Role of Aldehyde Oxidase in the Hepatic in Vitro Metabolism of 3-Methylindole in Pigs

Gonzalo J. Diaz*,† and E. James Squires

Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The metabolism of 3-hydroxy-3-methylindolenine (HMI), a recently discovered metabolite of 3-methylindole (3MI, skatole) produced by porcine liver microsomes, was investigated in vitro using porcine liver cytosol. HMI was rapidly metabolized to a single product, 3-hydroxy-3-methyloxindole (HMOI), by porcine cytosol. By the use of the selective inhibitors menadione and quinacrine, it was shown that the enzyme responsible for the oxidation of HMI into HMOI was aldehyde oxidase (AO; aldehyde:oxygen oxidoreductase, EC 1.2.3.1). The activity of AO in the conversion of HMI to HMOI was measured in a population of pigs ($n = 30$) with a wide range of 3MI levels in back fat (0.07−0.30 mg/kg). AO activity was found to be negatively correlated ($r = -0.70$; $P < 0.001$) with the level of 3MI in fat. The results of the present study suggest that AO plays an important role in the metabolism of 3MI in the pig and that its catalytic activity is related to an adequate 3MI clearance.

Keywords: *Aldehyde oxidase; 3-methylindole; skatole; pigs; metabolism*

INTRODUCTION

Aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1; AO) is a cytosolic metalloflavoprotein enzyme with a molecular mass of ~270 kDa (Krenitsky et al., 1974). AO is a homodimer composed of two identical subunits, each subunit containing FAD, molybdenum, and iron (as Fe/S clusters) as prosthetic groups in a 1:1:4 ratio (Rajagopalan, 1980). The enzyme catalyzes a unique reaction, involving oxidative hydroxylation of the substrate, in which the oxygen atom incorporated into the product is derived from water rather than molecular oxygen (O_2); hence, it functions as a true oxidase (Rajagopalan, 1980). The physiological role of AO is still unknown (Rajagopalan, 1980), but the enzyme can oxidize a variety of nitrogen-containing heterocyclic compounds as well as aliphatic and aromatic aldehydes (Beedham, 1985). Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2; XO) is a molybdenum hydroxylase related to AO (Beedham, 1985), which exhibits some overlapping substrate specificity with AO (Krenitsky et al., 1974).

AO was first isolated from porcine liver in 1940 (Rajagopalan and Handler, 1966), and activity of AO has been detected in liver cytosol from pigs, rabbits, humans, rat, and monkey (Rajagopalan and Handler, 1966; Rodrigues, 1994). AO is susceptible to strong inhibition by a variety of compounds including amytal, antimycin A, oligomycin, hormones such as estradiol and progesterone, several quinones, and noionic detergents such as Triton X-100 (Rajagopalan and Handler, 1966). Quinacrine is a competitive inhibitor ($K_i = 1.5 \times 10^{-6}$ M) against all substrates (Rajagopalan and Handler, 1964), whereas menadione has been successfully used in inhibition studies involving oxidative metabolism catalyzed by AO (Kaye et al., 1985; Rodrigues, 1994). XO is specifically inhibited by allopurinol, a drug that is used extensively in the treatment of gout in humans (Kaye et al., 1985; Krenitsky et al., 1974).

Skatole (3-methylindole; 3MI) is a naturally occurring microbial metabolite produced from tryptophan in the gastrointestinal tract of ruminants (Yokoyama and Carlson, 1979), humans (Fordtran et al., 1964), and pigs (Jensen et al., 1995). 3MI is a well-known acute pneumotoxin for cattle, and it has important implications for pig meat production. Whole (uncastrated) male pigs are used for meat production in several countries, due to a better feed conversion, improved carcass leanness, and a better composition of fatty acids compared with castrated pigs [reviewed by Babol and Squires (1995)]. However, 5−10% of the whole male pigs carry the so-called "boar taint" (a fecal-like odor liberated when the meat is cooked), and 3MI is one of the major contributors to boar taint (Lundström and Bonneau, 1996). It is not known why only a small percentage of a given population of pigs accumulates 3MI to a level that can be detected by humans. One possible explanation for this difference could be individual differences in the metabolism of 3MI (Lundström et al., 1995). In in vitro assays, 3MI accumulation in fat has shown to be significantly correlated with levels of cytochrome P450 2E1 (Squires and Lundström, 1997) and the rate of sulfation of the 3MI metabolite 6-hydroxy-3-methylindole (Babol et al., 1998).

One of the most important metabolites of 3MI produced by liver microsomal enzymes of several species is 3-hydroxy-3-methyloxindole (HMOI) (Skiles et al., 1989; Smith et al., 1993; Baek et al., 1997). Skordos et al. (1998) indicated that the formation of HMOI occurs through the oxidation of the 3MI metabolite, 3-hydroxy-3-methylindolenine (HMI), and postulated that this reaction is catalyzed by AO. However, experiments involving HMOI formation in the presence of selective inhibitors of AO were not conducted. Using porcine liver

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 6446; fax (519) 836-9873; e-mail gdiaz@aps.uoguelph.ca].

† Permanent address: Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia, Apartado Aéreo 76948, Santafé de Bogotá, Colombia.

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microsomes, Diaz et al. (1999) found that HMI is quantitatively the most important metabolite of porcine liver microsomes because it accounts for almost 50% of all 3MI metabolites produced. The elucidation of the metabolic fate of HMI is therefore significant in explaining the overall metabolism of 3MI in the pig.

The aim of the present study was to investigate the role of porcine liver AO in the oxidation of HMI into HMOI by using selective inhibitors of the enzyme and also to measure the enzyme activity in several samples of porcine cytosol using HMI as a substrate. The possible relationship between the hepatic activity of AO and the amount of 3MI present in the fat of pigs was also investigated.

MATERIALS AND METHODS

Chemicals. Menadione, quinacrine, and allopurinol were purchased from Sigma-Aldrich Canada (Oakville, ON). Authentic HMOI was graciously provided by Dr. G. S. Yost, Department of Pharmacology and Toxicology, University of Utah. HMI was produced using porcine liver microsomes, and it was isolated and purified using preparative HPLC as described before (Diaz et al., 1999). Isolated HMI was freeze-dried and kept in a desiccator at −20 °C until used.

Preparation of Porcine Liver Cytosol. Liver samples were taken from 30 intact male pigs obtained by back-crossing F3 European Wild Pig × Swedish Yorkshire boars with Swedish Yorkshire sows (Squires and Lundström, 1997). Liver samples were frozen in liquid nitrogen and stored at −80 °C. For the preparation of the cytosolic fraction, partially thawed liver samples were finely minced and homogenized with 4 volumes of 0.05 M Tris-HCl buffer, pH 7.4 (containing 0.15 M KCl, 1 mM EDTA, and 0.25 M sucrose) using an Ultra-Turax homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 10000*g* for 20 min, and the resulting supernatant was centrifuged again at 100000*g* for 60 min using a Beckman L5-50 ultracentrifuge (Beckman) to obtain the cytosolic fraction and the microsomal pellet. Cytosolic fractions were stored at −80 °C before analysis. Protein concentrations were determined according to the method of Smith et al. (1985) using bicinchoninic acid protein assay reagents purchased from Pierce Chemical Co. (Rockford, IL) and bovine serum albumin as standard.

Enzyme Assays. To investigate the role of AO in the conversion of HMI to HMOI, incubations containing HMI, porcine liver cytosol, and different concentrations of the selected AO inhibitors menadione and quinacrine were conducted. Each incubation was run in duplicate and was performed for three randomly selected cytosol porcine samples. HMOI formation was detected and quantitated by HPLC as described under Chromatographic Analysis. AO activity was measured as the formation of HMOI per minute per milligram of cytosolic protein. Assay mixtures contained 0.05 M sodium phosphate buffer, pH 7.4, with 5 mM $MgCl_2$ and 1 mM EDTA, 1 mg of cytosolic protein, and 1 μg of HMI in a final assay volume of 250 μL. For the inhibition experiments, different final concentrations of menadione (0, 2, 5, 10, 25, 50, and 100 μM) or quinacrine (0, 0.05, 0.1, 0.25, 0.5, and 1.0 mM) were tested in the assay mixture. Menadione was dissolved in ethanol (final assay concentration of 4%, v/v), which had no effect on activity in controls without inhibitor; quinacrine was dissolved in buffer. Incubations were carried out for 10 min at 37 °C in a shaking water bath; the reaction was stopped with 250 μL of ice-cold acetonitrile. After the addition of acetonitrile, the mixture was vortexed and centrifuged at 4000*g* for 15 min. A 400 μL aliquot of the clear supernatant was diluted with 400 μL of water, and 100 μL of the mixture was analyzed immediately by high-performance liquid chromatography (HPLC). Dilution with water was necessary to avoid leading of the chromatographic peaks. HMOI production was quantitated by using an external standard. Controls included incubations using boiled cytosol and incubations carried out without the addition of cytosol. Incubations run under the same conditions as described above were conducted using 0.1, 0.5, and 1.0 mM allopurinol to investigate the role of XO on the enzymatic conversion of HMI into HMOI.

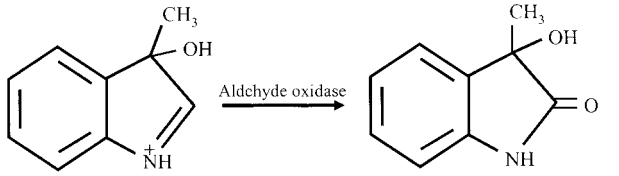

Figure 1. Oxidative conversion of HMI into HMOI catalyzed by AO.

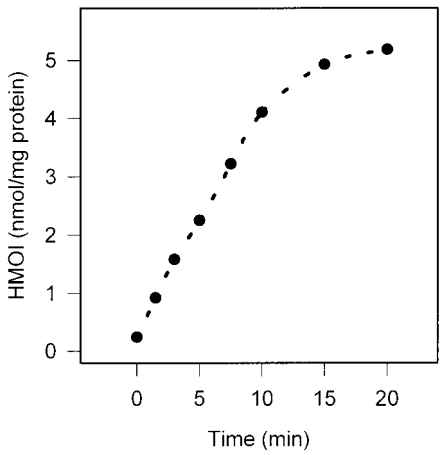

Figure 2. Formation of HMOI from HMI, catalyzed by porcine cytosol. Each data point represents the mean of duplicate assays performed for three pigs.

Chromatographic Analysis. HPLC was conducted using a Spectra-Physics system (Spectra-Physics, San Jose, CA) consisting of an SP8800 gradient pump, an SP8880 autosampler with a 100 μL injection loop, and an SP Spectra 100 UV detector. The HPLC method is a modification of a previously reported binary gradient system method (Baek et al., 1997). HMOI and HMI were separated using a reverse-phase Prodigy ODS, 5 μm, 250 × 4.6 mm column (Phenomenex, Torrance, CA). The mobile phase consisted of two solvents, A (0.01 M potassium dihydrogen phosphate buffer, pH 3.9) and B (acetonitrile), with the following gradients: 0 min, 90% A; 6 min, 80% A; 12 min, 70% A; 18 min, 30% A; 25 min, 10% A; 26 min, 90% A; 35 min, 90% A. All gradients were linear, and the flow rate was set at 1.2 mL/min. Absorbance was monitored at 250 nm. HPLC analysis was conducted immediately after the addition of acetonitrile, centrifugation of the incubation mixture, and dilution of the supernatant with water.

Measurement of 3MI Fat Content. For the quantitation of the 3MI fat content, a sample of subcutaneous backfat was taken from each of the pigs from which the liver samples were taken (n = 30), and each sample was analyzed using a colorimetric assay (Mortensen and Sørensen, 1984). Samples were taken after slaughter at ∼108 kg live weight and were kept at −20 °C until they were analyzed. All analyses were done in duplicate.

Statistical Analysis. Pearson correlation coefficients, linear regression analysis, and one-way ANOVA were computed using the Statistical Analysis System (SAS, 1995).

RESULTS

Porcine cytosol catalyzed the conversion of HMI to HMOI (Figure 1) in a time-dependent manner (Figure 2). Under these assay conditions, the formation of HMOI was found to be linear (r^2 = 0.995) for up to 10 min

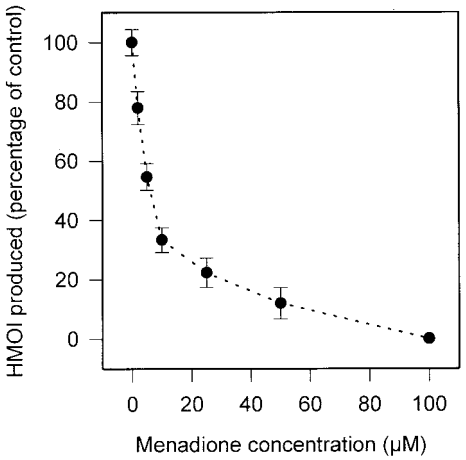

Figure 3. Inhibition of the formation of HMOI from HMI by menadione. Each data point represents the mean of duplicate assays performed for three pigs.

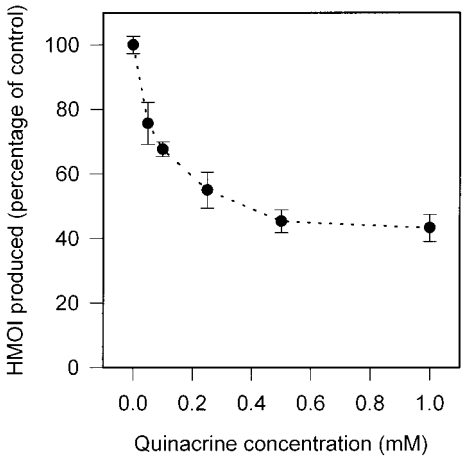

Figure 4. Inhibition of the formation of HMOI from HMI by quinacrine. Each data point represents the mean of duplicate assays performed for three pigs.

(Figure 2). No HMOI was formed when cytosol was boiled before the incubation or when no cytosol was added to the assay mixture. The total number of nanomoles found at the end of the incubations was equivalent to the number of HMI nanomoles added to the incubation. The addition of the AO inhibitors menadione or quinacrine to the incubation mixtures containing HMI and cytosolic protein decreased the formation of HMOI in a dose-dependent manner. When no inhibitor was added, the total amount of HMOI produced was considered to be 100%. When a concentration of 10 μM menadione was added to the incubations, only 33.3% of the HMOI formed in the absence of menadione was found; when the menadione concentration was increased to 100 μM, no HMOI was produced (Figure 3). At a concentration of 50 μM quinacrine, 75.5% of the control HMOI production was observed, and at 1 mM 43.4% of the control HMOI was found (Figure 4). Menadione was a more potent inhibitor of the reaction because even a concentration of quinacrine 10 times higher than that of menadione (1 mM versus 100 μM) was not enough to completely inhibit the conversion of HMI to HMOI. The addition of up to 1.0 mM allopurinol to the assay mixture did not affect the conversion of HMI to HMOI (data not shown).

The AO activity, estimated as nanomoles of HMOI produced per minute per milligram of cytosolic protein,

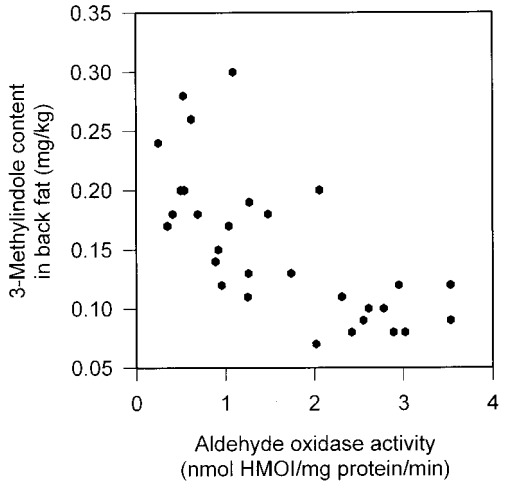

Figure 5. Plot of back fat 3MI content versus hepatic AO activity in pigs (n = 30). AO activity was measured as nanomoles of HMOI formed per milligram of cytosolic protein per minute.

Table 1. Hepatic Aldehyde Oxidase Activity in Pigs with Different 3-Methylindole Fat Contents[a]

degree of accumulation	3-MI fat content (mg/kg)	n	mean (± SD) 3-MI content (mg/kg)	mean (± SD) AO activity (nmol of HMOI/mg of protein/min)
high	≥0.2	7	0.24 ± 0.04[a]	0.80 ± 0.61[b]
moderate	0.11−0.19	15	0.15 ± 0.03[b]	1.40 ± 0.90[b]
low	≤0.1	8	0.09 ± 0.01[c]	2.73 ± 0.45[a]

[a] Within a column, means lacking a common superscript differ ($P < 0.05$).

versus the 3MI fat content of the 30 pigs used in this study is shown in Figure 5. The Pearson correlation coefficient between these two variables was found to be −0.70 ($P < 0.001$), whereas the determination coefficient was $r^2 = 0.49$.

The 3MI fat content in all samples ranged from 0.07 to 0.3 mg/kg and had a mean value of 0.15 mg/kg, whereas the AO activity ranged from 0.25 to 3.53 nmol of HMOI/mg of protein/min and had a mean value of 1.27 nmol of HMOI/mg of protein/min. The results were grouped in three categories according to the 3MI fat content of each pig as follows: high 3MI accumulators (0.2 mg/kg 3MI or more), moderate 3MI accumulators (0.11−0.19 mg/kg 3MI), and low accumulators (0.1 mg/kg 3MI or less). Lundström and Bonneau (1996) have suggested that levels of 3MI of ≥0.2−0.25 mg/kg cause unacceptable taint by sensory analysis. The mean values for 3MI fat content and AO activity for these three categories of pigs are shown in Table 1.

DISCUSSION

Menadione is a well-documented inhibitor of AO (Johns, 1967; Krenitzky et al., 1974; Rodrigues, 1994), and biochemical reactions sensitive to inhibition by menadione are attributed to AO (Beedham et al., 1995; Rashidi et al., 1997). Rodrigues (1994) found that at a concentration of 10 μM, menadione completely inhibited the oxidation of N^1-methylnicotinamide, the model substrate for AO. In the present experiment, a concentration of 10 μM menadione decreased the formation of HMOI by 56.7%, and at 100 μM menadione, no HMOI was formed, indicating a complete inhibition of the enzymatic activity. The inverse dose−response relation-

ship observed between HMOI production and menadione concentration strongly suggests that AO is the enzyme responsible for the biotransformation of HMI into HMOI in porcine cytosol. Quinacrine has been reported as being a competitive inhibitor ($K_i = 1.5 \times 10^{-6}$ M) of AO against all substrates (Rajagopalan and Handler, 1964). In the present trial, quinacrine was less potent than menadione in inhibiting the conversion of HMI into HMOI, but it also inhibited the reaction to a large extent. The inhibition of HMOI formation caused by quinacrine also suggests that the production of HMOI from HMI is catalyzed by AO. On the other hand, the lack of inhibition observed when allopurinol was added to the reaction mixture indicates that XO is not involved in the oxidative metabolism of HMI into HMOI.

N-heterocyclic cations constitute a major group of substrates for AO (Beedham, 1985). Quaternization of a ring nitrogen atom activates the heterocycle to nucleophilic substitution and enhances the reactivity of the compound toward enzyme-catalyzed attack (Beedham, 1985). HMI is a recently identified N-heterocyclic quaternized metabolite produced by porcine microsomal enzymes (Diaz et al., 1999), and therefore it constitutes a suitable substrate for AO-catalyzed oxidation. The results of the present study strongly suggest that AO activity present in the cytosol of pigs is responsible for the oxidation of HMI to form a more polar and stable metabolite, HMOI. HMOI can also be produced by microsomal enzymes (Diaz et al., 1999); however, in experiments conducted with mouse liver microsomes and cytosol, Skordos et al. (1998) found that only ~4.4% of the HMOI formed contained two atoms of molecular oxygen. This figure indicates that only ~4.4% of the HMOI produced in this system is derived from the 3MI-catalyzed oxidation mediated by microsomal enzymes (the oxygen atom introduced by AO does not come from molecular oxygen but from water). Therefore, even though HMOI can be formed by microsomal enzymes, it appears that almost all HMOI formed in a combined "microsomal−cytosolic" system is produced by the oxidation of HMI, catalyzed by AO.

When hepatic AO activity (measured as the formation of HMOI) was plotted against the 3MI fat content, a clear inverse relationship was observed (Figure 4). This finding suggests that hepatic AO activity is related to 3MI clearance. The relatively high determination coefficient ($r^2 = 0.49$) indicates that almost 50% of the variation in 3MI fat content is explained by the hepatic enzymatic activity of AO. The results shown in Table 1 also indicate that AO activity may be very significant in the adequate clearance of 3MI in the pig. High 3MI fat levels were associated with low enzymatic activity (mean values of 0.24 mg/kg 3MI and 0.80 nmol of HMOI/mg of protein/min, respectively), whereas low 3MI levels were associated with high enzymatic activity (mean values of 0.09 mg/kg 3MI and 2.73 nmol of HMOI/mg of protein/min, respectively). Pigs classified as high 3MI accumulators had a hepatic mean AO activity 3.4 times lower than those pigs classified as low accumulators ($P < 0.05$).

The results of the present study suggest that AO plays an important role in the metabolism of 3MI in the pig and that its catalytic activity is related to an adequate 3MI clearance. The enzymatic activity of AO in the pig might be used as a potential marker to identify pigs containing low levels of 3MI in the fat, which will eventually help to control "boar taint".

Menadione is customarily used as a source of vitamin K in swine diets (National Research Council, 1987). Recommended levels of inclusion are 2.5 mg/kg for grower diets and 2.0 mg/kg for finisher diets (Patience et al., 1995). Because menadione is a potent inhibitor of AO and the enzyme appears to be important in the metabolism of 3MI, care should be exercised so that excessive levels of menadione are not present in swine diets. It is possible that some of the sporadic episodes of "boar taint" could have been caused by high levels of menadione in the diet, resulting in high levels of 3MI in the fat of pigs. Studies are needed to determine whether the levels of menadione commonly used in practical pig diets are capable of inhibiting AO activity. Additionally, it has been observed that high levels of dietary copper lead to molybdenum deficiency and thus to low AO activity because molybdenum is a cofactor for this enzyme (Beedham, 1985). It is important to avoid excess copper levels in pig diets to avoid a decrease in the activity of AO and the potential occurrence of "boar taint" episodes.

ABBREVIATIONS USED

3MI, 3-methylindole; AO, aldehyde oxidase; XO, xanthine oxidase; HMI, 3-hydroxy-3-methylindolenine; HMOI, 3-hydroxy-3-methyloxindole.

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