Azoxystrobin at sub-cytotoxic concentrations disrupts intracellular zinc homeostasis: A flow cytometric analysis with rat thymic lymphocytes and fluorescent probes

Mai Shoji1, Masaki Asada1, Akihiko Matsumoto1, Haruki Nishino1, Ao Yi Xiang1, Mizuki Mizobuchi2, Naoki Kanematsu2, Hajime Miura1 and Norio Kamemura2

1Graduate School of Integrated Arts and Sciences, Tokushima University, Tokushima 770-8502, Japan
2Department of Food-Nutritional Sciences, Faculty of Life Sciences, Tokushima Bunri University, Tokushima 770-8514, Japan

(Received December 13, 2019; Accepted December 20, 2019)

ABSTRACT — Azoxystrobin is a broad-spectrum fungicide having a wide usage. However, the toxic effect of azoxystrobin in humans is not reported. In Japan, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit (2.5 mg/kg) in a shipment of an Australian barley used in different food products. Thus, there is a chance of azoxystrobin exposure through food to humans, and hence it is imperative to study the toxic effects of this compound. In this study, the toxic effect of azoxystrobin was evaluated to predict its adverse effects on human. Azoxystrobin at 3-30 µM (approximately 1.2-12.1 mg/L) raised the intracellular Zn\(^{2+}\) concentration of rat thymic lymphocytes. This increase was due to an influx of extracellular Zn\(^{2+}\) and a release of intracellular Zn\(^{2+}\). Azoxystrobin partially inhibited the temperature-dependent Zn\(^{2+}\) influx, thus jeopardizing the cellular Zn\(^{2+}\) homeostasis. Because Zn\(^{2+}\) is an important intracellular messenger in lymphocytes, this altered Zn\(^{2+}\) homeostasis might lead to adverse effects if the blood concentration of azoxystrobin reaches 3 µM or more in humans. However, by extrapolating the azoxystrobin pharmacokinetics data of rats to human, it can be predicted that such high blood concentration may be unlikely in humans.

Key words: Azoxystrobin, Intracellular zinc, Lymphocytes, Membrane potential

INTRODUCTION

Azoxystrobin is a broad-spectrum fungicide and its overall usage is increasing every year after the start of its sale (U.S. Geological Survey, 2016). This chemical agent blocks the electron transport chain in mitochondria resulting in inhibition of ATP production and higher oxidative stress in fungus (Sauter, 1995; Wong and Wilcox, 2001). Azoxystrobin is also toxic to different vertebrates and invertebrate species of both freshwater and marine systems (Rodrigues et al., 2013). Therefore, toxicological studies with azoxystrobin in non-target organisms involving mammals and particularly human draw attention (European Food Safety Authority, 2010; Kunz et al., 2017). To the best of our knowledge, there is no existing data to demonstrate the toxic effects of azoxystrobin at environmental concentrations in human. However, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit (2.5 mg/kg) in a shipment of an Australian barley (Minister of Agriculture, Forestry, and Fisheries of Japan, 2018). This particular barley was used in different food products and the manufacturing company voluntarily recalled its cereal products. In this study, the toxic effect of azoxystrobin was evaluated in an
in vitro model to predict its adverse effects on human.

Increased oxidative stress is found to be one of the adverse effects of azoxystrobin in some species of vertebrates and invertebrates (Rodrigues et al., 2013; Han et al., 2014, 2016). Oxidative stress can mobilize intracellular zinc (Zn²⁺) (McCord and Aizenman, 2014; Abiria et al., 2017). Therefore, there is a possibility that azoxystrobin leads to intracellular Zn²⁺ mobilization by oxidative stress resulting in the elevation of intracellular Zn²⁺ concentration. Zn²⁺ is released during the oxidative conversion of intracellular non-protein thiol to disulfide (Maret, 1995). To test this possibility, the effect of azoxystrobin on cellular content of non-protein thiols [(NPT)ᵢ] and intracellular Zn²⁺ concentration [(Zn²⁺)ᵢ] were studied using a flow cytometric technique coupled with appropriate fluorescent probes in lymphocytes isolated from rat thymus (thymocytes). Along with azoxystrobin, another postharvest fungicide pyrimethanil was also studied at the same concentration. It is to be mentioned that Zn²⁺ enters rat thymic lymphocytes through a temperature-sensitive pathway. The application of ZnCl₂ significantly increases the (Zn²⁺)ᵢ and this phenomenon is completely suppressed in cold condition (Takahashi et al., 2012; Fukunaga et al., 2015). The thymus gland is very active in the pre-adolescent and neonatal periods. Since there is a concern regarding the adverse effect of azoxystrobin in the pediatric population, any information pertaining to the adverse cellular changes in thymocytes by azoxystrobin may be of clinical importance.

MATERIALS AND METHODS

Chemicals

Azoxystrobin (standard purity of 99.8%) and pyrimethanil (standard purity of 98.9%) were procured from Wako Pure Chemicals (Tokyo, Japan). Propidium iodide (PI), 5-chloromethylfluorescein diacetate (5-CMF-DA), and FluoZin-3-AM were obtained from Invitrogen (Eugene, OR, USA). Diethylenetriamine-N,N,N',N'',N''-pentaaetic acid (DTPA; Dojindo Molecular Technologies, Inc.) and N,N,N',N''-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; Dojindo Molecular Technologies, Inc.) were used as extracellular and intracellular Zn²⁺ chelators respectively. The other reagents were obtained from Wako Pure Chemicals (Tokyo, Japan).

Cell preparation

The animal experiments were performed after obtaining approval from the committee of Tokushima University, Tokushima, Japan (approval no. 05279). The thymus glands of male Wistar rats (6-8-week-old) were triturated in chilled Tyrode’s solution (2-4°C) to dissociate thymic lymphocytes (thymocytes). The solution containing thymocytes (cell suspension) was stored at 36-37°C for at least 1 h before using in the subsequent experiments. It was noted that the cell suspension contained a small amount (216.9 ± 14.4 nM) of Zn²⁺ from the cell preparation (Sakanashi et al., 2009).

Thymocytes were used in this experiment for chemical cytotoxicity because these living cells with intact cell membranes can be easily isolated from thymus without enzymatic treatment. However, there is a technical limitation to obtain freshly dissociated thymocytes. During prolonged incubation, some cells may spontaneously undergo apoptosis. Hence, all the experimental works were completed within 6-7 hr after organ dissection to avoid spontaneous apoptosis of the cells. Since cell shrinkage is one of the parameters detected in the early stage of apoptosis (Bortner and Cidlowski, 1998), the forward scatter was used as a parameter to determine the cell size. The forward scatter and cell viability in control cells were kept unchanged during the entire experiment. Azoxystrobin (0.3-100 mM in 2 µL dimethyl sulfoxide) was added to the suspension (1998 µL) to prepare bath concentrations of 0.3-100 µM and the cells were treated with azoxystrobin for 1 hr. To test the effect of azoxystrobin on the temperature-sensitive Zn²⁺ pathway, the augmentation of FluoZin-3 fluorescence by applying 3 µM ZnCl₂ was examined in the presence and absence of 30 µM azoxystrobin.

Dimethyl sulfoxide (up to 0.3%) did not affect cell viability and the fluorescent cellular parameters studied. A sample (100 µL) from each cell suspension was analyzed by flow cytometry. Data acquisition from 2500-3000 cells took approximately 10-15 sec. The cellular parameters monitored for 2500 cells were similar to those examined for 10000 cells. Thus, 2500-3000 cells were deemed sufficient to study the cellular actions of azoxystrobin.

Measurements of cellular parameters with fluorescent probes

Zn²⁺ at a micromolar concentration significantly potentiates the cytotoxic action of H₂O₂ (Matsui et al., 2010). To examine whether azoxystrobin also has a similar property, the increase in the percentage of cell population exhibiting PI fluorescence by 300 µM H₂O₂ was compared in the presence and absence of 30 µM azoxystrobin.

Fluorescence was analyzed using a flow cytometer and software package (CytoACE-150; JASCO, Tokyo, Japan). Azoxystrobin at concentrations used in this study exhibited no fluorescence under the said experimental condi-
Disruption of cellular Zn\textsuperscript{2+} homeostasis by azoxystrobin

ions. PI (5 µM) was added to the suspension to assess cell lethality (percentage population of cells exhibiting PI fluorescence). The measurement of cellular non-protein thiols (NPT)i was performed using 1 µM 5-CMF-DA (Chikahisa et al., 1996). In order to monitor the change in intracellular Zn\textsuperscript{2+} concentration (Zn\textsuperscript{2+})i, the cells was incubated with 1 µM FluoZin-3-AM (Gee et al., 1989) for 50-60 min before initiating estimation.

FluoZin-3 fluorescence was monitored in the cells under extracellular Ca\textsuperscript{2+}-free condition because azoxystrobin also increases Ca\textsuperscript{2+} influx, as observed in our previous experiments (unpublished data on file). Azoxystrobin-induced changes in FluoZin-3 and 5-CMF fluorescence were examined only in the living cells with intact cell membranes that did not exhibit PI fluorescence. The excitation wavelength of all the probes (PI, 5-CMF, and FluoZin-3) was 488 nm, and emissions were detected at 530 ± 20 nm for 5-CMF and FluoZin-3, and at 600 ± 20 nm for PI. Each experimental series was conducted thrice unless stated otherwise.

**Statistical analysis**

The continuous variables were expressed as mean and standard deviation. Data were analyzed using Tukey’s multivariate method and a p value of < 0.05 was considered as statistically significant.

**RESULTS**

Impact on cell lethality by azoxystrobin and pyrimethanil

Treatment with 30 µM azoxystrobin and 30 µM pyrimethanil for 3 hr did not increase the population of cells exhibiting PI fluorescence, dead cells or cells with compromised cell membranes (Fig. 1). Thus, both these agents at 30 µM can be assumed to be non-cytotoxic.

![Fig. 1. Cytotoxic action of azoxystrobin on rat thymocytes. A: Changes in cytogram (forward scatter versus intensity of propidium fluorescence) after incubation with 30 µM azoxystrobin and 30 µM pyrimethanil for 3 hr. The cytogram consisted of 2000 cells. The dotted line under the cytogram indicates the cells exhibiting propidium fluorescence. B: The percentage population of cells exhibiting propidium fluorescence after 3 hr incubation with 30 µM azoxystrobin and 30 µM pyrimethanil. The column and bar indicate the mean and standard deviation of four samples.](image)
Changes in 5-CMF fluorescence by azoxystrobin and pyrimethanil

Although the cells were considered to be intact, there was a possibility that azoxystrobin and pyrimethanil at 30 µM concentration might affect some cellular parameters. To test this possibility, the effects of azoxystrobin and pyrimethanil on the (NPT)i were examined using 5-CMF-DA. 5-CMF fluorescence was significantly attenuated by the treatment with 30 µM azoxystrobin and 30 µM pyrimethanil for 3 hr (Fig. 2). The potency of azoxystrobin to reduce the (NPT)i was found to be greater than that of pyrimethanil.

Changes in FluoZin-3 fluorescence by azoxystrobin and pyrimethanil

The decrease in (NPT)i by azoxystrobin and pyrimethanil might indicate the conversion of thiols to disulfide leading to intracellular Zn²⁺ release from the cellular zinc...
stores. Therefore, the change of \((\text{Zn}^{2+})_i\) by azoxystrobin and pyrimethanil was estimated with FluoZin-3 fluorescence. The intensity of FluoZin-3 fluorescence was significantly increased by treatment with 30 µM azoxystrobin for 1 hr, but not by 30 µM pyrimethanil (Fig. 3). The FluoZin-3 fluorescence was significantly augmented by incubation with 3-30 µM azoxystrobin for 1-2 hr in a concentration-dependent manner (Fig. 4). The concentration of FluoZin-3 fluorescence observed at 1 hr after initiating incubation with azoxystrobin was similar to that observed at 2 hr.

**Characteristics of azoxystrobin-induced augmentation of FluoZin-3 fluorescence**

It was speculated that \((\text{Zn}^{2+})_i\) increased in the presence of 3-30 µM azoxystrobin. Therefore, the supply of increased \((\text{Zn}^{2+})_i\) was prevented by the use of DTPA, a chelator for extracellular Zn²⁺. The concentration of FluoZin-3 fluorescence in the control was significantly reduced by 10 µM DTPA. The addition of 30 µM azoxystrobin, however, still augmented the FluoZin-3 fluorescence under extracellular Zn²⁺-free conditions (Fig. 5). The intracellular Zn²⁺ chelator TPEN at 10 µM completely suppressed the FluoZin-3 response, which was elicited by azoxystrobin. Therefore, it is likely that the azoxystrobin-induced increase in \((\text{Zn}^{2+})_i\) was due to an influx of extracellular Zn²⁺ as well as a release of intracellular Zn²⁺. The augmentation of FluoZin-3 fluorescence by ZnCl₂ in the presence of azoxystrobin was significantly less than that in the absence of azoxystrobin (Fig. 6). Thus, azoxystrobin seems to possess an inhibitory action on temperature-sensitive Zn²⁺ influx.

**Increase in cell vulnerability to oxidative stress by azoxystrobin**

The increase in the percentage of cell population exhibiting PI fluorescence by 300 µM H₂O₂ was compared in the presence and absence of 30 µM azoxystrobin. The simultaneous application of azoxystrobin and H₂O₂ increased the population of PI-stained cells than azoxystrobin alone (Fig. 7).

## DISCUSSION

**Azoxystrobin-induced increase in intracellular Zn²⁺ concentration**

The elevated \((\text{Zn}^{2+})_i\) may be a common characteristic of toxin-induced oxidative stress. The intracellular Zn²⁺ release is linked with Zn²⁺-thiol/disulfide interchange (Maret, 1994) and oxidative stress converts thiols to disulfides resulting in a Zn²⁺ release. Azoxystrobin has been shown to induce oxidative stress in some vertebrates and invertebrate species (Rodrigues et al., 2013; Han et al., 2014, 2016). Therefore, it is not surprising that azoxystrobin increased \((\text{Zn}^{2+})_i\) even under extracellular Zn²⁺-free conditions. However, it may be interesting to note here that a temperature-sensitive Zn²⁺ influx that resulted by adding ZnCl₂ to the cell suspension was partially suppressed by azoxystrobin. The Zn²⁺ transporters of cell and organelle membranes regulate intracellular Zn²⁺ homeostasis (Hara et al., 2011), and azoxystrobin may inhibit one of them. Zn²⁺ is an intracellular signaling molecule in the lymphocytes (Hirano et al., 2008; Fukuda et al., 2011), and therefore, azoxystrobin is considered to jeopardize some cellular functions of lymphocytes resulting in immune malfunctions.

**Implication of this work**

The use of azoxystrobin is increasing every year (U.S. Geological Survey, 2016). In Japan, azoxystrobin was detected at a five-fold higher concentration than the normal upper limit of (2.5 mg/kg) in a shipment of an Aus-
In this in vitro study, the lowest concentration of azoxystrobin to increase (Zn\(^{2+}\)) was found to be 3 µM (equivalent to 1.2 mg/L). As azoxystrobin pharmacokinetic data in human and azoxystrobin amount in food products using barley are not available, it may not be appropriate to consider that the blood concentration of azoxystrobin will reach to 1.2 mg/L in human consumers. The pharmacokinetics of oral azoxystrobin was studied in rats (Shah and Ray, 2008), and it was revealed that more than 85% of the administered azoxystrobin gets excreted unchanged within 48 hr in feces (up to 80%) and urine (< 17%). Therefore, the oral absorption of azoxystrobin is low, and it also undergoes extensive metabolism. After extrapolating these data to human, it can be predicted that the blood concentration of azoxystrobin may not get too high after intake of foods containing azoxystrobin. Hence, an adverse effect from azoxystrobin in human is unlikely.

**ACKNOWLEDGMENTS**

The Japan Society for the Promotion of Science (Tokyo, Japan) provided a Grant-in-Aid for Scientific Research (C26340039) that was used in this study. We thank Yasuo Oyama for skillful assistance.

**REFERENCES**

Disruption of cellular Zn\(^{2+}\) homeostasis by azoxystrobin


