g* for 5 min, and the resulting supernatant was further centrifuged at 9800 × *g* for 10 min. The pellet was suspended in 10 ml medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES–KOH, pH 7.2 and centrifuged at 4500 × *g* for 15 min. The final mitochondrial pellet was suspended in 1 ml medium containing 250 mM sucrose and 10 mM HEPES–KOH, pH 7.2 and was used within 3 h. The mitochondrial protein concentration was determined by a biuret assay with BSA as the standard (Cain and Skilleter, 1987).

After each isolation procedure a respiratory analysis was conducted using a simple state 3 (ADP-activated)-state 4 (without ADP) transition with succinate as substrate and the respiratory control ratio (RCR) (state 3 divided by state 4) was calculated (Chance and Williams, 1956) to confirm that the isolated mitochondria were fully functional. The RCR of mitochondria was always in the range 4.3–5.5.

2.4. Mitochondrial respiration assay

Mitochondrial respiration was monitored using a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Glasgow, Scotland, UK), and respiratory parameters were determined according to Chance and Williams (1956). In total, one milligram mitochondrial protein was added to 1 ml respiration buffer containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES–KOH, pH 7.4, plus 0.5 mM EGTA and 10 mM K_2HPO_4, at 30 °C. Oxygen consumption was measured using 5 mM glutamate + 5 mM malate or 5 mM succinate (+50 nM rotenone) as respiratory substrates in the absence (state-4 respiration) or the presence of 400 nmol ADP (state-3 respiration).

2.5. Estimation of mitochondrial membrane potential ($\Delta\psi$)

The mitochondrial membrane potential ($\Delta\psi$) was estimated spectrofluorimetrically using a model RF-5301 PC Shimadzu fluorescence spectrophotometer (Tokyo, Japan) at 495/586 nm excitation/emission. Safranine O (10 μM) was used as a probe (Zanotti and Azzone, 1980). Mitochondria (2 mg protein) energized with 5 mM glutamate + 5 mM malate or 5 mM succinate (+50 nM rotenone) were incubated in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES–KOH, pH 7.4, and 0.5 mM EGTA (2 ml final volume).

2.6. ATP quantification

ATP levels were determined using the firefly luciferin–luciferase assay system (Lemasters and Hackenbrock, 1976). Mitochondria (1 mg protein) energized with 5 mM glutamate + 5 mM malate were incubated at 30 °C for 15 min in a medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4 (1 ml final volume). After incubation, the mitochondrial suspension (1 mg protein/ml) was centrifuged at 9000 × *g* for 5 min at 4 °C, and the pellet was treated with 1 ml ice-cold 1 M $HClO_4$. After centrifugation at 14,000 × *g* for 5 min at 4 °C, 100 μl aliquots of the supernatants were