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Human arylacetamide deacetylase hydrolyzes ketoconazole to trigger hepatocellular toxicity

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ABSTRACT

Ketoconazole (KC), an antifungal agent, rarely causes severe liver injury when orally administered. It has been reported that KC is mainly hydrolyzed to N-deacetyl ketoconazole (DAK), followed by the N-hydroxylation of DAK by flavin-containing monooxygenase (FMO). Although the metabolism of KC has been considered to be associated with hepatotoxicity, the responsible enzyme(s) remain unknown. The purpose of this study was to identify the responsible enzyme(s) for KC hydrolysis in humans and to clarify their relevance to KC-induced toxicity. Kinetic analysis and inhibition studies using human liver microsomes (HLM) and recombinant enzymes revealed that human arylacetamide deacetylase (AADAC) is responsible for KC hydrolysis to form DAK, and confirmed that FMO3 is the enzyme responsible for DAK N-hydroxylation. In HLM, the clearance of KC hydrolysis occurred to the same extent as DAK N-hydroxylation, which indicates that both processes are not rate-limiting pathways. Cytotoxicity of KC and DAK was evaluated using HepaRG cells and human primary hepatocytes. Treatment of HepaRG cells with DAK for 24 h showed cytotoxicity in a dose-dependent manner, whereas treatment with KC did not show due to the low expression of AADAC. Overexpression of AADAC in HepaRG cells with an adenovirus expression system elicited the cytotoxicity of KC. Cytotoxicity of KC in human primary hepatocytes was attenuated by diisopropylfluorophosphate, an AADAC inhibitor. In conclusion, the present study demonstrated that human AADAC hydrolyzes KC to trigger hepatocellular toxicity.

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1. Introduction

Ketoconazole (KC), a synthetic imidazole antifungal agent, is effective against fungal infections and is widely used as a topical antifungal. In addition, KC is orally administered for Cushing's syndrome [1,2], but hypoadrenalism may occur due to blocked adrenal steroidogenesis from inhibiting cytochrome P450s [3,4]. A major concern of oral ketoconazole is severe hepatotoxicity [5]. The US Food and Drug Administration recently limited usage of ketoconazole oral tablets, and the European Medicines Agency

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http://dx.doi.org/10.1016/j.bcp.2016.07.007 0006-2952/© 2016 Elsevier Inc. All rights reserved. recommended no use of oral ketoconazole due to potential severe liver injury.

In patients with KC-induced hepatotoxicity, hypersensitivity and eosinophilia were rarely observed with no allergic reactions. Therefore, cytotoxic mechanisms rather than immunotoxic mechanisms have been considered [5,6]. In general, not only the parent compound but also the metabolites should be considered when evaluating toxicity. KC can be hydrolyzed to deacetylketoconazole (DAK) by esterases and then metabolized to N-hydroxy DAK by flavin monooxygenase (FMO) (Fig. 1), which finally leads to a dialdehyde form [7–9]. Rodriguez and Acosta [10] evaluated the cytotoxicity of KC and DAK using rat hepatocytes by monitoring lactate dehydrogenase (LDH) leakage. They found that DAK showed a higher cytotoxicity than KC, and the cytotoxicity of DAK was exacerbated by *n*-octylamine, which is an activator of FMO enzymes. Next, the cytotoxicity was attenuated by methimazole, a competitive inhibitor of FMO enzymes, which implies that metabolism by esterases and FMO enzymes could be associated with ketoconazole-induced hepatotoxicity. It was reported that, among human FMO enzymes, human FMO1 and FMO3 could catalyze N-hydroxylation of DAK [9]. Yet the enzyme(s) responsible

Abbreviations: AADAC, arylacetamide deacetylase; BNPP, bis-(*p*-nitrophenyl) phosphate; CES, carboxylesterase; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; G6P, glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; HLM, human liver microsome; HPLC, high-performance liquid chromatography; KC, ketoconazole; LC–MS/MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; MRM, multiple-reaction monitoring; NADPH-GS, nicotine adenine dinucleotide phosphate-generating system; PMSF, phenylmethylsulfonyl fluoride.

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T. Fukami et al. / Biochemical Pharmacology xxx (2016) xxx-xxx



Fig. 1. Proposed metabolic pathways of KC to reactive metabolites.

for DAK formation from KC, the first step of metabolism of KC, remains unknown.

Representative esterases involved in drug hydrolysis in the human liver are carboxylesterase (CES) and arylacetamide deacetylase (AADAC) [11]. Human CES enzymes are classified into 5 isoforms, CES1–CES5. Among them, CES1 and CES2 are mainly involved in drug hydrolysis [12]. CES1 prefers compounds with small alcohol and large acyl moieties, whereas CES2 prefers compounds with large alcohol and small acyl moieties [13]. AADAC is a unique enzyme that prefers compounds with smaller acyl moieties than compounds preferred by CES1 and CES2 [13]. Based on these preferences, we assumed that AADAC is responsible for the hydrolysis of KC, which has an acetyl group. In this study, we tried to clarify which enzymes are responsible for KC hydrolysis in humans and investigate whether KC hydrolase is involved in KC-induced cytotoxicity using HepaRG cells.

2. Materials and methods

2.1. Materials

Diisopropyl fluorophosphate (DFP), pheynylmethylsulfonyl fluoride (PMSF), vinblastine sulfate, and calcium chloride (CaCl₂) were purchased from Wako Pure Chemical Industries (Osaka, Japan). KC, DAK, and telmisartan were purchased from Toronto Research Chemicals (Toronto, Canada). Bis-(*p*-nitrophenyl) phosphate (BNPP) and 2-chloro-3',4'-dimethoxybenzil (CDMB) were purchased from Sigma-Aldrich (St. Louis, MO). Glucose-6phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and β-nicotinamide adenine dinucleotide phosphate oxidized form (NADP⁺) were purchased from Oriental Yeast (Tokyo, Japan). Recombinant human AADAC, CES1, and CES2 expressed in baculovirus-infected insect cells were prepared as previously described [14,15]. Recombinant human FMO1, FMO3, and FMO5 expressed in baculovirus-infected insect cells were purchased from Corning (Corning, NY). Cell Counting Kit-8 (CCK-8) and Cytotoxicity LDH Assay Kit-WST were from Dojin Chemical Laboratories (Kumamoto, Japan). Human cryopreserved hepatocytes (lot# HC1-30 (African American, female, 47 years) and lot# HC2-20 (Caucasian, female, 47 years) were purchased from Xenotech LLC (Lenexa, KS). All other chemicals were the highest quality or analytical quality that could be obtained commercially.

2.2. Human tissues

Pooled human liver microsomes (HLM) (n = 50) and pooled human intestine microsomes (HIM) (n = 7) were purchased from Corning. Individual human renal microsomes (HRM) were

purchased from Tissue Transformation Technologies (Edison, MJ). Pooled human pulmonary microsomes (HPM) (n = 4) were purchased from Xenotech LLC (Kansas City, KS). Individual human liver samples obtained from 24 donors (16 Caucasians, 5 Hispanic, and 3 Black donors; 15 males, 9 females) were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan). HLM were prepared according to a previously reported method [16]. The use of human tissue samples was approved by the ethics committees of Kanazawa University (Kanazawa, Japan).

2.3. KC hydrolase activity

KC hydrolase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and various enzyme sources (HLM, HRM, or HPM: 1.0 mg/ml; HIM: 0.2 mg/ml; homogenates of Sf21 cells expressing AADAC, CES1, or CES2: 0.4 mg/ml). In a preliminary study, we confirmed that the KC hydrolase activity was linear with respect to protein concentration (HLM: <1.2 mg/ml; HIM: <0.4 mg/ml; homogenates of Sf21 cells expressing AADAC: <0.6 mg/ml) and incubation time (<3 h). KC was dissolved in methanol such that the final concentration of methanol in the incubation mixture was 1%. The reaction was initiated by adding KC (final concentration: 10 µM) after a 2-min pre-incubation at 37 °C. After a 3-h incubation at 37 °C, the reaction was terminated by adding 100 µl of ice-cold acetonitrile. After removing the protein by centrifugation at 20,380g for 5 min, a 5-µl aliquot of the supernatant was subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using LC equipment consisting of a HP1100 system, including a binary pump, an automatic sampler, and a column oven (Agilent Technologies, Santa Clara, CA), which was equipped with a Zorbax SB-C18 column (2.1×50 mm, 3.5μ m; Agilent Technologies). The column temperature was 25 °C. The mobile phase was 0.1% formic acid (A) and methanol, including 0.1% formic acid (B). The conditions for elution were as follows: 30–50% B (0-1.0 min), 50% B (1.0-14.0 min), 50 to 30% B (14.0-14.01 min), and 30% B (14.01-25 min). The flow rate was 0.2 ml/min. The LC system was connected to a PE Sciex API 2000 tandem mass spectrometer (Applied Biosystems, Foster City, CA), which was operated in positive electrospray ionization mode. The turbo gas was maintained at 550 °C. Nitrogen was used as the nebulizing, turbo, and curtain gas at 50, 80, and 40 psi, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as the collision gas. The collision energy was 121 V. Two m/z ion transitions were monitored in multiple reaction monitoring (MRM) mode: m/z 531.0 and 81.1 for KC and m/z 489.2 and 81.1 for DAK. Analytical data were processed using Analyst software (version 1.5; Applied Biosystems) in the API 2000 LC-MS/MS system. Quantification of DAK was obtained by comparing the peak area with that of an authentic standard.

2.4. Inhibition analysis of KC hydrolase activity

To examine the involvement of AADAC in KC hydrolysis in humans, an inhibition analysis was performed. DFP and BNPP are serine esterase inhibitors [17,18]. Vinblastine is a AADAC and CES2 inhibitor [19]. PMSF and CDMB are CES inhibitors [19,20]. Telmisartan is a CES2 inhibitor [19]. The final concentrations were 10 μ M for DFP and BNPP, 50 μ M for telmisartan, 100 μ M for vinblastine, PMSF, and CDMB. PMSF, vinblastine, and telmisartan, which were dissolved in DMSO such that the final concentration of the organic solvent in the incubation mixture was 2%. The other inhibitors were dissolved in distilled water. The experimental procedure and conditions were the same as above.

2.5. Other hydrolase activities

Flutamide [21], indiplon [22], phenacetin [15], rifampicin [23], fenofibrate [13], and irinotecan [24] hydrolase activities were determined according to our previous reports. Individual HLM from 24 donors were used as the enzyme sources. The protein concentrations were as follows: fenofibrate and irinotecan hydrolase activities, 0.2 mg/ml; flutamide, phenacetin, and indiplon hydrolase activities, 0.4 mg/ml; rifampicin hydrolase activity, 0.5 mg/ml. The substrate concentrations were as follows: 500 μ M flutamide, 50 μ M indiplon, 1 μ M phenacetin, 50 μ M rifampicin, 5 μ M fenofibrate and 2 μ M irinotecan.

2.6. DAK N-hydroxylase activity

The DAK N-hydroxylase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) containing 100 mM potassium phosphate buffer (pH 7.4 or pH 8.8), various enzyme sources (HLM: 0.5 mg/ml; recombinant FMO1, FMO3: 0.02 mg/ml, recombinant FMO5: 0.075 mg/ml) and DAK (final concentration of 300 μ M). DAK was dissolved in methanol such that the final concentration of methanol in the incubation mixture was 1%. In a preliminary study, we confirmed that the DAK *N*-hydroxylase activity was linear with respect to protein concentration (HLM: <0.5 mg/ml; recombinant FMO1 and FMO3: <0.08 mg/ml; recombinant FMO5: <0.1 mg/ml) and incubation time (HLM: <10 min; recombinant FMO1 and FMO3: <5 min; recombinant FMO5: <60 min). The reaction was initiated by the addition of an NADPH-GS after a 2-min pre-incubation at 37 °C. After a 10-min (HLM), 3-min (recombinant FMO1 and FMO3), or 45-min (recombinant FMO5) incubation at 37 °C, the reaction was terminated by the addition of 100 µl of ice-cold acetonitrile. After removing the protein by centrifugation at 20,380g for 5 min, a 5-µl aliquot of the supernatant was subjected to LC-MS/MS. The LC-MS/MS apparatus and conditions were the same as described above except that the conditions for elusion were as follows: 20-90% B (0-3 min), 90% B (3-11 min), 90 to 20% B (11-11.01 min), and 20% B (11.01-18 min). The collision energy was 40 V. Two m/z ion transitions were monitored in MRM mode: *m*/*z* 505.2 and 81.0 for *N*-hydroxy DAK.

2.7. Identification of N-hydroxy DAK

Because an authentic standard of *N*-hydroxy DAK was not commercially available, LC–MS/MS analysis was performed to examine whether the formed metabolite was *N*-hydroxy DAK. The samples were prepared as described above except that 100 mM potassium phosphate buffer (pH 8.8), recombinant FMO3 (0.2 mg/ml), and DAK (250 μ M) were used. In the single-quadrupole mode (Q1-scan) with LC–MS/MS, full-scan spectra were acquired with a scan range of *m*/*z* 50–550. Subsequently, in the product ion scan mode, Q1 spectra at *m*/*z* 505.2 was monitored and fragment ions were scanned in the range of *m*/*z* 50–550.

2.8. Quantification of N-hydroxy DAK

The *N*-hydroxy DAK formation was quantified based on the decreased amount of DAK. DAK (50 μ M) was incubated with recombinant FMO3 (0.2 mg/ml) for 0, 1, 2, and 3 min. The decreased amounts of DAK were quantified and applied to the peaks of formed *N*-hydroxy DAK. After determining the peak area per known content of *N*-hydroxy DAK, the value was used to calculate the amount of *N*-hydroxy DAK formed in the incubation mixture.

2.9. Kinetic analyses

The kinetic analyses of KC hydrolysis and DAK *N*-hydroxylation were performed with $2-150 \mu$ M and $2-500 \mu$ M of reaction mixtures, respectively. The kinetic parameters were estimated from a curve fitted using a computer program designed for nonlinear regression analysis (KaleidaGraph; Synergy Software, Reading, PA). The following equations were used:

Michaelis–Menten equation : $V = V \max * [S]/(Km + [S])$

Substrate inhibition equation : $V = V \max * [S] / (Km + [S] + [S]^2 / Ki)$

where *V* is the velocity of the reaction, *S* is the substrate concentration, *K*m is the Michaelis–Menten constant, *V*max is the maximum velocity, and *K*i is the substrate inhibition constant.

2.10. HepaRG cell culture

Human hepatoma-derived HepaRG cells obtained from Biopredic International (Rennes, France) were grown according to the manufacturer's instructions as follows: the cells were seeded in culture dishes and maintained in growth medium (William's E Medium with no glutamine (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 µg/ml insulin, and 5×10^{-5} M hydrocortisone) at 37 °C under an atmosphere of 5% CO₂ and 95% air. The medium was renewed every 3 days. After 14 days, the cells were detached by gentle trypsinization and seeded in a 24-well plate at 0.55×10^5 cells/well with the growth medium. After 14 days, the culture medium was changed to a differentiation medium (growth medium containing 2% DMSO) and the cell culture was maintained for 14 days to differentiate the cells.

2.11. Overexpression of human AADAC in HepaRG cells

Differentiated HepaRG cells were infected with recombinant adenoviruses expressing AADAC (AdAADAC) or green fluorescent protein (AdGFP) at MOI 2.5. The AdAADAC and AdGFP were constructed in previous studies [23,25]. After 24 h, the medium was changed to an adenovirus-free medium, and the cells were incubated for 72 h. To confirm overexpression of AADAC in HepaRG cells, flutamide hydrolase activity *in cellulo* was measured as an AADAC marker activity as follows: The cells were incubated with 500 μ M flutamide for 1 h, and the medium was collected to measure the FLU-1 concentration with high-performance liquid chromatography (HPLC) as previously described [21]. The protein concentrations of total cell homogenates were determined according to the method of Bradford [26] using γ -globulin as the standard.

2.12. Cytotoxicity assay using HepaRG cells

To evaluate the cytotoxicity of KC and DAK, a WST-8 (2-(2-meth oxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-te trazolium monosodium salt) assay, which is a modified MTT assay, was performed as follows: the differentiated HepaRG cells were treated with 0, 10, 25, and 50 μ M KC or DAK, which were dissolved in methanol such that the final concentration of methanol in the medium was 2%. After 24 h, CCK-8 reagent was added and the absorbance of the medium at 450 nm was measured. The effect of human AADAC on KC-induced cytotoxicity was evaluated as follows: The differentiated HepaRG cells infected with AdAADAC or AdGFP at MOI 2.5 for 24 h were treated with 10, 25, and 50 μ M KC for 24, 48, or 72 h. CCK-8 reagent was added, and the

4

absorbance of the medium at 450 nm was measured. The percent cell viability was calculated by comparing the absorbance of the treated cells with the control cells.

2.13. Cytotoxicity assay using human primary hepatocytes

The effects of AADAC on KC-induced cytotoxicity were also evaluated using human cryopreserved hepatocytes (lots HC1-30 and HC2-20) (Xenotech LLC) as follows: Human hepatocytes (3×10^4 cells/well) were seeded into 96-well plates. After 3-h incubation, the medium was changed to hepatocyte culture medium (epidermal growth factor-, antibiotics-, and fatty acid-free bovine serum albumin (FAF–BSA)-free) containing 30 μ M KC. DFP (10 μ M) was also treated as an AADAC inhibitor. After 48-h incubation, LDH release was measured photometrically at 492 nm using a kit. The maximum LDH release was measured after the addition of the lysis buffer attached to the kit. The percentage of LDH release was calculated by comparing the absorbance to the maximum LDH release of the control cells.

2.14. Statistical analysis

Comparisons of the two groups were made with an unpaired, two-tailed Student's *t*-test. A value of p < 0.05 was considered to be statistically significant.

3. Results

3.1. KC hydrolase activities in human tissue microsomes and recombinant esterases

To investigate which tissues show KC hydrolysis activity, human liver microsomes (HLM), human intestinal microsomes (HIM), human renal microsomes (HRM), and human pulmonary microsomes (HPM) were used as enzyme sources because these organs include drug-metabolizing enzymes. As shown in Fig. 2A, HLM $(10.2 \pm 0.824 \text{ pmol/min/mg protein})$ and HIM $(15.4 \pm 0.676 \text{ pmol/})$ min/mg protein) showed prominent activity at a substrate concentration of $10 \mu M$, whereas HRM (0.0398 ± 0.0117 pmol/ min/mg protein) and HPM (0.0843 ± 0.00689 pmol/min/mg protein) showed marginal activity. Representative esterases that are involved in drug hydrolysis are CES1, CES2, and AADAC. Human CES1 is predominantly expressed in the liver, but not in the intestines. Human CES2 is highly expressed in the liver, intestines, and kidneys. Accordingly, CES enzymes could be excluded from candidate enzymes catalyzing KC hydrolysis. In contrast, human AADAC is highly expressed in the liver and intestines, but not in kidneys and lungs [21]. Thus, we investigated whether human AADAC could catalyze KC hydrolysis, and found that recombinant AADAC showed high activity $(21.0 \pm 0.453 \text{ pmol/min/mg protein, at } 10 \,\mu\text{M of KC})$. In contrast, recombinant human CES1 (0.152 ± 0.0235 pmol/min/ mg protein) and CES2 (0.0984 ± 0.0315 pmol/min/mg protein) showed marginal activity that was close to the activity of the mock sample (0.0847 ± 0.0275 pmol/min/mg protein). Thus, it was demonstrated that AADAC has the potential to catalyze KC hydrolysis.

To confirm the responsibility of AADAC for KC hydrolysis in human tissues, kinetic analyses were performed using HLM (Fig. 2B), HIM (Fig. 2C) and recombinant AADAC (Fig. 2D). The kinetics were all fitted to a Michaelis–Menten kinetics curve. The Km value of recombinant AADAC was similar to the Km values for HLM and HIM (Table 1), indicating that AADAC could be an enzyme responsible for KC hydrolysis.

3.2. Inhibitory profiles of KC hydrolase activity in HLM and recombinant AADAC

An inhibition study was performed to examine whether AADAC is the principal enzyme responsible for KC hydrolysis in the human liver (Fig. 3). The KC hydrolase activity in HLM was potently inhibited by 10 μ M DFP and 100 μ M vinblastine and moderately



Fig. 2. KC hydrolase activities by human tissue microsomes and recombinant esterases. (A) KC hydrolase activities in HLM, HIM, HRM, HPM and recombinant CES1, CES2, and AADAC at a substrate concentration of 10 μ M. (B–D) Kinetic analyses of KC hydrolase activities in (B) HLM, (C) HIM, and (D) recombinant AADAC. Each column and data point represent the mean ± SD of three replicates.

 Table 1

 Kinetic parameters of KC hydrolase activities for HLM, HIM and recombinant AADAC.

Enzyme	Km μM	Vmax pmol/min/mg protein	CLint μL/min/mg protein
HLM	17.5 ± 1.63	28.3 ± 0.114	1.63 ± 0.147
HIM	10.9 ± 0.747	30.7 ± 0.981	2.89 ± 0.301
Recombinant AADAC	6.93 ± 0.105	35.3 ± 0.865	5.10 ± 0.201

Data are expressed as the mean ± SD of three replicates.



Fig. 3. Effects of chemical inhibitors on KC hydrolase activity in HLM and recombinant AADAC. The substrate concentration was 10 μ M. The control activity values in HLM and recombinant AADAC were 7.61 pmol/min/mg protein and 16.7 pmol/min/mg protein, respectively. Each column represents the mean ± SD of three replicates.

inhibited by 10 μ M BNPP but was not inhibited by 100 μ M PMSF, 100 μ M CDMB, and 50 μ M telmisartan (Fig. 3). We previously reported that the inhibitory potencies of PMSF and CDMB on AADAC activity were weak, although inhibition of CES1 and CES2 activities was potent [19,21]. Vinblastine is a strong inhibitor of AADAC and CES2 and telmisartan is a specific inhibitor of CES2 [19]. In addition, KC hydrolase activity was not affected by CaCl₂, which is an activator of PON. Of particular interest was that the inhibition profile observed in HLM was quite similar to the profile for recombinant AADAC (Fig. 3). The results suggest that AADAC is primarily responsible for KC hydrolysis in the human liver.

3.3. Correlation analyses between the hydrolase activities of KC and known substrates of esterases

The KC hydrolase activities in microsomes from a panel of 24 individual human livers were measured at a 10 μ M substrate concentration. The activities ranged from 0.388 to 10.4 pmol/min/mg protein (mean ± SD: 6.29 ± 1.97 pmol/min/mg protein) with 27-fold interindividual variability. KC hydrolase activity was significantly (*P* < 0.001) correlated with the hydrolase activities of flutamide, indiplon, phenacetin, and rifampicin, which are all specific substrates of AADAC (Fig. 4A–D). No correlation was observed between the hydrolase activities of KC and fenofibrate (*P* = 0.189) and irinotecan (*P* = 0.398 (Fig. 4E and F)), which are marker substrates for human CES1 and CES2, respectively [13]. These results further suggest that AADAC is an enzyme responsible for KC hydrolysis.

3.4. N-Hydroxylation of DAK in human liver

It has been reported that, among human FMO enzymes, human FMO1 and FMO3 could catalyze *N*-hydroxylation of DAK [9]. To evaluate the *N*-hydroxylation of DAK in HLM, the conditions for LC–MS/MS analysis were first constructed using recombinant FMO3. DAK was incubated with recombinant human FMO3 and

an NADPH-GS and three peaks (7.03, 7.54, and 7.95 min) were observed by Q1-scan at m/z 50–550 (Fig. 5A). The mass spectrum of the peaks at 7.03 and 7.95 min showed ions m/z 489.2 and 531.0, respectively, which correspond to DAK and KC, respectively (data not shown). The peak of KC was detected in both the presence and absence of NADPH (Fig. 5A). In fact, we confirmed KC contaminants in the commercially available DAK (0.352%) with LC–MS/MS (data not shown). The peak at 7.54 min showed an ion m/z 505.2, and the peak was not observed in the absence of NADPH-GS (Fig. 5A). The product ion scan of m/z 505.2 revealed a fragment at 487.1 (Fig. 5B), which corresponded to a product that loses a hydroxyl group form of *N*-hydroxy DAK. Thus, the peak at 7.54 min was assumed to be *N*-hydroxy DAK.

The activity was measured using recombinant FMO enzymes (FMO1, FMO3, and FMO5) that were commercially available. FMO1 and FMO3 enzymes showed high activity (FMO1: $39.3 \pm 2.39 \text{ nmol/min/mg}$ protein; FMO3: $37.6 \pm 1.75 \text{ nmol/min/mg}$ protein at a substrate concentration of 300μ M) as previously reported [9], whereas FMO5 showed marginal activity (0.674 ± 0.0180 nmol/min/mg protein) (Fig. 5C). When HLM were incubated with DAK and an NADPH-generating system, the peak of *N*-hydroxy DAK was detected, and the activity in HLM was 0.520 ± 0.00698 nmol/min/mg protein at a substrate concentration of 300μ M (Fig. 5C). Human FMO1 is predominantly expressed in the kidneys but not in the adult liver [27,28]. Therefore, it was suggested that the hydroxylation of DAK in the human liver is primarily catalyzed by FMO3.

3.5. Kinetic analyses of DAK hydroxylase activities in HLM and recombinant FMO3

Kinetic analyses using HLM (Fig. 5D) and recombinant FMO3 (Fig. 5E) were performed at pH 7.4 and pH 8.8, which reflect the physiological and optimal conditions for FMO, respectively. The DAK *N*-hydroxylase activity in HLM and recombinant FMO3 at pH 8.8 were higher than the activity at pH 7.4 (Fig. 5D and E). The activity in HLM was fitted to a Michaelis-Menten kinetics curve, whereas the activity in recombinant FMO3 appeared to exhibit substrate inhibition kinetics. Because the activity for FMO3 was not fitted to inhibition kinetics, the apparent kinetic parameters were estimated up to 300 μ M using the Michaelis-Menten equation. The *K*m value of recombinant FMO3 was similar to the *K*m value in HLM under both pH conditions (Table 2). These results further support the idea that FMO3 could be the major enzyme responsible for *N*-hydroxylation of DAK in the human liver.

3.6. Effects of AADAC on KC-induced cytotoxicity

It has been reported that DAK shows more potent cytotoxicity than KC in rat primary hepatocytes [10]. We first evaluated the cytotoxicity of KC and DAK toward human liver-derived HepaRG cells, which express FMO3 at a similar level to human primary hepatocytes [29], with a WST-8 assay. As shown in Fig. 6A, DAK showed potent cytotoxicity in a dose-dependent manner with an EC50 value of 22.8 μ M, whereas KC showed no cytotoxicity up to 50 μ M. This result is supported by previous studies using rat primary hepatocytes [10].

We surmised that the hydrolysis of KC to DAK by AADAC would be critical for KC-induced toxicity. When we examined the expression of AADAC in HepaRG cells by evaluating flutamide hydrolase activity at 500 μ M, which is the optimal concentration to measure the specific activity of AADAC [21], the activity (41.1 ± 8.76 pmol/min/mg protein) was much lower than the activity in human primary hepatocytes (410–679 pmol/min/mg protein) [23] (Fig. 6B). To increase the AADAC expression in

T. Fukami et al. / Biochemical Pharmacology xxx (2016) xxx-xxx



Fig. 4. Correlation analyses between KC hydrolase activity and (A) flutamide (B) indiplon, (C) phenacetin, (D) rifampicin, (E) fenofibrate, and (F) irinotecan hydrolase activities in a panel of HLM samples from 24 donors. The significance of the correlation between them was investigated with the Spearman rank correlation method.

HepaRG cells, the cells were infected with AdAADAC, which resulted in 831 ± 51.1 pmol/min/mg protein of flutamide hydrolase activity. This outcome was comparable to the activity observed in human primary hepatocytes (Fig. 6B). AdGFP (MOI 2.5)-infected HepaRG cells showed 45.3 ± 6.10 pmol/min/mg protein of flutamide hydrolase activity, which indicates there were no effects of adenovirus infection on AADAC activity. The viability of HepaRG cells infected with AdAADAC or AdGFP after the treatment with various concentrations of KC for 24, 28, and 72 h was evaluated (Fig. 6C). The viability of HepaRG cells infected with AdAADAC decreased in dose- and time-dependent manners, whereas the viability of control cells was hardly affected.

The effect of the KC hydrolysis by AADAC on KC-induced cytotoxicity was also evaluated using 2 lots of human primary hepatocytes (Fig. 7). Incubation with 30 μ M KC for 48 h resulted in 54.0 ± 3.46% (lot# HC1-30) and 45.4 ± 4.57% (lot# HC2-20) of LDH leakage, but the LDH release was attenuated (25.2 ± 5.61% and 24.0 ± 3.33% of LDH release, respectively) in the presence of 10 μ M DFP, which is a potent AADAC inhibitor. We confirmed that 10 μ M DFP did not affect the LDH release (data not shown). Considering together with the results of HepaRG cells, the significance of AADAC-dependent metabolism in KC-induced cytotoxicity was clearly indicated.

4. Discussion

KC is widely used for treatment of fungal infections. Because hypoadrenalism and hepatotoxicity are adverse reactions of KC, a number of countries recommended that KC not be administered orally. Although the mechanism of hypoadrenalism is suggested to be blocked adrenal steroidogenesis through inhibition of cytochrome P450s, the mechanism of hepatotoxicity has yet to be determined. Drug-induced hepatotoxicity is often caused via the production of a reactive metabolite(s). Identifying the enzymes involved in the metabolic activation of drugs would help to understand this mechanism of drug toxicity and may help researchers develop better processes for drug development. In this study, we sought to identify the enzyme(s) responsible for the metabolism of KC in humans to obtain some insight into the KC hepatotoxicity.

First, we found that high KC hydrolase activity was detected in HLM and HIM (Fig. 2A). KC hydrolase activity was detected with recombinant AADAC but not with recombinant CES1 and CES2 (Fig. 2A). The Km values of KC hydrolysis by recombinant AADAC were similar to those in HLM and HIM (Fig. 2B–D, Table 1). These results suggest that AADAC is responsible for KC hydrolysis. The responsibility of human AADAC in KC hydrolysis in human liver was confirmed with an inhibition study (Fig. 3). We previously found that AADAC prefers compounds with quite small acyl moiety [13]. Because KC also possesses an acetyl moiety, it is conceivable that AADAC can hydrolyze KC. Other potential enzyme candidates were examined, and there were no contributions of BCHE and PON to KC hydrolysis because human plasma expressing BCHE and PON did not show any KC hydrolase activity (data not shown).

The responsibility of AADAC for KC hydrolysis in human liver was also confirmed by the fact that KC hydrolase activity in a panel of 24 individual HLM samples was significantly correlated with the hydrolase activities of flutamide, indiplon, phenacetin, and rifampicin, which are specifically hydrolyzed by human AADAC [15,21–23]. It should be noted that although flutamide is hydrolyzed by CES2 at relatively low substrate concentration, it is hydrolyzed primarily by AADAC [30] at a concentration of 500 μ M, which was used in this study. HLM samples showed a 27-fold interindividual variability in KC hydrolase activity was from a liver genotyped as the homozygote of *AADAC*^{*3} (data not shown), which produces inactive AADAC enzyme [31]. Thus, the variability in KC hydrolysis could partly be due to genetic polymorphisms of AADAC.

A previous study of recombinant enzymes reported that human FMO1 and FMO3 catalyze *N*-hydroxylation of DAK [9]. We con-

T. Fukami et al./Biochemical Pharmacology xxx (2016) xxx-xxx



Fig. 5. DAK *N*-hydroxylase activities in recombinant FMO enzymes and HLM. (A, B) Detection of *N*-hydroxy DAK using the LC–MS/MS system. (A) Recombinant FMO3 was incubated with 250 μ M DAK in the presence or absence of NADPH-CS. Reaction mixture was injected into the LC–MS/MS and monitored in Q1 scan mode (range: *m*/z 50–550). (B) Recombinant FMO3 was incubated with 250 μ M DAK and NADPH-CS. The supernatant of the reaction mixture was concentrated and injected into LC–MS/MS and monitored in product ion scan mode (upper: Q1 scan, *m*/z = 505.2, lower: Q3 scan, range: *m*/z 50–550). (C) DAK hydroxylase activities in recombinant FMO3, and FMO5, and HLM at a substrate concentration of 300 μ M. (D, E) Kinetic analyses of DAK hydroxylase activities in (D) HLM and (E) recombinant FMO3 at pH 8.8 and pH 7.4. Each column and data point represent the mean ± SD of three replicates.

Table 2

Kinetic parameters of DAK hydroxylase activities in HLM and recombinant FMO3 at pH 7.4 and pH 8.8.

Enzyme	Km μM	Vmax nmol/min/mg protein	CLint μL/min/mg protein
HLM (pH 8.8)	111 ± 2.91	0.667 ± 0.0126	6.01 ± 0.171
HLM (pH 7.4)	216 ± 11.9	0.318 ± 0.00112	1.47 ± 0.0775
Recombinant FMO3 (pH 8.8)	70.3 ± 2.36	51.6 ± 1.13	734 ± 40.0
Recombinant FMO3 (pH 7.4)	235 ± 26.6	20.6 ± 0.922	87.9 ± 7.07

Data are expressed as the mean ± SD of three replicates.

firmed these results and found that FMO5 showed marginal activity (Fig. 5C). FMO3 and FMO5 are expressed in the human liver, whereas FMO1 is expressed only in the kidneys [32]. The expression level of FMO3 in human liver seems to be 1.9–9.6 folds higher than that of FMO5 [33]. Thus, FMO3 would be responsible for *N*-hydroxylation in the human liver. The responsibility of FMO for DAK *N*-hydroxylation in the human liver was supported by the fact that the activity was higher at pH 8.8, which is an optimal pH for FMO, rather than at pH 7.4 (Fig. 5D) and the *K*m values for *N*-hydroxylation of DAK in HLM and recombinant FMO3 were

close (Table 2, Fig. 5D and E). The kinetics in HLM were fitted to a Michaelis-Menten equation, whereas the kinetics of recombinant FMO3 had a substrate inhibition pattern. Such phenomena may arise due to the differences in the membrane environment in human liver and Sf9 cells.

Finally, we sought to investigate the involvement of AADAC in KC-induced hepatocellular toxicity using HepaRG cells and human primary hepatocytes. A previous study [10] suggested that FMO3 was required to elicit the toxicity of KC. In this study, differentiated HepaRG cells (a human hepatoma-derived cell line) were chosen in addition to human primary hepatocytes, because they retain the expression of drug-metabolizing enzymes, nuclear receptors, and drug transporters [34–36]. As for FMO3, it has been reported that the level of FMO3 in HepaRG cells was close to the level in human primary hepatocytes [29]. Because the AADAC level in HepaRG cells was about one tenth of the level in primary human hepatocytes, which was assumed by flutamide hydrolase activity, HepaRG cells were infected with AdAADAC (Fig. 6B), resulting that interestingly, KC caused cytotoxicity of AdAADAC-infected HepaRG cells in a dose- and time-dependent manner (Fig. 6C). The result was consistent with the fact that DAK showed more potent cytotoxicity than KC in HepaRG cells (Fig. 6A). Furthermore, the effect of AADAC on KC-induced toxicity was evaluated using human primary hepa-

T. Fukami et al. / Biochemical Pharmacology xxx (2016) xxx-xxx



Fig. 6. Effects of KC hydrolysis by AADAC on KC-induced cytotoxicity in HepaRG cells. (A) Effects of KC and DAK on the viability of HepaRG cells. HepaRG cells were treated with 10, 25, and 50 µM of KC and DAK for 24 h. ^{••} *P* < 0.001, compared with KC treatment. (B) Flutamide hydrolase activities in HepaRG cells infected with AdGFP or AdAADAC and human cryopreserved primary hepatocytes (lots 82 and 100). Flutamide hydrolase activity was measured at a concentration of 500 µM. NT means no transfection. Data for the human primary hepatocytes were from Nakajima et al. [23]. (C) Effect of KC on viability of HepaRG cells infected with AdGFP or AdAADAC. HepaRG cells infected with 10, 25, or 50 µM of KC for 24 h, 48 h, and 72 h. Cell viability was evaluated for KC-induced cytotoxicity. *P* < 0.05, ^{••}*P* < 0.001, compared with AdGFP infected HepaRG cells. Each column and data point represents the mean ± SD of three replicates.



Fig. 7. Effects of KC hydrolysis by AADAC on KC-induced cytotoxicity in human primary hepatocytes. Human primary hepatocytes (lots HC1-30 and HC2-20) were treated with 30 μ M of KC in the presence or absence of DFP for 48 h. LDH release was evaluated for KC-induced cytotoxicity. ^{**}*P* < 0.005 and ^{***}*P* < 0.001, compared with no treatment of DFP. Each column represents the mean ± SD of three replicates.

tocytes, resulting that DFP, a potent AADAC inhibitor, attenuated the KC-induced cytotoxicity (Fig. 7). Because LD_{50} values of KC for 48-h incubation in human primary hepatocytes were reported to be 27.9–45.6 μ M [37], we chose 30 μ M of KC concentration, which was assumed to show 50% of cytotoxicity. In fact, 54.0 ± 3.46% and 45.4 ± 4.57% of LDH leakage in 2 lots of human

primarily hepatocytes for 48-h incubation were observed. Cytotoxicity of KC was obviously different between HepaRG cells and human primary hepatocytes. A recent study [37] also reported that KC did not reveal cytotoxicity in HepG2 and HepaRG cells (LD₅₀ values: >50 µM, for 48- and 72-h incubation), whereas there was cytotoxicity in cryopreserved human primary hepatocytes (LD₅₀: 27.9-45.6 µM for 48-h incubation; 1.82-18.7 µM for 72-h incubation). The difference in the sensitivity would be due to the difference in the level of AADAC. The role of AADAC on KC-induced liver injury may have to be evaluated on in vivo using experimental animals such as mouse and rat. There are species differences in substrate specificity of AADAC [16]. For example, rifamycins such as rifampicin, rifabutin, and rifapentine are hydrolyzed by human AADAC, but not by mouse and rat AADAC [16]. In addition, specific inhibitors used on in vivo study for AADAC are still unknown. Because of these backgrounds, we decided to evaluate the involvement of human AADAC in KC-induced liver injury on in vitro in this study. Our overexpression system of AADAC in HepaRG cells or inhibition of AADAC in human primary hepatocytes would be a useful tool to evaluate the significance of AADAC in cellular functions.

In conclusion, we found that human AADAC is responsible for the hydrolysis of KC and elicits hepatocellular toxicity. A large interindividual variability has been found in AADAC expression in the human liver, although the regulation mechanisms of the constitutive or inducible expression of AADAC have yet to be clarified. In addition, it is possible that exogenous and endogenous compounds might modulate AADAC activity. Therefore, some intrinsic and extrinsic factors, which could modulate AADAC expression or activity, may be one of the causes of determining the susceptibility to KC toxicity.

Conflict of interest

The authors declare no conflict of interest.

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