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***In vitro* chronic effects on hERG channel caused by the marine biotoxin Azaspiracid-2**

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Abstract

Azaspiracids (AZAs) are marine biotoxins produced by the dinoflagellate *Azadinium spinosum* that accumulate in many shellfish species. Azaspiracid poisoning caused by AZA-contaminated seafood consumption is primarily manifested by diarrhea in humans. To protect human health, AZA-1, AZA-2 and AZA-3 content in seafood has been regulated by food safety authorities in many countries. Recently AZAs have been reported as a low/moderate hERG channel blockers. Furthermore AZA-2 has been related to arrhythmia appearance in rats, suggesting potential heart toxicity. In this study AZA-2 *in vitro* effects on hERG channel after chronic exposure are analyzed to further explore potential cardiotoxicity. The amount of hERG channel in the plasma membrane, hERG channel trafficking and hERG currents were evaluated up to 12 h of toxin exposure. In these conditions AZA-2 caused an increase of hERG levels in the plasma membrane, probably related to hERG retrograde trafficking impairment. Although this alteration did not translate into an increase of hERG channel-related current, more studies will be necessary to understand its mechanism and to know what consequences could have *in vivo*. These findings suggest that azaspiracids might have chronic cardiotoxicity related to hERG channel trafficking and they should not be overlooked when evaluating the threat to human health.

Keywords

automated patch clamp; Azaspiracid; cardiotoxicity; chronic; hERG; trafficking

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INTRODUCTION

Azaspiracids (AZAs) are marine biotoxins produced by the dinoflagellate *Azadinium spinosum* [1] that accumulate through the food chain in many shellfish species. These compounds were discovered in 1995 after a human intoxication episode in The Netherlands due to ingestion of contaminated mussels from Killary Harbor, Ireland [2, 3]. Since that episode, AZAs distribution expanded globally, appearing in several locations around the world [4]. Azaspiracid poisoning (AZP) is characterized predominantly by gastrointestinal symptoms like nausea, diarrhea, vomiting and stomach cramps. At the present time, more than 20 different analogues have been described [4], and three of them, AZA-1, AZA-2 and AZA-3, are regulated by food safety authorities in many countries owing to the human health risk [5].

AZAs *in vivo* toxicology studies showed that AZA-1 damaged several organs [6, 7] and might induce the appearance of lung tumors [7, 8]. Also, AZA-2 has been recently described as a toxin with acute arrhythmogenic potential *in vivo* [9]. Regarding *in vitro* studies, many cell biology alterations have been related to this toxin group, among others, cell viability reduction, cytoskeleton damage, apoptosis activation, calcium influx pathway modulation and human *ether-a-go-go*-related gene (hERG) channel block [4, 10-12].

HERG encodes the cardiac potassium channel (Kv 11.1) responsible for a critical repolarization current in the ventricular action potential (AP), the rapid delayed rectifier K⁺ current (I_{Kr}) [13]. HERG channel dysfunction is linked to ventricular repolarization disorders, being specially important the long QT syndrome (LQTS), a cardiac alteration based on delayed repolarizations and whose consequences may be syncope episodes, TdP (Torsades de Pointes) arrhythmias and sudden cardiac death [5, 13, 14]. Drugs can produce hERG alterations by different mechanisms such as direct channel block or disruption of channel trafficking. Blockage of I_{Kr} current is an acute effect that is usually evidenced in minutes, but the alteration of hERG trafficking is a chronic effect that often takes hours or even days to occur. Trafficking disruptions may alter hERG channel expression on the cell membrane surface leading to current reduction, an effect similar to acute blockage of channel current [15]. Therefore, the same consequences in the repolarization function can occur due to channel blockage or trafficking dysregulation. Currently, many drugs are known to cause direct channel block [16, 17], however, evidence of drugs disrupting hERG trafficking [18, 19] or even compounds that can exert both effects [20-22] is also accumulating. The relevance of analyzing channel blockage and trafficking is increasing nowadays [15, 23].

AZA-2 has been characterized as a moderate/low hERG channel blocker [9, 24], however there is no information about this toxin's effects on hERG channel after long-term exposure. Based on the fact that human exposure to marine toxins can be chronic and that channel trafficking effects usually appear at lower concentrations than direct block effects [15], the aim of this study was to evaluate the chronic effects of AZA-2 on the amount of hERG channel on the extracellular side of the cell membrane and their functional implications.

MATERIAL AND METHODS

Reagents

AZA-2 (Figure 1) was synthesized by Nicolaou and co-workers as previously described [25, 26]. Annexin V-FITC apoptosis detection kit was purchased from Immunostep (Salamanca, Spain). Nut Mix F-12 W/GLUTAMAX-I medium, fetal bovine serum (FBS), geneticin, trypsin/EDTA, Dulbecco's phosphate-buffered saline (DPBS), phosphate-buffered saline (PBS), Cy3® goat anti-rabbit IgG (H+L), Tris-glycine SDS sample buffer 2x, Novex® sharp protein standard and Novex®tris glycine gels (8 %, 1.5 mm, 10 well) were purchased from Invitrogen® (Madrid, Spain). Bovine serum albumin (BSA), β -mercaptoethanol, dimethylsulfoxide (DMSO), staurosporine, paraformaldehyde, trypan blue solution and monoclonal anti- β -tubulin I clone SAP.4G5 antibody were from Sigma-Aldrich Química S.A. (Madrid, Spain). Detachin™ was purchased from Labclinics (Barcelona, Spain). Anti-Kv 11.1 (HERG) (extracellular) was from Alomone Labs (Jerusalem, Israel). The anti-hERG antibody HERG (H-175) was from Santacruz Biotechnology, Inc. (Santa Cruz, CA). Immobilon®-FL transfer membrane and peroxidase conjugated goat anti-rabbit IgG was from Millipore® Iberica S.A. (Madrid, Spain). ECL mouse IgG HRP-linked whole Ab (from sheep) was purchased from GE Healthcare (Barcelona, Spain). SuperSignal® West Femto maximum sensitivity substrate and SuperSignal® West Pico chemiluminescent substrate were from Fisher Scientific (Madrid, Spain). IonFlux extracellular solution (145 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose (mM), EC solution), IonFlux intracellular solution (60 KCl, 70 KF, 15 NaCl, 5 HEPES, 5 EGTA (mM), IC solution) and IonFlux 16-96 well plates were obtained from Fluxion Biosciences Inc. (South San Francisco, CA). TBST was 50 mM Tris base, 96 mM NaCl and 0.05% Tween-20.

Cell line culture

A Precision™ hERG CHO (Chinese hamster ovary) Recombinant cell line (Millipore® Iberica S.A., Madrid, Spain) was used. HERG CHO cells were grown in Nut Mix F-12 W/GLUTAMAX-I medium supplemented with 10% FBS and 400 μ g/ml of geneticin (complete culture media) in a humidified 5% CO₂ incubator at 37 °C. They were fed every 24–36 h and passaged every 2–3 days. The cell culture confluence was always between 60 and 80% and the cell viability was always higher than 99%. Prior to the experiments, cell cultures were placed for two days at 30 °C in a humidified 5% CO₂ incubator in order to maximize the amount of hERG channel in the plasma membrane.

Imaging flow cytometry

Toxin and/or carrier were added at the desired concentrations to hERG-CHO cultures for 6 or 12 h at 37 °C. After washing the cells with warm DPBS, they were dissociated from the flask with warm Detachin™ for 10 min at 37 °C. Then the cells were centrifuged at 900 rpm and 4 °C for 5 min and the cell pellet was washed again with complete culture media in the same conditions. Subsequently, two washes with cold DPBS and one with cold annexin-V binding buffer followed. All samples were first incubated with FITC-annexin-V diluted (5:100) in annexin-V binding buffer for 15 min at 22 °C in the dark. After two washes with cold DPBS, they were fixed with 2% paraformaldehyde in DPBS for 15 min on ice. Non-specific binding was blocked with 4% BSA in DPBS for 30 min on ice. The cells were then

incubated with a 1:100 dilution of the anti-Kv 11.1 antibody (extracellular hERG) in 4%BSA/DPBS for 60 min at 37 °C and, after two washes with DPBS; they were incubated with Cy3® goat anti-rabbit IgG (1:200) in the same conditions. Finally the cell samples were washed twice with PBS and passed through a 50 µm nylon mesh filter. For flow cytometry analysis 2.5×10^6 cells/ml in 50 µl of PBS was used. Flow cytometry data were acquired using an ImageStream multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA). The intensity of the 488 nm laser was fixed at 20 mW. Cell classifiers were used for eliminating debris and clusters. The minimal number of events captured was 5000. Quantitative measurements were done using the data analysis software IDEAS (Amnis Corporation, Seattle, WA). After matrix compensation, only singlets and focused cells were used to generate final data. The results are reported as population median.

Automated patch clamp

The effect of long exposures to AZA-2 on hERG channel activity was tested using an IonFlux 16 automated patch clamp system (Fluxion Biosciences Inc, South San Francisco, CA). Current measurement experiments were performed in 16-well IonFlux plates that have 8 pairs of cell traps, each trap endowed with 20 trapping sites placed in parallel, so the measured current is the sum of an ensemble of 20 cells.

Previously, the cells were incubated with carrier (DMSO) alone or toxin at the desired concentrations for 6 or 12 h at 37 °C. Before every experiment, the cells were washed with toxin-free medium and placed for one hr at 30 °C in a humidified 5% CO₂ incubator to remove the toxin and allow them to recover from acute block. Then the cells were washed twice with 5 ml of warm DPBS and detached by incubation with 5 ml of warm Detachin™ solution for 10 min at 37 °C. Immediately, 5 ml of warm CHO serum-free culture medium supplemented with 25 mM HEPES was added and the cell suspension was centrifuged at 700 rpm and 19 °C for 5 min. Cell viability was determined using trypan blue. Viability was always higher than 99%. The cell pellet was suspended in CHO serum-free medium supplemented with 25 mM HEPES and centrifuged again in the same conditions. Finally the cells were washed twice with EC solution and suspended at a concentration of 20×10^6 cells/ml in EC before delivery in the appropriate IonFlux plate well.

Experimental protocols previously optimized for hERG-CHO cells were used for cell trapping and whole cell configuration [27]. The voltage protocol to record hERG currents was as follows: cells were clamped at -80 mV for 100 ms, pulsed to -100mV for 90 ms and to -50mV for 50 ms, then depolarized to +20 mV for 5 s and repolarized to -50 mV for 5 s, and finally returned to -80mV. The hERG peak tail current (current of interest, COI) was measured for 300 s after compound application in the second -50 mV step. Current data were accepted if cell resistances in each trap were at least 2 MΩ, along all the duration of the experiment. All experiments were done at room temperature (22 °C).

Western blot analysis

HERG channel protein levels in hERG-CHO cells were evaluated by western blot analysis using a specific antibody. Cell cultures were incubated with toxin and/or carrier (DMSO) at the desired concentrations for 6 or 12 h at 37 °C. Then, the cells were harvested by scraping

and, after two washes with DPBS, they were centrifuged at 1000 rpm and 4 °C for 5 min. The cell pellets were lysed immediately by adding 1× sample buffer with 2.5% β-mercaptoethanol and boiling for 5 min. Cell proteins were separated by electrophoresis in 8% SDS polyacrylamide gels and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked overnight in 5% nonfat dry milk in TBST. HERG channel proteins were detected by incubation of the membranes with an anti-hERG (H-175) antibody (1:20000) for 60 min with constant shaking. After two washes, the membranes were incubated with an HRP-labeled goat anti-rabbit IgG antibody (1:15000) for 60 min with constant shaking. Detection was done with the HRP chemiluminescent West Femto maximum sensitivity substrate following the manufacturer instructions. The membranes were re-blotted for normalization of hERG expression levels to a reference protein, β-Tubulin. Chemiluminescence was detected using a Dyversity Imaging System (Syngene, Cambridge, UK) and quantified with the Genetools software from Syngene (Cambridge, UK).

Data analysis

Data were plotted as mean± SEM. Statistical significance was determined using t test for unpaired data and ANOVA was used for multiple comparisons. $P < 0.05$ was considered for significance.

RESULTS

AZA-2 effects on hERG amount in the plasma membrane

A CHO cell line stably expressing hERG was used to study the effect of long exposures to AZA-2 on hERG amount in the plasma membrane. The cells were incubated with 50 or 200 nM AZA-2 for 6 or 12 h. Control cells were incubated in the same conditions with carrier (DMSO) in the absence of toxin. After treatment with the toxin, the cells were incubated with annexin-V to detect apoptotic events, followed by immunodetection of surface hERG. Cells treated with AZA-2 for 6 h seemed to undergo a slight increase of surface hERG levels versus carrier controls, however these results were not statistically different (Figures 2A and 2C). In these experiments, annexin-V-related fluorescence was similar in AZA-2-treated and control cells (Figure 2C). Conversely, after 12 h of incubation 50 and 200 nM AZA-2 induced a statistically significant increase of hERG channels on the cell surface when compared to carrier controls (Figures 2B and 2D). In fact, the intensity of hERG-related fluorescence observed in cells treated with 50 and 200 nM AZA-2 for 12 h was, respectively, 2.9 and 3.8 times higher than carrier control intensity. The same cells did not show an increase of annexin-V-related staining (Figure 2D), indicating that the cells were not undergoing apoptosis at that moment. Staurosporine, a compound that causes apoptosis of CHO cells [28], was used to explore the relationship of apoptosis with the increase of plasma membrane hERG. The cells were incubated with staurosporine at 0.5 and 1 μM for 12 and 24 h. A concentration of 0.5 μM staurosporine did not cause an increase of hERG- or annexin-V related fluorescence at any time. However, 1 μM Staurosporine induced a statistically significant increase of annexin-V staining without statistically significant changes in hERG-related fluorescence levels. In fact, the levels of annexin-V in 1 μM staurosporine-treated cells were 1.2 and 1.7 times the levels of carrier- treated cells at 12 and

24 h, being the levels of hERG-related fluorescence in the same cells 1.1 and 1.2, respectively.

AZA-2 effects on hERG trafficking

Western blot was used in order to distinguish if the increase of plasma membrane hERG channels observed after long AZA-2 treatments is due to a general increase of hERG protein levels or to trafficking dysregulation. hERG channels typically appear as two protein bands of 135 and 155 kDa, identified as the immature core-glycosylated channel and the mature fully-glycosylated channel, respectively [29]. The intensity of the 135 and 155 kDa bands was compared for toxin-treated and control cells. β -tubulin was used for normalization. hERG-CHO cells were treated with 50 and 200 nM AZA-2 for 6 and 12 h. Control cells were incubated with carrier (DMSO) in the absence of toxin following the same experimental protocol. Immunodetection of hERG by western blot showed the presence of two bands of 135 and 155 kDa in the whole cell lysates (Figure 3A). The intensity of the 135 kDa band, corresponding to the core-glycosylated form, was decreased in cells treated with AZA-2, but statistical significance was obtained only at 12 h (Figure 3B and 3C). In these conditions the levels of core-glycosylated hERG in 50 nM and 200 nM AZA-2 treated cells were reduced 1.5 and 1.7 times, respectively. The fully-glycosylated 155 kDa band seemed slightly increased in AZA-2-treated cells at 12 h, but these results were not statistically different versus control levels (Figure 3B and 3C).

AZA-2 effects on hERG currents after long-term exposure

The functional relevance of the increased amount of hERG channel in the plasma membrane was explored using automated patch clamp techniques. hERG-CHO cells were incubated with 50 nM AZA-2 for 6 and 12 h. Then the culture medium was replaced by toxin-free medium to wash out the toxin and avoid acute hERG block. The cells were allowed to recover from medium replacement for 1 h and lifted for patch clamp experiments. Control cells followed the same procedure in the absence of toxin. The voltage protocol shown in Figure 4A was used to activate hERG channels and trigger I_{Kr} current (Figure 4B). The amplitude of I_{Kr} was slightly increased in AZA-2-treated cells at 6 and 12 h but the results were not statistically different versus control (Figure 4C).

DISCUSSION

In vitro studies recently published have described AZAs as moderate/low potency hERG channel blockers [9, 24]. Currently, published evidence indicates that hERG channel liabilities can be produced by more mechanisms than only direct block [15, 18, 23]. One of the additional mechanisms considered to affect the channel activity is hERG trafficking. The alterations of hERG trafficking are being evaluated for many drugs regarded as potentially cardiotoxic, due to their acute blocking activity or *in vivo* heart dysfunctions [18, 30, 31]. In fact, a screening study for drug-induced hERG alterations reveals that almost 40% of the drugs that block I_{Kr} also have trafficking effects [32]. Therefore, considering that AZA-2 has been described as a hERG channel blocker and that it has been linked to the appearance of arrhythmias in rats [9, 24], its effect on hERG trafficking contributes additional evidence for potential cardiotoxicity. Actually, AZA-2 induces a significant increase of hERG channel

levels on the cell surface of hERG-CHO cells. Azaspiracids are known to trigger apoptosis in many cell lines [33, 34], and therefore the alterations of surface hERG levels might be due to the apoptotic process. However, surface hERG channels are clearly increased without any evidence of annexin-V externalization, which is considered a relatively early marker of apoptosis [35, 36]. Additionally, apoptosis is not necessarily linked to alterations of plasma membrane hERG, because staurosporine, a well-known apoptosis inducer in CHO cells [28], did not cause a change of surface hERG amount before or after the appearance of externalized annexin-V. These results suggest that the AZA-2-induced increase of hERG in the plasma membrane occurs before apoptosis.

Alterations of hERG density at the cell surface have been associated with functional implications on I_{Kr} current [23, 37]. Surprisingly, no significant alterations of this current were evidenced after exposure to AZA-2 for 6 and 12 h, although these experiments were not statistically powered to detect small current changes. In any case, the amplitude of I_{Kr} in cells treated with AZA-2 for 12 h seems to be slightly higher than in control cells. Considering the four-fold increase of surface hERG in these conditions, a marked increase of hERG currents would be expected. The lack of alterations in these currents suggests that most of these channels are not functional.

The number of hERG channels on the cell surface reflects a balance between its production and trafficking to the plasma membrane and its degradation [38]. hERG channels are present as two protein forms depending on its maturation level, the immature core-glycosylated channel protein, located in the reticulum and Golgi, and the mature fully-glycosylated channel protein, which is externalized to the plasma membrane where it is responsible for I_{Kr} [29]. Therefore, core-glycosylated, fully-glycosylated and plasma membrane hERG data provide information about different aspects of hERG trafficking. Based on the balance between production and degradation, the increase of external channels could be a consequence of a higher rate of channel production or a lower rate of internalization/degradation. However, higher synthesis of hERG in stable expression cell models, commonly used for trafficking studies [19, 31], would not be physiologically relevant. Our western blot results demonstrated that the elevated surface hERG in AZA-treated cells is not due to an overall higher amount of hERG, or an increase of hERG production. Actually, the amount of immature hERG is reduced by the presence of the toxin. In spite of this reduction of immature core-glycosylated hERG, fully-glycosylated hERG levels detected by western blot are not significantly affected by AZA-2 treatment. An increase of fully-glycosylated hERG might be expected considering the four-fold increase of surface hERG. However, fully-glycosylated hERG is generated in the Golgi and externalized to the plasma membrane in transport vesicles [38, 39]. Therefore, the fully-glycosylated hERG pool detected by western blot (Golgi, transport vesicles and plasma membrane) is bigger than the surface hERG pool detected by flow cytometry. In other words, surface hERG would represent a small subpopulation within fully-glycosylated hERG. The increase of plasma membrane hERG may be disguised in western blot results by the amount of not-yet-externalized fully-glycosylated hERG. In any case, if channel production is not increased, impairment of channel internalization/degradation must be responsible for elevated surface hERG. Very recently, regulation of hERG channel degradation through a caveolin-3/ ubiquitin ligase Nedd4-2-related pathway has been

described in a heterologous expression system and neonatal rat ventricular myocytes [40]. Caveolin-mediated endocytosis is dependent on membrane cholesterol and it is blocked by cholesterol depletion [41]. Actually, cholesterol content clearly influences cardiac ion channel endocytosis [38]. Interestingly, AZA-1 has been related to a reduction of total levels of cellular cholesterol and also to an increase of the amount of other proteins in the plasma membrane, probably through inhibition of retrieval by endocytosis [42]. These effects of AZAs may be involved in the increase of hERG in the plasma membrane induced by AZA-2 through an inhibition of channel internalization. However, it is often difficult to dissect direct effects of the toxin from preapoptotic or very early apoptotic changes, being more complicated because AZAs' target is unknown. Therefore, more studies will be necessary to clarify AZAs' exact mechanism of action to cause these effects.

In summary, *in vitro* chronic treatment with AZA-2 induces an increase of hERG channels in the plasma membrane. This alteration is probably due to an inhibition of channel retrograde trafficking, which causes reduction of channel internalization. Even though no functional increase of hERG mediated current was observed, more studies are needed to better understand the exact mechanism by which this toxin can exert this effect, in addition to the consequences these *in vitro* effects may have *in vivo* after chronic exposure to the toxin. These findings suggest that azaspiracids might have a chronic effect on hERG channel and they should be considered to evaluate the cardiovascular risk of these toxins.

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Abbreviations

AZAs	azaspiracids
AZP	azaspiracid poisoning
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EC	extracellular
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
hERG	human ether-a-go-go related gene

IC	intracellular
I_{Kr}	rapid delayed rectifier K ⁺ current
PBS	phosphate-buffered saline
RT	room temperature
SDS	sodium dodecyl sulfate
TdP	torsades de pointes

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Figure 1.
Chemical structure of AZA-2

Figure 2. AZA-2 effects on surface hERG channel

Surface hERG was detected by flow cytometry using a specific antibody. Simultaneously, Annexin-V staining was used in all experiments as an apoptosis marker. Representative histograms showing hERG-related fluorescence in cells treated with 200 nM AZA-2 (black line) and control cells (grey line) for 6 h (A) and for 12 h (B). HERG- and annexin-V-related fluorescence intensity in cells treated with 50 and 200 nM AZA-2 and control cells for 6 h (C) and 12 h (D)(media $\pm$ SEM, n=5, * statistically different versus control).

Figure 3. AZA-2 effects on core-glycosylated and fully-glycosylated hERG

CHO cells stably expressing hERG were treated with 50 or 200 nMAZA-2 for 6 or 12 h. Control cells followed the same procedure in the absence of toxin. HERG channel was detected by immunoblotting of whole cell lysates. Representative images of hERG immunoblot and tubulin re-blot (A). The upper panel shows the 135 and 155 kDa bands corresponding to the immature core-glycosylated and the mature fully-glycosylated channel proteins respectively. Bottom panel shows re-blot for β-tubulin in the same cells. Core-glycosylated and fully-glycosylated hERG channels in control cells and cells were treated with AZA-2 for 6 h (B) and 12 h (C). Data are shown as ratio of 135 kDa or 155 kDa band intensity to β-tubulin band intensity (mean ± SEM, n=6, * statistically different versus control).

Figure 4. HERG channel activity after long exposure to AZA-2

CHO cells stably expressing hERG were treated with 50 or 200 nM AZA-2 for 6 or 12 h. Each experiment had control cells incubated only with carrier. Automated patch clamp experiments were performed following a voltage protocol to elicit I_{kr} activation (A). HERG currents (B, representative current trace observed for cells treated with 50 nM AZA-2 for 12 h) were recorded for 5 min. Mean currents (pA) were obtained for control cells and cells treated with 50 nM AZA-2 after 6 or 12 h of incubation (C) (mean $\pm$ SEM, $n=3$).