Aldehyde Oxidase Importance In Vivo in Xenobiotic Metabolism: Imidacloprid Nitroreduction in Mice

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Aldehyde oxidase (AOX) metabolizes many xenobiotics in vitro, but its importance in vivo is usually unknown relative to cytochrome P450s (CYPs) and other detoxification systems. Currently, the most important insecticides are neonicotinoids, which are metabolized in vitro by AOX on reduction of the nitro-imino group and by CYPs via oxidation reactions. The goal of this study was to establish the relative importance of AOX and CYPs in vivo using the mouse model. The procedure was to reduce liver AOX activity by providing tungsten or hydralazine in the drinking water or to use the AOX-deficient DBA/2 mouse strain. None of these approaches reduced CYP activity measured in vitro with an isozyme nonspecific substrate. Liver AOX activity was reduced by 45% with tungsten and 61% with hydralazine and 81% in AOX-deficient mice relative to controls. When mice were treated ip with the major neonicotinoid imidacloprid (IMI), metabolism by CYP oxidation reactions was not appreciably affected, whereas the AOX-generated nitrosoguanidine metabolite was decreased by 30% with tungsten and 56% with hydralazine and 86% in the AOX-deficient mice. The other IMI nitroreduction metabolite, desnitro-IMI, was decreased by 55%, 65%, and 81% with tungsten, hydralazine, and in the AOX-deficient mice, respectively. Thus, decreasing liver AOX activity by three quite different procedures gave a corresponding decrease for in vivo reductive metabolites in the liver of IMI-treated mice. Possible AOX involvement in IMI nitroreduction or sensitivity between the two strains. This is the first study to establish the in vivo relevance of AOX in neonicotinoid metabolism in mammals and one of the first for xenobiotics in general.

Key Words: neonicotinoid; imidacloprid; aldehyde oxidase; nitroreduction.

Neonicotinoids account for about 25% of the total worldwide insecticide market. Led by imidacloprid (IMI) and thiamethoxam, they are the most important class of insecticides in protecting crops from pests (Jeschke et al., 2011). The nitro substituent on neonicotinoids is important relative to their potency and selectivity for the insect nicotinic acetylcholine receptor (nAChR). From the seven commercial neonicotinoids, approximately 100 metabolites have been identified in plants and mammals, some of which are bioactivated and can interact with the mammalian nAChR (e.g., desnitro-IMI [IMI-NH]) (Ford and Casida, 2006a, b, 2008).

In vitro studies have indicated the importance of cytochrome P450s (CYPs) in IMI oxidation and aldehyde oxidase (AOX) in IMI reduction. IMI is oxidized to the 5-hydroxy (IMI-5-OH) and olefin (IMI-ole) metabolites and reduced to the nitrosoguanidine (IMI-NNO), aminoguanidine (IMI-NNH₂), IMI-NH, and IMI-urea metabolites by a variety of human CYP isozymes (Fig. 1) (Schulz-Jander and Casida, 2002; Schulz-Jander et al., 2002). However, the nitroreduction reaction can also occur with rabbit liver cytosol independent of NADPH (Dick et al., 2005; Schulz-Jander and Casida, 2002). The nitroguanidine IMI and other nitroguanidine neonicotinoids (clothianidin and dinofeturan, Supplementary fig. 1) are reduced by the cytosolic molybdo-flavoenzyme AOX to the nitrosoguanidine and aminoguanidine metabolites (e.g., IMI-NNO and IMI-NNH₂) in the presence of electron donor substrates such as N-methylnicotinamide (NMN) (Dick et al., 2005). Although thiamethoxam is a nitroguanidine neonicotinoid, it is a poor substrate for AOX (Dick et al., 2005). In vivo, selective inhibitors established that neonicotinoids are metabolized by CYPs to their hydroxylated metabolites including IMI-5-OH (Shi et al., 2009), but AOX involvement was not determined. The role of AOX in neonicotinoid metabolism has yet to be established in vivo, especially in the oxidative- and CYP-rich environment of the liver.

AOX is important in xenobiotic metabolism. Recent focus has been mainly on in vitro metabolism of pharmaceuticals containing aldehyde, nitro, or N-heterocyclic moieties (Kitamura et al., 2006; Pryde et al., 2010). This enzyme is...
expressed mainly in liver but is also present in many other tissues with variations in activity depending on species, gender, age, drug usage, and disease states (Al-Salmy, 2002; Beedham, 1987; Garattini et al., 2008; Pryde et al., 2010). Tungsten (Rivera et al., 2005) or hydralazine (Crichtley et al., 1994; Johnson et al., 1985) in the diet or drinking water results in reduced AOX activity in guinea pigs, rabbits, and mice. There are even notable differences in AOX activity between strains of mice (Al-Salmy, 2002), e.g., compared with CD-1 mice, the DBA/2 strain is deficient in the expression of AOX homologue 1 (AOH1) and homologue 2 and has reduced expression of AOX1 (Vila et al., 2004). Because AOH1 and AOX1 are the primary AOX genes expressed in mouse liver (Garattini et al., 2008), DBA/2 mice are an appropriate AOX-deficient model for studies on in vivo mammalian xenobiotic metabolism. The wide range of inter- and intraspecies AOX activities may result in different rates of neonicotinoid metabolism and detoxification in mammals and insects. Despite the increasing significance of AOX, there have been very few studies examining the in vivo contribution of this enzyme to xenobiotic metabolism. Mice can serve as a surrogate for humans because AOX activity in IMI nitroreduction in vitro is comparable between these two species (Dick et al., 2005). This study uses chemical inhibitors and genetic deficiency for mice and Drosophila melanogaster to evaluate the relevance of AOX in neonicotinoid metabolism in vivo.

MATERIALS AND METHODS

Chemicals

IMI, thiacloprid (THI), NMN, sodium tungstate dihydrate, hydralazine hydrochloride, p-dimethylaminomchloraldehyde (DMAC), and 7-ethoxycoumarin were from Sigma-Aldrich (St Louis, MO); PBS, pH 7.4, was from Invitrogen (Grand Island, NY).

Organisms

AOX-expressing systems were compared with AOX-inhibited or -deficient systems (mice and Drosophila) in IMI metabolism. Male Swiss Webster (25–35 g), DBA/2 (20–21 g), and CD-1 (30–38 g) mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed and maintained according to the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and procedures were performed under an Institutional Animal Care and Use Committee-approved protocol. Animals were housed in a temperature-controlled room (18°C–26°C) under a 12-h light-dark cycle. Food and water were provided ad libitum. Experiments involved three mouse treatment sets: (1) Swiss Webster (control) versus tungsten-treated Swiss Webster, (2) Swiss Webster (control) versus hydralazine-treated Swiss Webster, and (3) CD-1 (control) versus DBA/2 (AOX-deficient). All mice within each treatment set were of same age, and the treatment set involved the same number of mice per treatment (n = 5–13). Swiss Webster mice were used in sets 1 and 2 because this strain was used in earlier in vivo IMI metabolism studies (Ford and Casida, 2006a). CD-1 mice were used as controls for comparison with DBA/2 mice in set 3 because these two strains were used in earlier studies in comparing AOX expression (Vila et al., 2004). AOX-expressing (AOX+ /+) (wild-type Oregon R strain) and AOX-deficient (AOX− /−) (ty2 Pol+ Aldox-1st Sb− /−) Drosophila were obtained from Carolina Biological Supply Company (Burlington, NC).

Mouse Studies

Treatments. Swiss Webster mice were used for studies involving tungsten or hydralazine treatment compared with controls. For the tungsten study, control mice were given regular drinking water, and the treatment group was given drinking water supplemented with tungsten (0.7 mg/ml) for 14 days. For the hydralazine study, control mice were given drinking water containing 5 mM potassium phosphate, pH 6. and the treatment group was given a solution of hydralazine hydrochloride (0.1 mg/ml) in 5 mM potassium phosphate, pH 6, for 7 days. DBA/2 and CD-1 mice received regular drinking water. Following these treatment schedules, the mice were administered either IMI (ip, 10 mg/kg) in dimethylsulfoxide (1 μl/g body weight) or carrier solvent alone. Livers were removed 1 h after IMI or dimethylsulfoxide treatments and analyzed for AOX activity, CYP activity, and AOX metabolite levels as described below.

Liver enzyme assays. Mouse liver cytosol and microsomes were prepared by homogenizing liver (250 mg) in ice-cold PBS (17.7 ml) using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) followed by centrifugation of the homogenate at 1000 × g for 10 min and then the supernatant at 10,000 × g for 30 min. An aliquot of the 10,000 × g supernatant was recovered for AOX activity analysis, and the remainder was centrifuged at 100,000 × g for 1 h to collect the CYP-containing microsomal pellet fraction, which was resuspended in PBS for protein measurement (Bradford, 1976) and the CYP activity assay. The oxidative activity of AOX was assayed spectrophotometrically using DMAC as a substrate (Maia and Mira, 2002). Mouse liver cytosol (15 μl, 14–20 mg/ml protein) was added to 50 μl DMAC solution (200 μl in PBS) and the reaction monitored by an absorbance decrease using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) at 398 nm for 5 min with an average control value of −18.4 μM/OD/min.

7-Ethoxycoumarin is a broad-spectrum substrate used to measure the activity of many CYP enzymes by monitoring the oxidation to 7-hydroxycoumarin (Waxman and Chang, 2007). Microsomes (20 μl, 8 mg/ml protein in PBS) were mixed with 50 mM 7-ethoxycoumarin (4 μl in methanol) in assay buffer (156 μl, 100 mM potassium phosphate, pH 7.4, containing 20% [vol/vol] glycerol and 0.1 mM EDTA) and prewarmed at 37°C for 5 min. After addition of 10 μM NADPH (20 μl in assay buffer), reactions were incubated at 37°C for 30 min in a shaking water bath. Ice-cold 2M HCl (25 μl) was added to stop reactions, and the mixture was vortexed and placed on ice. Samples were extracted with chloroform (450 μl), briefly vortexed, and then centrifuged at 3000 × g for 5 min. The organic phase (bottom layer) was removed (300 μl), added to 30 mM sodium borate (1 mM, pH 9.2), and vortexed. Following centrifugation at 3000 × g for 5 min, the upper layer was recovered and plated (200 μl) on a Costar 96-well black plate and fluorescence read at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a SpectraMax M2 Microplate Reader (Molecular Devices) with an average control value of 11.2 nmol 7-hydroxycoumarin/mg protein.

Liver IMI metabolite analysis. Metabolites were recovered for analysis by homogenizing liver (500 mg) in ice-cold acetonitrile (ACN) (2 ml containing 10 nmol THI as an internal standard) and centrifuging at 900 × g for 15 min. The supernatant was evaporated to dryness under nitrogen at 25°C, reconstituted in 10:90:0.1 ACN/water/formic acid (HCO2H) (300 μl), and filtered through 0.2 μm nylon for liquid chromatography–mass spectrometry (LC/MS) analysis. For metabolite analyses in all experimental groups (except tungsten and its control set), an Agilent 1100 series LC was used with a Waters LCT Premier XE mass spectrometer as described previously (Swenson and Casida, 2013). For the tungsten set, metabolites were quantified by selected reaction monitoring using an Agilent G6410B QQQ instrument with a Gemini reverse-phase C-18 column (50×4.6 mm, 5 μm). For LC separation, mobile phase A consisted of 95:5 water/methanol and mobile phase B consisted of 60:35:5 isopropanol/methanol/water, both containing 0.1% HCOH. Samples (10 μl) were injected into the LC starting with a flow rate of 0.1 ml/min at 0% B for 5 min and increasing to 100% B (at 0.4 ml/min) by 20 min and held for 5 min (at 0.5 ml/min), followed by 0% B from 28 to 35 min. tR values for analytes were verified with analytical standards. Nitroreduction and oxidation (Fig. 1) metabolite levels were quantified by comparing peak areas with the THI internal standard.
**Drosophila Studies**

**In vitro metabolism and analysis.** *Drosophila* were frozen at −80°C and homogenized using a mortar and pestle (120 mg/1 ml ice-cold PBS). The AOX-containing supernatant was collected after centrifugation at 16,000 × g for 30 s. To verify AOX−/− *Drosophila* had negligible AOX activity, cytosol was assayed with DMAC. In order to obtain detectable levels of IMI reductive metabolites, saturating conditions of IMI and the cofactor, NMN, were used (Dick et al., 2006). An aliquot of the cytosolic supernatant (1 mg protein) was incubated with IMI (1 mM) and N-methylnicotinamide (10 mM) in PBS (200 μl total volume) for 20 min at 37°C in a shaking water bath. Ice-cold ACN (400 μl containing 10 nmol THI as an internal standard) was added to terminate reactions and incubated on ice for 10 min. Following centrifugation at 16,000 × g for 5 min, the supernatant was evaporated to dryness under nitrogen and analyzed by LC/MS.

**Sensitivity.** *Drosophila* adults (15–20) were placed in glass test tubes (16 × 100 mm) containing filter paper strips (10 × 80 mm) and covered with parafilm. Solutions of IMI (5 μg in 50 μl water) were injected through the parafilm onto the filter paper of each vial, and *Drosophila* were monitored for adverse effects (twitching, immobilization, or death) at 15 and 165 min. The percentage of adversely affected *Drosophila* was used to determine sensitivity to IMI.

**Statistical Analysis**

Within each mouse treatment set, experiments were performed in at least triplicate (including controls) and reported as percent of control (mean) ± SE. Significant differences between AOX-expressing and AOX-inhibited or -deficient groups were analyzed by Student’s *t*-test using Microsoft Excel. A *p* value < 0.05 was considered statistically significant. For the correlation analyses, the Pearson correlation coefficient (*r*) and *r*² were calculated using R software (version 2.15.2).

**RESULTS**

**AOX Activity Was Reduced in Tungsten- and Hydralazine-Treated Mice and the DBA/2 Strain**

The first goal was to generate or obtain mice with reduced cytosolic AOX activity but normal microsomal CYP activity. Two diagnostic inhibitors, tungsten and hydralazine, were evaluated in 7- or 14-day treatments for *in vivo* AOX inhibition by measuring the oxidation of DMAC by liver cytosol. Tungsten or hydralazine treatment did not result in any signs of apparent toxicity or changes in body weight or water consumption. Cytosolic AOX activity in tungsten (14-day drinking water) and hydralazine (7-day drinking water) treated mice was significantly reduced: 45 ± 4% and 61 ± 3% less than control mice, respectively (Fig. 2A). DBA/2 mice, a strain known to be deficient in liver AOX activity, had significantly lower (81 ± 2% less) liver cytosolic AOX activity compared with CD-1 mice (Fig. 2A). In further studies not detailed here, two specific and potent *in vitro* AOX inhibitors, raloxifene and menadione (Obach et al., 2004), did not reduce AOX activity in Swiss Webster mice as analyzed 15–90 min after ip treatment (25–40 mg/kg raloxifene; 25 mg/kg menadione). Additionally, IMI treatment (ip, 10 mg/kg for 1 h) of Swiss Webster, CD-1, or DBA/2 mice did not affect AOX activity compared with treatment of mice with carrier solvent alone.

![FIG. 1. Partial metabolic pathways for IMI to its reduction and oxidation metabolites. Structures of other nitroguanidine neonicotinoids, clothianidin, dinotefuran, and thiamethoxam, are shown in Supplementary figure 1. AOX produces IMI-NNO and IMI-NNH₃ in vitro. IMI-ole is shown as an oxidative product, because it is proposed to be formed from IMI-5-OH or IMI-4-OH (Roberts and Hutson, 1999).](image1)

![FIG. 2. Effect of tungsten or hydralazine treatment or AOX deficiency (DBA/2 mice) compared with control mice (Swiss Webster given regular drinking water or CD-1 mice) on (A) liver cytosolic AOX activity using DMAC as the substrate and (B) liver microsomal CYP activity using 7-ethoxycoumarin as the substrate. Values represent the mean ± SE as percent of the control. n = 5 (tungsten or AOX deficient), n = 13 (hydralazine). **p < 0.01 or ***p < 0.001 compared with control.](image2)
CYP Activity Was Not Altered in Tungsten- and Hydralazine-Treated Mice and the DBA/2 Strain

To evaluate whether the candidate AOX inhibitor treatments or mouse strain differences affected CYP activity, the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin was monitored in liver microsomal fractions. Tungsten or hydralazine treatment had no significant effect on liver microsomal CYP activity compared with control mice (Fig. 2B). CYP activity was also not significantly different between DBA/2 and CD-1 mice (Fig. 2B) or from IMI treatment as above.

IMI-NNO and IMI-NH as Nitroreduction Metabolites

To test whether the mice with reduced AOX activity also had decreased metabolism of the IMI nitroguanidine substituent, both reduction and oxidation metabolites in liver were analyzed by LC/MS (Figs. 1 and 3). For this study, IMI nitroreduction metabolites included IMI-NNO and IMI-NH. Although studies by Dick et al. (2005) reported in vitro AOX-catalyzed IMI nitroreduction to IMI-NNO and IMI-NNH₂, the latter metabolite was not detected here by LC/MS analysis likely due to its high reactivity with aldehyde-containing solvent impurities or liver components (Dick et al., 2005, 2006). Oxidation metabolites of IMI included IMI-5-OH and IMI-ole (Fig. 1). A further nitroreduction metabolite IMI-urea was consistently detected in Drosophila in vitro reactions.

Reduced AOX Activity Resulted in Decreased IMI Nitroreduction But Not Oxidation in IMI-Treated Mice

Tungsten and hydralazine treatments not only resulted in significantly less AOX activity (Fig. 2A) but also decreased IMI nitroreduction. Tungsten treatment resulted in 30 ± 15% less IMI-NNO and 55 ± 6% less IMI-NH production, and hydralazine treatment resulted in 56 ± 5% less IMI-NNO and 65 ± 5% less IMI-NH relative to controls (Fig. 4A). Compared with CD-1 mice, DBA/2 mice formed 86 ± 1% less IMI-NNO and 81 ± 3% less IMI-NH (Fig. 4A). All differences were significant relative to controls except the IMI-NNO levels after tungsten treatment. Levels of IMI and IMI oxidation metabolites, IMI-5-OH and IMI-ole, were not significantly affected by either hydralazine or tungsten treatment or in DBA/2 mice (Fig. 4B). This strain difference in reduction versus oxidation is readily apparent on comparing the LC/MS chromatograms in Figure 3.

IMI Metabolism and Sensitivity Were Not Affected in AOX−/− Drosophila

The final studies considered whether insect AOX is important in IMI nitroreduction and detoxification. Drosophila were used as a model organism to define IMI metabolism and sensitivity. When tested for AOX activity using the DMAC assay, AOX−/− Drosophila had less than 1% of the activity of AOX+/+ insects. Incubations of IMI and NMN with homogenate cytosol from AOX++ or AOX−/− Drosophila produced comparable

![Figure 3](image_url)

**FIG. 3.** Representative LC/MS chromatograms of liver metabolites from AOX (CD-1) mice versus AOX-deficient (DBA/2) mice showing (A and B) differences in AOX-dependent reductive metabolites (IMI-NH m/z 211, tᵱ 8.0 min; IMI-NNO m/z 240, tᵱ 20.9 min) and (C and D) similarities in AOX-independent oxidative metabolites (IMI-ole m/z 254, tᵱ 21.3 min; IMI-5-OH m/z 272, tᵱ 21.9 min).
levels of IMI nitroreduction metabolites (IMI-NNO, IMI-NH, and IMI-urea) (Supplementary fig. 2A). IMI metabolite levels were independent of NMN, further verifying that their formation was not via AOX. For sensitivity assays, 5 μg IMI was chosen as a discriminating dose, resulting in intermediate toxicity (symptoms) that could be easily monitored over time. Although there was considerable variability in response, there was no significant difference between AOX+/- and AOX−/− Drosophila in the sensitivity to IMI (Supplementary fig. 2B).

**DISCUSSION**

AOX is a potentially important factor in drug metabolism, with many studies examining its in vitro inhibition and the proposed effects on xenobiotic action (Garattini et al., 2008; Obach, 2004; Pryde et al., 2010). There is a wide range of AOX activity between species with rabbits, monkeys, and humans the highest, mice intermediate, and rats and dogs having the lowest activity (Pryde et al., 2010). This same species-dependent relationship is also observed for in vitro IMI nitroreduction by liver cytosol (Dick et al., 2005).

Tungsten and hydralazine treatments provide a way to reduce AOX activity in vivo in mammals to evaluate its relevance in xenobiotic metabolism (Critchley et al., 2005). Tungsten replaces molybdenum at the active center of AOX, rendering it inactive (Rivera et al., 2005), but the mechanism of AOX inactivation by hydralazine is unknown (Johnson et al., 1985). The goal of this study was to reduce AOX activity without affecting CYP activity in vivo. The level of AOX inhibition by tungsten treatment in mice (45%) was less than that by hydralazine (61%), a difference reflected in their effect on IMI metabolism. Hydralazine treatment resulted in significantly reduced IMI metabolism to IMI-NNO and IMI-NH, but tungsten treatment only significantly reduced IMI metabolism to IMI-NH.

There are four AOX genes in mice with two of the variants being expressed in the liver, AOH1 and AOX1. DBA/2 mice are completely deficient in the expression of AOH1 and have low expression of AOX1 compared with CD-1 mice (Vila et al., 2004). Our data also establish that DBA/2 mice have significantly lower AOX activity in the liver and further show that the reduced AOX activity decreased IMI metabolism to IMI-NNO and IMI-NH but not to IMI-5-OH or IMI-ole.

The AOX-generated IMI metabolites are not all detoxification products. IMI-NH is a likely contributor to the nicotinic effects of IMI. It is over 300 times more potent than IMI at the mammalian nAChR (vertebrate α4β2 IC₅₀ = 8.2nM for IMI-NH; 2600nM for IMI), and the mouse ip toxicity is also increased several fold (Chao and Casida, 1997; Tomizawa and Casida, 2003, 2005). IMI-NNO retains insecticidal activity (Nauen et al., 1998), and as an N-nitroso compound, it was subjected to extensive toxicological tests and cleared of potential problems (Advisory Committee on Pesticides, 1993). Our study concludes that reduced AOX activity is tightly correlated with reduced IMI metabolism to IMI-NNO and IMI-NH (Fig. 5), indicating that these products are mostly from AOX, not CYPs. Based on the metabolic sequence and relevant correlations, IMI-NH is mostly formed via IMI-NNO rather than another pathway.

Insect AOX is implicated in insecticide detoxification and resistance (Hemingway et al., 2000). However, AOX+/- and AOX−/− Drosophila did not display any differences in IMI nitroreduction or sensitivity. These limited data suggest a greater importance of AOX for the nitroreduction of IMI in mice rather than Drosophila. This species specificity is not surprising because the proposed Drosophila orthologs to mouse AOX1 and AOH1 (Garattini et al., 2008) have 30% or less sequence identity (online DRSC Integrative Ortholog Prediction Tool) (Hu et al., 2011), which may contribute to differences in substrate selectivity and activity.

AOX is the most important mouse IMI hepatic nitroreductase in vivo. Mouse AOX activity is similar to that of humans in metabolizing IMI and some other substrates and therefore is a preferred model for human AOX. Although our studies
used ip treatment, IMI-NH and IMI-NNO are produced in vivo when rats or mice are exposed to IMI orally (Advisory Committee on Pesticides, 1993) and may be relevant to people with dietary exposure. Differences in AOX expression and nitroreduction may result in species differences in toxicity and residue dissipation. This is the first conclusive evidence of AOX, not just CYP, involvement in neonicotinoid metabolism in vivo and more generally is also one of the first studies showing the toxicological importance of AOX in in vivo xenobiotic metabolism.

**SUPPLEMENTARY DATA**

Supplementary data are available online at [http://toxsci.oxfordjournals.org/](http://toxsci.oxfordjournals.org/).

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