Developmental Expression of Aldehyde Dehydrogenase in Rat: a Comparison of Liver and Lung Development

Miyoung Yoon, Michael C. Madden, and Hugh A. Barton

Metabolism is one of the major determinants for age-related changes in susceptibility to chemicals. Aldehydes are highly reactive molecules present in the environment that also can be produced during biotransformation of xenobiotics and endogenous metabolism. Although the lung is a major target for aldehyde toxicity, early development of aldehyde dehydrogenases (ALDHs) in lung has been poorly studied. The expression of ALDH in liver and lung across ages (postnatal day 1, 8, 22, and 60) was investigated in Wistar-Han rats. In adult, the majority of hepatic ALDH activity was found in mitochondria, while cytosolic ALDH activity was the highest contributor in lung. Total aldehyde oxidation capability in liver increases with age, but stays constant in lung. These overall developmental profiles of ALDH expression in a tissue appear to be determined by the different composition of ALDH isoforms within the tissue and their independent temporal and tissue-specific development. ALDH2 showed the most notable tissue-specific development. Hepatic ALDH2 was increased with age, while the pulmonary form did not. ALDH1 was at its maximum value at postnatal day 1 (PND1) and decreased thereafter both in liver and lung. ALDH3 increased with age in liver and lung, although ALDH3A1 was only detectible in lung. Collectively, the present study indicates that, in the case of aldehyde exposure, the in vivo responses would be tissue and age dependent.

Key Words: aldehyde dehydrogenase; postnatal development; liver versus lung comparison; propanal; hexanal; benzaldehyde.

Age-related changes in susceptibility have been a great concern for predicting therapeutic and/or toxic effects of chemicals in early life from adult data. Developmental changes in metabolism are among the major determinants of differential responses to chemicals between young organisms and adults. Many phase I and phase II enzymes have been reported to be expressed pre- and postnatally (Cresteil, 1987; Hines and McCarver, 2002; McCarver and Hines, 2002), but they do not develop at equal rates. The variable patterns of maturation depend on the type of enzyme, isoforms within the specific enzyme family, and tissues in which the enzymes are expressed. There is very little information regarding the age-dependent changes in the metabolic capacity of the lung, so it is often assumed to parallel changes in hepatic metabolism with lower amount of activity (Sarangapani et al., 2003). Although the liver is generally the most important organ for xenobiotic metabolism, other tissues such as the lung can be important depending on the chemical of interest such as aldehydes.

Several aldehydes, including formaldehyde, acetaldehyde, acrolein, and propanal are listed among the 188 U.S. EPA hazardous air pollutants (U.S. EPA, 1999), and additive or synergistic health effects are possible if they are mixed with particulate matter (PM) (Jakab et al., 1992). Ambient aldehydes are derived from many sources, including combustion processes (e.g., diesel exhaust), outgassing (e.g., particleboard), and atmospheric transformation processes (Suh et al., 2000). Aldehydes can arise from endogenous sources in lung via lipid peroxidation (Kappus, 1991) or reactions of ozone with lung lining fluids (Frampton et al., 1999). Aldehydes may be ingested from food as well (Feron et al., 1991), though it is uncertain how much is transported to the lung via this route of intake. Acetaldehyde inhalation can induce lung function decrements in asthmatics (Myou et al., 1993) and may potentiate other asthma symptoms. Some studies have implied that children are more susceptible to formaldehyde exposure, as indicated by the increased risk of asthma and chronic bronchitis (Krzyzanowski et al., 1990; Rumchev et al., 2002).

One of the most important pathways for aldehyde detoxication is their oxidation by aldehyde dehydrogenase (ALDH). The ALDH level appears to be important in determining the dosage of aldehyde delivered to the target tissue (Stanek and Morris, 1999). Thus, lower level of ALDH at earlier ages could suggest the potential for greater toxicity. Few toxicity studies have been
carried out with inhalation exposures to aldehydes during developmental period. Therefore it was of interest to characterize whether aldehyde dehydrogenase (ALDH) activities were higher or lower in young animals.

In rats, at least seven families of ALDHs are present. They exhibit rather broad substrate specificity, but may not be entirely redundant because they tend to exhibit specificity for distribution among tissues, cell types, and subcellular locations. Among ALDHs, classes 1, 2, and 3 have been reported to be the most important in the metabolism of exogenous aldehydes, including those produced from the bioactivation of certain xenobiotics (Sophos and Vasiliou, 2003). Other ALDH classes have rather narrow specificity for endogenous aldehydes generated during normal intermediary metabolism including retinoic acid biosynthesis, the metabolism of γ-aminobutyric acid (GABA) and folate (Vasiliou et al., 2000). Members of ALDH1 family are mainly located in cytosol where, in addition to metabolizing xenobiotics, they participate in retinoic acid synthesis, which is essential in growth, development, and maintenance of normal cellular differentiation (Vasiliou et al., 2000). ALDH1A1 shows constitutive expression in several tissues, with the highest level found in liver (Vasiliou et al., 2000). ALDH1A2 is an extrahaesthetic form with the highest mRNA expression in testis of rats (Wang et al., 1996). Class 2 ALDH is a mitochondrial enzyme expressed in a larger number of tissues than ALDH1, with the highest expression in liver and intermediate levels in kidney and lung in rat (Dipple and Crabb, 1993). ALDH2 plays a major role in acetaldehyde detoxication following ethanol metabolism in vivo (Klyosov et al., 1996), and its polymorphism has been attributed as a cause of alcohol sensitivity (Goedde and Agarwal, 1987). ALDH2 participates in one of the major metabolic pathways for the removal of 4-hydroxynonenal from lipid peroxidation, thereby playing a role in protecting cells against the oxidative stress (Mitchell and Petersen, 1987; Ohsawa et al., 2003). ALDH2 also appears to be involved in the metabolism of reactive aldehyde intermediates including methoxyacetaldehyde formed from 2-methoxyethanol (Kitagawa et al., 2000) and chloroacetaldehyde generated from the metabolic activation of vinyl chloride (Sharpe and Carter, 1993). ALDH2 deficiency is associated with the incidence of esophageal and other upper aerodigestive tract cancers and is a risk factor for ethanol-induced bronchoconstriction in asthmatics (Sladek, 2003). Class 3 ALDHs oxidize toxic aldehydes formed by lipid peroxidation. ALDH3A1 is constitutively expressed in the cytosol of several extrahepatic tissues including cornea, stomach, and lung (Dunn et al., 1988; Vasiliou et al., 2000). In liver, ALDH3A1 is observed when induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin or polycyclic aromatic hydrocarbons. Increased ALDH3A1 expression has been reported in several tumors (Lindahl, 1992). ALDH3A2 is constitutively expressed in virtually all tissues (Dunn et al., 1988; Vasiliou et al., 2000).

Developmental studies on extrahepatic ALDH expression have been performed in rat, including stomach (Bhat, 1998; Picchiottino and Lee, 2002), skin, eye (Pappas et al., 1997), intestine (Bhat, 1998), and kidney (Bhat et al., 1998), focusing on selected isoforms, particularly those relevant to retinoic acid synthesis. Knowledge of the development of individual isoforms in liver is incomplete, although several studies have provided information about hepatic ALDH development (Cao et al., 1989; Horton and Mills, 1979; Sjoblom et al., 1978). The expression of ALDH in the lung has been poorly investigated compared to the liver and other extrahepatic tissues, although the lung is a major target for toxicity from inhaled aldehydes.

In the present study, aldehyde dehydrogenase expression was investigated during tissue development in both rat liver and lung by measurement of activity with four aldehyde substrates in four subcellular fractions and the determination of ALDH protein expression level with 1D and 2D Western blot analysis.

MATERIALS AND METHODS

Animals. Timed-pregnant and adult female Wistar-Han IGS rats were obtained from Charles River Laboratory (Raleigh, NC). The animals were maintained according to the NRC Guide for the Care and Use of Laboratory Animals within a AAALAC-approved animal facility. Animals were housed individually and maintained on a 12-h light/dark cycle at ambient temperature (22°C) and relative humidity (50 ± 10%) and were provided Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. Timed-pregnant dams were randomly assigned to three different groups to produce pups for postnatal day (PND)1, 8, or 22. Female pups from a given litter were allowed to suckle freely until they were sacrificed. The day of birth was designated as PND0. PND60 animals were purchased separately and considered as adults. Animals were sacrificed by decapitation. Liver and lung were removed without perfusion, followed by immediate freezing in liquid nitrogen and storage at −80°C until analysis. Tissues from appropriate numbers of PND1 (n = 10), PND8 (n = 4) and PND22 (n = 3) pups were pooled to provide sufficient material for analysis. The number of litters used in a given pool was kept as small as possible. Adult tissues were used individually to prepare subcellular fractions.

Reagents. Propanol (97%), hexanal (98%), benzaldehyde (99.5+%), sodium deoxycholate, thioruea, pyrazole, rotenone, NAD, and NADP were obtained from Sigma-Aldrich Co. (St. Louis, MO). Sodium pyrophosphate was from Fisher Chemicals (Fair Lawn, NJ). Urea, 2D clean-up kit, dithirotretol (DTT), 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propane sulfonate (CHAPS), IPG buffer pH 3–10, immobilized pH gradient (IPG) strips (Immobiline Drystrip, 11 cm, pH 3–10), and destack rehydration solution were from Amersham Biosciences AB, (Uppsala, Sweden). LDS sample buffer (2% lithium dodecyl sulfate, 10% glycerol, 0.51 mM EDTA. 0.22 mM SERVA® Blue G250, and 0.175 mM phenol red in 247 mM Tris buffer, pH 8.5), precast 4–12% Bis-Tris gel, and Magic Mark® XP Western standard were from Invitrogen Co. (Carlsbad, CA). Precast 10% Bis-Tris gel was from Bio-Rad Laboratories Inc. (Hercules, CA). Western lightning chemiluminescence reagent plus was from PerkinElmer Inc. (Wellesley, MA). Goat polyclonal anti-ALDH1A1 antibody (made against a synthetic peptide with a specific sequence within human ALDH1A1 protein) and horseradish peroxidase-conjugated anti-rabbit and anti-goat IgG antibody were obtained from Abcam, Ltd. (Cambridge, UK). Rabbit polyclonal anti-human ALDH2 antibody and rabbit polyclonal anti-ALDH3 antibody were generous gifts from Dr. Henry Weiner (Purdue University, West Lafayette, IN) and Dr. Ronald Lindahl.
TABLE 1

Assay Conditions to Distinguish ALDH Isoforms

<table>
<thead>
<tr>
<th>Subcellular fractions</th>
<th>Substrate</th>
<th>ALDH1</th>
<th>(p-)ALDH2</th>
<th>ALDH3A1</th>
<th>ALDH3A2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Propanal 10 mM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>Refer to citations in each subcellular fraction</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 μM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 μM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Propanal 10 mM</td>
<td>–</td>
<td>+/+</td>
<td>–</td>
<td>–</td>
<td>(DiFabio et al., 2003; Goedde and Agarwal, 1990; Klyosov, 1996; Shah et al., 1991; Yin et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 μM</td>
<td>–</td>
<td>+/+</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 μM</td>
<td>–</td>
<td>+/+</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>Propanal 10 mM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>–</td>
<td>(Goedde and Agarwal, 1990; Kathmann et al., 2000; Klyosov, 1996; Marselos and Lindahl, 1988; Wang et al., 1996; Yin et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 μM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 μM</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzaldehyde 5 mM &amp;</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>Propanal 10 mM</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td>(Goedde and Agarwal, 1990; Martini and Murray, 1996; Yoshida et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 μM</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 μM</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/-</td>
<td></td>
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</tbody>
</table>

Note. +/-: The isoform is present in the subcellular fraction and responsible for the given aldehyde metabolism. –: The isoform is not present in the subcellular fraction.

Preparation of subcellular fractions. Each subcellular fraction was prepared by differential centrifugation. After weighing, liver tissue was homogenized in 250 mM sucrose, 5 mM Tris–HCl, and 0.5 mM EDTA buffer (pH 7.2) with a Teflon-glass homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min at 4°C, and the supernatant (1000 × g supernatant, S1) was further centrifuged at 8,000 × g for 15 min at 4°C. The 8,000 × g pellet was washed once and resuspended in homogenizing medium containing 0.3% sodium deoxycholate (mitochondrial fraction). The post-mitochondrial supernatant was centrifuged at 100,000 × g in 4°C. Supernatant was saved as the cytosol fraction, and the microsomal pellet was washed once and resuspended in homogenizing buffer. Lung tissue was minced with Polytron homogenizer first, and then the same procedure described above was followed. Each subcellular fraction was used immediately after the preparation for enzyme activity measurement or stored at −80°C until use for the 1D or 2D electrophoresis.

Measurement of aldehyde dehydrogenase (ALDH) activity. In the present study, four different subcellular fractions combined with four different aldehyde substrates were used to discriminate among the different ALDH isoforms. ALDH isoforms (class 1–3) currently known to participate in the metabolism of propanal, hexanal, or benzaldehyde at given concentrations in each subcellular fraction are summarized in Table 1 based on their kinetic properties and their expression sites within cells. For isoform specific activity measurements, 50 μM propanal for mitochondrial ALDH2, 50 μM hexanal for microsomal ALDH3A2, and 5 mM benzaldehyde (with NADP) for cytosolic ALDH3A1 were used. Otherwise the given aldehyde metabolism may represent more than one ALDH isoform as described in Table1. General (nonspecific) ALDH activity in each subcellular fraction was represented by 10 mM propanal metabolism.

ALDH activity was measured based on the method by Nelson and Lipsky (1995) with minor modifications. Subcellular fraction protein was preincubated with incomplete reaction mixture containing 1 mM NAD(P), 1 mM pyrazole, and 2 μM rotenone in 50 mM sodium pyrophosphate buffer (pH 8.8) for 10 min at room temperature. Reaction was started by adding aldehyde substrate. The final volume of reaction mixture was 300 μl. The absorbance change at 340 nm due to the reduction of NAD(P) was monitored over time. NAD(P)H formation was proportional to incubation time, and ALDH activity was expressed as nmol NAD(P)H formed per min per mg protein based on the linear portion of the reaction curve. Propanal was dissolved in sodium pyrophosphate buffer. Hexanal and benzaldehyde were dissolved in methanol (final concentration 0.1 (v/v, 25 μM) or 1%, respectively). Benzaldehyde metabolism was assayed with NADP as a cofactor. The blank rate was measured with the same amount of the buffer or 0.1 or 1% methanol in buffer without aldehyde substrate. The presence of methanol did not alter the blank rate. For immunoinhibition of benzaldehyde metabolism in liver or lung cytosol, ALDH3 antibody was incubated at 1:100 dilution with cytosol at 4°C for 30 min prior to the assay procedure described above. The amount of protein used in the assay was kept the same for each subcellular fraction from all four age groups. Protein concentration was determined by the Lowry method with bovine serum albumin as a standard. (Lowry et al., 1951).

1D electrophoresis. ALDH1 and ALDH2 isoforms in S1 and cytosol fraction were detected by the ALDH1/2 antibody. BLAST (Basic Local Alignment Search Tool) searching for the antigen sequence for ALDH1A1 antibody from Abcam, Ltd. (Cambridge, UK) found it could recognize multiple rat ALDH isoforms including ALDH1A2, ALDH2 precursor (p-ALDH2), ALDH2, ALDH1B1, phenobarbital-inducible ALDH1, ALDH1A1, and ALDH1A3, so we designated this antibody as ALDH1/2 in this study (the computation was performed at the Swiss Institute of Bioinformatics using the BLAST network service, http://www.expasy.org/tools/blast/). ALDH1/2 antibody revealed two bands in S1 and cytosol by 1D Western blots.
The lower-molecular-weight band was considered as ALDH2 or p-ALDH2 protein, since only this band cross-detected with ALDH2-specific antibody. Since there was no standard protein available for rat ALDH proteins, adult liver mitochondrial (for ALDH2), adult lung cytosol (for ALDH3A1), or adult liver microsomal (for ALDH3A2) protein pooled from six individual animals was used as a positive control and to construct a standard curve for quantitation relative to adult levels. Subcellular protein samples were heated at 70°C for 10 min in LDS sample buffer with 1% DTT and separated by SDS–PAGE using 4–12% Bis-Tris gel (NuPAGE system, Invitrogen, Co., Carlsbad, CA). Proteins were transferred to a nitrocellulose membrane, and the immunodetection was performed according to Abcam’s (Cambridge, UK) protocol. ALDH1/2 antibody was used at 1 µg/ml concentration. ALDH2 and ALDH3 antibodies were used at 1:4000 and 1:5000 dilutions, respectively. Bands were visualized by enhanced chemiluminescence (ECL) detection, and the density was determined by Kodak Digital Science 1D image analysis software (version 3.0, New Haven, CT). The intensities of the ALDH2 immuno-reactive bands were calculated as adult liver equivalents converted from the standard curve using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Western blotting with anti-ALDH2 antibody showed two bands very close together in liver mitochondria at approximately molecular weight 51–53 kD, but only the lower molecular weight bands were observed in lung mitochondria. The additive combination of the two bands in the standards and in liver samples was used for the conversion of intensities to adult equivalents, but only the lower band was used for the calculation of lung values. ALDH3A1 and ALDH3A2 band densities were also converted to adult lung (3A1) or liver (3A2) equivalents, according to the same method used in ALDH2 calculation. ALDH1 band densities were not converted to adult equivalents, but age comparisons among different age groups were performed.

2D Western blot analysis. 1000 × g supernatant (S1) protein from the liver or lung tissue samples (200 µg) was cleaned using 2D clean-up kit (Amersham Biosciences AB, Uppsala, Sweden) and resuspended in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer (pH 3–10), and 0.07% DTT). IPG strips (11 cm, pH 3–10) were rehydrated with S1 samples in destreak rehydration solution containing 0.5% IPG buffer (pH 3–10) for 11 h at 50 V. Isoelectric focusing (IEF) was performed using IPGPhor (Amersham Biosciences AB, Uppsala, Sweden). The running condition was 250-V gradient for 1 h, 500-V gradient for 1 h, 1000-V gradient for 1 h, 8000-V gradient for 2 h, and 8000-V step-n-hold for 5 h at 20 min. After IEF, IPG strips were equilibrated with 1% DTT in LDS sample buffer for 30 min at room temperature or stored at −80°C until use. After SDS–PAGE electrophoresis (10% bis-Tris gel with Bio-Rad’s Criterion XT equipment), gels were used for Western transfer to nitrocellulose membranes according to Bio-Rad instruction. Immunodetection was followed using the same procedure in the 1D electrophoresis with ALDH1/2 antibody. Three groups of spots were revealed by ALDH1/2 antibody in 2D Western blots and group 3 was ALDH2 proteins judged by immunodetection with ALDH2-specific antibody.

Data analysis. All values were expressed as the mean ± SEM. Statistical comparison among the four different age groups was made by one-way ANOVA, followed by Tukey-Kramer multiple comparison test. All statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and the acceptable level of significance was established at p < 0.05.

RESULTS

Developmental expression of ALDH in liver and lung was investigated in Wistar-Han rats in three ways: enzyme activity measurement and Western blot analysis with one-dimensional (1D) or two-dimensional (2D) electrophoresis. Comparisons were made across ages for specific isoforms expression and total (i.e., nonspecific or general) aldehyde metabolism capacity using 10 mM propanal metabolism as an indicator.

### TABLE 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALDH (nmol/min/mg protein)</td>
<td>Protein (mg/g tissue)</td>
</tr>
<tr>
<td>S1</td>
<td>Propanal 10 mM</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 µM</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 µM</td>
<td>13.3 ± 0.5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Propanal 10 mM</td>
<td>23.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 µM</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 µM</td>
<td>20.2 ± 1.6</td>
</tr>
<tr>
<td>Cytosol</td>
<td>Propanal 10 mM</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 µM</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 µM</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Benzaldehyde 5 mM</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td>Propanal 10 mM</td>
<td>17.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Propanal 50 µM</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Hexanal 50 µM</td>
<td>33.0 ± 1.7</td>
</tr>
</tbody>
</table>

Note. Each value represents the mean ± SEM for six individual animals.

*Value calculated from specific activity (ALDH activity/mg subcellular protein) and the protein amount in the tissue.

*Not assayed in this study.

*Not detected.
ALDH activities were determined in adult liver and lung subcellular fractions, including 1000 × g supernatant (S1), mitochondria, cytosol, and microsomes (Table 2). Specific activity (i.e., ALDH activity per mg subcellular protein) with 10 mM propanal was the highest in the mitochondria compared to other subcellular fractions both in liver and lung (one-way ANOVA followed by Tukey’s multiple comparison test). However, the distribution of aldehyde dehydrogenation ability within a tissue (i.e., ALDH activity per g tissue), measured with 10 mM propanal, showed that the majority was located in the mitochondria in liver, while the cytosol was the highest contributor in lung relative to other subcellular fractions (one-way ANOVA followed by Tukey’s multiple comparison test). Specific activity of ALDH in each subcellular fraction was scaled up to the activity per gram whole tissue to estimate the contribution of each subcellular ALDH to the total aldehyde metabolism capacity in a tissue. Protein contents of liver and lung subcellular fractions from PND1, 8, 22, and 60 animals are summarized in Table 3. Hepatic mitochondrial and microsomal protein content was significantly low in PND1 animals and rapidly increased during the first week after birth (Table 3). Lung also showed low mitochondrial and microsomal protein on PND1, which increased more gradually with age compared to liver.

ALDH activity in S1 fraction was measured with propanal (10 mM or 50 μM) and hexanal (50 μM) (Table 4). Hepatic total ALDH activity showed a gradual increase with age. At PND1 it was 62% of the adult, and a rapid increase occurred during the first week after birth up to PND22. No age-dependent change was observed in lung total ALDH activity, which was already similar to adult level on PND1. The pattern of development of ALDH activity in S1 varied depending on the aldehyde substrate. Especially, ALDH activity toward 50 μM propanal in lung showed a completely different development pattern from that of ALDH with 10 mM propanal or 50 μM hexanal. The activity declined by nearly two-fold from PND1 to PND8 and remained low until PND22 and recovered to the PND1 level by PND60.

Temporal differences in the development between ALDH1 and ALDH2 were investigated with 1D or 2D electrophoresis followed by Western blotting with ALDH antibody (Fig. 1). Levels of ALDH1 determined by 1D Western blotting were similar at PND1 and PND60 in liver and lung (Figs. 1A and 1B). By contrast, ALDH2 increased approximately three-fold in liver from PND1 to PND60, while a smaller increase was observed in lung. Two-dimensional Western blots revealed three major groups of spots in both PND1 and PND60 liver S1 (Figs. 1C and 1D). Group 1 was relatively darker in PND1 (Fig. 1C), while the relative density of group 2 and group 3 were increased in PND60 compared to PND1 sample (Fig. 1D).

Western blotting of the same 2D blots in Figures 1C and 1D with ALDH2-specific antibody demonstrated that all spots in group 3, but no spots in groups 1 and 2, were ALDH2 proteins in liver S1 fraction (data not shown). Two-dimensional Western blots of lung ALDH proteins were also performed, but one of the protein spots was so dominant on 2D gel separation that the expression of other spots could not be clearly interpreted; furthermore, it overlapped with some ALDH spots (data not shown).

ALDH activity and protein expression in mitochondria, cytosol, and microsomes in liver and lung of different ages were measured to investigate the individual development pattern of ALDH isoforms of interest in this study. Western blot analysis showed that ALDH2 protein gradually increased with age in liver mitochondria (Fig. 2D). Increased ALDH2 protein expression across ages was correlated with ALDH...
activity measured with 50 µM propanal, 50 µM hexanal, or 10 mM propanal as substrates (Fig. 2D). In the lung, ALDH2 protein expression showed a U-shaped curve with age (Fig. 2E). ALDH activity in lung mitochondria was also correlated with ALDH2 protein change, showing a similar decline from PND1 to PND22 and then an increase by PND60 with both 50 µM and 10 mM propanal substrate (Fig. 2E).

Lung and lung cytosol fractions were used to investigate ALDH1, ALDH2, and ALDH3A1 protein amount and activity development. Western blotting with ALDH1/2 antibody revealed two bands in each liver cytosol of different ages (Fig. 3A). ALDH1 protein expression was highest at PND1 and dramatically declined with age both in liver and lung cytosol (Figs. 3C and 3D). ALDH2 precursor, p-ALDH2 protein (thought to be present in cytosol before importing into mitochondria), however, showed a gradual increase with age, maturing around PND22 in liver cytosol (Fig. 3C). No p-ALDH2 band was detected in lung cytosol until PND22 (Fig. 3D), and the relative density of p-ALDH2 to ALDH1 band in lung cytosol was much lighter than in liver (Fig. 3B). The development of p-ALDH2 protein showed a correlation with ALDH activity measured with 50 µM propanal in liver cytosol (Fig. 3C). The activity with 10 mM propanal development was correlated with neither ALDH1 nor ALDH2 protein expression in liver (Fig. 3C). Neither ALDH1 nor ALDH2 protein expression was fully reflected by the developmental patterns of ALDH activity in lung cytosol, regardless of which aldehydes were used as substrates (Fig. 3D).

ALDH3A1 protein in lung cytosol detected with Western blotting rapidly increased after PND8 (Fig. 3E). Trace amounts of ALDH3A1 were detected in liver cytosol in all ages (blot not shown). ALDH3A1 activity in cytosol was measured with 5 mM benzaldehyde as substrate and NADP as cofactor (Fig. 3E). ALDH3A1 activity was detected only in adult lung cytosol (Fig. 3E). A small amount of ALDH activity was found in liver cytosol of all ages with 5 mM benzaldehyde, but this was not immunoinhibited by ALDH3 antibody (Student’s t-test). Control benzaldehyde metabolism rates (nmol/min/mg protein), expressed as the mean ± SEM, were 0.4 ± 0.1, 0.5 ± 0.01, 0.8 ± 0.1, and 1.9 ± 0.3 for PND1, PND8, PND22, and PND60 liver, respectively. The metabolism rates with the presence of ALDH3 antibody were 0.3 ± 0.01, 0.5 ± 0.02, 0.8 ± 0.1, and 2.6 ± 0.7 for PND1, PND8, PND22, and PND60 liver, respectively. Benzaldehyde metabolism in lung cytosol was significantly diminished by ALDH3 antibody. Control metabolism was 1.7 ± 0.1 in PND60 lung, but only 0.3 ± 0.1 was detected with the presence of ALDH3 antibody (Student’s t-test, p < 0.0001).

A gradual increase in ALDH3 protein in microsomes (ALDH3A2) was found in liver across ages (Fig. 4A). A similar increase in hepatic microsomal ALDH activity across ages was found with 50 µM hexanal or 10 mM propanal as substrates (Fig. 4A). In lung, however, ALDH3A2 protein was fully expressed at adult level by PND8 (Fig. 4B). ALDH activity measured as 50 µM hexanal or 10 mM propanal
metabolism in microsomes was only fully developed after PND22 (Fig. 4B).

**DISCUSSION**

The primary objective of this study was to quantitatively describe the age-related differences in aldehyde metabolism in the rat and compare the development patterns between liver and lung. This objective requires characterizing the adult, for which we used PND60 for comparison with earlier ages.

In adult animals, evaluation of total ALDH activity in the liver and lung found the highest specific activity in mitochondria. We estimated the total ALDH with substrates metabolized by multiple isoforms, such as 10 mM propanal and hexanal (Table 1). Both mitochondria and cytosol are major contributors to ALDH activity in the liver and lung. ALDH activities were measured in liver (D) and lung (E) mitochondrial fractions from PND1, 8, 22 and 60 animals with 50 μM propanal and 10 mM propanal as substrates. Hexanal (50 μM) was used only with liver mitochondria. Each specific activity value represents the mean ± SEM for 3 pools (PND1, PND8, and PND22) or for 6 individual tissues (PND60). Each value in D and E was normalized by its corresponding adult value from Table 2. α, β, γ Values are significantly different from one another among different age groups for oxidation of a specific aldehyde substrate if not sharing the same superscript (One-way ANOVA, followed by Tukey-Kramer multiple comparison test, p < 0.05).

FIG. 2. Developmental Profiles of ALDH Activity and Protein Expression in Mitochondria. ALDH2 protein was detected using Western blot analysis with ALDH2 antibody in liver or lung mitochondria. Ten micrograms of adult lung mitochondrial protein from 5 individual animals were loaded in lane 1–5 (A). Lanes 6–9 contain 0.5, 2, 6, and 15 μg of liver mitochondria pooled from 6 adults, used as standards for relative quantitation (A). Liver mitochondrial protein (2 μg each, B) and lung mitochondrial protein (5 μg each, C) were loaded in triplicate with PND1 in lanes 3, 6, and 9, PND8 in lanes 2, 5, and 8, and PND22 in lanes 1, 4, and 7. Lanes 10–12 contain 0.2, 0.5 and 2 μg standards (B and C). ALDH2 band density from A, B, and C were normalized by the corresponding adult value and plotted in D and E (Each value represents the mean ± SEM of triplicate measurements for the pooled sample of PND1, 8, and 22, or for 5 or 6 individual samples for PND60). ALDH activities were measured in liver (D) and lung (E) mitochondrial fractions from PND1, 8, 22 and 60 animals with 50 μM propanal and 10 mM propanal as substrates. Hexanal (50 μM) was used only with liver mitochondria. Each specific activity value represents the mean ± SEM for 3 pools (PND1, PND8, and PND22) or for 6 individual tissues (PND60). Each value in D and E was normalized by its corresponding adult value from Table 2. α, β, γ Values are significantly different from one another among different age groups for oxidation of a specific aldehyde substrate if not sharing the same superscript (One-way ANOVA, followed by Tukey-Kramer multiple comparison test, p < 0.05).
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to the total activity estimated per gram of tissue, with lung cytosol actually being a somewhat larger contributor than mitochondria. The subcellular distribution of ALDH activities in lung is reported for the first time here. No appreciable strain difference has been reported regarding the subcellular distribution of hepatic ALDH (or the expression of isoforms) in rodents. Lindahl and Evces (1984) showed the subcellular distribution of ALDH activities in liver is very similar among different strains of rats except one highly inbred strain. Also, no qualitative difference in the expression pattern of hepatic ALDH isoforms was found between two strains of mice (Cheung et al., 2003). However, species difference in hepatic ALDH expression is obvious among different animal species including humans (Lindahl and Evces, 1984; Tsutsumi et al., 1988). The main difference between human and animals is related to cytosolic ALDH forms, while the properties of mitochondrial and microsomal ALDH are very similar across species (Klyosov et al., 1996; Tsutsumi et al., 1988). Information on species or strain differences in the expression of extrahepatic ALDHs is limited. Cheung et al. (2003) showed a species-specific expression of skin ALDH3.

Total metabolic capacity values need to be interpreted with care because liver is dominantly hepatocytes, while lung activity resides in only a few cell types, for example in the Clara cells and type II epithelial cells. Although even hepatic ALDHs are reported to show small but distinct heterogeneous intralobular distribution in adult rats (Maly and Sasse, 1987; Yamazaki et al., 1988), pulmonary ALDHs show more limited distribution, being highly localized within the Clara cells of the distal bronchioles in adult rats (Bogdanffy et al., 1986). However, no information is available regarding the localization of ALDHs within the lung during development in rats. Several enzymes are reported to show distribution changes during lung development in rats, such as CYP2B and NADPH1 reductase (Ji et al., 1995), γ-glutamyltransferase (Oakes et al., 1997), and protein phosphatase 2A (Xue et al., 1998). These changes were suggested to arise from the roles of enzymes shifting from protection against blood-borne xenobiotics in fetus to air-borne toxicants in adult lung (Ji et al., 1995). During mouse lung development, the expression of Aldh1 and Raldh2 was reported to change within the lung anatomical system in association with their roles in metabolizing developmental signaling molecules (i.e., retinaldehyde) (Hind et al., 2002). Thus, it is quite possible that the expression of rat pulmonary ALDHs is not limited to the same cell types as in adults during development. Although we used the total lung tissue to estimate the whole-lung aldehyde oxidation ability, the site-specific expression of ALDH within the lung remains to be characterized. Additionally, the deposition of aldehyde may not be uniform over the surface of the lungs, e.g., as predicted for formaldehyde uptake in the human lung (Overton et al., 2001), which may play a role in the metabolic fate and the rate of catabolism of the aldehyde.

The current study showed that the lung and liver ALDH exhibit tissue-specific development. The developmental profile of pulmonary ALDH is described and compared with the liver for the first time here. Hepatic total ALDH activity clearly shows an age-related increase after birth, whereas pulmonary total ALDH activity remains constant during development, with neonatal lung already showing aldehyde oxidation ability comparable to the adult. Hepatic ALDH activity was previously reported to appear before birth and rapidly increase at the end of gestation, resulting in 50% adult activity at PND1, similar to our result (Sjöblom et al., 1978). Because of the overlapping substrate specificity of ALDH enzymes, the development of each ALDH isoform could be important, as well as that of the total aldehyde dehydrogenation capacity, to predict the metabolism of a given aldehyde at certain developmental stages. Several findings in whole-tissue homogenates in our study suggest that ALDH isoforms develop in a diverse way. The developmental patterns of ALDH activity with 50 μM propanal, which is metabolized by ALDH1 and ALDH2, differ from the overall trends. The comparison of ALDH1 and ALDH2 expression in whole-tissue homogenate indicates that the role of ALDH2 is increased in adults both in liver and lung, while the reverse was true for ALDH1 (Fig. 1).

These whole-tissue findings led us to investigate the temporal differences in ALDH isoform development using three subcellular fractions, each of which is known to possess one major ALDH isoform (Table 1). We observed an age-dependent increase in hepatic mitochondrial ALDH2 protein and activity, similar to previous reports (Horton and Mills, 1979; Sjöblom et al., 1978). The fainter higher-molecular-weight band observed in the 1D blot of ALDH2 proteins could be the p-ALDH2 containing a 19-amino acid mitochondria leader sequence (Farres et al., 1988) and/or a cross-reacting protein (Fig. 2B). This band pattern in the rat liver was reported in another study (Dipple and Crabb, 1993). The lung ALDH2 development was completely different from that observed in liver. It showed a decline and then increase during the first 60 days of life.

In liver and lung cytosol, ALDH1 decreased dramatically with age. Protein consistent with p-ALDH2 also was detected in cytosol (thought to be present in cytosol before importing into mitochondria), and the expression was increased with age in both liver and lung. This precursor seems to contribute greatly in liver cytosol aldehyde metabolism, as activity with propanal (50 μM) mirrored the p-ALDH2 protein. This may be explained by a much higher affinity of ALDH2 toward propanal compared to ALDH1 (Klyosov et al., 1996). In lung, however, ALDH1 seems to play a role in the 50 μM propanal metabolism before p-ALDH2 appears. The initial decreasing phase of activity is likely associated with the rapid fall of ALDH1 protein expression during the early postnatal period, and the recovery shown after PND22 with the beginning of p-ALDH2 expression. ALDH activity with other aldehyde substrates in liver followed neither ALDH1 protein development nor that of p-ALDH2, indicating the combined role of ALDH1 and p-ALDH2 for metabolism of these aldehydes in
Developmental Profiles of ALDH Activity and Protein Expression in Cytosol. Western blot analysis was performed with liver or lung cytosol using ALDH1/2 or ALDH3 antibody. Twenty micrograms of liver (A) or lung (B) cytosolic protein were loaded in duplicate from pooled tissues (lanes 1 and 5: PND1, lanes 2 and 6: PND8, lanes 3 and 7: PND22, lanes 4 and 8: PND60) and the obtained band densities were plotted in C and D (Each value represents the mean ± SEM of duplicate measurements for the pooled sample of PND1, PND8, PND22 and PND60). Fifteen micrograms of liver or lung cytosolic protein from pooled PND1, 8, 22 and 60 samples were used for ALDH3 detection (blots not shown) and the mean ± SEM of each band density from PND1, 8, 22 and 60 was plotted in E. ALDH activity was measured in liver (C) and lung cytosol (D) from PND1, 8, 22 and 60 animals with 50 μM propanal, 50 μM hexanal and 10 mM propanal as substrates. Benzaldehyde (5 mM) with NADP cofactor was used to measure ALDH3A1 activity in lung cytosol (E). Each specific activity value represents the mean ± SEM for 3 pools (PND1, PND8, and PND22) or for 6 individual tissues (PND60) and was normalized by its corresponding adult value from Table 2.a,b,c,d Values are significantly different from one another among different age groups for oxidation of a specific aldehyde substrate if not sharing the same superscript (One-way ANOVA, followed by Tukey-Kramer multiple comparison test, p < 0.05). ND indicates that the value was not detectible in the present experimental condition.
cytosol during development. Unlike in the liver, these activities in lung changed similarly across ages with 50 μM propanal metabolism, although the later developmental phase is likely associated with the increase in ALDH3A1 in addition to p-ALDH2. Current results on the ALDH1 development show some discrepancies from previous studies. Ogura et al. (2004) reported U-shaped retinal dehydrogenase activity (ALDH1) development, with the minimum at PND17 in rat. However, Bhat et al. (1998) were unable to detect any retinal dehydrogenase activity, mRNA, or protein expression in neonatal rat liver up to PND42. They also showed the lung retinal dehydrogenase activity was very low until PND10, but increased gradually with age along with its mRNA and the protein content in rat (Bhat et al., 1998). Possible explanations for the discrepancy include the complexity of class 1 ALDH, which comprises at least three isoforms (1A1, 1A2, and 1A3) (Vasiliou et al., 2000). The relative contribution of each isoform might be different for the oxidation of propanal versus retinal due to different affinities and independent development of ALDH1 isoforms. Ogura et al. (2004) supported the independent development of ALDH1 isoforms by showing an increase in ALDH1A1 mRNA, but a decrease in ALDH1A2 mRNA expression with age in neonatal rat liver up to PND27.

The ALDH3A1 activity was only detectable in adult lung cytosol. This was not surprising, since ALDH3A1 is known to be constitutively expressed in several extrahepatic tissues including lung, but not in normal rat liver (Dunn et al., 1988). The role of ALDH3A1 in aldehyde metabolism in lung during postnatal development could not be excluded, since we detected ALDH3A1 protein expression, albeit the activity was under detection limit. Although information is limited regarding the control of cytosolic ALDH3 expression, it might be related with greater needs for tissue protection, given that a common characteristic of tissues with constitutive expression of cytosolic ALDH3 is that they are “portals of entry” for environmental components including potentially harmful chemicals. The lung and cornea are directly exposed to oxygen, so they are at high risk from reactive oxygen species and potential oxidative damage. Indeed, cellular oxygen levels are involved in the regulation of the constitutive expression of ALDH3 (Reisdorph and Lindahl, 2001), and it is believed to play a role in protecting the eye from UV and UV-induced oxidative damage (Vasiliou et al., 2000). According to Pappas et al. (1997), cytosolic ALDH3 was shown to be increased sharply to adult level within 5 days after birth in rat eye and skin. In our study, lung ALDH3A1 showed a similar age-dependent increase, albeit the onset of expression is slower than in the eye. The mechanism of developmental control of cytosolic ALDH3 remains to be clarified.

Development of another ALDH3 isoform, ALDH3A2, showed an age-dependent increase in activity with age in liver microsomes, which correlated with the amount of protein. A similar pattern of activity development was seen in a previous study (Horton and Mills, 1979). Although a similar trend was seen in lung ALDH3A2 development, there was a discrepancy between catalytic activities and levels of enzyme protein. The reason for the presence of inactive proteins in lung microsomes during development is unclear.

FIG. 4. Developmental Profiles of ALDH Activity and Protein Expression in Microsomes. Western blot analysis was performed with liver and lung microsomes using ALDH3 antibody. Twenty micrograms of microsomal protein were loaded in duplicate from pooled PND1, 8, 22 and 60 samples (blots not shown) and the mean ± SEM of each band density was plotted in A and B. ALDH activity was measured in liver (A) and lung microsomes (B) from PND1, 8, 22 and 60 animals with 50 μM hexanal and 10 mM propanal as substrates. Each specific activity value represents the mean ± SEM for 3 pools (PND1, PND8, and PND22) or for 6 individual tissues (PND60) and was normalized by its corresponding adult value from Table 2. a,b,c Values are significantly different from one another among different age groups for oxidation of a specific aldehyde substrate if not sharing the same superscript (One-way ANOVA, followed by Tukey-Kramer multiple comparison test, p < 0.05).
Currently, few direct studies have been performed which examine how the sensitivity to aldehyde exposure would be influenced by the changes in ALDH levels in vivo. Nasal ALDHs are reported to play roles in determining the delivered dosage of acetaldehyde to the target in vivo (Stanek and Morris, 1999), which may influence the extent of acetaldehyde exposure to the target tissue. Several in vitro studies have demonstrated that in different systems the toxicity of aldehydes (which are substrates of different ALDHs) can be modulated by increased or decreased expression of the appropriate ALDH. For instance, one study with the lung cancer cell lines showed that the suppression of ALDH1 and ALDH3A1 activity can lead to increased cytotoxicity to acetaldehyde (Moreb et al., 2005). The inactivation of mitochondrial and cytosol ALDHs by a chemical inhibitor markedly increased the susceptibility of rat hepatocytes to decadienal and cinnamaldehyde (Niknahad et al., 2003a,b). In addition to exogenous aldehydes, endogenous aldehydes may be important in the removal of reactive aldehydes produced from during the exposure to other chemicals (e.g., ozone) (Madden et al., 1993; Vasiliou et al., 2000). Neonatal rats and postweanling rabbits were shown to be more sensitive to ozone-induced effects such as an increase in prostaglandin E2 production compared to adults (Gunnison et al., 1990), possibly suggesting that the capability to metabolize aldehydes may be important in mediating ozone-induced toxic responses across ages.

There have been increasing attempts to quantitatively describe differences across ages that may contribute susceptibility to chemical exposures. Although age-specific pharmacokinetic differences can be major determinants for the susceptibility to environmental chemicals, it is often difficult to obtain pharmacokinetic parameters in young animals compared to adults. In vitro studies using tissues of animals of different ages represent one approach to obtain biochemical parameters such as metabolic information for incorporation into a predictive physiologically based pharmacokinetic (PBPK) life stage model. Age-specific changes in metabolism can be used as a modifier of Vmax measured in adults to estimate Vmax in younger organisms, when Km is considered constant with age because the same protein is active across ages. However, the matter is complicated in the case of aldehydes because of the presence of multiple isoforms active in the metabolism of a given aldehyde. If the relative contribution of each isoform for a given aldehyde were known, the age-specific development of ALDH isoforms described here would be more useful to extrapolate aldehyde metabolism in adults to the young.

In summary, the differences in the composition of ALDH isoforms between liver and lung suggest that aldehyde-metabolizing capacity may respond differently to environmental and/or physiological factors in the two tissues. The development of total ALDH activity was age dependent in liver, but total pulmonary activity was fully expressed at birth. The total ALDH level across age is determined by the sum of the individual ALDH isoforms levels during development, and there was no single specific isoform which dominantly determines total ALDH development pattern. The relative contribution of each isoform changed with age in both liver and lung due to the independent development of isoforms. Further studies are needed to elucidate the relationship between the ALDH development and the windows of susceptibility to the hepatic and/or pulmonary toxicants.

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