Toxicology in Vitro 27 (2013) 570–579

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

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

The role of mitochondria and biotransformation in abamectin-induced cytotoxicity in isolated rat hepatocytes

Marcos A. Maioli [a], Hyllana C.D. de Medeiros [a], Marieli Guelfi [a], Vitor Trinca [b], Flávia T.V. Pereira [b], Fábio E. Mingatto [a,*]

[a] Laboratório de Bioquímica Metabólica e Toxicológica (LaBMeT), UNESP – Univ Estadual Paulista, Campus de Dracena, 17900-000 Dracena, SP, Brazil
[b] Laboratório de Morfofisiologia da Placenta e Embrião (L@MPE), UNESP – Univ Estadual Paulista, Campus de Dracena, 17900-000 Dracena, SP, Brazil

ARTICLE INFO

Article history:
Received 17 July 2012
Accepted 29 October 2012
Available online 6 November 2012

Keywords:
Abamectin
Hepatotoxicity
Calcium
ATP
Necrosis

ABSTRACT

Abamectin (ABA), which belongs to the family of avermectins, is used as a parasiticide; however, ABA poisoning can impair liver function. In a previous study using isolated rat liver mitochondria, we observed that ABA inhibited the activity of adenine nucleotide translocator and F_oF_1-ATPase. The aim of this study was to characterize the mechanism of ABA toxicity in isolated rat hepatocytes and to evaluate whether this effect is dependent on its metabolism. The toxicity of ABA was assessed by monitoring oxygen consumption and mitochondrial membrane potential, intracellular ATP concentration, cell viability, intracellular Ca^{2+} homeostasis, release of cytochrome *c*, caspase 3 activity and necrotic cell death. ABA reduces cellular respiration in cells energized with glutamate and malate or succinate. The hepatocytes that were previously incubated with proadifen, a cytochrome P450 inhibitor, are more sensitive to the compound as observed by a rapid decrease in the mitochondrial membrane potential accompanied by reductions in ATP concentration and cell viability and a disruption of intracellular Ca^{2+} homeostasis followed by necrosis. Our results indicate that ABA biotransformation reduces its toxicity, and its toxic action is related to the inhibition of mitochondrial activity, which leads to decreased synthesis of ATP followed by cell death.

1. Introduction

Avermectins are metabolites derived from the fermentation of the fungi *Streptomyces avermitilis*; these metabolites belong to the family of macrocyclic lactones and exhibit extraordinarily potent anthelmintic activity (Burg et al., 1979; Fisher and Mrozik, 1989). Abamectin (ABA) is a mixture of avermectins containing $\geqslant$80% B_{1a} and $\leqslant$20% B_{1b} (Meister, 1992; Zeng et al., 1996; Agarwal, 1998). Avermectin B_{1a} and B_{1b} differ chemically by the presence of a methylene or ethylene group at C-26 (Zeng et al., 1996). According to Hayes and Laws (1990), these molecules have similar biological activities and toxicological properties. ABA is widely used because of its potent anthelmintic and insecticidal action and wide spectrum of action. ABA is also used as an insecticide to control citrus, nut culture and household pests, such as fire ants (Elbetieha and Daas, 2003). In veterinary medicine, ABA is administered to animals in a systematic way to control endoparasites and ectoparasites (Shoop et al., 1995).

The mechanism of ABA action is related to its effect on the γ-aminobutyric acid (GABA) system and Cl^- channels. GABA receptors are responsible for regulating the neural basal tone of the brain (Turner and Schaeffer, 1989) and are in virtually all neurons of the central nervous system (CNS). The symptoms of ABA poisoning exhibited in laboratory animals include pupil dilation, vomiting, convulsions and/or tremors and coma (Lankas and Gordon, 1989). In addition, some studies have reported genotoxic effects of ABA (Molinari et al., 2010).

As demonstrated by the *in vivo* studies (Lowenstein et al., 1996; Hsu et al., 2001) and the *in vitro* study conducted with isolated hepatocytes (El-Shenawy, 2010), the liver can also be affected by ABA. ABA caused an increase in the concentration of the enzyme aspartate aminotransferase (AST) in serum *in vivo* and an increase in the concentration of AST and alanine aminotransferase (ALT) *in vitro*, which are used as indicators of damage to the hepatic parenchymal cells (Klaassen and Eaton, 1991). We previously demonstrated that ABA inhibits the activity of F_oF_1-ATPase and adenine nucleotide translocator (ANT) when added at micromolar concentrations to isolated rat liver mitochondria, an effect associated with significantly reduced ATP synthesis (Castanha Zanoli et al., 2012).

F_oF_1-ATPase is an enzyme present in the inner mitochondrial membrane that is responsible by ATP synthesis driven by the proton electrochemical gradient generated in the respiratory chain. The main components of the enzyme are F_o, an integral membrane protein that works as a proton channel, and F_1, a hydrophilic moiety which contains the catalytic and regulatory sites (Hatefi,

* Corresponding author. Tel.: +55 18 3821 8158; fax: +55 18 3821 8208.
E-mail address: fmingatto@dracena.unesp.br (F.E. Mingatto).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.10.017

1993; Pedersen, 1996). ANT is other important component of the mitochondrial machinery of ATP synthesis because of its intrinsic adenine nucleotide translocase activity. ANT has been involved in both pathological (mitochondrial permeability transition formation/regulation and cell death) and physiological (adenine nucleotide exchange) mitochondrial events, making it a prime target for drug-induced toxicity (Oliveira and Wallace, 2006).

The xenobiotic metabolism in the liver is accomplished by cytochrome P450 and its main function is to increase the polarity of these substances, so excretion occurs more easily (Oga, 2008). However, this process is responsible for the toxic effects of numerous chemical compounds. The metabolites may cause adverse effects in the animal (Ioannides and Lewis, 2004; Mingatto et al., 2007; Maioli et al., 2011) by changing a fundamental cellular component (mitochondria, for example) at the cellular and molecular level, thus modulating its function (Meyer and Kulkarni, 2001).

Due to the important functions of the liver in animals and previous studies that indicated the occurrence of liver damage after the use of ABA, this study aims to characterize the mechanisms of ABA toxicity on parameters related to bioenergetics and cell death and determine whether the toxicity induced by the compound is due to a possible activation following its metabolism in the liver.

2. Materials and methods

2.1. Chemicals

Abamectin, containing 92% avermectin B_{1a} and 8% avermectin B_{1b}, was kindly supplied by the company Ourofino Agribusiness (Cravinhos, SP, Brazil), proadifen was purchased from Sigma–Aldrich (St. Louis, MO, USA), and sodium pentobarbital was a gift from Cristália (Itapira, SP, Brazil). All other reagents were of the highest commercially available grade. Abamectin and proadifen were dissolved in anhydrous dimethyl sulfoxide (DMSO). All stock solutions were prepared using glass-distilled deionized water.

2.2. Animals

Male Wistar rats aged 7–8 weeks and weighing approximately 200 g, were used in this study. The animals, which were 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 and incubation of hepatocytes

For the surgical procedure, the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The hepatocytes were isolated by a collagenase perfusion of the liver as described previously (Guguen-Guillouzo, 1992). The hepatocyte viability after isolation was determined by Trypan blue (0.16%) uptake, and the initial cell viability in all experiments was more than 85%. The hepatocytes were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM Hepes and 0.1% bovine serum albumin (BSA), and maintained at 4 °C. The cells (1×10^6/mL) were incubated in 25-mL Erlenmeyer flasks, which were maintained under constant agitation (30 rpm) at 37 °C under a 95% O_2 and 5% CO_2 atmosphere. The reactions in the experiments of cell viability, cellular ATP content, mitochondrial membrane potential, release of cytochrome *c*, caspase 3 activity and necrotic cell death were initiated by the addition of abamectin (ABA) at concentrations of 25, 50, 75 and 100 μM. Aliquots (1 mL) of the suspension were removed from the mixture at appropriate times for the determination of cell death and biochemical parameters. In some experiments, the cells were incubated with 100 μM proadifen 15 min before the addition of ABA.

2.4. Oxygen uptake

Oxygen uptake by the isolated hepatocytes was monitored using a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Glasgow, Scotland, UK). The respiration buffer contained 250 mM sucrose, 2 mM KH_2PO_4, 10 mM HEPES, pH 7.2, 0.5 mM EGTA, 0.5% BSA, and 5 mM $MgCl_2$, at 37 °C. The cells were treated with 0.002% digitonin, and state 4 and state 3 mitochondrial respiration rates were measured in the presence of 1 μg/mL oligomycin and 2 mM ADP, respectively (Moreadith and Fisckum, 1984). ABA at concentrations of 5, 10, 15 and 25 μM was added to the medium immediately after the initiation of state 3 or state 4 respirations.

2.5. Mitochondrial membrane potential

The mitochondrial membrane potential was determined using the fluorescent probe TMRM (tetramethyrodamine, methyl ester). The cell suspensions incubated with different concentrations of abamectin were collected and centrifuged at 50*g* for 5 min. The pellet was suspended and incubated for 10 min at 37 °C with TMRM solution at a final concentration of 6.6 μM. After the incubation, the samples were centrifuged twice at 50*g* for 5 min, and the pellet was suspended with 1 ml of Triton X-100, 0.1% (v/v). Subsequently, the samples were centrifuged at 2000*g* for 5 min, and the fluorescence of the TMRM captured and retained by the mitochondria was determined in the supernatant using a fluorescence spectrophotometer RF-5301 PC (Shimadzu, Tokyo, Japan) at excitation and emission wavelengths of 485 and 590 nm, respectively. The results are expressed as a percentage of the fluorescence intensity over the control group.

2.6. Cellular ATP content

Cellular ATP content was determined by the firefly luciferin–luciferase assay. The cell suspension was centrifuged at 50*g* for 5 min at 4 °C, and the pellet containing the hepatocytes was treated with 1 mL of ice-cold 1 M $HClO_4$. After centrifugation at 2000*g* for 10 min at 4 °C, aliquots (100 μL) of the supernatant were neutralized with 65 μL of 2 M KOH, suspended in 100 mM Tris–HCl, pH 7.8 (1 mL final volume), and centrifuged again. Bioluminescence was measured in the supernatant with a Sigma–Aldrich assay kit according to the manufacturer's instructions using a SIRIUS Luminometer (Berthold, Pforzheim, Germany).

2.7. Evaluation of cell viability

Cell viability was assessed by the leakage of alanine transaminase (ALT) and aspartate transaminase (AST) from hepatocytes. After incubation with ABA at concentrations of 25, 50, 75 and 100 μM the cell suspensions were collected at time 0, 30, 60, 90 and 120 min and centrifuged (50*g* for 5 min). The presence of ALT and AST in the supernatant was determined using Enzyme Activity Assay Kits (Bioclin, Quibasa, Brazil) according to the manufacturer's instructions. The absorbance was measured at 340 nm with a spectrophotometer DU-800 (Beckman Coulter, Fullerton, CA, USA). Enzyme activity in the supernatant is expressed as a percentage of the total activity, which was determined by lysing the cells with 0.5% Triton X-100.