Cytosolic and localized inhibition of phosphodiesterase by atrazine in swine tissue homogenates

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Abstract

Atrazine (ATR) significantly inhibited phosphodiesterase (PDE) in crude homogenates of swine heart, brain, and lung, but not liver or kidney tissues. Except for heart, PDE activities in the cytosolic fraction of the tissue homogenates were not affected by ATR. The inhibition of the PDE activity in the cytosol from heart homogenate was not significantly different between ATR and a non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX). Dixon plots of the crude tissue homogenates showed that heart and brain were inhibited via two different mechanisms (competitive or mixed inhibition, and noncompetitive inhibition, respectively), suggesting that ATR may be a semi-specific PDE inhibitor. Furthermore, in crude tissue homogenates, ATR did not inhibit PDE as effectively as IBMX suggesting that there are ATR-susceptible and ATR-nonsusceptible forms of PDE. Association constants for ATR were 55 μM for heart and 310 μM for brain. The stability of the activity of PDE was affected by freezing, requiring the use of only freshly prepared tissue homogenates.

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1. Introduction

Atrazine (ATR) (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is one of the most commonly used herbicides in the United States (Rossi, 2003). It has been reported that the administration of ATR at 30 mg/kg b.w. results in the delayed onset of puberty in rats (Ashby et al., 2002), disruption of organogenesis at concentrations between 10 and 20 mg/L in zebrafish (Wiegand et al., 2001), and a reduction in the size of gray tree frogs at metamorphosis was observed at exposures of 200 μg/L and greater (Diana et al., 2000). ATR may affect the proper maturation of animals by acting as an endocrine disruptor, but it does not affect the estrogen/estrogen receptor-α or estrogen receptor-β binding equilibria (Allran and Karasov, 2000; Carr et al., 2003; Sanderson et al., 2002).

Alternatively, endocrine disruption may occur by changes in the ability of the central nervous system to properly generate hormonal signals (Cooper et al., 2000; Stoker et al., 2002).

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger whose proper regulation is necessary for the appropriate management of cellular processes. Phosphodiesterase (PDE) terminates intracellular second messenger signaling by converting cAMP to 5′-adenosine monophosphate (5′-AMP). Previously, we reported competitive inhibition of a commercially available PDE by ATR (Roberge et al., 2004). Inhibition of PDEs may explain many of the effects of ATR observed in vivo, although little has been done to explore this connection (Sanderson et al., 2001). More than 50 different PDE proteins are known, and their distributions among tissues vary widely, while sub-cellular localization of PDEs can be dependent on conditions within the cell (Houslay and Adams, 2003; Mehats et al., 2002). The distribution of ATR and its metabolites in animal tissue has been
studied extensively, but work relating to the sub-cellular distribution is lacking. To determine the action of ATR on animals, an understanding of its localized effects on PDEs in specific tissues and at the sub-cellular level is necessary. This paper discusses the effects of ATR on swine tissue and the sub-cellular distribution of PDEs affected by ATR. Swine tissues were chosen based on the following: swine are considered to be a good mammalian test species, the size of the tissues, and the ready availability of fresh samples.

2. Experimental

2.1. Tissue homogenization

Tissues (lung, liver, brain, kidney, and heart) were collected at slaughter from three adult (90–110 kg) Yorkshire swine of mixed sex on different days and transported on ice for immediate homogenization (Animal and Range Science Department, North Dakota State University, Fargo, ND). Homogenization was carried out using the modified method of Geoffroy et al. (1999). Briefly, 5 g of tissue were homogenized in 20 mL of ice cold buffer [0.25 M sucrose, 2 mM NaF, 10 μM sodium orthovanadate, 20 mM sodium molybdate, 20 mM tris–HCl (pH 7.4), 0.4 mM EGTA, 1.6 mM EDTA, 20 mM 2-mercaptoethanol, 0.1 mM benzamidine, 2 mM phenylmethanesulfonyl fluoride, 2.5 μg/mL leupeptin, 2.5 μg/mL pepstatin A, 50 μg/mL trypsin inhibitor, 5 μg/mL aprotinin (Sigma–Aldrich, Milwaukee, WI)] with a Teflon pestle (Ehrenreich et al., 1973; Geoffroy et al., 1999). Aliquots of the crude homogenate were saved for PDE activity assays. Tissue homogenates were fractionated at 4 °C using centrifugation (Lodish et al., 2004). Aliquots of the supernatant and pellet were saved on ice after each spin (600 g × 10 min, 15,000 g × 5 min, 100,000 g × 60 min) resulting in a total of seven tissue fractions. The protein concentration of the crude homogenate and the 100,000 g supernate were determined using the Bradford method (Bradford, 1976). Aliquots of a single animal were frozen at −80 °C for one week then thawed at room temperature to determine the stability of PDE activity after freezing. Results for the crude tissue homogenates and the cytosolic fraction (100,000 g × 60 min) are presented here.

2.2. PDE activity

PDE activities were determined immediately after centrifugation using radiolabeled 14C-cAMP (MC-157, Moravek Biochemicals, Brea, CA) as the substrate. To determine the effect of freezing on PDE activity, frozen aliquots of homogenates were thawed and assayed along with paired fresh homogenates. Incubations of the homogenized tissue fractions were carried out at 30 °C for 20 min. In order to determine the PDE activity, crude tissue homogenates were diluted 1:10 in 10 mM Trizma, pH 7.4, but the cytosolic fractions were used as prepared. Incubations occurred in covered black flat-bottomed 96-well plates (3686, Corning, Corning, NY) with a total reaction volume of 25 μL [20 μL of 1:10 crude tissue homogenate, 2 μL of 14C-cAMP and 3 μL of 18 MΩ water (Millipore, Bedford, MA) or 3 μL of 100 μg/mL 3-isobutyl-1-methylxanthine in 20% MeOH (IBMX; Sigma–Aldrich) or 3 μL of 100 μg/mL ATR in 20% MeOH (Sigma–Aldrich, note: use of controls are described later in Section 3.3)]. Reactions were stopped by the addition of 5 μL of 55% trichloracetic acid (Sigma–Aldrich; Butcher and Sutherland, 1962). The radiolabeled cAMP and 5'-AMP product were separated on silica gel plastic TLC sheets (EM-5748-7, EM Science, Gibbstown, NJ; Higashida et al., 2002). Radioactivity was quantified using a Bioscan System 200 Imaging Scanner (Bioscan, Washington, DC). Enzymatic activities were calculated from the specific activity of the radiolabeled substrate spotted on the plate (56 mCi/mmol, 0.01 mCi/mL) and the amount of radioactivity in the substrate or product peak relative to the total amount of radioactivity present. The Student’s t-test was used to assign significance at the 95% confidence interval.

2.3. Dixon plots

Experiments used to construct Dixon plots were modified in order to enhance the detection of differences in the hydrolysis of 14C-cAMP. Briefly, 40 μL of the 1:10 crude homogenate, 10 μL of 100 μg/mL ATR in 20% MeOH, and 2 μL of cAMP (1.8 nmol/mL, 0.9 nmol/mL, or 0.45 nmol/mL) were placed in covered black flat-bottomed 96-well plates (Corning, Corning, NY). Incubations occurred for 20 min and were stopped by the addition of 5 μL of 55% trichloracetic acid. Separation and quantification of substrate and product were accomplished by TLC as described previously. Dixon plots were constructed by plotting the inverse of the reaction rate against the concentration of the inhibitor at three different concentrations of substrate using Microsoft Excel®.

3. Results

3.1. Effects of freezing on PDE activity

In order to determine the suitability of freezing freshly prepared tissue homogenates for later use, the activity of homogenates was determined before and after freezing. Significant differences existed between the activities of the fresh homogenates and homogenates that had been frozen (Fig. 1, P < 0.05). Phosphodiesterase activities of the fresh preparations were greater for the crude homogenates of lung and brain than frozen homogenates, while those of liver and heart were lower. Freezing did not significantly affect the activity of phosphodiesterase from kidney. Only fresh tissue preparations were used for subsequent experiments, in order to monitor mechanistic effects more closely resembling the in vivo situation instead of artifacts introduced by freezing and thawing of the enzyme.
3.2. Animal-to-animal variation

Tissue PDE activities of the crude homogenate were different among the three swine \((P < 0.05, \text{ data not shown})\). However, rank order of the cAMP hydrolyzing ability in the presence of the inhibitors was the same for each of the three animals in each of the tissues. Expressed as a percentage of basal values, protein normalized differences in PDE activities were significantly different only for liver tissues among the three animals; none of the other tissues were significantly different \((P < 0.05)\). This allowed for the pooling of the results from the three animals.

3.3. Crude homogenate activity

Homogenates (crude and isolated) were tested in the presence of 20% MeOH in Milli-Q water and in the presence of Milli-Q water to determine if the 20% MeOH inhibited PDE. The 20% MeOH treatments did not significantly affect the ability of PDE to hydrolyze cAMP \((P < 0.05)\). Fresh tissue fractions from brain had the greatest activity \((126 \text{ pmol/min/mg protein, Fig. 3})\), while liver exhibited the least \((13.6 \text{ pmol/min/mg protein})\). All activities, including lung, kidney, and heart \((20.1, 72.3, \text{ and } 77.2 \text{ pmol/min/mg protein})\) were reproducible from tissue homogenates of the same animal \((\text{SEM} < 10\%)\). Homogenates were incubated in the presence of IBMX \((\text{a universal PDE inhibitor})\) and ATR in order to determine if ATR could inhibit PDE \((\text{Fig. 3})\). IBMX is a known universal PDE inhibitor and was used as a positive control. IBMX significantly inhibited PDEs in all tissue homogenates but to a different extent in each preparation \((P < 0.05)\). ATR inhibited PDEs in brain, lung, and heart, but had no significant effect on liver or kidney \((P < 0.05)\). IBMX was a better inhibitor in lung and brain when compared to ATR. In heart, the rate of hydrolysis of cAMP was similar in the presence of either inhibitor \((P < 0.05)\). Tissues harvested from all three animals were in agreement concerning significance or non-significance when comparing the basal PDE activities to the PDE activities in the presence of ATR. Because of the consistency in trends among the three animals, data from a single animal will be presented in figures in order to facilitate discussion of absolute values.

3.4. Cytosolic activity

PDE activities in cytosolic fractions were assayed in the presence of IBMX and ATR \((\text{Fig. 4})\). Uninhibited liver exhibited the lowest cytosolic activity \((126 \text{ pmol/min/mg protein})\), while brain exhibited the highest \((286 \text{ pmol/min/mg protein})\). IBMX significantly inhibited cytosolic PDE in every tissue except kidney, while only heart cytosolic PDE was significantly inhibited by ATR. Similar to the inhibition in the crude homogenate, ATR and IBMX inhibition were not significantly different from one another in the cytosolic fraction of heart homogenate.

3.5. Dixon plots

Dixon plots were constructed in order to determine the mode of inhibition of PDE by ATR in crude heart and
brain homogenates (Fig. 5). Plots were linear and intersected left of the $y$-axis indicating that the inhibition of PDE was competitive, noncompetitive, or mixed. The intersection of the plot for heart intersected above the $x$-axis, which suggests a competitive or mixed type inhibition, while that of brain was near the $x$-axis indicating non-competitive inhibition. A vertical line drawn from the intersection of the plots to the $x$-axis produced the association constant ($K_a$) for ATR with the different tissue homogenates (heart = 55 $\mu$M and brain = 310 $\mu$M).

4. Discussion

It has been common practice when studying PDEs to freeze the preparation after homogenization and isolation (Geoffroy et al., 1999). However, the integrity of PDE activity in frozen crude homogenates has not been reported. Differences in the activities of the tissue preparations before and after freezing range from an increased activity of 174% for liver to a decrease of 52% for lung (14% and 7.2% hydrolysis of the substrate respectively; Fig. 1). These data are not surprising since PDE activities have been shown to deviate positively or negatively after freezing (Ho et al., 1976). Since little is known about the mechanisms behind these processes, only fresh tissue fractions were used in subsequent studies, to ensure that the PDE activity and inhibition characteristics were obtained without confounding factors. Comparing the protein normalized activities, as a percent reduction in the presence of inhibitors, allowed for statistical conclusions to be drawn. However, animal-to-animal variability should be taken into account when comparing PDE activity in the presence of potential inhibitors.

Activities presented were for crude homogenates; thus, the PDE activities, normalized for the mass of protein, appear lower than those found in the literature (Geoffroy et al., 2001; Kotera et al., 2004; Messner et al., 1979). Work with the PDEs has normally focused on the isolation and characterization of a specific PDE family or isoform. This requires homogenates to undergo significant cleanup in order to separate PDE from the components of the original tissue. PDEs were not isolated from other tissue components for two reasons. First, the goal of this work was to assess the potential of ATR to inhibit all forms of PDE in
the homogenized tissue; therefore the potential selective loss of any of the PDEs due to the sample preparation procedure was deemed unacceptable. Second, by working with whole tissue homogenates, ATR had the potential to interact with all of the matrix components. Consideration of interactions with other cellular components allowed for the possibility that ATR could be bound to other constituents or degraded in the tissues and more closely mimic in vivo conditions.

ATR significantly inhibited PDEs in the crude homogenates of the lung, brain, and heart (Fig. 3; \( P < 0.05 \)). Inhibition of PDE by ATR was not as effective in the crude tissue homogenates as inhibition by IBMX, except for heart. This indicated that ATR was either a specific PDE inhibitor, or that the homogenization conditions did not necessarily render metabolic processes inoperable. Metabolized forms of ATR were less effective at inhibiting PDE than the parent compound (Roberge et al., 2004). The cytochrome P-450 family of enzymes mediate the metabolism of ATR in vertebrates (Adams et al., 1990; Eisler, 2000; Lu et al., 1998). The possibility exists that ATR could have been metabolized during the study period because the presence of the less efficacious ATR metabolites cannot be necessarily discounted. However, in the cases of lung and brain, the cytosolic fraction had less activity than the crude homogenate on a per volume basis, which indicated that PDEs were removed by centrifugation. Using centrifugation, it would not be expected that the cytochrome P-450 enzymes would be more concentrated in the cytosolic fraction, which would be necessary to explain the data obtained from the brain and lung cytosol preparations compared to the crude homogenates. Furthermore, when the reactions were run with individual tissue preparations over a 40-min time span, 10-min increments produced linear plots of activity, which demonstrated that ATR was not degraded during the course of the reaction, and that metabolic processes were not a confounding factor in these experiments (data not shown).

Different PDE forms are localized within the sub-cellular matrix (Geoffroy et al., 1999; Ho et al., 1976; Jin et al., 1998; Marko et al., 2002). The only cytosolic fraction that exhibited an ATR-sensitive PDE activity was from the heart (Fig. 4). ATR reduced the activity of this fraction by 37%, indicating that PDEs insensitive to ATR inhibition were present in lung, kidney, liver, and brain cytosols. Moreover, the significant decreases in crude homogenate activities as a result of ATR can be attributed to localized forms of PDE susceptible to ATR inhibition in some tissues (lung and brain), while a non-localized form of ATR-susceptible PDE is present in the cytosol of others (heart). ATR was either somewhat selective and inhibited multiple forms of PDE, or ATR was a selective PDE inhibitor and the particular ATR-susceptible form of PDE was localized in lung and brain but not in heart tissue. The PDE activity of the cytosolic fraction of heart was not significantly different between IBMX and ATR inhibited reactions, which indicated that the same PDE isoforms were inhibited equally by both compounds.

Dixon plots were used to determine the mode of inhibition of PDE in crude tissue homogenates by ATR. The presence of ATR in homogenates from both heart and brain significantly inhibited PDE activity and the average association constants for ATR were 55 \( \mu \)M and 310 \( \mu \)M, respectively. Intersection of the Dixon plots for heart homogenates were above the \( x \)-axis, which indicated either a competitive or mixed type of inhibition. Plots from the brain crude homogenates intersected close to the \( x \)-axis, and indicated that the inhibition may be noncompetitive. There has been no attempt to isolate and characterize the different types of PDEs in these systems. The mechanistic determinations (Dixon plots) are important in describing the types of inhibition that may be present in animal tissue resulting in a physiological response. Demonstration of PDE specific inhibition brings into question the role of ATR in sub-cellular PDE fractions, which may have a significant effect on the biology of the cell as a whole. Because the suggested modes of inhibition do not agree, ATR may act on various PDEs by different mechanisms.

Localization and transient localization of PDE has been documented on a family- and isoform-specific basis (Jurcivius et al., 2003; Kotera et al., 2004). In the present study, repeatable tissue-specific cytosolic PDE activity (heart) indicated a non-localized ATR-susceptible PDE, while crude homogenates of brain and lung indicated a localized ATR-susceptible PDE. Crude tissue homogenates of liver and kidney indicated that ATR-nonsusceptible forms of PDE were present. Furthermore, results from Dixon plots of the crude tissue homogenates showed that ATR inhibited PDE from different tissues via different mechanisms, suggesting that ATR was not an indiscriminate PDE inhibitor, but may be selective for certain PDE isoforms.

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