Article

Are hydrogen peroxide, hypochlorite and peracetic acid potential endocrine disruptors?

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Abstract: Arylsulfatase and β-glucuronidase are two important enzymes in human, which play important role on dynamic equilibrium of steroidal estrogens. This work probably for the first time reported that hydrogen peroxide (H_2O_2), hypochlorite and peracetic acid (PAA) could effectively inhibit the activities of arylsulfatase and/or β-glucuronidase. The 50 percent of inhibitions (IC_{50}) of H_2O_2, and PAA on arylsulfatase were found to be 142.90±9.00, 91.83±10.01, and 43.46±2.92 μM, respectively. The corresponding respective IC_{50} of hypochlorite and PAA on β-glucuronidase were 704.90±41.40 and 23.26±0.82 μM, while H_2O_2 showed no inhibition on β-glucuronidase. It was further revealed that the inhibition of hypochlorite on both arylsulfatase and β-glucuronidase was irreversible. On the contrary, the inhibition by H_2O_2 and PAA was reversible. Moreover, it was found that the inhibitions of arylsulfatase and/or β-glucuronidase by these three chemicals were pH-dependent, among which the inhibition by H_2O_2 was competitive and non-competitive for PAA. In general, H_2O_2 and hypochlorite can be endogenously produced in human, which suggested that the two compounds are potential endocrine disruption compounds (EDCs) as they can cause endocrine disruption via inhibition of arylsulfatase and β-glucuronidase. This work further indicated that any agent that can

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induce production of H_2O_2 or hypochlorite in human is potential EDC, which explains why some EDCs with very weak or no estrogenic potency can cause endocrine disruption that confirmed in epidemiological studies.

Keywords: Arylsulfatase; β-glucuronidase; Hydrogen peroxide; Hypochlorite; Inhibition

1. Introduction

Arylsulfatase and β-glucuronidase have been widely present in many organisms including humans, which are able to catalyze the hydrolysis of sulfates/glucuronides (Dzialoszynski et al., 1967; Meroni et al., 1997; Posey et al, 1977; Taha et al., 2019a). The important physiological functions of the two enzymes are to maintain dynamic equilibrium of natural estrogens in human body. Normally, only about 2 to 3 percent of natural estrogens are free estrogens in human, while the rest are sulfate or glucuronide form (Gruber et al., 2002; Zhao et al., 2014). The free natural estrogens play important roles in stimulating growth, blood flow, water retention in sexual organs, neuro and vaso protection, reduction of bone loss, while the conjugated estrogens act as reservoir (Gruber et al., 2002; Reed et al., 2005). Natural free estrogens in human are accurately regulated, and once the equilibrium was disrupted this may trigger severe outcome. For example, humans with significantly higher urinary arylsulfatase/β-glucuronidase activity results in higher free estrogens in body, which have been thought to be the main reason for the development of many cancers including breast cancer, stomach cancer, ovarian cancer, colonic cancer, etc.(Almandil et al., 2019; Chapman et al., 2002; Rath et al., 2004; Taha et al., 2019b). Therefore, inhibition of arylsulfatase/β-glucuronidase had been explored as one of the effective strategies for such cancer treatment, among which STX64 has been applied in clinical trials (Walaszek et al., 1990; Reed et al., 2005).

Arylsulfatase and β-glucuronidase have been reported to be two extreme enzymes, which can endure high concentrations of mercury dichloride, sodium azide, ethanol and EDTA (Zhang et al., 2020). As sanitizers for various medical and non-medical applications, H_2O_2, hypochlorite, and PAA have been commonly used (Hidalgo et al., 2002; Huang et al., 2004; Hugo et al., 1995; Loo et al., 2012) . Among these, H_2O_2 and hypochlorite are also important compounds, which can be endogenously produced by cellular enzymatic reactions in human, which are important signal biomarker in human (Bekeschus et al., 2014; Forman et al., 2016;Koide et al., 2011; Sato et al., 2013). This work is the first to report that H_2O_2, hypochlorite and PAA can effectively inhibit arylsulfatase and/or β-glucuronidase, which may indicate important physiological functions.

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2. Materials and Methods

2.1 Enzymes and chemicals

Potassium 4-nitrophenyl sulfate (*p*NPS, purity>98%), 4-Nitrophenyl-β-D-glucuronide (*p*NPG, purity>98%) and p-nitrophenol (*p*NP, spectrophotometric grade), arylsulfatase (≥10,000 units/g solid) from *Helix pomatia* (Type H-1, catalogue number: S9626), β-glucuronidase (≥100,000 units/g solid) from *Helix pomatia* (Type H-1, catalogue number: G0751) were purchased from Sigma-Aldrich (Shanghai, China). H_2O_2 (30%) was purchased from Guangzhou chemical reagent factory (Guangzhou, China). NaClO (available chlorine, 8.0%, w/w) was purchased from ANPEL (Shanghai, China). The NaClO concentration was calibrated with the standard method of GB/T 19106 (2013). PAA (13%, w/w) including solution I and II was purchased from GHTECH (Shantou, China). Before using, fresh PPA was obtained by mixing the solution I and II in the proportion of 1:1 (v/v), and it was used within 2 days. This PAA concentration was calibrated according to the standard method of GB/T 19104 (2008). The other reagents not listed were purchased from Aladdin (Shanghai, China).

Stock solutions of arylsulfatase and β-glucuronidase were prepared at the concentration of 10 U/mL by dissolving enzyme powder into 0.5 M Tris-HCl buffer (pH=7.0). The *p*NPS stock solution was prepared with 0.5 M acetate buffer (pH 5.8), while *p*NPG was prepared with 0.5 M phosphate buffer (pH 7.0), both of which were at a concentrations of 5 mM. All solutions were prepared with reagent grade chemicals and ultrapure water (18.2 MΩ/cm).

2.2 Enzyme activity

The activity of arylsulfatase was measured according to Zhang et al. (2020). In simple, the arylsulfatase activity was determined by measuring the amount of *p*NP produced from *p*NPS. In the analysis, the assay mixture solution (3 mL) containing 0.5 mL diluted arylsulfatase enzyme, 0.5 mL of 5 mM *p*NPS, and 2 mL of 0.5 M acetate buffer (pH=5.8) was incubated at 37 ℃ for 1 h. The reaction was stopped by adding 2 mL of 0.5 M NaOH solution to the mixture, and the *p*NP released from the *p*NPS was quantified spectrophotometrically at a wavelength of 400 nm (UV spectrophotometer, CANY 723, Shanghai, China). Each experiment was performed in triplicate.

The β-glucuronidase activity was determined according to the method described in Zhang et al. (2020). Briefly, the β-glucuronidase activity was determined by measuring the amount of *p*NP released from *p*NPG. The reaction mixture solution (1 mL), contained 0.6 mL of 0.1 M phosphate buffer (pH 7.0), 0.2 mL diluted β-glucuronidase enzyme and 0.2 mL of 5 mM *p*NPG. The above reaction mixture solution was incubated for 1 h at 37 ℃. The reaction was stopped by adding 1 mL of 0.5 M NaOH and the *p*NP deconjugated from *p*NPG was quantified spectrophotometrically at a wavelength of 400 nm. Each experiment was performed in triplicate.

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Arylsulfatase/β-glucuronidase enzyme activity (U) was defined as the absorbance equivalent of 1 μmol *p*NP produced per hour per milliliter of enzyme solution at 37°C. For the convenience of comparison, relative activity is used, and the maximal enzyme activity is arbitrarily set to 100%. Limit of detection (LOD) of the spectrophotometric method was calculated according to the three times of the standard deviation (SD), which was obtained from nine repeated determinations of the lowest *p*NP concentration used for the standard calibration curve (Yuan et al., 2016; 2017). LOD of this method for *p*NP was 31 μg/L, which is equal to 0.22 U/L for arylsulfatase and β-glucuronidase.

2.3 Inhibitory assays

Inhibition experiments on arylsulfatase or β-glucuronidase by different inhibitors were performed with different concentrations, and the inhibitor solution was freshly prepared before use. For arylsulfatase inhibition experiment, the reaction mixture solution (3.5 mL) contained 2 mL of 0.1 M acetate buffer (pH 5.8), 0.5 mL of diluted arylsulfatase enzyme, 0.5 mL of 5 mM *p*NPS and 0.5 mL of water as the control or 0.5 mL of inhibitor (H_2O_2, NaClO or PAA) with different concentrations. The addition concentrations of H_2O_2 and NaClO in the reaction mixture were 1, 5, 10, 50, 100, 500, 1000, 5000, and 10000 μM, respectively, while the concentrations of PAA were 1, 5, 10, 50, 100, 500, and 1000 μM, respectively. The reaction mixture solution was mixed and incubated for 1 h at 37 ℃. The *p*NP deconjugated from *p*NPS was measured at a wave length of 400 nm after the reaction was stopped by adding 1.5 mL of 0.5 M NaOH. Each experiment was performed in triplicate.

For *β*-glucuronidase inhibition experiment, the reaction mixture solution (1.2 mL) contained 0.6 mL of 0.1 M phosphate buffer (pH 7.0), 0.2 mL of diluted arylsulfatase enzyme, 0.2 mL of 5 mM *p*NPG and 0.2 mL of water as the control or 0.2 mL of H_2O_2, NaClO or PAA. The addition concentrations of H_2O_2 in the reaction mixture were 1, 5, 10, 50, 100, 500, 1000, 5000, and 10000 μM, respectively, while the concentrations of NaClO and PAA were the same with 1, 5, 10, 50, 100, 500, and 1000 μM, respectively. The reaction mixture solution was mixed and incubated for 1 h at 37℃. The *p*NP deconjugated from *p*NPG was measured at a wave length of 400 nm after the reaction was stopped by adding 0.8 mL of 0.5 M NaOH. Each experiment was performed in triplicate.

To check the inhibition differences at different pH conditions, different buffer solutions were used as listed. 0.1 M acetate buffer (pH 5.0-5.8), 0.1 M citric acid/0.1 M sodium citrate (pH 5.0-6.0), 0.5 M Tris–HCl (pH 7.0-8.0), and 0.1 M phosphate buffer (pH 6.0-8.0).

Inhibition percent was calculated as shown in equation (1):

$$I\ (\%) = 100 - 100 * (A / C) \quad (1)$$

Where I is the inhibition percent of the enzyme activity by an inhibitor, A is the measured enzyme activity with an inhibitor at different concentrations, C is the measured activity without addition of an inhibitor. Inhibition dose-response curve was plotted with Graphpad Prism 8 software, and IC_{50} was simultaneously obtained, which

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means the half inhibition concentration of an inhibitor.

3 Results

3.1 Inhibition dose-response curves

Figures 1 and 2 showed the inhibition dose-response curves of H_2O_2, hypochlorite and PAA on arysulfatase and β-glucuronidase. It is evident that hypochlorite and PAA could effectively inhibit both arysulfatase and β-glucuronidase, while H_2O_2 was only effective for inhibiting arylsulfatase. The respective IC_{50} of H_2O_2, hypochlorite, and PAA on arylsulfatase were determined to be 142.90±9.00, 91.83±10.01, and 43.46± 2.92 μM, while 704.90 ± 41.40 and 23.26 ± 0.82 μM for the inhibition of β-glucuronidase for hypochlorite and PAA. These clearly suggested that PAA had the strongest inhibitory effect on arylsulfatase, follows by hypochlorite, with H_2O_2 being the least. Meanwhile, the inhibitory effect of PAA on β-glucuronidase was much stronger than that of hypochlorite. Interestingly, the inhibitory effect of hypochlorite on arylsulfatase was significantly stronger than that on β-glucuronidase. In contrast, the inhibitory effect of PAA on β-glucuronidase appeared to be stronger than that on arylsulfatase. However, it should be noted that the maximum inhibition degree of PAA on β-glucuronidase was only about 60%.

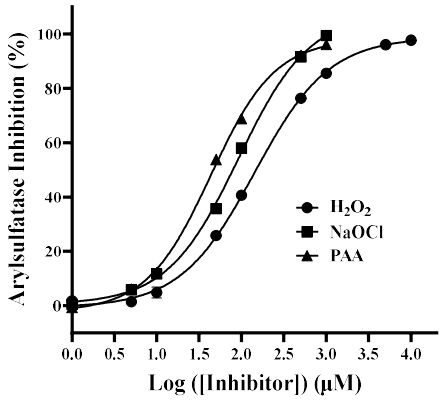

Fig. 1 Dose-response curves of H_2O_2, NaClO and PAA on arylsulfatase

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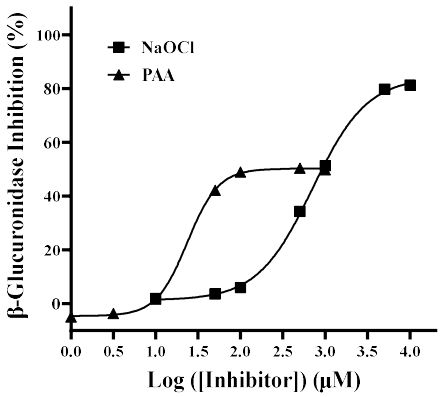

Fig. 2 Dose-response curves of NaClO and PAA on β-glucuronidase. Data for H_2O_2 is not drawn as it showed no inhibition on β-glucuronidase.

3.2 Effect of pH on enzyme inhibition

As H_2O_2 and PAA belong to weak acids and hypochlorite is a weak alkaline, it is reasonable to consider that their dissociation degrees are all pH-dependent, and subsequently affect their inhibitory powers to arysulfatase and β-glucuronidase. On the other hand, it has been known that both arysulfatase and β-glucuronidase could be completely inhibited at high above 11 or below 3 (Zhang et al., 2020). Thus, in this study, the pH studied was controlled in the range of 5-8. As can be seen in Fig. 3, there was no significant differences in the inhibition of arylsulfatase by H_2O_2 in the pH range of 5 to 7, while reduced inhibition was observed under weak alkaline condition of pH 8.

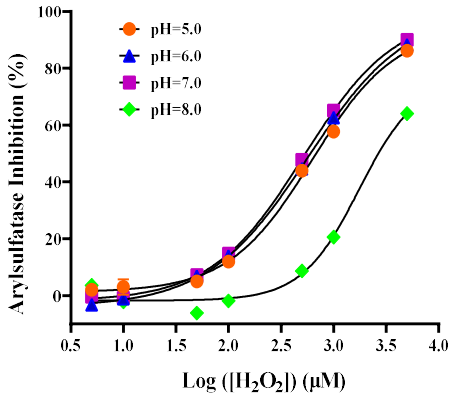

Fig. 3 Dose-response curves of H_2O_2 on arylsulfatase under different pH conditions.

Under acidic condition, hypochlorite can react with hydrogen ion by forming hypochlorous acid (HOCl) which may have different inhibition power to of the enzymes studied (data is not shown). It appears from Fig. 4A that the inhibition of

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hypochlorite on arylsulfatase was much stronger at pH=5.0 than at pH=5.8. Moreover, its IC_{50} at pH=5.0 (i.e. 7.64±0.61 μM) was 12 times lower than that at pH=5.8 (i.e. 91.83±10.01 μM). Similar trend was observed in Fig. 4B for β-glucuronidase. These seemed to suggest that acidic condition was making hypochlorite more inhibitory towards arysulfatase and β-glucuronidase. The inhibitory effects of PAA on arylsulfatase at different pH were shown in Fig. 5. It seemed that inhibition tended to decrease with increasing pH, e.g. nearly no inhibition was observed at pH 8 with a PAA concentration below 1 mM. Similar to arylsulfatase, the inhibitory of PAA on β-glucuronidase was insignificant at pH 5 and 6 (Fig. 5B).

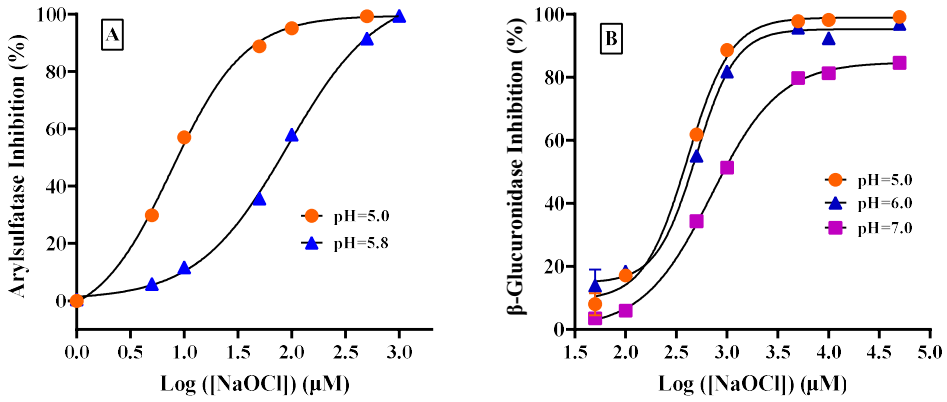

Fig. 4 Dose-response curves of NaClO on arylsulfatase (A) and β-glucuronidase (B) under different pH conditions.

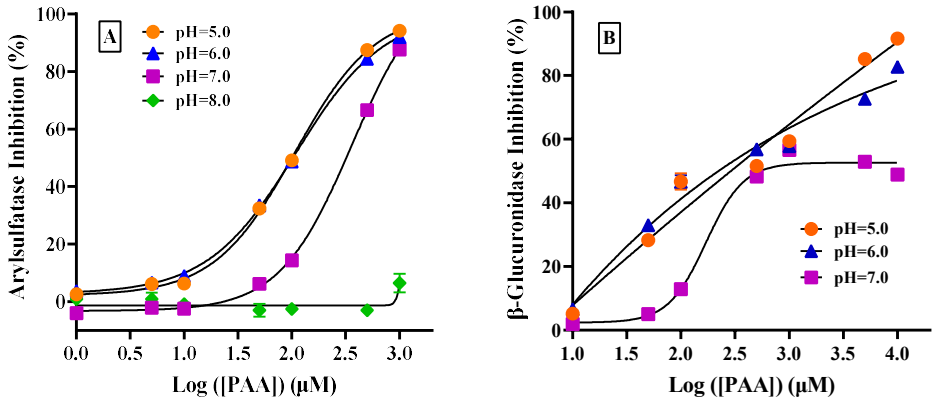

Fig. 5 Dose-response curves of PAA on arylsulfatase (A) and β-glucuronidase (B) under different pH conditions.

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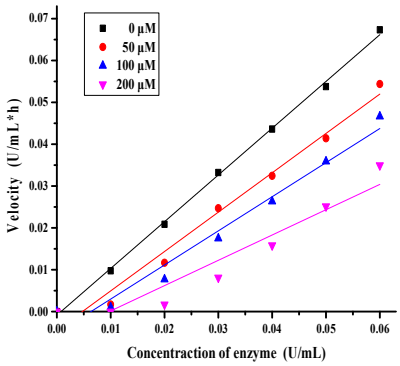
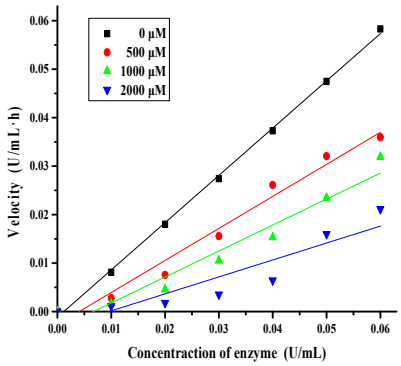
3.3 Inhibition mechanism

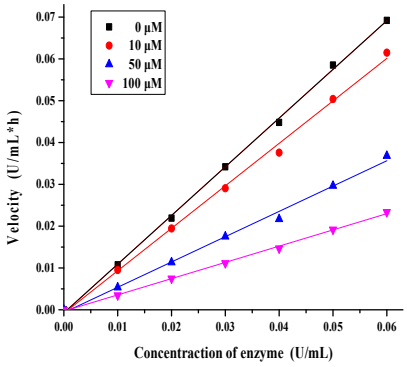
To explore possible inhibition mechanisms of arysulfatase and β-glucuronidase by H2O2, hypochlorite and PAA at different pH, the relationship between enzyme activity and individual inhibitor concentration was presented in Fig 6. It was found that the inhibitions of H_2O_2 and PAA on arylsulfatase should be reversible, while the inhibition of hypochlorite on arylsulfatase was irreversible. Similar to arylsulfatase, the inhibition of PAA on β-glucuronidase was reversible, while the inhibition of hypochlorite was irreversible. To further determine the inhibition kinetics, Lineweaver-Burk plot was plotted in Fig.7, showing that the inhibition on arylsulfatase by H2O2 was competitive, while non-competitive for the inhibitions of PAA on arylsulfatase and β-glucuronidase.



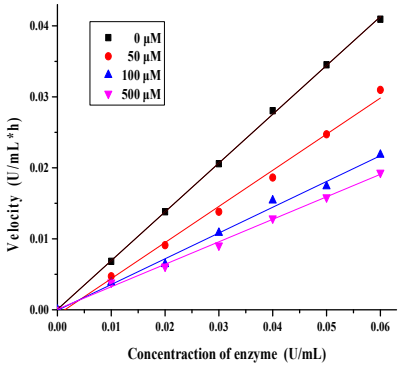
NaOCl —— Arylsulfatase



NaOCl —— β-Glucuronidase



PAA —— Arylsulfatase



PAA —— β-Glucuronidase

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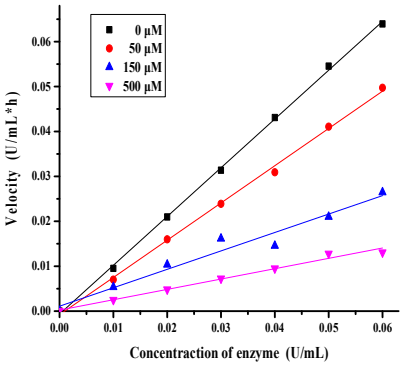

H_2O_2 —— Arylsulfatase

Fig. 6 Inhibitions of H_2O_2, NaClO and PAA with different concentrations on arylsulfatase and/ or β-glucuronidase.

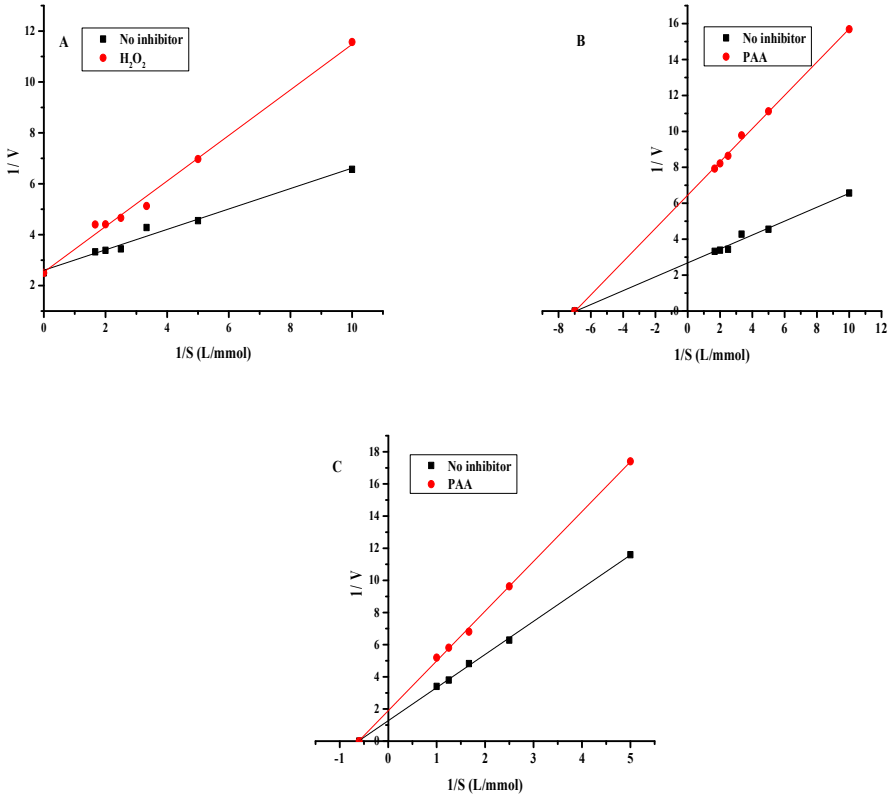

Fig. 7 Lineweaver-Burk plots of the inhibition reaction of arylsulfatase in the presence of H_2O_2 (A), PAA (B). Lineweaver-Burk plots of the inhibition reaction of β-glucuronidase in the presence of PAA (C).

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3.4 Significance of this work

Endocrine disrupting compounds (EDCs) are, by definition, natural or synthetic agents that can mimic, enhance, or inhibit the action of endogenous hormones that are responsible for maintaining homeostasis and controlling normal development (Melnick, 1999; U.SEPA, 1997). The most commonly investigated EDCs are those that can mimic the effects of steroid hormones, which often show different estrogenic potencies that can be determined with different bioassays (Bovee et al., 2004; Liu et al., 2009; 2010). With the definition of estrogenic potency, it is convenient to identify which are strong EDCs, while which are weak. The potential adverse effect of each EDC depends on both its estrogenic potency and concentration, thus the concept of estrogen equivalence (EEQ) has been widely adopted (Luo et al., 2018; 2020; Wang et al., 2020).. This above criterion well describes the potential adverse effect of one individual EDC at any given concentration. However, many EDCs cannot be well explained with the above conception. For example, di-2-(ethyl hexyl) phthalate, well-known as DEHP, the estrogenic potency of which was as low as 10^{-7}, suggesting an extremely weak EDC (Luo et al., 2018). Considering its possible human exposure level to normal population, DEHP as an EDC unlikely pose potential adverse effects to human. Nevertheless, epidemiological studies have shown that DEHP can pose different adverse effects on human including reproductive, developmental and cardiovascular systems (Benjamin et al., 2017; Kay et al., 2013; Mariana et al., 2016; Mathieu-Denoncourt et al., 2015).. Another example is cadmium, which possesses no estrogenic potency at all, but it is a well-known EDC (Luo et al., 2014; Paschoalini et al., 2019; Stasenko et al., 2010).

Many studies have shown that both DEHP and cadmium could induce production of H_2O_2 in human (Ghosh etal., 2010; Mates et al., 2010; Matovic et al.,2015; Shen et al., 2019; Szymanska-Chabowska et al., 2009), while existence of H_2O_2 can further induce hypochlorite (Yap et al., 2007). Based on the fact that H_2O_2 and hypochlorite can effectively inhibit arylsulfatase and/or β-glucuronidase, this may suggest that DEHP and cadmium can disturb human endocrine system via production of H_2O_2 and hypochlorite. This may well explain why DEHP and cadmium with very weak or no estrogenic potency can act as two EDCs that can pose adverse effects to human. Not limited to DEHP and cadmium, any agent that can induce production of H_2O_2 and hypochlorite in human or animals is potential EDC.

Table 1 Hydrogen peroxide in human reported by different studies

Number	Objective	Sample type	Sample size	Concentration (μM)	Reference
1	Man	Plasma	1	4.825	Yamamoto et al., 1987
2	Man	Plasma	1	5.5	Yamamoto and Ames, 1987
3	Men	Plasma	17	5.85-7.15	Deskur et al., 1998
4	Men	Plasma	50	2.14-3.15	Lacy et al., 1998a
5	Men and women	Plasma	236	0.61-6.79	Lacy et al., 1998b

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6	Children	Plasma	--	1.4-2	Li et al., 2010
7	Men and Women	Plasma	60	2.5-6.2	Banerjee et al., 2003
8	Men and Women	Plasma	30	21-113	Eierusz-Wysocka et al., 1995
9	Men and Women	Plasma	53	30.5-50.3	Kazmierczak et al., 1995
10	Pregnant women	Plasma	31	50.1-66.9	Tsukimori et al., 2008
11	Men	Blood	6	114-577(288)	Varma et al., 1990
12	Men	Plasma	6	13-57(34)	Varma et al., 1990

Considering the importance of H_2O_2 and hypochlorite in human body, studies relating their human blood concentration were summarized. As shown in Table 1, the reported concentrations of H_2O_2 in human blood samples varied greatly, which ranged from 0.1-6050 μM. Among these, some exceeded the IC_{50} that reported in this work for arylsulfatase and β-glucuronidase, which clearly suggest that H_2O_2 in human body can act as an EDC via inhibition of arylsulfatase. Compared to H_2O_2, hypochlorite showed much stronger inhibition to arylsulfatase, and showed simultaneously inhibition to β-glucuronidase, which are all pH-dependent. As hypochlorite can also be endogenously produced through peroxidation of chloride ion by the catalysis of enzyme myeloperoxidase in leukocytes including macrophages, monocytes and neutrophils, the adverse effect of any potential EDC via induction of H_2O_2 and hypochlorite, is likely enhanced when production of hypochlorite is at more favorable condition. It should be noted that due to instability of H_2O_2 in human blood as well as the matrix interferences, not all the concentration data summarized in Table 1 is corrected (Forman et al., 2016). However, it is still believable that H_2O_2 and hypochlorite in human can act as two special EDCs via inhibition of arylsulfatase and/or β-glucuronidase, which further suggests that any agent that can induce production of H_2O_2 or hypochlorite is potential EDC. To the best of our knowledge, this is the first report that H_2O_2, hypochlorite, and PAA can effectively inhibit arylsulfatase and/or β-glucuronidase, which further suggest some EDCs may act as endocrine disruptors via induction of H_2O_2 and hypochlorite.

5. Conclusions

It was demonstrated for the first time that H_2O_2, hypochlorite, and PAA could effectively inhibit arylsulfatase and/or β-glucuronidase. The following conclusions can be drawn.

1) Hypochlorite and PAA at the concentration level of μm could significantly inhibit both arylsulfatase and β-glucuronidase, while H_2O_2 was only effective for inhibiting arylsulfatase.

2) The inhibition of H_2O_2 and PAA to arylsulfatase and β-glucuronidase was found to be reversible, while irreversible inhibition for hypochlorite.

3) H_2O_2, hypochlorite, and PAA may behave as special endocrine disruptors through

inhibition of arylsulfatase and/or β-glucuronidase. This indeed led to extended family of known endocrine disruptors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgement

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