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Research Article

Age-Dependent Change of Hepatic Flavin Containing Monooxygenase and Aldehyde Oxidase Activities in Chickens

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Abstract

Flavin containing monooxygenases (FMO) and aldehyde oxidases (AO) are two important groups of non-cytochrome P450 enzymes in animals and humans. Age-dependent hepatic activities of those enzymes were evaluated in male Ross 708 broiler chickensat age 1, 7, 14, 28 and 56 days. FMO activity was significantly higher in chicken livers at age of day 1, declined rapidly to the lowest level measured at age of day 14 and then gradually increased. Meanwhile hepatic AO was low in chicken at age of day 1 and gradually increased. The overall AO activity in chicken liver was low compared to its hepatic FMO or CYP450 reported previously. Since FMO and AO activities are age-dependent in chicken livers, the impact of age on endogenous and exogenous metabolism by hepatic FMO and AO should be considered.

ABBREVIATIONS

AO: Aldehyde Oxidase; BCA: Bicinchoninic Acid; CYP450: Cytochrome P450; FMO: Flavin Containing Monooxygenase; IC_{50}: Half Maximal Inhibitory Concentration; LC/MS: Liquid Chromatography Mass Spectrometry; MRM: Multiple Reaction Monitoring; NADPH: Reduced β-nicotinamide Adenine Dinucleotide 2′-Phosphate; RCF: Relative Centrifuge Force; SULT: Sulfotransferase; UGT: Uridine Diphosphate Glucuronosyl Transferase

INTRODUCTION

Hepatic metabolizing enzymes play important roles in the metabolism of endogenous and exogenous molecules in animals and humans. Flavin containing monooxygenases (FMO) and aldehyde oxidases (AO) are two important groups of non-cytochrome P450 (CYP450) oxygenases and have unique characteristics in drug metabolism. Microsomal FMO catalyze NADPH-dependent oxygenation of soft nucleophilic heteroatoms, such as N or S, of compounds. FMO often produce distinct metabolites although both CYP450 and FMO can oxidize many of the same substrates. FMO are not induced and rarely inhibited. They generally convert lipophilic nucleophiles to more polar and readily excreted metabolites with reduced pharmacological activity [1]. AO are cytosolic enzymes and not NADPH dependent. AO act on a wide range of substrates with different chemical structures and functionalities, especially nitrogen-containing heterocyclic compounds such as pyridines, diazines, benzimidazole, purines and a variety of other fused heteroaromatic compounds [2,3].

Different FMO and AO isoforms have been identified among different species. FMO3 is the only FMO isoform currently identified in chicken [4]. Most attention on FMO3 in chickens is on metabolism of trimethylamine in laying hens. Trimethylamine results in fishy taint in eggs [5-7]. An understanding of the development of FMO3 in chicken livers is limited. AO1 is the only AO isoform identified and isolated from chicken [8]. Unlike humans and rodents, whose AO1 is predominantly expressed in the livers and lungs, the highest expression of AO1 in chicken is in the kidneys and heart [9]. Information regarding AO1 in chicken livers is limited.

The purpose of this investigation was to assess age-dependent developmental patterns of hepatic FMO and AO activities in chickens. FMO activity in chicken liver microsomes and AO activity in chicken liver cytosols from the livers of 5-ages of chickens, 1, 7, 14, 28 and 56 days were measured using selective substrates of FMO and AO.

MATERIALS AND METHODS

Chemicals

O^6-benzylguanine, benzydamine hydrochloride, benzydamine N-oxide hydrogen maleate, raloxifene hydrochloride, estradiol, chlorpromazine hydrochloride, clomiphene citrate, tamoxifen citrate, methimazole and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO). 8-oxo-benzylguanine was bought from Toronto Research Chemicals (Canada).

Animal work

All animal work was conducted under the oversight of the Institutional Animal Care and Use Committee (IACUC), and according to local, state, and national regulations. Ross 708 broiler raising and husbandry conditions were conducted as described previously [10]. Male chickens were randomly selected for liver collection at days 1, 7, 14, 28 and 56 post-hatch. The numbers of male chickens selected at each sampling day were 64, 32, 16, 8 and 6, respectively, considering chicken liver weights for preparation. Liver collection was performed as described previously [10].

Liver microsomes and cytosol preparation

Male chicken livers collected at days 1, 7, 14, 28 and 56 were processed for liver microsomes and cytosols. Every eight livers collected on day 1 and every two livers collected on day 7 were randomly combined for processing due to liver weights. Chicken livers collected on other days were processed individually. Three sets of liver samples collected on the aforementioned days were processed to liver powder using a SPEXSamplePrep 6870 Freezer/Mill (Metuchen, NJ) as described previously [10].

Weighed chicken liver powder was then prepared to liver microsomes and cytosols as described previously [10].

Protein content measurement

Protein content in chicken microsomes and cytosols was measured using the Thermo Scientific Pierce BCA protein assay kit (Rockford, IL) and a BMG Labtech PHERA star FS plate reader (Ortenberg, Germany) described previously [10].

FMO activity measurement

FMO activity was determined by N-oxidation of benzydamine in chicken liver microsomes. Chicken liver microsomes (0.1 mg protein/mL) were pre-incubated with NADPH (final concentration 1mM) in pH 7.4 phosphate (100 mM with10 μM $MgCl_2$) buffer in 96-well plates at 41°C for 3 min. The reaction was initiated by adding a series of concentrations of benzydamine (0, 2.5, 5, 10, 25, 50, 100 and 200 μM). Organic solvents (methanol or acetonitrile) were 0.5% in all incubations. The reactions were terminated with cold acetonitrile after 10 min. The mixtures were centrifuged at 4°C and 20,000 RCF in an Eppendorf 5417R centrifuge (Hauppauge, NY) for 20 min. The supernatants were analyzed for the formation of benzydamine N-oxide.

FMO activity deactivation and inhibition

Thermo-deactivation of FMO activity was measured by pre-incubating chicken liver microsomes (0.1 mg protein/mL) in pH 7.4 phosphate (100 mM with10 μM $MgCl_2$) without NADPH at 49°C for 5 min. The mixtures were allowed to cool down in ice. Then, NADPH was added and benzydamineN-oxidation was performed using the above procedure.

Chemical inhibition of FMO activity was only performed in the liver microsomes from the chickens of 1-day, 14-days and 56-days old groups. Chicken liver microsomes (0.1 mg protein/mL) were pre-incubated with NADPH (final concentration 1mM) and 50 μM Clomiphene or 100 μM Tamoxifen or 200 μM Methimazole in pH 7.4 phosphate (100 mM with10 μM $MgCl_2$) buffer in 96-well plates at 41°C for 3 min. Then 200 μM benzydamine was added to start reactions as described above. The inhibition was demonstrated by percent reduction of formed benzydamine N-oxide relative to the reaction without the inhibitors.

AO activity measurement

AO activity was determined by oxidation of O^6-benzylguanine in chicken liver cytosol. A series of concentrations (0, 2.5, 5, 10, 25, 50, 100 and 250 μM) of O^6-benzylguanine were pre-incubated in pH 7.4 phosphate (100 mM with10 μM $MgCl_2$) buffer at 41°C for 3 min. The metabolizing reaction was initiated by adding chicken liver cytosols (0.1 mg protein/mL). The reactions were terminated after 10 min with cold acetonitrile. The mixtures were centrifuged at 4 °C and 20,000 RCF in an Eppendorf 5417R centrifuge for 20 min. The supernatants were then analyzed for the formation of 8-oxobenzylguanine.

Aldehyde oxidase activity inhibition

Chemical inhibition of AO activity was only performed in liver cytosols from the chickens of the 56-days old group. O^6-benzylguanine (250 μM) was pre-incubated in pH 7.4 phosphate (100 mM with10 μM $MgCl_2$) buffer with 100 μM raloxifene or 200 μM menadione or 200 μM allopurinol in 96-well plates at 41°C for 3 min. The reaction was initiated by adding the chicken liver cytosols (0.1 mg protein/mL). Then the reactions were terminated as above for analysis of 8-oxobenzylguanine formation.

Liquid chromatography-mass spectrometry (LC/MS) analysis

Benzydamine N-oxide and 8-oxobenzylguanine were analyzed by an LC/MS system consisting of a Waters AcquityUPLC and an Applied Biosystem API 4000 Q trap mass spectrometer. Separation was accomplished using an AcquityUPLCBEHC18 column (1.7 μm, 2.1 x 50 mm) eluted with a 2 min gradient using acetonitrile and water with 0.1 percent formic acid as mobile phases at a flow rate of 0.4 mL/min and a column temperature of 40 °C. The MRM transitions of benzydamine N-oxide and 8-oxobenzylguanine were from m/z 326 to 265 and 258 to 91, respectively, using positive electrospray ionization mode on the mass spectrometer. The formed metabolites were quantified with calibration standards from 0 to 5000 nM.

Data processing

The apparent Michaelis-Menten kinetic parameters, V_{max} and K_m, were obtained from a nonlinear regression fitting of metabolite formation rates against incubation concentrations of benzydamine or O^6-benzylguanine using SigmaPlot v 9.0 (Systat Software, Inc., San Jose, CA). FMO and AO activities were expressed using the intrinsic clearance, the ratio of enzymatic parameters V_{max}/K_m with the unit μL/min/mg protein.

RESULTS

FMO activity was measured by N-oxidation of benzydamine in chicken liver microsomes. The oxidation was illustrated in an age-dependent manner in chicken livers (Figure 1). The hepatic activity was 71.0 ± 1.6 μL/min/mgin chickens immediately post hatch, rapidly declined to its lowest (9.8 ± 2.5 μL/min/mg) at age of 14-days, and then gradually increased to 43.4 ± 8.3 μL/min/mg at the age of 56-days.

Pre-heating for 5 min at 49 °C reduced FMO activity to 74, 51, 0, 49 and 68 percent in liver microsomes of chickens at ages of 1, 7, 14, 28 and 56-days. This thermal deactivation was more effective when FMO activity was high but limited when the activity was low (Figure 1). FMO selective inhibitors, clomiphene, tamoxifen and methimazole at their given concentrations demonstrated equivalent or higher inhibition on benzydamine N-oxidation than pre-heating in chicken liver microsomes (Figure 2), especially in the liver microsomes with the lowest FMO activity at age of 14-days. Methimazole at 200 μM showed the most effective inhibition on chicken hepatic FMO activity in all tested ages.

Hepatic AO activity in chickens was monitored using O^6-benzylguanine oxidation. Age had an impact on AO activity in chicken livers (Figure 3). The lowest hepatic activity was detected at the age immediate post hatch and then increased with age of chickens. Although hepatic AO activity was low compared to FMO in chicken livers across the ages, it was obviously detectable.

Inhibition of O^6-benzylguanine oxidation was evaluated with AO inhibitors, raloxifene and menadione, and a xanthine oxidase inhibitor allopurinol in the chicken liver cytosols of age 56-days. Raloxifene at 100μM resulted in about 40 percent inhibition of this oxidation. But menadione and allopurinol demonstrated minimal or no inhibition at all (Figure 4).

The apparent K_m values of benzydamine N-oxidation and O^6-benzylguanine oxidation in chicken livers are demonstrated in Table 1.

DISCUSSION

N-oxidation on the tertiary nitrogen of benzydamine is a distinct reaction of FMO. This metabolic reaction has been used to assess hepatic FMO activities in a variety of species, including livestock such as pig and cattle [11-16]. O^6-Benzylguanine can be metabolized to 8-oxo-benzylguanine by CYP450 and AO. This metabolism occurs in liver cytosols predominantly by AO in the absence of NADPH [17,18].

Age has significant impact on hepatic FMO activity in chickens. The high FMO activity immediately post hatch and then rapid reduction afterwards are similar to the activities of CYP450 and phase II conjugate enzymes (UGT and SULT) in chickens [10,19]. This unique feature is quite different from rodents or mammals. The early embryonic development of chicken livers may play important roles on such phenomenon [10]. After the activity reduced to the lowest, it then gradually increased with age. The husbandry conditions and diets seem no impact on this phenomenon [10]. Chicken hepatic FMO is crucial in detoxification of natural toxicants, such as pyrrolizidine alkaloids produced by a variety of plant species. FMO has more contributions than CYP450 in metabolism of such natural toxicants in chickens [20]. In laying hens, FMO has played a critical role in eliminating trimethylamine by N-oxidation to prevent fishy-eggs due to accumulation of this molecule in egg yolks [5,21,22]. Since hepatic FMO activity changes with age, the FMO dependent metabolism of endogenous and exogenous compounds should be impacted by age of chickens.

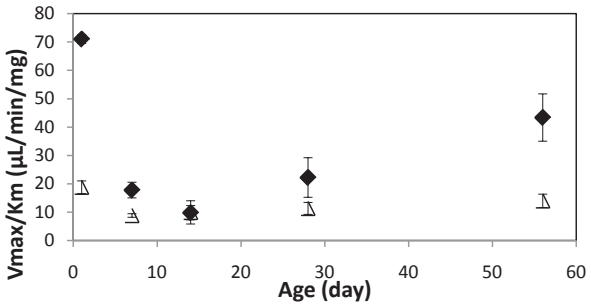

Figure 1 Impact of age on hepatic FMO activity in chickens (n=3 at each age). The filled symbols are incubated at 41 °C and empty symbols are pre-heated at 49 °C for 5 min before 41 °C incubation.

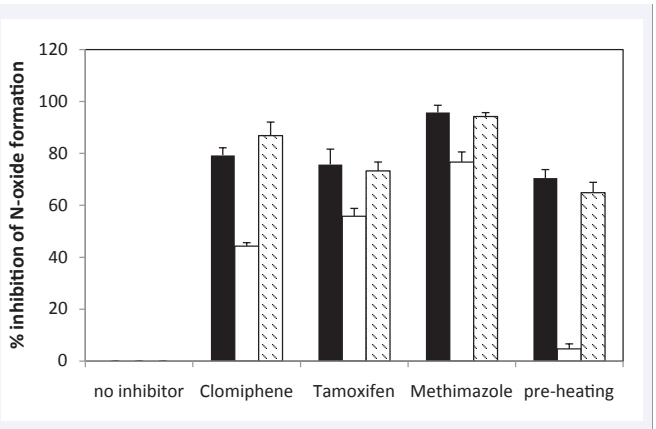

Figure 2 Chemical and thermal inhibition on benzydamine N-oxidation in the liver microsomes of chickens at ages of 1 (black-filled bars), 14 (empty bars) and 56 (gradient line-filled bars) days (n=3 at each age), inhibited with 50 μM clomiphene, or 100 μM tamoxifen, or 200 μM methimazole, or pre-heating at 49 °C for 5 min.

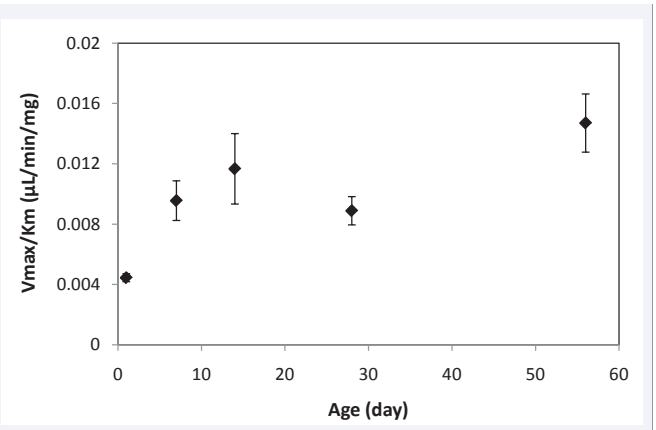

Figure 3 Impact of age on hepatic AO activity in chickens (n=3 at each age).

Inhibition of N-oxidation by thermal deactivation at absence of NADPH is a distinct procedure to distinguish CYP450 and FMO activities in liver microsomes [1]. Effectiveness of this thermal deactivation is at least partially FMO activity-dependent as demonstrated in this study. A similar phenomenon has been observed in a species comparison. Pre-heating at 45 °C for 5 min eliminates almost 100 percent of benzydamine N-oxidation

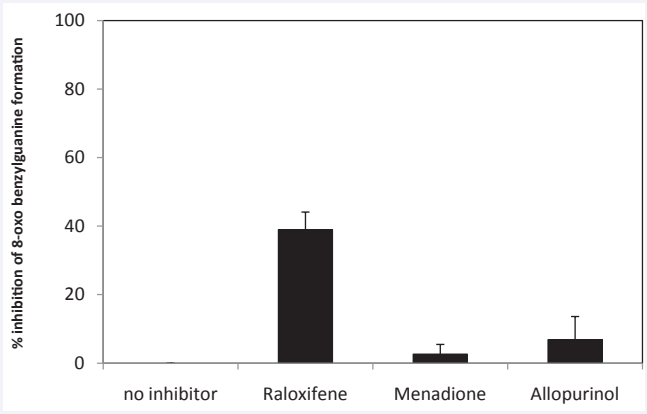

Figure 4 Chemical inhibition of O6-Benzylguanine oxidation in liver cytosol of chickens at ages of 56 days (n=3 at the age), inhibited with 100 μM raloxifene, or 200 μM menadione, or 200 μM allopurinol.

Table 1: Hepatic FMO and AO substrates, metabolites and Km values of chickens.

Enzyme	Substrate	Metabolite	Km (μM)
FMO	Benzydamine	Benzydamine N-oxide	38.2 ± 11.5
AO	O^6-benzylguanine	8-oxobenzylguanine	188 ± 64

in dog and rat liver microsomes but only 60 to 80 percent of the activity in human and monkey liver microsomes while the intrinsic clearance of benzydamine through N-oxidation is 1700, 280, 9.2 and 11 μL/min/mg in dog, rat, human and monkey liver microsomes, respectively [16]. FMO enzymes may be less thermal labile when their activity or density is low at endoplasmic reticulum of liver cells. Chicken hepatic FMO may be relatively more thermal stable since the average body temperature of chickens is 41 °C which is higher than mammals and rodents.

Chemical inhibition is more effective to chicken hepatic FMO. Methimazole, tamoxifen and clomiphene are FMO inhibitors. Methimazoleat 200 μM is more competitive than tamoxifen at 100 μM and clomiphene at 50 μM in inhibition of human FMO3[23], which is consistent in FMO inhibition in chicken liver microsomes of this study. Selectivity of different FMO inhibitors is species or FMO isoform dependent. For example, the lack of inhibition on benzydamine N-oxidation by methimazoleto 500 μM has been observed in porcine liver microsomes [14] in which FMO1 and FMO3 are both expressed [15]. In this study methimazoleat 200 μM was most effective in the inhibition of chicken hepatic FMO although the percent inhibition was somehow FMO activity-dependent.

Hepatic AO levels in chicken livers were low but detectable across ages from day 1 to 56 using O^6-benzylguanine oxidation. The lower AO activity immediately post hatch and its increase with age of chickens are different from the developmental patterns of other hepatic metabolizing enzymes in chickens [10,19]. This implies that chicken hepatic AO may only be developed near or after hatch. The low hepatic AO activity is consistent with the AO1 mRNA expression measurement in different tissues of chickens. AO1 mRNA has been detectable only in chicken kidneys and hearts but not in chicken livers using Northern blot, although the much more sensitive RT-PCR method highlights the transcript in other chicken organs, including the liver [9]. Hepatic AO activity in chickens is much lower than in rodents and most mammals [24]. However this enzyme still contributes to metabolism of endogenous molecules, such as vitamin A_1 and A_2 [25], or exogenous compounds, such as neonicotinoid insecticides [24] and coumarinrodenticides [26] in chicken livers. Chicken hepatic AO may also be involved in the metabolism of olaquindox, a antimicrobal feed additive in chicken feed [27].

Allopurinol is a standard xanthine oxidase inhibitor with reported IC_{50} values of 0.2 to 50 μM in a variety of in vitro assays [28]. Menadione is an AO inhibitor with an IC_{50} value 0.2 μM for human AO [29]. Menadione inhibits O^6-benzylguanine oxidation effectively in human liver cytosolic fractions but allopurinol does [17]. However it has been observed that menadione inhibits an AO mediated metabolism of bisdesoxyolaquindoxin rat and pig liver cytosols at 100 μM but not in chicken liver cytosol at all [27]. In this study, both menadione and allopurinol at 200 uM had limited or no inhibition on O^6-Benzylguanine oxidation. Raloxifene is a potent and highly selective uncompetitive AO inhibitor. Its Ki values are about 1 nM for several AO mediated metabolisms [29]. In human liver cytosol, raloxifene (50 μM) can inhibit AO oxidation to 100 percent [30]. In this study, O^6-Benzylguanine oxidation was only inhibited to 40 percent by raloxifene at 100 μM in chicken liver cytosols. This probably indicates the difference of selectivity of that AO inhibitors to O^6-benzylguanine oxidation in chickens and other species.

In summary, hepatic FMO and AO activities are age-dependent in chickens. High FMO activity at hatch followed by rapid reduction is similar to other metabolizing enzymes in chicken livers. The age pattern of hepatic AO activity is different from other metabolizing enzymes in chickens besides the overall low activity across ages. Age has an obvious impact on hepatic FMO and AO activities in chicken livers and the implications of those differences on the metabolism of endogenous and exogenous compounds should be further evaluated.

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