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Imidacloprid affects rat liver mitochondrial bioenergetics by inhibiting F_oF_1-ATP synthase activity

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ABSTRACT

Imidacloprid (IMD) is a neonicotinoid insecticide widely used in crops, pets, and on farm animals for pest control. Several studies were conducted examining the adverse effects of IMD on animals often exhibiting hepatic damage. The aim of this study was to determine the effects of IMD on bioenergetics of mitochondria isolated from rat liver. Imidacloprid (50–200 μM) produced a concentration-dependent decrease in oxygen consumption and ATP production without markedly affecting mitochondrial membrane potential (MMP). Oxygen consumption experiments showed that IMD did not significantly affect the respiratory chain, and this was similar to findings with oligomycin and carboxyatractyloside, suggesting a direct action on F_oF_1-ATP synthase and/or the adenine nucleotide translocator (ANT). Imidacloprid inhibited F_oF_1-ATP synthase activity only in disrupted mitochondria and induced a partial inhibition of ADP-stimulated depolarization of the MMP. Our results indicate that IMD interacts specifically with F_oF_1-ATP synthase resulting in functional inhibition of the enzyme with consequent impairment of mitochondrial bioenergetics. These effects of IMD on mitochondrial bioenergetics may be related to adverse effects of this insecticide on the liver.

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Introduction

Pesticides are extensively used in agriculture and are increasingly present in the environment. These compounds are often persistent and bioaccumulate through biological chains, such as soil-plant-food or water-organisms-food (Preston 2002), and have undergone risk assessments for human health (Cochran and Ross 2017).

Imidacloprid (IMD) is a systemic neonicotinoid insecticide that kills insects via ingestion or contact, interfering with the nervous system through blockade of acetylcholine post-synaptic receptors (nAChR) (Tomizawa, Lee, and Casida 2005). This insecticide is widely used for control of various insects in the treatment of seeds, soil, and crops, and even for the control of fleas on domestic animals, flies in farm animals stables and lice on sheep (Bayer 2018; Fossen 2006; Hutchinson, Jacobs, and Mencke 2001; Pospischil 2002). Because this compound is highly selective for nAChR in insects, IMD should not be a serious toxic agent for mammals; however, cases of poisoning have occurred in humans due to occupational exposure or misuse of products containing IMD (Han, Tian, and Shen 2018; Kumar, Verma, and Kumar 2013; Mohamed et al. 2009).

A number of studies were conducted on the influence of IMD on animals including rats (Kapoor et al. 2014; Mohany et al. 2011; Toor, Sangha, and Khera 2013), mice (Arfat et al. 2014), broiler chickens (Kammon et al. 2010), fish (Desai and Parikh 2013; Qadir et al. 2014), and bovids (Kaur, Sandhu, and Kaur 2006), demonstrating that the compound has the capacity to damage the liver. However, the mechanisms underlying the adverse actions in hepatic tissue remain to be determined. Further, there are also some reports indicating neurotoxic (Abou-Donia et al. 2008; Bal et al. 2010; Lonare et al. 2014), genotoxic (Demsia et al. 2007; Kataria et al. 2016; Stivaktakis et al. 2016), and reproductive (Bal et al. 2012a, 2012b) effects attributed to IMD.

The liver plays a key role in metabolism as this organ receives large amounts of nutrients and

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xenobiotics that are absorbed through the digestive tract and portal vein. Among the major functions of the liver are the uptake of amino acids, lipids, carbohydrates, and vitamins, with subsequent storage, metabolic conversion, and release into blood and bile (Guillouzo 1998). The liver is also the main organ involved in the biotransformation of exogenous substances (xenobiotics), with the capacity to convert hydrophobic compounds into water soluble metabolites, which are more readily eliminated by the organism. This process of biotransformation (detoxification) is carried out mainly by the cytochrome P450 enzymatic system (Danielson 2002; Ioannides 2002).

Mitochondria are responsible for the synthesis of almost all of the ATP that is required for maintaining cellular structure and function. These organelles represent a preferential and critical target for the action of drugs, toxins, or their reactive metabolites. The adverse effects on mitochondria might occur through direct and indirect mechanisms, leading to mitochondrial dysfunction, including changes to electron transport and oxidative phosphorylation, mitochondrial permeability transition, calcium transport, the oxidative state, and a series of other events leading to ATP depletion and cell death (Dykens et al. 2008; Li et al. 2012; Nadanaciva et al. 2007; Tavares et al. 2015). Thus, it is important to examine the effects of IMD on parameters related to mitochondrial bioenergetics using isolated organelles from rat liver in order to determine mechanisms underlying hepatotoxicity induced by this insecticide.

Methods

Chemicals

IMD was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest commercially available grade. IMD concentrations used in the experiments were selected following preliminary mitochondrial respiration assay. Dimethyl sulfoxide (DMSO) utilized to dissolve IMD exerted no marked effect on the assays. The volume of DMSO added never exceeded 0.5% of the total media volume. All stock solutions were prepared using glass-distilled deionized water.

Animals

Male Wistar rats weighing approximately 200 g were employed in this study. The animals, obtained from the Central Bioterium of the São Paulo State University (Unesp), Botucatu, SP, Brazil, were maintained with a maximum of 4 rats per cage under standard lab conditions, while water and food were provided *ad libitum*. The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the Unesp, College of Agricultural and Technological Sciences, Dracena, SP, Brazil (Approval number 30/2015.R2).

Isolation of intact and disrupted rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen et al. 1978). Animals were euthanized by decapitation under ether narcosis, and the liver was immediately excised, sliced in 50 ml medium containing 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES-KOH, pH 7.2, and homogenized three times for 15 sec at 1-min intervals with a Potter-Elvehjem homogenizer. Homogenate was centrifuged at 770 × *g* for 5 min, and resulting supernatant was further centrifuged at 9,800 × *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 4,500 × *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 used within 3 hr. The mitochondrial protein concentration was determined by a biuret assay with BSA as the standard (Cain and Skilleter 1987).

Disrupted mitochondria were obtained by heat shock treatment after three consecutive cycles of freezing in liquid nitrogen and thawing in a water bath heated to 37°C. The membrane fragments were kept at 4°C and used in assessment of mitochondrial enzymatic activity within 3 hr.

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 (1955). One mg 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 plus 5 mM malate or 5 mM succinate (plus 50 nM rotenone) as respiratory substrates in the absence (state 4 respiration) or the presence of 400 nmol ADP (state 3 respiration).

Estimation of mitochondrial membrane potential (Δψ)

The mitochondrial membrane potential (Δψ) (MMP) was estimated spectrofluorimetrically using a fluorescence spectrophotometer (Shimadzu RF-5301 PC, Tokyo, Japan) using 495/586 nm as the excitation/emission wavelength. Safranine O (10 μM) was used as a probe (Zanotti and Azzone 1980). Mitochondria (2 mg protein) in the presence of 5 mM glutamate plus 5 mM malate 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), at 30°C.

ATP quantification

ATP levels were determined in mitochondria in the presence of 5 mM glutamate plus 5 mM malate using the firefly luciferin-luciferase assay system (Lemasters and Hackenbrock 1976). The incubation medium contained 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 (1 ml final volume). The reaction was started by addition of mitochondria (1 mg protein), the medium was shaken, and after 10 min of incubation in the presence of IMD, the suspension was centrifuged at 9,000 × *g* for 5 min at 4°C, and 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 neutralized with 5 M KOH, suspended in 100 mM TRIS-HCl, pH 7.8 (1 ml final volume), and centrifuged at 15,000 × *g* for 15 min. The supernatant was processed with a Sigma-Aldrich assay kit (Catalog Number FLAA) according to the manufacturer's instructions and measured using a SIRIUS Luminometer (Berthold, Pforzheim, Germany).

Mitochondrial F_OF_1-ATP synthase activity

Mitochondrial F_oF_1-ATP synthase activity was measured in intact-uncoupled and freeze/thaw-disrupted mitochondria according to method of Bracht, Ishii-Iwamoto, and Salgueiro-Pagadigorria (2003), with modifications. Intact mitochondria (0.5 mg protein/ml) were incubated in a medium containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.4, plus 0.2 mM EGTA, a chelating agent, and 5 mM ATP for 20 min at 37°C, in the presence of 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), in a final volume of 0.5 ml. When disrupted mitochondria were utilized as enzyme source, medium contained 20 mM TRIS-HCl (pH 7.4) and 5 mM ATP. The reaction was started by addition of 5 mM ATP and terminated by addition of ice-cold 5% trichloroacetic acid. F_oF_1-ATP synthase activity was evaluated by measuring released inorganic phosphate, as described by Fiske and Subbarow (1925), at 700 nm using a DU-800 spectrophotometer (Beckman Coulter, Fullerton, CA). Results were expressed as nmol Pi/min/mg protein. Sensitivity to oligomycin (1 μg/ml), an inhibitor of proton translocation in F_oF_1-ATP synthase, was tested in all mitochondrial suspensions.

Inhibition of ADP-induced depolarization of Δψ

Inhibition of ADP-induced depolarization of Δψ was performed according to O'Brien, Oliveira, and Wallace (2008) with modifications. Freshly isolated mitochondria (2 mg protein) were added in a medium containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES-KOH, pH 7.4, plus 0.5 mM EGTA, 5 mM glutamate plus 5 mM malate, and 10 μM safranine O (2 ml final volume) at 30°C. Then, 200 μM IMD or 1 μg/ml oligomycin or 5 μM carboxyatractyloside (cATR) were added 0.5 min before adding 400 nmol ADP. ADP-induced depolarization describes the change and recovery in Δψ upon addition of ADP. The amplitude of depolarization induced by ADP was measured in the presence and absence of the test compounds.

Statistical analysis

Data are expressed as mean ± SEM, and statistical differences were calculated using one-way analysis of variance (ANOVA) followed by the Dunnett's test using GraphPad Prism, v 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Effects of IMD on mitochondrial respiration

Mitochondrial oxygen (O_2) consumption was monitored in the presence of varying concentrations of IMD. The parameters assessed were state 3 respiration (consumption of oxygen in the presence of respiratory substrate and ADP) and state 4 respiration (consumption of oxygen after ADP has been exhausted). At the concentrations tested (50–200 μM), IMD inhibited state 3 respiration of mitochondria in a concentration-dependent manner. This effect was observed when mitochondria were in the presence of either glutamate plus malate, the respiratory chain site I substrates (Figure 1A), or succinate, a respiratory chain site II substrate (Figure 1B). The compound did not stimulate state 4 respiration, indicating that IMD does not appear to act as an uncoupler (data not shown).

Subsequent experiments with carbonyl cyanide m-chlorophenylhydrazone (CCCP)-stimulated mitochondrial respiration were performed to examine the inhibitory influence of the compound on respiratory chain or ATP synthase. IMD did not markedly block CCCP-uncoupled respiration, indicating that only oxidative phosphorylation was inhibited (Figure 2). The same behavior was noted with oligomycin (F_oF_1-ATP synthase inhibitor) and carboxyatractyloside (ANT inhibitor).

Influence of IMD on mitochondrial membrane potential (Δψ)

Figure 3 illustrates the effect of IMD (50–200 μM) on Δψ of glutamate plus malate-energized rat hepatic mitochondria. IMD did not significantly dissipate the Δψ. At the end of the experiment, 1 μM CCCP (uncoupler) was added as a positive control, and the mitochondrial membrane electrical potential dissipated.

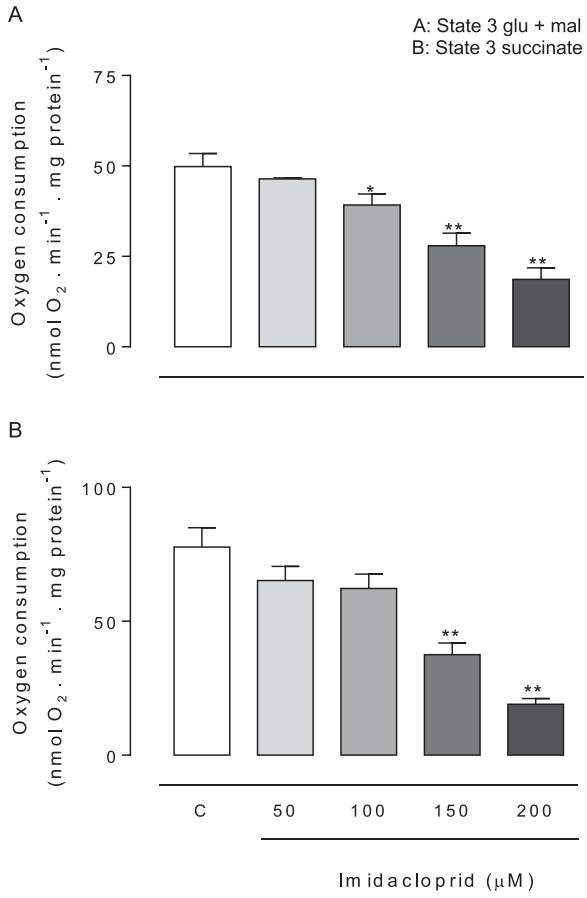

Figure 1. Effect of IMD (50–200 μM) on the state 3 respiration rate of 5 mM glutamate plus 5 mM malate (A) and 5 mM succinate-energized (B) rat liver mitochondria (1 mg protein), in a respiration medium (1 ml final volume) described in Methods. State 3 respiration was induced with 400 nmol ADP. Values represent the mean ± SEM of three experiments with different mitochondrial preparations. C: control, without the addition of IMD. *Significantly different from control ($P < 0.05$), **Significantly different from control ($P < 0.01$).

Effect of IMD on mitochondrial ATP levels

The effect of IMD (50–200 μM) on mitochondrial ATP levels was assessed using the respiratory assay conditions 10 min after mitochondria were incubated with the compound (Figure 4). In agreement with the mitochondrial respiration results, IMD produced a significant concentration-dependent decrease in mitochondrial ATP levels.

Influence of IMD on F_OF_1-ATP synthase activity

The effects of IMD (50–200 μM) on F_oF_1-ATP synthase activity were determined in intact-uncoupled mitochondria in the presence of CCCP,

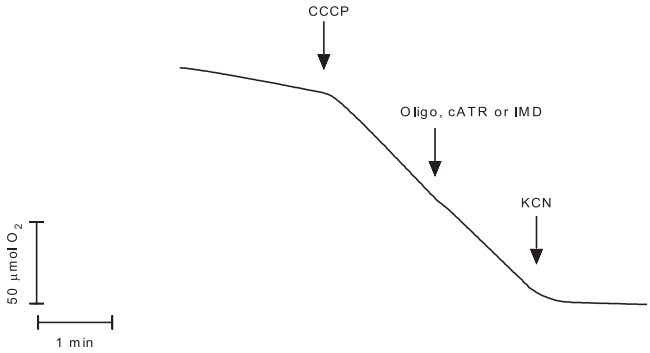

Figure 2. Comparative effect of IMD (200 µM), Oligo: oligomycin (1 µg/ml), cATR: carboxyatractyloside (1 µM) and KCN: potassium cyanide (1 µM) on the respiration of rat liver mitochondria energized with 5 mM glutamate plus 5 mM malate, after stimulation with the uncoupler CCCP (1 µM). The assay conditions are the same as the mitochondrial respiration described in Methods. Arrows indicate the addition of compounds. The trace is representative of those obtained with three independent experiments for each tested compound carried out on three different mitochondrial preparations.

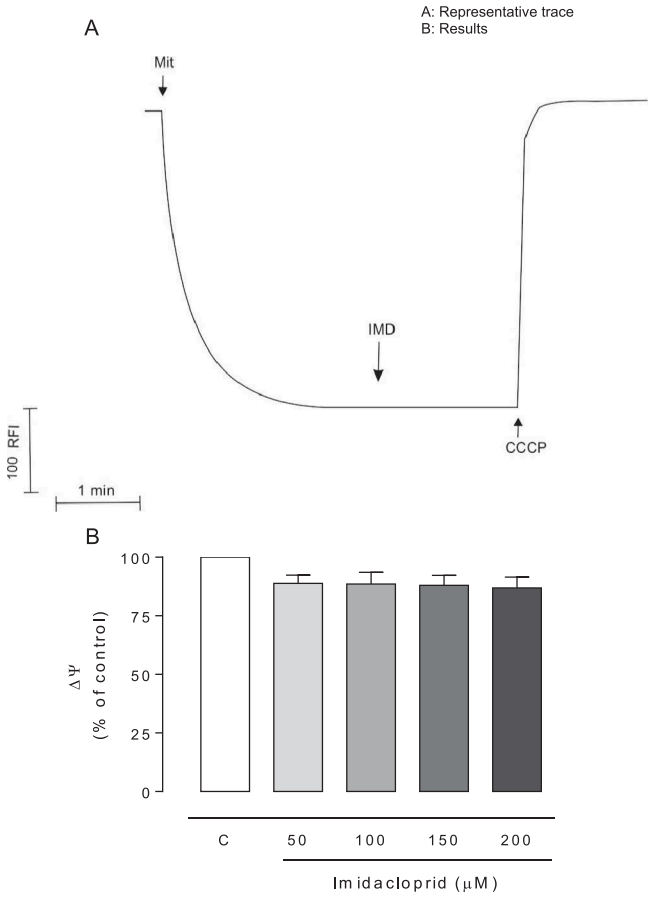

Figure 3. Influence of IMD (50–200 µM) on the membrane potential of 5 mM glutamate plus 5 mM malate-energized rat liver mitochondria (2 mg), in a medium (2 ml final volume) described in Methods. This experiment was performed in state 4 conditions, that is, in the absence of exogenous ADP. Figure A is representative of a membrane potential experiment, and the values in Figure B represent the mean ± SEM of three experiments with different mitochondrial preparations. C: control, without addition of IMD. RFI: relative fluorescence intensity.

and in freeze/thaw-disrupted mitochondria (Figure 5A and 5B, respectively). The F_oF_1-ATP synthase activity of uncoupled mitochondria was not markedly affected by IMD (Figure 5A). In disrupted mitochondria, IMD significantly reduced F_oF_1-ATP synthase activity in a concentration-dependent manner (Figure 5B).

Effect of IMD on ADP-induced depolarization of Δψ

The purpose of this assay was to determine whether IMD inhibits ADP-induced depolarization of Δψ by interference with ANT. Carboxyatractyloside was used as a positive control for direct ANT inhibition. IMD (200 µM) initiated only a partial inhibition of ADP-stimulated depolarization of Δψ, an effect similar to that found with oligomycin (1 µg/ml) (Figure 6).

Discussion

The effects of IMD on the bioenergetics of mitochondria isolated from rat liver were determined to assess the potential involvement of mitochondria in hepatic injury induced by this insecticide. Mitochondria are responsible for most of the energy generated and used by cells through oxidative phosphorylation (Nicholls and Ferguson 2013). The energy released by oxidation of substrates in the respiratory chain is utilized to transport protons across the inner membrane, supporting the proton motive force that drives ATP synthesis by F_oF_1-ATP synthase or complex V, which consists of two functional proteins: F_1, situated in the mitochondrial matrix, and F_o, located in the inner mitochondrial membrane. Several investigators reported that various compounds are capable of producing alterations in oxidative phosphorylation (Bridges et al. 2014; Maioli et al. 2012; Wallace and Starkov 2000). Among these are inhibitors that interfere with ATP synthesis often operating in respiratory chain complexes or in F_oF_1-ATP synthase (Nicholls and Ferguson 2013; Zheng and Ramirez 2000).

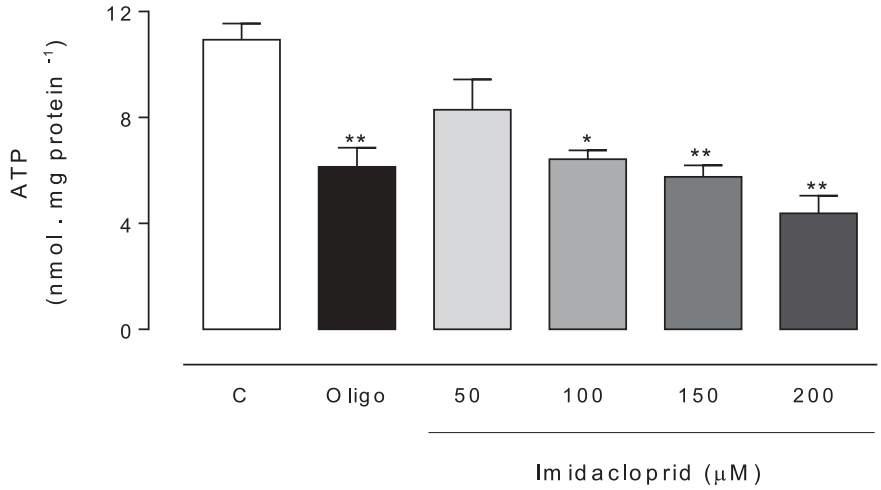

Figure 4. Effect of IMD (50–200 μM) on the ATP levels in 5 mM glutamate plus 5 mM malate-energized rat liver mitochondria (1 mg), in a medium (1 ml final volume) described in Methods, using the luciferin-luciferase system. Values represent the mean ± SEM of three experiments with different mitochondrial preparations. C: control, without the addition of IMD. Oligo: oligomycin 1 μg/ml. *Significantly different from control ($P < 0.05$), **Significantly different from control ($P < 0.01$).

Data obtained using mitochondria incubated in the presence of glutamate plus malate (electron donors for complex I) and succinate (electron donor for complex II) showed that IMD inhibits state 3 respiration in a concentration -dependent manner ranging from 100–200 μM in complex I and 150–200 μM in complex II. According to Chance and Williams (1955), state 3 respiration involves mitochondria, ADP and a respiratory substrate, and the rate of ADP phosphorylation is the limiting factor of the process. The blockade observed in the two complexes may be attributed to the direct action of IMD in the respiratory chain or from an inhibitory effect on F_oF_1-ATP synthase or the adenine nucleotide translocator (ANT).

To distinguish whether the blocking effect of IMD on state 3 respiration is due to inhibition of the electron transport chain or oxidative phosphorylation, a respiration test stimulated by the uncoupler CCCP was performed. If blockade occurs in the electron transport chain, uncoupler-stimulated O_2 consumption might be inhibited. If the tested compound rather acts on oxidative phosphorylation, inhibition of CCCP-uncoupled respiration is not possible. Mitochondrial O_2 consumption was not blocked by IMD, but inhibited by KCN (respiratory chain complex IV inhibitor), indicating that the inhibition of state 3 respiration by this insecticide may not occur by direct action on the respiratory chain. Thus, IMD may act as an inhibitor of F_oF_1-ATP synthase and/or ANT, since this effect is similar to that noted with oligomycin, a specific inhibitor of F_oF_1-ATP synthase, and carboxyatractyloside (cATR), an inhibitor of ANT.

IMD did not dissipate the $\Delta\psi$. Since this experiment was performed in state 4 respiring mitochondria, that is, in the absence of exogenous ADP, $\Delta\psi$ may only be dissipated by an uncoupler, which is not the case for IMD. Evidence indicates an absence of stimulation in the state 4 respiration and affirms the previously proposed hypothesis that IMD behaves similarly to oligomycin and/or cATR.

Mitochondria are responsible for producing most of the ATP needed for energy-requiring reactions in eukaryotic cells. Interference in the synthesis or use of mitochondrial ATP is the mechanism by which many xenobiotics produce acute or chronic toxicity (Meyer and Kulkarni 2001). IMD significantly inhibited synthesis of ATP in a concentration-dependent manner, starting at 100 μM. Similar results of IMD affecting mitochondrial bioenergetics were obtained in another study by our group on mitochondria isolated from the heads and thoraces of Africanized honey bees (Nicodemo et al. 2014).

Two experiments were performed to determine the possible effect of IMD on F_oF_1-ATP synthase and/or ANT. In the first, the influence of the insecticide on ADP-induced depolarization of the

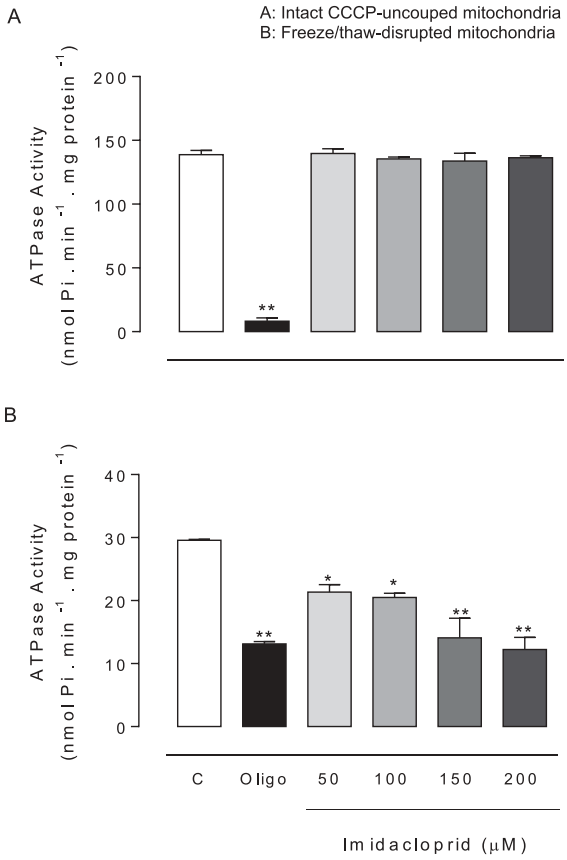

Figure 5. Influence of IMD (50–200 μM) on F_oF_1-ATP synthase activity in intact CCCP-uncoupled (A) and in freeze/thaw-disrupted (B) rat liver mitochondria. Intact mitochondria (0.5 mg protein) were incubated in a medium (0.5 ml final volume) described in Methods. Disrupted mitochondria (0.5 mg protein) were incubated in a medium (0.5 mL final volume) also described in Material and Methods. F_oF_1-ATP synthase activity was evaluated by measuring released inorganic phosphate. Values represent the mean ± SEM of three experiments with different mitochondrial preparations. C: control, without the addition of IMD. Oligo: oligomycin 1 μg/ml. *Significantly different from control ($P < 0.05$), **Significantly different from control ($P < 0.01$).

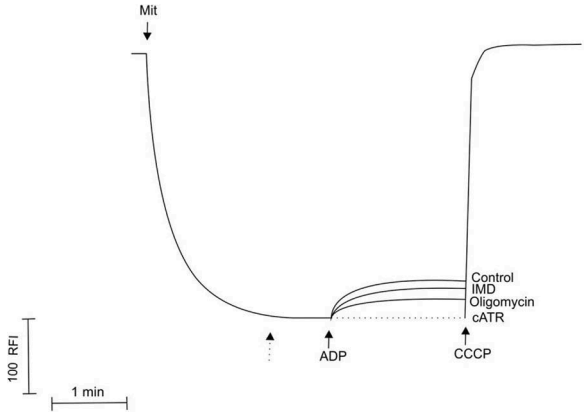

Figure 6. Comparative effect of IMD (200 μM), oligomycin (1 μg/ml) and carboxyatractyloside (cATR) (5 μM) on ADP (400 nmol)-induced depolarization of Δψ. Mitochondria (2 mg protein) were added in a medium (2 ml final volume) described in Methods, plus 5 mM glutamate plus 5 mM malate and 10 μM safranine O. Dotted arrow: Addition of IMD, oligomycin or cATR. Control: without the addition of tested compounds. RFI: relative fluorescence intensity. The trace is representative of those obtained with three independent experiments for each tested compound carried out on three different mitochondrial preparations.

Δψ was evaluated using oligomycin (specific inhibitor of the F_o component of F_oF_1-ATP synthase) and cATR (ANT specific inhibitor). When cATR was added prior to ADP, depolarization of the formed potential was completely inhibited because the ANTs were blocked; thus, ADP was not transported to the mitochondrial matrix. When oligomycin was added prior to ADP, only partial depolarization of the Δψ occurred, since the transport of adenine nucleotides by ANT was limited in this condition. Therefore, IMD exerted a similar effect to oligomycin, indicating that it only acts on F_oF_1-ATP synthase.

To confirm the IMD-induced inhibition of F_oF_1-ATP synthase, the effect on enzyme activity was assessed using intact-uncoupled and freeze/thaw-disrupted mitochondria with an excess of ATP, a condition that drives the enzyme to operate in the reverse direction, thus hydrolyzing ATP (Bracht, Ishii-Iwamoto, and Salgueiro-Pagadigorria 2003). IMD significantly diminished the activity of F_oF_1-ATP synthase in a concentration-dependent manner, but only in disrupted mitochondria. A possible explanation for this observed difference is that in CCCP-uncoupled mitochondria, the overall structure of the organelle is preserved, while in the disrupted mitochondria, mitochondrial structure is destroyed, and F_oF_1-ATP synthase is exposed. This suggests that disruption of the mitochondrial structure enables IMD to interact with the enzyme, most likely targeting the F_1 portion. Further studies are necessary to clarify the exact mechanism by which IMD interacts with complex V. This effect, together with the inhibition of state 3 respiration and the assays performed in ADP-induced depolarization of Δψ, indicates that the insecticide inhibits F_oF_1-ATP synthase.

The high specificity for receptors in insects was considered to possess highly selective toxicity to insects and relative sparing of animals. However, studies of the effects of IMD on rodents, chicken, fish, and bovids noted hepatotoxicity. Animals exposed to different doses of the insecticide exhibited signs and symptoms characteristic of liver damage, such as hemorrhage, hepatocyte degeneration, central venous congestion and dilatation, hepatic sinusoids (Kammon et al. 2010; Mohany et al. 2011; Toor, Sangha, and Khera 2013), pycnotic nuclei and leukocyte infiltration, as well as hypertrophied blood vessels, cytoplasmic lesions, and liver necrosis (Arfat et al. 2014), with consequent release of enzymes aspartate aminotransferase and alanine aminotransferase in the extracellular medium (Arfat et al. 2014; Desai and Parikh 2013; Duzguner and Erdogan 2012; Kapoor et al. 2014; Mohany et al. 2011; Qadir et al. 2014; Soujanya et al. 2013; Toor, Sangha, and Khera 2013).

Previous studies demonstrated that exposure to IMD poses a potential risk to farm animals and humans. Shridhar (2010) reported a clinical toxicity episode in which 8 buffaloes died after drinking water from a pond contaminated with IMD. Data on human exposure to IMD are related to occupational exposure (Agarwal and Srinivas 2007; Calumpang and Medina 1996; Kumar, Verma, and Kumar 2013) and case reports of self-poisoning (David, George, and Peter 2007; Huang et al. 2006; Lin et al. 2013; Mohamed et al. 2009; Proença et al. 2005; Wu, Lin, and Cheng 2001). Generally, mild clinical effects such as tachycardia, hypertension, nausea, vomiting, headache, and diarrhoea occur, but more serious sequelae, including respiratory failure, seizures (Agarwal and Srinivas 2007; Mohamed et al. 2009; Wu, Lin, and Cheng 2001), and even death (Huang et al. 2006; Iyyadurai, George, and Peter 2010; Proença et al. 2005; Shadnia and Moghaddam 2008) were reported.

In addition to the effects on animals described above, the widespred use of neonicotinoid insecticides, such as IMD, was suggested as a factor that might contribute to the decline of bee species (Goulson 2013; van der Sluijs et al. 2013). Since IMD is a systemic insecticide, to reduce its quantity in the environment, some management methods such as insecticide application as a seed dressing were adopted, avoiding spray treatments in the fields. In this way, recent studies using the quantitative weight of evidence (QWoE) methodology to assess higher-tier studies on the effects of IMD on honey bees concluded that IMD, as it is currently used as a seed treatment and with good agricultural practice, does not present a significant risk to honey bees at the level of the colony (Solomon and Stephenson 2017; Stephenson and Solomon 2017).

Thus, despite the use of IMD seeming to be apparently safe for animals, the misuse, intentional ingestion, and accidental exposure to this compound were noted to produce damage to animals and humans, but the exact mechanisms underlying IMD poisoning are still unknown. Our results suggest that IMD may induce changes in mitochondrial bioenergetics and that the enzyme F_oF_1-ATP synthase is a target of the adverse action of this insecticide. Because inhibition of energy metabolism may affect several processes important for cell maintenance, it is conceivable that the liver mitochondrial energy production changes described in the present investigation might contribute to IMD-mediated hepatotoxicity. In addition, some of the general signs of toxicity that were observed in IMD poisoning in animals and humans may partly be the consequence of a diminished energy production in tissues, as the effects reported may certainly not be restricted to liver mitochondria.

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