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Arecoline inhibits the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced cytochrome P450 1A1 activation in human hepatoma cells

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Abstract

In the present study, we investigated the effect of arecoline, a major areca nut alkaloid, on the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced activation of cytochrome P4501A1 (CYP1A1) in a human hepatoma cell line Huh-7. We treated Huh-7 cells with 10 nM TCDD in the presence of different concentrations of arecoline (50–300 μM). Our results indicated that arecoline attenuated the TCDD-induced CYP1A1 enzyme activation with an inhibitory effect on cell proliferation. By using real-time RT-PCR, we demonstrated that arecoline inhibited the TCDD-induced activations of CYP1A1 and AhR repressor (AhRR) mRNA expression in a similar pattern. Our results revealed that arecoline inhibited AhR mRNA expression with no direct effect on CYP1A1 enzyme activity. Therefore, in our present study, the observed inhibitory effect of arecoline on CYP1A1 activation was not due to the up-regulation of AhRR or direct inhibitory effect on CYP1A1. Taken together, here we have demonstrated that arecoline attenuates the TCDD-induced CYP1A1 activation mainly via down-regulation of AhR expression in human hepatoma cells, suggesting the possible involvement of arecoline in the AhR-mediated metabolism of environmental toxicants in liver.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxins; Areca nut; Arecoline; Cytochrome P450 1A1; Human hepatoma

1. Introduction

Areca nut, or betel nut, is one of the most widely used psychoactive substances, with several hundred million users worldwide, predominantly in southern Asia [1]. Taiwanese betel quid chewers have an average of 14 to 23 betel quids per day [2]. In general, Taiwanese betel quids include an entire fresh green areca fruit (containing husk), *piper betle* (leaf or inflorescence), and slaked lime paste. The slaked lime paste is either white (white lime paste), containing no additive, or brown (red lime paste) due to the addition of catechu, an extract of *Acacia catechu*.

Areca nut contains many polyphenols and several alkaloids. Areca nut extract has demonstrated to be cytotoxic or genotoxic *in vitro* [3,4]. Recently, areca nut has been classified as a human carcinogen (Group 1) in association mainly with oral, pharyngeal, and esophageal cancers [5], as well as with liver cancer [6–9]. Powdered areca nut placed in the oral cavity of human volunteers gives rise to a rapid appearance of arecoline, the major alkaloid in areca nuts, in blood plasma, indicating systemic absorption of this alkaloid [5]. Arecoline has been shown to induce human buccal mucosal fibroblast proliferation and collagen synthesis *in vitro* [10,11]. Moreover, a recent study has revealed that cytochrome P450 (CYPs) efficiently activate betel quid-specific *N*-nitrosamines, thus resulting in mutagenic activation of the *N*-nitrosamines in *S. typhimurium* YG7108 [12].

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Like most of persistent organic pollutants, the lipophilic and ubiquitous features of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can result in its bioaccumulation along food chains and ultimately in humans. TCDD can cause immune suppression, birth defects, and carcinogenesis [13]. There have been significant increases in cancer incidence and mortality from lymphohematopoietic neoplasm reported in TCDD contaminated areas [14–16]. It has been well recognized that the toxic effects of TCDD are mediated by interaction with cytosolic AhR, a helix-loop-helix transcription factor. AhR plays a pivotal role in mediating a broad range of distinct toxic responses induced by polyhalogenated and polycyclic aromatic hydrocarbons [17].

Upon TCDD binding, the ligand-activated AhR translocates into the nucleus and forms a heterodimer with the aromatic hydrocarbon nuclear translocator (ARNT/HIF-1β) [18], and hence mediates gene expression via binding on the *cis*-acting dioxin responsive element (DRE) [19–22]. The ligand-activated AhR induces gene expression of AhR repression (AhRR), which in turn inhibits AhR function by competing with AhR via dimerizing with ARNT and binding to the xenobiotic responsive element (XRE) sequence [23]. Thus, AhR and AhRR form a regulatory circuit in the xenobiotic signal transduction pathway and provide a mechanism for regulating AhR function.

Genetic and biochemical studies have revealed that AhR is necessary for most of the TCDD-induced toxic effects [21,24] and that genetic polymorphism of CYP1A1 and susceptibility to oral squamous cell carcinoma and oral precancer lesions are associated with smoking/betel-quid use [25]. These studies suggest the involvement of betel quid in carcinogenesis, possibly via interfering with the AhR-mediated detoxification activities. However, few studies have investigated the underlying mechanism for CYP1A1 expression regulated by interaction between TCDD and betel quid. The objective of this study is to investigate the *in vitro* effect of betel quid on the TCDD-induced CYP1A1 expression in human hepatoma cells. In this study, we employed arecoline as a model compound for betel-quid chewing. Our results suggest that arecoline inhibits the TCDD-induced CYP1A1 activation mainly via down-regulation of AhR expression.

2. Materials and methods

2.1. Chemicals and antibodies

TCDD was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Arecoline, ethoxyresorufin, resorufin, salicylamide, and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The human hepatoma cell line Huh-7 was kindly provided by Dr. Chiung-Tong Chen (National Health Research Institutes, Taiwan). Recombinant human CYP1A1 protein was obtained from Gentest (Gentest, Woburn, MA, USA).

2.2. Cell culture and treatments

Huh-7 cells were maintained in DMEM supplemented with fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100 units/ml), and sodium bicarbonate (3.7 g/L) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Huh-7 cells, seeded on 24-well plates at 90% confluence, were left untreated or treated with 10 nM TCDD in the presence of different concentrations of arecoline (50, 100, 200, and 300 μM) for 24 h.

2.3. Ethoxyresorufin-O-deethylase (EROD) assay

After treatment, the medium was removed and the wells were washed twice with fresh medium. The EROD activity was determined as previously described [26,27] using 10 μM of ethoxyresorufin in DMEM medium as the substrate of CYP1A1 enzyme, in the presence of 1.5 mM of salicylamide to inhibit conjugating enzymes. After incubation for 30 min at 37 °C, fluorescence was measured by using a Fluoroskan multi-well fluorescence plate reader (Labsystems, Helsinki, Finland), with excitation at 530 nm and emission at 590 nm. Resorufin standard curve was used to convert fluorescence to pmole of resorufin formed. The CYP1A1 enzyme activity was defined as resorufin formation (in pmole) per 100 min of reaction time (pmole resorufin/100 min).

2.4. Cytotoxicity assay

Huh-7 cells were seeded at a concentration of 3×10^4 cells/well in 96-well dishes, and MTT cytotoxicity assay was performed as previously described in detail [28].

2.5. RNA extraction and real-time RT-PCR

Total RNA was extracted by using the REzol reagent (PROtech Technologies, Tainan, Taiwan) following the manufacturer's instructions, except that nuclease-free DNase I digestion (TaKaRa Bio. Inc., Otsu, Shiga, Japan) was performed to eliminate genomic DNA contamination. Purified RNA was reverse transcribed using oligo-deoxythymidine as a primer. Gene-specific cDNA was amplified from first-strand cDNA using a pair of gene-specific primers and the LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostics GmbH, Mannheim, Germany). These gene-specific primer pairs (Table 1) were designed by using the software LightCycler Probe Design (Roche). Gene expression was relatively quantified by calibration against standard curves generated with serial dilutions of the first-strand cDNA mix [29]. In this study, CYP1A1, AhR, and AhRR were normalized to β-actin.

Table 1
Primer sequences

Gene/promoter	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
CYP1A1	ggagctagacacagtga	accgatacacttccgc
AhR	tcttaggctcagcgtc	gctcggtcttcggtat
AhRR	tgaccttgtccttgaccc	ccatcctcactgtgctttc
β-Actin	acaccccagccatgtacg	tggtggtgaagctgtagcc

2.6. Direct effect of arecoline on recombinant CYP1A1 enzyme activity

Recombinant human CYP1A1 protein (1.25 pmol) were mixed with different concentrations of arecoline (50, 100, 200, and 300 μM) in a total volume of 250 μl of sodium phosphate buffer (0.1 M, pH 7.4) containing 10 μM ethoxyresorufin and 10 mM $MgCl_2$. The reaction was initiated by adding 1 mM NADPH. The reaction mixture was incubated at 37 °C for 30 min and then the reaction was quickly quenched on ice. The CYP1A1 enzyme activity was determined by using EROD assay as described above.

2.7. Statistics

Each experiment was performed independently in triplicate for at least three times. Student's *t*-test was used to determine statistical significance of the difference between experimental groups. Differences were considered statistically significant when p was less than 0.05.

3. Results

3.1. Arecoline inhibits the TCDD-induced CYP1A1 enzyme activation

To address the arecoline effect on CYP1A1 enzyme activation induced by 10 nM TCDD, we treated Huh-7 cells with different concentrations of arecoline (50, 100, 200, and 300 μM) for 24 h in the presence or absence of 10 nM TCDD. As shown in Fig. 1a, our data showed that TCDD alone caused a significant increase in CYP1A1 enzyme activity and that arecoline (50, 100, 200, and 300 μM) significantly inhibited the TCDD-induced CYP1A1 activation (by 14%, 24%, 42%, and 66%, respectively) in a dose-dependent manner. Meanwhile, treatment with arecoline alone up to 300 μM caused no marked effect on CYP1A1 activity.

We also determined the cytotoxic effect of arecoline and/or TCDD on Huh-7 subjected to the same treatments as described in Fig. 1a by using MTT assay. As shown in Fig. 1b, arecoline (50, 100, 200, and 300 μM) caused decreases in survival rate (by 1%, 15%, 23%, and 22%, respectively), however with no statistical significance, except in treatment with arecoline up to 300 μM. Our data also showed that TCDD (10 nM) exhibited no significant cytotoxicity in Huh-7 cells in combined treatments with or without arecoline. Moreover, under microscope, no marked increase in cell death was observed in all treatments, suggesting that the observed decreases in survival rate might be resulted from inhibition of cell proliferation by arecoline.

3.2. Arecoline inhibits the TCDD-induced CYP1A1 mRNA transcription

In order to evaluate the arecoline effect on activation of CYP1A1 mRNA transcription induced by 10 nM TCDD, Huh-7 cells were left untreated or treated with 10 nM TCDD in the presence of various concentrations of arecoline (100, 200, and 300 μM) for 24 h. Then we analyzed CYP1A1 mRNA expression by using real-time RT-PCR. As shown in Fig. 2a, our data revealed that arecoline (100, 200, and 300 μM) significantly attenuated the 10 nM TCDD-induced CYP1A1 mRNA expression (by 46%, 53%, and 76%, respectively) in a dose-dependent manner. Meanwhile, arecoline alone caused no detectable effect on basal level of CYP1A1 mRNA expression.

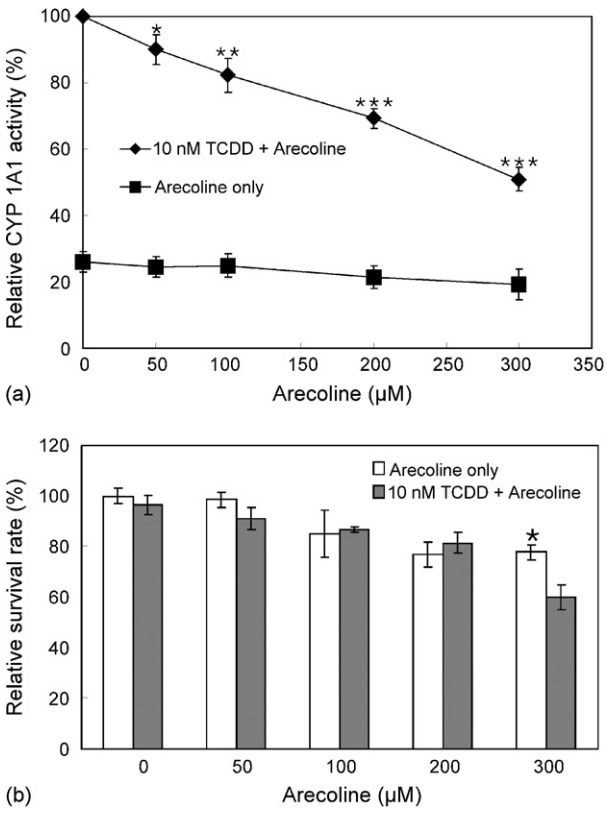

Fig. 1. Arecoline inhibits the TCDD-induced CYP1A1 enzyme activation with an inhibitory effect on cell proliferation. Huh-7 cells were treated with or without 10 nM TCDD in the presence of different concentrations of arecoline (50, 100, 200, and 300 μM) for 24 h. (a) After treatments, CYP1A1 activities were determined by using EROD assay. CYP1A1 activities are presented as means ± S.E., $n = 11$, and are expressed as a percentage of CYP1A1 activity as compared with that treated with 10 nM TCDD alone. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* that treated with 10 nM TCDD alone. (b) Cytotoxicity was determined by using MTT assay. Survival rates are presented as means ± S.E., $n = 3$, and are expressed as relative survival rates as compared with that of untreated control. $^{*}P < 0.05$ *vs.* the respective untreated control.

3.3. Arecoline inhibits the TCDD-induced AhRR mRNA transcription

In addition to CYP1A1, we also analyzed another TCDD-responsive/AhR-mediated gene, AhRR, since there was a possibility that arecoline inhibited CYP1A1 expression via up-regulation of AhRR expression. To clarify the possibility, we treated Huh-7 cells with various concentrations of arecoline for 24 h in the presence or absence of 10 nM TCDD. As shown in Fig. 2b, we demonstrated that arecoline (100, 200, and 300 μM) attenuated the TCDD-induced AhRR mRNA expression (by

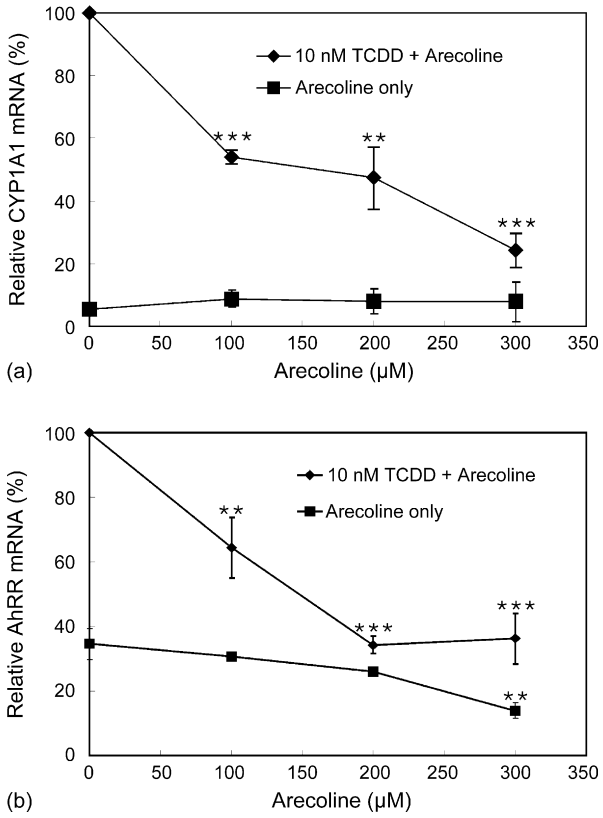

Fig. 2. Arecoline inhibits the TCDD-induced CYP1A1 and AhRR mRNA expression. Huh-7 cells were treated with or without 10 nM TCDD in the presence of different concentrations of arecoline (100, 200, and 300 μM) for 24 h. (a) After treatment, CYP1A1 mRNA expression was determined by using quantitative real-time RT-PCR. CYP1A1 mRNA levels are presented as means ± S.E., $n = 3$, and are expressed as a percentage of CYP1A1 mRNA as compared with that treated with 10 nM TCDD alone. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* that treated with 10 nM TCDD alone. (b) AhRR mRNA expression was determined by using quantitative real-time RT-PCR. AhRR mRNA levels are presented as means ± S.E., $n = 4$, and are expressed as a percentage of AhRR mRNA as compared with that treated with 10 nM TCDD alone. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the respective untreated control.

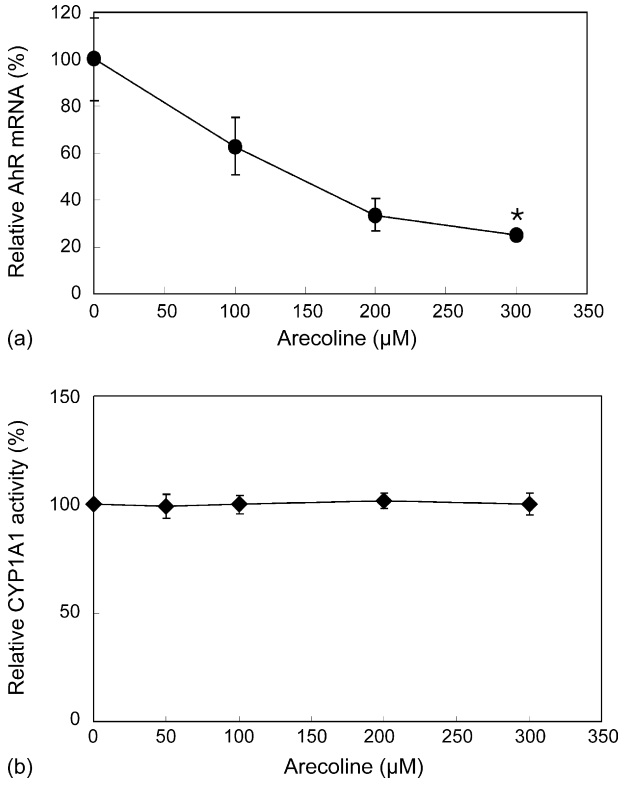

Fig. 3. Arecoline inhibits AhR mRNA expression with no direct effect on CYP1A1 enzyme activity. (a) Huh-7 cells were treated with different concentrations of arecoline (100, 200, and 300 μM) for 24 h. After treatment, AhR mRNA expression was determined by using quantitative real-time RT-PCR. AhR mRNA levels are presented as means ± S.E., $n = 3$, and are expressed as a percentage of AhR mRNA as compared with that of untreated control. $^{*}P < 0.05$ *vs.* the untreated control. (b) For analyzing direct effect of arecoline on CYP1A1 enzyme activity, recombinant human CYP1A1 protein was treated with different concentrations of arecoline (50, 100, 200, and 300 μM) in sodium phosphate buffer (0.1 M, pH 7.4) with 10 μM ethoxyresorufin and 10 mM $MgCl_2$. The reaction was determined as described in detail in Section 2.

36%, 66%, and 64%, respectively). Meanwhile, treatment with 300 μM arecoline alone also caused a significant decrease in AhRR mRNA expression as compared with that of untreated control.

3.4. Arecoline inhibits AhR mRNA expression and exhibits no direct effect on CYP1A1 enzyme activity

To further explore the mechanism by which arecoline inhibited the TCDD-induced CYP1A1 enzyme activation, we treated Huh-7 cells with various concentrations of arecoline for 24 h and then we analyzed AhR mRNA expression by using real-time RT-PCR. As shown in Fig. 3a, our data indicated that treatments of Huh-7 with 100, 200, and 300 μM caused decreases in AhR mRNA expression by 37%, 66%, and 75% ($P < 0.05$), respectively.

To further examine if arecoline could directly inhibit CYP1A1 activity *in vitro*, we incubated recombinant human CYP1A1 enzyme with different concentrations of arecoline (50, 100, 200, and 300 μM) for 30 min and then analyzed the CYP1A1 enzyme activities of those treated samples by using EROD assay. Our data indicated that treatment with arecoline ≤ 300 μM exhibited no direct effect on the recombinant human CYP1A1 enzyme activity (Fig. 3b).

4. Discussion

Both TCDD [30] and betel nut [5] have been classified as Group 1 human carcinogens by the International Agency for Research on Cancer (IARC). TCDD is a persistent, widespread, toxic environmental contaminant, which is a potent inducer for aryl hydrocarbon hydroxylase activity in the liver and other tissues. TCDD induces hydroxylase activity by increasing the transcription rate of CYP1A1 gene [31]. It is noteworthy that CYPs have been demonstrated to play critical roles in mutagenic activation of the areca-specific *N*-nitrosamines [12]. Chemical nitrosation of arecoline, one of the major alkaloids in areca nuts, leads to the formation of areca-specific *N*-nitrosamines such as 3-(*N*-nitrosomethylamino)propionitrile, 3-

(*N*-nitrosomethylamino)propioaldehyde, *N*-nitrosoguvacoline, and *N*-nitrosoguvacine [32].

In the present study, we employed two model compounds (arecoline and TCDD) to evaluate the combined effects of betel-quid chewing and environmental dioxin on regulation of AhR-mediated gene expression in human hepatocytes. Our data revealed that arecoline $\leq$300 μM inhibited the TCDD-induced CYP1A1 enzyme activation (Fig. 1a) and mRNA expression (Fig. 2a) in a dose-dependent manner. Meanwhile, we also showed that arecoline up to 300 μM could cause a mild inhibition of survival rates (Fig. 1b). No increase in cell death and no change in cell morphology were observed under microscope in our present study, suggesting that the inhibitory effect of arecoline on survival rate was possibly due to its inhibitory effect on cell proliferation. Therefore, the observed inhibitory effect of arecoline on the TCDD-induced CYP1A1 activation is not due to cytotoxicity induced by arecoline. Moreover, arecoline alone exhibited no detectable effect on the basal levels of enzyme activity (Fig. 1a) and mRNA expression (Fig. 2a) of CYP1A1, suggesting that arecoline itself or its cellular metabolites did not affect the basal CYP1A1 activity and gene expression in Huh-7 cells.

A previous study has revealed that another TCDD-responsive/AhR-mediated gene, AhRR, constitutively forms heterodimer with ARNT [23]. The AhRR/ARNT heterodimer functions as a negative transcriptional repressor by competitively binding to the XRE in the promoter region of CYP1A1 gene, thus resulting in down-regulation of CYP1A1 activity [23]. However, our present study showed that arecoline inhibited the TCDD-induced mRNA expressions of both CYP1A1 and AhRR in a similar pattern (Fig. 2). Our results rule out the possible transcriptional repressor role of AhRR in down-regulation of CYP1A1 by arecoline, suggesting that arecoline inhibits the TCDD-induced activations of CYP1A1 and AhRR possibly via a common regulation mechanism.

Furthermore, to address the arecoline effect on cellular level of AhR, we treated Huh-7 cells with different concentrations of arecoline (100, 200, and 300 μM) for 24 h, and then we analyzed AhR mRNA expression by using real-time RT-PCR. Our data showed that arecoline caused marked decreases in AhR mRNA expression in a dose-dependent manner (Fig. 3a). The observed inhibitory effect of arecoline on activations of CYP1A1 enzyme (Fig. 1a) and CYP1A1 mRNA (Fig. 2a) is highly correlated with its down-regulation of AhR mRNA expression. On the basis of these findings, it is suggested that arecoline attenuates the TCDD-induced CYP1A1 gene expression via down-regulation of AhR mRNA expression.

It has been shown that resveratrol, an AhR antagonist found in grapes, is able to directly inhibit CYP1A1/EROD activation induced by benzo[a]pyrene [33]. Therefore, we also established an *in vitro* assay system for determining the direct effect of arecoline on recombinant CYP1A1 enzyme activity. As shown in Fig. 3b, it was demonstrated that treatment of Huh-7 cells with arecoline up to 300 μM exhibited no direct effect on CYP1A1 enzyme activity. Our present study suggests that the inhibitory effect of arecoline on TCDD-induced CYP1A1 activation is mediated mainly via down-regulation AhR expression.

Permeability of human buccal and vaginal mucosa to alkaloids from areca nut has been demonstrated previously [34]. It has also been reported that the highest concentration of arecoline detected in saliva during areca quid chewing is 89.9 μg/ml (580 μM) [35]. Thus, it is reasonable that the arecoline concentrations (50–300 μM) adopted in this study are able to represent the arecoline concentrations found in gingival tissues and fluids of areca chewers. However, data of arecoline concentration in plasma of areca chewers is still limited in the meantime.

Halogenated aromatic hydrocarbons, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs), have been found widespread in the environment. Areca nut is one of the most widely used psychoactive substances, with several hundred million users worldwide, predominantly in southern Asia, including Taiwan. However, there are few studies investigating the combined effect of betel quid and TCDD on liver toxicity. Thus, it is of extreme importance to reveal the possible health impact resulting from co-exposure to these two substances. In this study, we provide the first evidence showing the inhibitory effect of arecoline on the TCDD-induced AhR-mediated gene expression, which plays a critical role in the detoxification of environmental toxicants in the liver. On the basis of our results, we demonstrate that arecoline attenuates the TCDD-induced CYP1A1 activation mainly via down-regulation of AhR expression.

5. Conclusions

In the present study, we investigate the effect of arecoline on induction of CYP1A1 activity by TCDD in human hepatoma cells. Our data indicate that arecoline inhibits the TCDD-induced CYP1A1 activation/expression in a dose-dependent manner. We also demonstrate that the treatment exhibits a similar inhibitory effect on another TCDD- responsive/AhR-mediated gene, AhRR. We find that arecoline causes decreases in AhR mRNA expression in a dose-dependent manner, however, with no direct effect on CYP1A1 enzyme activity. Therefore, in our present study, the observed inhibitory effect of arecoline on CYP1A1 activation is not due to the upregulation of AhRR or direct inhibitory effect on CYP1A1. Taken together, our results suggest that arecoline attenuates the TCDD-induced CYP1A1 activation mainly via down-regulation of AhR expression in human hepatoma cells.

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