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Original Article

Purinergic Receptor Antagonist A438079 Disrupts Benzo[a] pyrene-Mediated IL-1β, CYP1A and CYP1B Transcript Induction Pathway in Zebrafish: Insights into Possible Mechanisms

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Abstract

The deleterious effects of benzo[a]pyrene are mainly due to its metabolites generated by CYP1 metabolism. P2X7 is an important member of purinergic receptors involved in diverse cell signaling cascades, as the inflammasome pathway. Receptor blocking has beneficial effects in several models of inflammatory and neurological diseases. In our study, we show for the first time that the A438079, a "selective" P2X7 antagonist, promotes a downregulation in IL-1β, CYP1A1 and CYP1B1 gene transcription in *Danio rerio* gills exposed to benzo[a]pyrene. Our results show a modulation of IL-1β and CYP1 mRNAs, suggesting a possible novel mechanism involving the P2X7 receptor in benzo[a]pyrene-mediated CYP1 induction. Nevertheless, as A438079 was also proven to block the membrane ATP channel pannexin-1, the effects of this compound on downregulating CYPs transcription would also be due to a disruption of Ca^{2+} influx necessary to activate CYPs transcription by the AhR-Arnt pathway.

Keywords: P2X7, benzo[a]pyrene, zebrafish, CYP1A1, CYP1B1, gene transcription, A438079

INTRODUCTION

The P2X7 receptor is an important member of the purinergic P2X family of ATP-activated ion channels involved in a wide number of pathophysiological conditions, including activation of the NLRP3 inflammasome and neurotransmitter signaling (Burnstock, 2017; Di Virgilio *et al.*, 2017). The chemical compound A438079 (CAS 899507-36-9) is considered a P2X7-selective antagonist that virtually does not act on other purinergic receptors (McGaraughty *et al.*, 2007). Several types of cell injuries caused by different conditions related to immune system activation (e.g., fibrosis, apoptosis, and oxidative stress) can be efficiently prevented by this compound (Huang *et al.*, 2014; da Silva *et al.*, 2014; Deng *et al.*, 2021; Santos *et al.*, 2022). The scientific literature has pointed to the role of NLRP3 inflammasome on the hepatic injury process caused by acetaminophen (APAP) (Hoque *et al.*, 2012) and that the use of A438079 has shown to be protective against this deleterious process (Xie *et al.,* 2013). In this case, it was shown that the NLRP3 inflammasome blockade was not involved in this protective mechanism, but the inhibition of the activities of cytochrome P450 (CYP) enzymes was responsible for hepatic protection by decreasing the generation of APAP toxic metabolites (Xie et al., 2013).

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Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) frequently found in aquatic environments contaminated with petroleum derivatives. BaP causes a complex variety of toxic effects in exposed animals, and its cellular metabolism is initiated by binding to the Aryl Hydrocarbon Receptor (AhR) that induces the expression of CYP enzymes, mainly CYP1A1 and CYP1B1 (IARC 2010; 2012). These CYPs are the main enzymes involved in phase I biotransformation reactions of most PAHs, causing a modification of its structure by inserting an oxygen atom from molecular oxygen. BaP exposure can also result in inflammatory effects in different animal models (Henke *et al.*, 2016; Maikawa *et al.*, 2018, Kumar *et al*., 2022), including zebrafish (Tarasco *et al*., 2022), e.g., by stimulating the production of cytokines such as interleukin 1-beta (IL-1β). Due to the observed inhibitory actions of A438079 on the catalytic activities of CYP enzymes involved in the APAP metabolism (Xie *et al*., 2013), in this study we were interested in evaluating if A438079 would also interfere with the transcription of genes involved in BaP metabolism (CYP1A1 and CYP1B1), in gills of zebrafish. Given the stimulatory effects of BaP on the immune response of animals, we were also interested to check if A438079 treatment affects IL-1β transcript levels in response to BaP exposure in the fish. We hypothesize that in addition to decreasing CYP1A activity (as proposed by Xie *et al.*, 2013), P2X7 blockade also results in reduced levels of CYP1A1 and CYP1A2. Moreover, A438079 treatment decreases IL-1β response to BaP, evidencing that P2X7 receptor is involved in the inflammatory response to BaP.

MATERIAL AND METHODS

All the chemicals (BaP, A438079) used in this study were purchased from Sigma-Aldrich™, USA. Wild-type zebrafish were purchased from an aquarium shop, and were acclimated at laboratorial conditions (well water at pH 7.4, continuous aeration, 26-28 °C) for 30 days before the BaP exposure experiment. Animals were kept in aquariums at a density of 5 animals/3L and fed *ad libitum* once time every day. During the acclimation period, the water was changed every three days, and no mortality occurred. For the BaP exposure experiment, three experimental groups were used: Control (injected intraperitoneally with saline, the same vehicle used to dissolve and inject A438079), BaP (exposed to a single concentration of 100 µg/L BaP in water; static exposure), and A438079+BaP (animals injected intraperitoneally with 160 µg/g of the antagonist A438079 and then exposed to a single concentration of 100 µg/L BaP). A438079 concentration used in this study was chosen based on previous *in vitro* tests in which IL-1β transcript levels were reduced in zebrafish, due to the well-known involvement of P2X7 receptors on the activation of IL-1β (Ferrari *et al.*, 2006; Lenertz *et al*, 2011; Wan *et al,* 2016) (data not shown)

In total, 75 adult zebrafish were used (25 animals per group in 5 aquariums of 3 L (5 replicates of a pool of 5 fish each). At the end of the BaP exposure period (6 days), the animals were euthanized, and gills were collected and frozen in liquid nitrogen. Injections and cryoanesthesia to euthanasia were performed according to Kinkel *et al.* (2010). All procedures were conducted in accordance with ethical principles in animal experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and consistent with international laws in countries such as the USA, England, Canada, or Europe, which deal with ethics and animal welfare. Total RNA of the gills was extracted with Trizol, Invitrogen™, USA, according to the manufacturer´s instructions. cDNA strands were synthetized from the RNA samples using the High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems™, USA. Real-Time Quantitative PCR (qPCR) was performed to quantify the transcription levels of CYP1A1, CYP1B1, and IL-1β genes, using the EF-1α as endogenous control and the comparative $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). The statistical analysis was performed using the software Statistica 8. The normality and homogeneity of the data were tested by Shapiro-Wilk and Levene test, respectively, resulting in nonparametric data, and therefore the Kruskal-Wallis test was used, followed by multiple comparisons of mean ranks. The level of statistical significance was of $p<0.05$. Moreover, microsomal preparations of gills of the BaP-exposed zebrafish were incubated for 30 min at 37 °C with increasing concentrations of A438079 to check any inhibitory effect on ethoxy-resorufin-O-deethylase (EROD) activity (indicative of CYP1A enzyme).

RESULTS AND DISCUSSION

The levels of CYP1A1, CYP1B1 and IL-1β transcripts of the animals from the exposure experiment are shown in table 1. A typical response of the P2X7 activation is the release of IL-1β (Ferrari et al., 2011). Levels of IL-1β in the BaP group were 3.5-fold higher than in the control group ($p<0.05$), which suggest the activation of an inflammatory response. In contrary, the gills of animals from A438079+BaP group presented 5-fold lower levels of IL-1β transcription compared to control group ($p<0.05$). This result is consistent with a general downregulation of proteic inflammatory markers, including IL-1β, in mice intraperitoneally injected with A740003, another selective P2X7 antagonist (Wu *et al.,* 2017a). In another study, A438079 treatment also showed to reduce IL-1β production in an *ex vivo* human tonsil histoculture infected with HIV, which agrees with our results (Soare *et al.,* 2018). Similarly, A438079 reduced IL-1β release in a mouse model of autoimmune exocrinopathy, also confirming our findings (Khalafalla *et al.,* 2017). However, decreases in IL-1β protein levels due to P2X7 blockade could be a consequence of post-transcriptional events, as proposed

by Lombardi *et al.* (2017). In general, the effects of P2X7 blockade by antagonist compounds on IL-1β mRNA transcript levels are more related to an inhibition of its upregulation (Wu *et al.,* 2017b). Interestingly, our results suggest a long-term effect of A438079 on the immune system, considering that the gill samples were collected 6 days after A438079 injection. These results also evidence the involvement of P2X7 receptors in the mechanism of inflammatory response stimulated by BaP exposure.

Of particular importance, the transcript levels of CYP1A1 and CYP1B1 of zebrafish from the BaP group increased about 266 and 20 times, respectively, when compared to the control group ($p<0.05$; Table 1), which is consistent with known BaP effects (IARC 2010; 2012). However, BaP effect on upregulating mRNA levels of both CYP1A1 and CYP1B1 was drastically lower in the presence of A438079, being increased only 89 and 2 times, respectively, compared to the control group. Moreover, the A438079+BaP group presented significant lower gene transcript levels compared to the group exposed only to BaP (Table 1). Furthermore, A438079 also inhibited EROD activity in a concentration-dependent manner (Table 2), which agrees with the inhibition of CYP1A2 and CYP2E1 activities by A438079 in rat liver, as previously reported (Xie et al., 2013). An inhibitory effect of another selective P2X7 antagonist (JNJ-47965567) was also recently demonstrated on the activities of some CYP human enzyme family members, such as CYP1A2 and CYP3A4 (Bhattacharya *et al*., 2013).

Table 1. Relative gene expression in zebrafish gills exposed to Benzo[a]pyrene with or without P2X7 antagonist A438079.

Groups	*CYP1A1*	*CYP1B1*	*IL-1β*
Control	1.08 ± 0.09	1.01 ± 0.02	1.02 ± 0.02
BaP	266.42 ± 55.34[a]	20.09 ± 7.55[a]	3.45 ± 1.00[a]
A438079 + BaP	89.13 ±15.71[a,b]	2.49 ± 1.28[a,b]	0.22 ± 0.028[a,b]

a: statistical difference compared to control ($p<0.01$). b: statistical difference compared to the BaP group ($p<0.01$).

Table 2. In vitro EROD inhibition by different concentrations of A438079 (A43) on microsomal preparations of gills of the BaP-exposed zebrafish.

Groups	% Response
Control	6.6
BaP	100.0
Bap + A43 (100 nM)	53.3
Bap + A43 (1 μM)	15.6
Bap + A43 (10 μM)	16.0
Bap + A43 (100 μM)	5.5
Bap + A43 (1 mM)	1.7

Although our results agree with the inhibition of CYP activities by A438079 previously described (Xie *et al.,* 2013), effects on CYPs transcript levels have never been reported before, indicating that the P2X7 antagonist A438079 was able to reduce CYP1A1 and CYP1B1 transcription, even after 6 days of BaP exposure. These results suggest a possible participation of P2X7 in the regulation of AhR-mediated activation of certain CYP transcripts, although mechanisms involved in such effects remains to be further investigated. Some hypothesis to explain these effects could be formulated. For example, it has been shown that microtubule disrupters, such as colchicine, can alter the expression of different CYPs, including CYP1A1 (Dvorák *et al.,* 2006), and this mechanism seems to be caused by changes in the cell cycle. Colchicine has also antagonistic effects on the P2X7 receptor (Marques-da-Silva *et al.,* 2011), which may reinforce the idea that A438079 has similar effects, promoting changes in the gene transcription of CYP enzymes found here.

Moreover, it is well established that PAHs such as BaP can bind to AhR, thus modulating CYP1A1 expression (Hockley *et al.,* 2007). However, an alternative pathway for CYP induction was proposed by Wohak and colleagues (Wohak *et al.*, 2016), based on a previous study that revealed the upregulation of CYP3A4 expression by p53 (Goldstein *et al.,* 2013). The authors showed that BaP-induced CYP1A1 expression are also modulated by p53 binding to the response element in the CYP1A1 promoter region. These findings are also supported by the fact that exposure to BaP increases p53 protein levels (He *et al.,* 2016), and CYP1A1 expression (Baird *et al.*, 2005). Moreover, previous findings have shown that p53 expression

and its activation can be mediated by P2X7 receptors (Kim et al., 2011), a mechanism that also involves the up-regulation of IL-1β. Activation of Nf-kβ is known to be dependent on IL-1β increase (Lawrence et al., 2009), thus any reduction in concentrations of this interleukin due to P2X7 blockade would result in less Nf-kβ activation. A438079 was recently shown to decreased NF-κB activation (Wan et al, 2016; Huang et al., 2017; Deng et al., 2021) and activity (Huang et al. 2014); therefore, it can be also postulated that A438079 decreased CYP1 induction in BaP-treated zebrafish due to a decreased Nf-kβ-mediated AhR transcription, an effect that can be also related to decreased IL-1β (Figure 1).

Finally, despite A438079 is considered a "selective" antagonist of P2X7 purinergic receptors, it was proven that this compound can also bind and block the ATP release channel pannexin-1 in vertebrate cells (Qiu & Dahl, 2009), including zebrafish (Kurtenbach et al., 2013). Pannexin-1 forms nonjunctional channels (pannexon) that open upon depolarization and in response to purinergic receptor stimulation, causing the release of ATP from a stimulated cell to act on membrane purinergic receptors, including P2X7, of adjacent non-excitable cells (Dahl, 2015). ATP release can elevate Ca^{2+} in adjacent cells through activation of both metabotropic and ionotropic purinergic P2 receptors

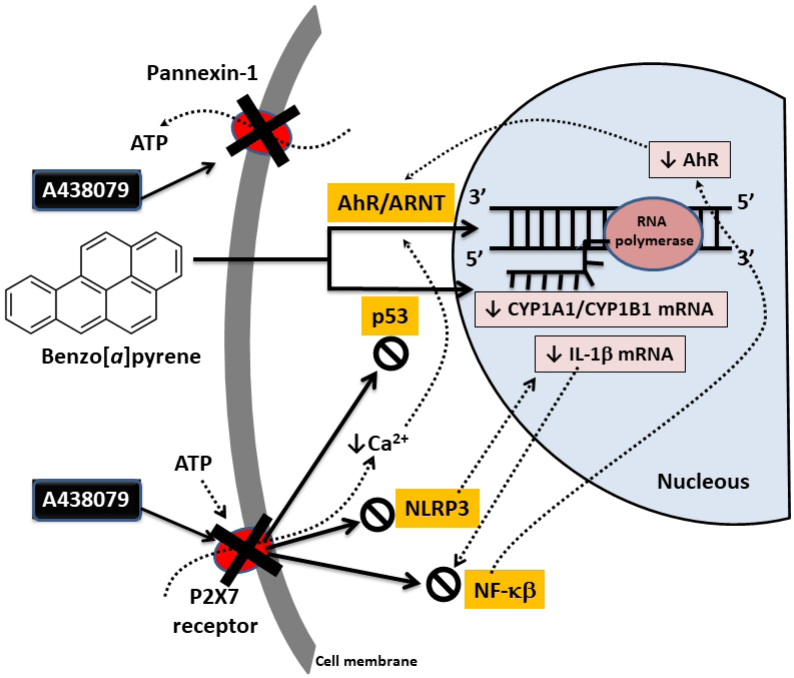

Figure 1: Possible routes affected by A438079 treatment in zebrafish resulting in decreased expression of CYP1A, CYP1B and IL-1b. Some insights on possible effects of P2X7 blockade by A438079 are: a) P2X7 blockade causes a decrease in p53 expression, attenuating its potentiation effect on BaP-AhR-mediated induction of CYP1A and CYP1B transcription; b) P2X7 blockade decrease NLRP3 inflamasome activation, resulting in decreased IL-1b transcription; c) P2X7 blockade reduce NFkb activation, an effect that could be potentiated by reduced levels of IL-1b, resulting in diminished AhR transcription. All these effects can be potentiated by a possible additional blockade of pannexin-1 channels, reducing extracellular levels of ATP and thus decreasing purinergic signaling.

can influence DNA adduct formation from BaP in human cells, confirming a role for p53 in the cytochrome P450 (CYP) 1A1-mediated biotransformation of BaP in vitro (Krais *et al*., 2016). It is well known that activation of the P2X7 receptor is able to upregulate p53 levels in cells (Lenertz *et al.,* 2011) due to its pro-apoptotic role. Furthermore, it was recently demonstrated that the P2X7 receptor blockade by A438079 significantly reduces p53 protein levels during cisplatin exposure (Zhang *et al*., 2015). Interestingly, p53 was also shown to stimulate P2X7 expression (Tung *et al*., 2021; Cuthbertson & Sluyter 2022), suggesting a positive feedback loop of control of both components (P2X7 and p53). Hence, the p53-induced CYP1A1 pathway may have an important P2X7-related role in the BaP-induced CYP expression. Taking into account the involvement of p53 in the activation of CYP transcription, it may be suggested that BaP-induced CYPs is in part due to the AhR pathway but also due to the p53 mechanism (Figure 1). This would explain why CYP1A1 and CYP1B1 transcript levels were higher than control levels even after BaP+A438079 treatment, despite significantly lower than the levels observed in animals exposed only to BaP.

Moreover, it is well established that the transcription of AhR, which is essential for CYP induction, is dependent on the activity of the nuclear factor kappa B (Nf-kβ) (Tian *et al*., 2002). In accordance to this, Vogel *et al*. (2014) showed that the inhibition of Nf-kβ in cells led to a decrease in TCDD-mediated CYP1A induction in mice, due to a decrease in AhR transcription. Nf-kβ is a key transcription factor in regulating the immune system and inflammatory responses,

(Iglesias & Spray, 2012). Compounds like TCDD, BaP, and 7,12-dimethylbenz[a]anthracene, three known CYP1A1 inducers, have been reported to increase Ca^{2+} levels in the human mammary epithelial cell line MCF-10A (Tannheimer *et al.,* 1997; 1999) and in mouse testicular TM4 Sertoli cells (Zhang *et al.*, 2022). Changes in free intracellular Ca^{2+} cause alterations in the activity of several kinases involved in the phosphorylation of key signaling proteins that modulate gene transcription. Previous investigations revealed that DNA binding by human and mouse AhR-Arnt heterodimers requires phosphorylation of both proteins, whereas formation of AhR-Arnt heterodimers requires phosphorylation of Arnt only (Pongratz *et al*., 1991; Berghard *et al.*, 1993). Moreover, Long *et al.* (1999) showed that a protein kinase C-mediated event is required for the AhR to form a functional transcriptional complex that leads to trans-activation. Therefore, intracellular Ca^{2+} might be a key factor in the induction of CYP1A1 by various compounds. Thus, it can be additionally rationalized that P2X7 and pannexin-1 ATP channel blockade by A438079 disrupts the Ca^{2+} signaling process necessary for the activation of the AhR-Arnt signaling pathway, resulting in a decrease in the transcription of CYP genes (Figure 1).

CONCLUSION

The main finding of this study was the interference of A438079 on the BaP-mediated CYP1A1, CYP1B1 and IL-β gene upregulation, which could suggest the involvement of P2X7 receptors in the modulation of these genes in zebrafish. Based on these results, we hypothesize that the substantial lower upregulation of CYPs in the BaP+A438079 group was due to lower AhR activation due to diminished Ca^{2+} influx and/or reduced Nf-kβ-mediated AhR transcription in combination with a possible impairment of the p53 pathway, effects that resulted from the blockade of P2X7 receptor and pannexin-1 ATP release channel. The main implication of these results is that despite the pharmacological blockade of P2X7 may result in interesting anti-inflammatory effects, it impairs CYP-mediated biotransformation, which would reduce the capacity of organisms to metabolize xenobiotics and pharmaceutical drugs. However, these insights require further experiments to better clarify such mechanisms.

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