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The Ontogeny and Population Variability of Human Hepatic NADPH Dehydrogenase Quinone Oxido-Reductase 1 (NQO1)

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ABSTRACT

The NADPH dehydrogenase quinone oxido-reductase 1 (NQO1) enzyme is an antioxidant and metabolic enzyme that performs two electron reduction of quinones and other chemicals. Based on the physiologic role(s) of NQO1, we hypothesized that expression and activity of this enzyme would vary with age and other demographic variables. Cytosols from 117 archived human livers were investigated for changes in NQO1 with age, sex, obesity, and ethnicity. Protein expression but not activity of NQO1 was weakly negatively correlated with age (Spearman r = -0.2, P = 0.03). No sex differences were observed for either protein expression or activity and for ethnicity; Caucasians had greater NQO1 activity than Asians (P < 0.05). Overweight children had statistically significantly higher NQO1 activity as compared with ideal weight children (P < 0.05) although this difference was not observed in adults. These findings establish that NQO1 is approximately as active in children as adults. However, modeled NQO1 clearance (both allometric and physiologically based pharmacokinetics) predicted maturation at 23 to 26 years. This is almost certainly an overestimate, with error in the model resulting from a small sample size and inability to scale for age-related changes in hepatic cellularity and/or cytosolic protein content, and indicates a delay in reaching maximum clearance through the NQO1 pathway that is affected by physiologic development as much, or more than, biochemical development. Obesity may increase hepatic NQO1 activity in children, which is likely a protective mechanism in oxidative stress, but may also have significant implications for drug and chemical disposition in obese children.

Introduction

Throughout the human lifespan, hepatic capacity for drug and chemical disposition can change dynamically. This may be a result of changes in the expression and activity of metabolic and transport proteins as well as physiologic changes that occur in the liver with aging (Tajiri and Shimizu, 2013; Coughtrie, 2015; Miyagi and Long-Boyle, 2015). The body’s capacity for chemical, hormonal, and nutrient homeostasis is regulated through a complex series of physiologic and biochemical interactions. We are progressively beginning to determine the enzyme expression patterns that are associated with different life stages, such as how neonates differ from children, adults, and the elderly (Tajiri and Shimizu, 2013; Coughtrie, 2015; Miyagi and Long-Boyle, 2015).

The NADPH dehydrogenase quinone oxido-reductase 1 (NQO1) enzyme (EC 1.6.5.2) is a cytosolic protein that performs a two-electron reduction converting quinone species to hydroquinones (Dinkova-Kostova and Talalay, 2010). In doing so, NQO1 prevents the one electron reduction producing semiquinone radicals and therefore mediates a detoxification reaction (Dinkova-Kostova and Talalay, 2010). The crystal structure of NQO1 was elucidated in 2000, confirming that the cofactors NADP and NADPH share the same binding site as the substrates, suggesting a ping-pong mechanism of catalysis (Faig et al., 2000). The NQO1 enzyme is critical for bioreductive activation of antitumor drugs such as mitomycin C, where higher levels of NQO1 are present in tumors due to hypoxic induction, hence tumors showing reduced levels of NQO1 activity do not respond as well to treatment (Siegel et al., 2012). Moreover, NQO1 can reduce toxic metabolites of therapeutic drugs such as the quinone imine metabolites of diclofenac and mefenamic acid, thereby providing a protective path for detoxication (Vredenburg et al., 2014). Associations between NQO1 polymorphisms, in particular the low-activity C609T variant, and childhood diseases such as asthma and leukemia have been demonstrated (David et al., 2003; Guha et al., 2008; Goodrich et al., 2009; Vijayakrishnan and Houlston, 2010; Li and Zhou, 2014). In the adult population, increased cardiovascular and cancer risks (Han et al., 2009; Martin et al., 2009; Kolesar et al., 2011; Lin et al., 2014) as well as increasing severity of Alzheimer’s disease in the elderly have also been linked to NQO1 polymorphisms (Tsvetkov et al., 2011). Given these associations with childhood diseases and...
diseases of aging, the NQO1 enzyme is understudied with respect to ontogeny. A single previous study performed in 27 pediatric liver samples demonstrated no differences in NQO1 activity with age, although a weak trend toward increasing activity from birth was observed (Miyagi et al., 2009). In that study, protein levels were not analyzed, and comparisons to adults or the elderly were not performed. Hence, it is unknown whether the susceptibility to disease reported is from constitutively low (polymorphic), developmentally low (pediatric) or declining (elderly) NQO1 activities, or a combination of these factors.

In our present study, we have characterized the expression and activity of NQO1 in the human liver from birth to old age, including pharmacokinetic modeling to predict hepatic clearance (CL) maturation through NQO1 pathways. Because NQO1 has been associated with diseases of childhood and of aging, we hypothesized that the activity of the enzyme would be altered constitutively with the aging process, thus providing us with insight into developmental aspects of enzyme regulation. Additionally, we investigated the impact of covariates sex, ethnicity, and obesity and the potential influence these factors may have on NQO1.

Materials and Methods

Sample Archive and Preparation. An archive of 117 human liver cytosols was collected from commercial (Cellz Direct, Carlsbad, CA; PuraCyp, Carlsbad CA; and Xenotech, Lenexa, KS) and biorepository sources (Hawai'i Biobank, Honolulu, HI, and National Disease Research Interchange, Bethesda, MD). This study was approved as exempt by the University of Hawai'i Institutional Review Board on Human Ethics.

Commercial samples were kept at −80°C until use. Tissue samples collected from the biorepositories were processed to prepare cytosolic fractions as follows: tissues were suspended in 0.1 M Tris-HCl with 5 mM MgCl₂ and 2 mM phenylmethylsulfonyl fluoride and homogenized using a mechanical homogenizer (Tissue Tearor, Cole-Palmer, Vernon Hills, IL). The total lysate was subsequently centrifuged for 20 minutes at 10,000g and the supernatant centrifuged for 1 hour at 100,000g to purify the cytosolic fraction. The protein content of all cytosolic fractions was measured using the bicinchoninic acid method and bovine serum albumin as standard (Smith et al., 1985).

Western Blot for NQO1 Protein Presence. Detection of NQO1 protein by immunoblotting was performed using SDS-PAGE gels (10%) to resolve 10 µg of liver cytosol. Each sample was analyzed on at least two separate gels. The proteins were transferred onto polyvinylidene fluoride membranes using a semidry system at 12 V for 45 minutes. Membranes were blocked with 5% nonfat milk powder in phosphate-buffered saline with 0.05% Tween-20 (PBST) for 1 hour at room temperature. Primary polyclonal rabbit anti-NQO1 (ab34173; Abcam, Cambridge, MA) was added at 1:1000 for 16 hours at 4°C. Membranes were then washed 3 times for 10 minutes in PBST and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody at 1:5000 (donkey anti-rabbit; Cedarlane, Burlington, Canada) at room temperature. Membranes were then washed 3 times for 10 minutes in PBST and finally incubated for 1 minute in enhanced chemiluminescence solution before detection on X-ray film.

A representative pooled liver S9 lysate (10 µg) was included on every blot to normalize the expression across different membranes. Samples were semi-quantified using Image J version 1.48 (http://imagej.nih.gov/ij/). Briefly, images were scanned as .tiff files, opened in Image J, and converted to 32-bit gray images. An equal sized square box was drawn, and the mean gray values were determined for the appropriate band. The background (mean of three readings per blot) was subtracted from each band. Liver cytosol expression was normalized to the pooled liver S9 lysate on its own membrane, and the expression relative to the control was reported.

Biochemical Determination of NQO1 Activity. The method for analyzing NQO1 activity was performed as previously described elsewhere (Esumi et al., 1972) using 40 µM 2,6-dichloroindophenol (DCPIP, C₁₂H₇Cl₂NO₂) as the substrate, with or without the NQO1 inhibitor dicoumarol (C₁₉H₁₂O₆, 20 µM final concentration), and the cofactor NADPH at a 200 µM final concentration. Protein levels were 0.2 mg in the well. The reaction absorbance was monitored at λ = 600 nm, every 5 seconds for 5 minutes, and the extinction coefficient ε = 21 mM⁻¹ cm⁻¹ was used for calculating specific activities. All reactions were monitored for the same length of time (5 minutes) within the 5-minute window, consisting of a linear regression containing 36 data points. During this time less than 10% of substrate was metabolized. Positive controls were a pooled (n = 200) adult human S9 liver samples and showed intraday coefficient of variation 8.1% and interday 10.2%.

Pharmacokinetic Modeling and Scaling. To evaluate hepatic clearance, we modeled the enzyme kinetic modeled using both the well-stirred (eq. 1) and the parallel tube (eq. 2) equations:

\[ CL_{\text{hepatic}}(\frac{h}{l}) = \frac{Q_{\text{hepatic}} \times f_e \times CL_{\text{int}}}{Q_{\text{hepatic}} + f_e \times CL_{\text{int}}} \] (eq. 1)

\[ CL_{\text{hepatic}}(\frac{h}{l}) = \frac{Q_{\text{hepatic}}}{1 - e^{\left(-\frac{f_e \times CL_{\text{int}}}{Q_{\text{hepatic}}}\right)}} \] (eq. 2)

The intrinsic clearance \( CL_{\text{int}}(V_{\text{max}}/K_{\text{m}}) \) was calculated with experimental \( V_{\text{max}} \) (maximum rate of reaction) and the published \( K_{\text{m}} \) (substrate concentration at half maximum velocity) for DCPIP of 88 µM (Preussch et al., 1991). Hepatic blood flow (\( Q_{\text{hepatic}} \)) of 901 h⁻¹, an average adult liver size of 1500 g, and the cytosol conversion of 80.7 mg/g of liver (Cubitt et al., 2011) were used.

The unbound fraction of DCPIP in the incubation (\( f_{\text{inc}} \)) was determined experimentally with equilibrium dialysis. Briefly, Micro Float-A-Lyzer dialysis devices (Spectrum Laboratories, Rancho Domínguez, CA) with 10 K molecular mass cutoff and 100-200 µl capacity were used, as per the manufacturer’s instructions. The assay solution (200 µl) consisting of heat-inactivated cytosol, buffer, bovine serum albumin, DCPIP, and NADPH was dialyzed against 2500 volumes (0.5 l) of buffer for 18 hours with continuous stirring. The cytosol used was pooled from 10 individual adults, randomly taken from our archive. The demographics of the pool were three female, seven male: eight Caucasian, one African American, and one Hispanic, age range 20 to 87 years (mean 56.1 ± 26.4 years). The final concentration of protein was 0.2 mg. Three dialysis experiments were performed, with each experiment assayed in triplicate using a spectrophotometer ( Molecular Devices, Sunnyvale, CA) at λ = 600 nm. The comparison was made to the optical density of the same incubation with DCPIP freshly added. The \( f_{\text{inc}} \) was defined as the optical density of DCPIP added to the incubation at time 0 minus the optical density of solution dialyzed for 18 hours divided by the optical density at time 0. The \( f_{\text{inc}} \) for DCPIP was 0.595 ± 0.14 with an intraexperiment coefficient of variation of 8.99% and interexperiment coefficient of variation of 23.8%.

To scale for hepatic clearance in children, both allometry and physiologically based pharmacokinetic (PBPK) analyses were used. Equation 3 shows the allometric model used to predict \( CL_{\text{pediatric}} \):

\[ CL_{\text{pediatric}}(\frac{h}{l}) = CL_{\text{hepatic}} \times \left(\frac{W_c}{W_{\text{std}}}\right)^3 \] (eq. 3)

where \( W_c \) is the weight of the child and \( W_{\text{std}} \) is the standard average weight of an adult (70 kg) (EFSA Scientific Committee, 2012).

The activity data were also analyzed using PBPK models derived from SimCYP Pediatric (Certara, St. Louis, MO). For pediatric samples, a previously published model (Miyagi and Collier, 2011) was used with exceptions. The present model differs from the published structure due to the inclusion of a pediatric body surface area (BSA) calculation (eq. 4) to account for obesity (Johnson et al., 2005; Johnson et al., 2006). As DCPIP is a weak acid, binding to human serum albumin was assumed, and the unbound fraction for DCPIP was modified by pediatric albumin levels using eq. 8 where \( P_{\text{BSA}} \) is 44 g/l (McNamara and Alcorn, 2002). The \( CL_{\text{int}} \) was calculated for both pediatric and adult models using eq. 9 with the experimental \( V_{\text{max}} \) divided by \( K_{\text{m}} \) and scaled using the cytosolic protein per gram of liver (CPPGL) of 80.7 mg/g of liver (Cubitt et al., 2011) and liver volume (eq. 5). The adult cytosolic protein value was used for pediatric samples because a pediatric scalar for cytosolic protein per gram of liver is not currently available. Pediatr \( Q_{\text{hepatic}} \) was calculated using eq. 6 (Miyagi and Collier, 2011).
Ontogeny of Hepatic NQO1

NQO1 Protein Determination. The protein expression of NQO1 was determined by Western blot analysis (Fig. 1A) in 108 samples and resulted in a median of 1.41 mean pixel density normalized to pooled S9 liver sample, and ranged from no detection (n = 2) to 15.9 mean pixel density. When the data were analyzed with age as a continuous variable, there was a statistically significant negative correlation between age and protein expression (Spearman r = −0.2, P = 0.03, Fig. 1B). This was primarily driven by the adult population (21–64 years) with a statistically significant negative correlation (Spearman r = −0.34, P = 0.005), but no correlation was observed for children or the elderly. These data suggest that age accounts for 4% of the variation in overall data but 12% in the adult age group.

When the individuals were grouped into children (≤20 years), adults (21–64 years), and elderly (≥65 years), no statistically significant differences were observed between the groups (Fig. 1C). Categorizing “children” as under the age of 21 is in compliance with U.S. National Institutes of Health guidelines, although the World Health Organization generally categorizes children as 18 or under. There were only two individuals over the age of 18 (aged 19 and 20); if these two individuals were excluded from the analysis, the outcomes did not change significantly. Therefore, to best fit the widest definition of “pediatric” and increase power, 20 years and under was used throughout.

There were also no detectible differences measured between sexes or ethnicities (Fig. 1, D and E). A statistically significantly lower expression of NQO1 was detected in obese adults compared with ideal weight (P < 0.05) or overweight individuals (P < 0.01, Fig. 1F). It is not appropriate to include children in the analyses as their categories use percentiles instead of BMI to assign a weight group (see Materials and Methods). When children were analyzed separately there were

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Population demographics</th>
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<tbody>
<tr>
<td>Age &amp; Group (range, yr)</td>
<td>n (Mean ± S.D.)</td>
</tr>
<tr>
<td>Population: 0.04–87</td>
<td>117 (41.3 ± 23.8)</td>
</tr>
<tr>
<td>Asian</td>
<td>11</td>
</tr>
<tr>
<td>Native Hawaiian</td>
<td>4</td>
</tr>
<tr>
<td>African American</td>
<td>9</td>
</tr>
<tr>
<td>Hispanic</td>
<td>8</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>2</td>
</tr>
<tr>
<td>Pediatric: ≤20</td>
<td>29 (7.6 ± 7.2)</td>
</tr>
<tr>
<td>Native Hawaiian</td>
<td>2</td>
</tr>
<tr>
<td>African American</td>
<td>6</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>1</td>
</tr>
<tr>
<td>Adult: 20–64</td>
<td>71 (47 ± 11)</td>
</tr>
<tr>
<td>Asian</td>
<td>11</td>
</tr>
<tr>
<td>Native Hawaiian</td>
<td>1</td>
</tr>
<tr>
<td>African American</td>
<td>3</td>
</tr>
<tr>
<td>Hispanic</td>
<td>5</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>1</td>
</tr>
<tr>
<td>Caucasian</td>
<td>16</td>
</tr>
<tr>
<td>Native Hawaiian</td>
<td>1</td>
</tr>
<tr>
<td>African American</td>
<td>3</td>
</tr>
<tr>
<td>Hispanic</td>
<td>5</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>1</td>
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U test (binary outcomes). Fitting of the Gaussian curve was performed under medium criteria where iteration occurs up to 1000 times until the fit moves by less than 0.001%. The curve fit was unweighted and unconstrained. For data with continuous variables, a line was fitted using linear least-squares regression with 95% confidence intervals (dotted lines). Comparison of model fits for preferred model was with F tests and Akaike’s informative criteria (AIC).
no statistically significant differences between groups within the child population, although a trend of higher protein levels in both underweight and overweight children compared with ideal weight was noticed (Fig. 1G).

**NQO1 Activity.** Analysis of NQO1 activity indicates no statistically significant differences between children (≤20 years), adults (21–64), or the elderly (≥65, Fig. 2A), with a median (range) of 2.95 (0.79–20.51), 4.11 (1.71–19.05), and 3.69 (1.37–13.14) nmol/min/mg protein, respectively. Using age as a continuous variable, there were also no correlations of NQO1 activities with age for children, adults, the elderly, or the entire population. If all adults (20–89) are included in the analysis, a modest but statistically significant correlation of declining NQO1 activities with age is observed (r = −0.29, P = 0.01), and, although significant, age only accounts for 8% of the observed variability in this age bracket.

Although some evidence is present to suggest that the activity of NQO1 may decline in old age, when a plateau-to-1-phase decay model (loss of activity at some age) was compared with a horizontal line (no change in NQO1 activity from 13 days to 89 years) for these data, both the F test (F = 1.068) and AIC with correction for finite sample size (AICc = −3.165) rejected the decay model in favor of a horizontal line (Fig. 2B). Therefore, we contend that after birth, NQO1 biochemical activities are constant with age throughout life. The amount of NQO1 protein detected by Western blot analysis correlated significantly with NQO1 activity (Spearman’s r = 0.47, P < 0.0001; Fig. 2C), and 22% of the variation in activity is accounted for by variation in NQO1 specific content.

Further study of the distribution frequency of NQO1 activities in this population shows a similar skewness in the data toward individuals with higher activities, which is apparent in the column graph comparisons in Figs. 1 and 2. The curve-fit for the population distribution (Fig. 3) has an amplitude of 21.48 ± 1.438 individuals, which is in very good agreement with the maximum raw frequency of 28 individuals. The curve
fit is considered very good-to-excellent with $R^2 = 0.93$, and a runs test indicated that the residuals did not deviate significantly from the model.

Moreover, when assessing the goodness of fit, the curve-fit identified the high frequency of the bin 3.0–3.9 nmol/min/mg protein as an outlier to the fitted curve with apparently higher numbers of individuals than ought to occur. This demonstrates that the frequency analysis is sensitive for detecting polymorphisms conferring slow metabolizer phenotypes (which should occur in around 12 individuals in this cohort based on published data, see Discussion). The model was adjusted to eliminate the effect of the outliers on fit and returned a definite positive skew to an extensive metabolizer phenotype.

The mean activity score in the population was $3.48 \pm 1.62$ nmol/min/mg protein. Using the fitted averages there are 18 individuals with activities $>4$ S.D. of the mean (representing 15% of the population; $P = 0.006$). This implies that the positive skew/extensive metabolizer phenotype is real, and occurs at equal or greater frequencies than genetic polymorphisms. We speculate this is due to environmental responsiveness of NQO1 (as will be discussed).

Sex was not associated with differences in NQO1 activity in children, adults, or the elderly, nor when all age groups were combined. However, with regards to ethnicity the activity of NQO1 was statistically significantly lower in Asian as compared with Caucasian adults ($P < 0.05$; Fig. 4A), and it approached significance in Asians as compared with all other ethnic groups. No statistically significant differences between individual ethnic groups were observed for children (Fig. 4B), likely due to lack of power. When children and adults were combined, the Kruskal-Wallis test returned a result that ethnicity was a statistically significant source of variability ($P = 0.04$), but no individual group was significantly different compared with each other using post hoc analysis (Fig. 4C).

For measures of obesity, underweight was associated with greater NQO1 activities in adults compared with the ideal weight, overweight, and obese categories although this was not statistically significant due to the small sample size (underweight $n = 3$; Fig. 4E). In children, when NQO1 activity was categorized into underweight ($<5$th percentile BMI for age), ideal weight (5 to 85th percentile BMI for age), or overweight ($>85$th percentile BMI for age), overweight children had a statistically significantly higher NQO1 activity than the ideal weight children (Mann-Whitney $U$ test, $P = 0.037$; Fig. 4F). It is not appropriate to combine adult and pediatric data sets for further analysis because the method of calculating obesity status differs between the two groups.

**Pharmacokinetics Modeling of NQO1.** Using allometric modeling and DCPIP as the substrate for NQO1, the maximum mean clearance reached 4.36 ± 0.37 l/h, and the predicted age of NQO1 maturity is 23 ± 1.8 years (Fig. 5A). A one-phase exponential growth curve fit better than a sigmoidal curve or straight line, with an $R^2 = 0.116$, absolute sum of squares = 1074, and sum of squares = 3.06. The well-stirred and parallel tube model produced near identical results, only differing after the third decimal place.

The PBPK analysis using the well-stirred model predicted a maximum clearance of 4.055 ± 0.307 l/h, and the predicted age of NQO1 maturity at 26.0 years of age (Fig. 5B). A one-phase exponential association curve was used with an $R^2 = 0.296$, absolute sum of squares = 407, and sum of squares = 2.082. Although the PBPK parallel tube model predicted a maximum clearance of 4.175 ± 0.322 l/h, with the predicted age of NQO1 maturity at 24.9 years (one-phase exponential association curve with $R^2 = 0.269$, absolute sum of...
squares = 470, and sum of squares = 2.238). The models tested for maturation were line, sigmoidal curve, and one-phase exponential association, with one-phase exponential association being the best fit according to AICc \( \Delta AICc = 16.74, \) with 99.98% probability it is correct; parallel tube, exponential compared with line, \( \Delta AICc = 15 \) with 99.95% probability). The sigmoidal curve was ambiguous, and no comparative statistical data could be generated.

Even though we contend that NQO1 is a class II developmental enzyme biochemically, it would appear that the combination of biochemical and physiologic development serves to delay complete maturation of hepatic clearance through the NQO1 pathway.

**Discussion**

The data presented herein demonstrate that activity of the enzyme NQO1 does not appear to be developmentally regulated, with levels of hepatic activity as high in neonates and children as they are in adults. Under the classification system originally proposed by Greengard (1969, 1977), where class I is “higher in fetal life;” class II is “constant throughout life;” and class III is “develops after birth,” we suggest that NQO1 be classified as a class II ontogenetic enzyme in the human liver. Notably, Greengard (1969, 1977) primarily described and investigated metabolic enzymes involved in biochemical pathways such as gluconeogenesis and glycolysis, with very few studies on xenobiotic-metabolizing enzymes. Her classifications primarily revolved around cross-species comparisons.

For the highly conserved enzymes of endogenous biochemical metabolism, it is unsurprising that considerable cross-species similarities or identical functions/roles/regulation occur. However, it is recognized that for enzymes considered to be primarily xenobiotic metabolizing considerable evolutionary divergence has occurred after speciation. This has resulted in, for example, certain cytochrome P450 enzymes (e.g., CYP3A4, 3A5, and 3A7) being expressed in humans but not other species such as rodents (Gonzalez and Nebert, 1990). Specifically with respect to NQO1, all vertebrates carry the NQO1 gene, and this produces the same or a strikingly similar protein with quinone oxido-reductase

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**Fig. 4.** NQO1 activity compared with ethnicity and weight. The activity of NQO1 was determined by DCPIP metabolism. Activity was compared with (A) ethnicity of adult population, (B) ethnicity of the pediatric population, (C) ethnicity of the whole population, and (D) weight as a continuous variable for adults. The dotted line represents the 95% confidence interval. (E) Weight categories for adults and (F) weight categories for children (horizontal lines = median). *\( P < 0.05, \) Mann-Whitney \( U \) test. AA, African American; IW, ideal weight; NH, Native Hawaiian; OB, obese; OW, overweight; UW, underweight.

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**Fig. 5.** Pharmacokinetic modeling of NQO1 with DCPIP. Using (A) allometric and (B) PBPK scaling the hepatic clearance was determined for DCPIP. Well-stirred (\( u \), continuous line) and parallel tube (\( s \), dashed line) analyses are shown.
functions that can be readily up-regulated under conditions of oxidative stress as is constitutively part of the Ah-Receptor battery (Vasiliiou et al., 2006).

Despite the presence of true NQO1 orthologs across species and the undoubted identical cross-species effects of hypoxia, species-specific genetic regulation as well as some differences in environmental activation/suppression based on nuclear receptor and signaling factor presence would be expected. Hence, the early Greengard classification system with its pan-species applicability in development may be considered too simplistic for xenobiotic metabolizing enzymes. With respect to this point, a recent review by Hines (2008) has suggested that the classification system itself (classes I, II, and III) can also be applied to describe the ontogeny of xenobiotic-metabolizing enzymes in humans. Although cross-species comparisons are almost certainly doubtful, the classification system and its descriptors seem, at least to us, to be appropriate as long as the species of interest is clearly noted.

Another interesting finding was that in the adult population there were significantly lower protein levels of NQO1 in obese individuals compared with ideal weight and overweight adults although activity did not differ. There was no difference in protein expression between ideal weight and overweight adults, which was somewhat surprising as it suggests only extremes (BMI >30) inhibit NQO1 expression. Conversely, we detected higher NQO1 activity and protein in overweight children as compared with ideal weight children. Comparing the data between children and adults is not appropriate because overweight and obesity are defined differently in these two populations. A larger data set would be needed to determine the mechanism by which higher hepatic NQO1 protein and activity levels occur in overweight children. Such data could be particularly useful to the clinicians and researchers who focus on pediatric cancer, nonalcoholic fatty liver disease, and oxidative stress. Recent studies have shown a link between obesity and oxidative stress in humans and mice (Cheng et al., 2008; Chartoumpekis et al., 2013; Martinez-Hernandez et al., 2015), and this may be one of the reasons for higher expression of NQO1 in overweight children.

One plausible explanation for the variability in our findings is that NQO1 is a polymorphic enzyme with low-activity variants (NQO1*2 and NQO1*3) that result in a slow metabolizer phenotype. The finding that Asians (here “Asian” represents Japanese, Filipino, Korean, and Chinese descent) have significantly lower NQO1 activity than Caucasians likely relates to a high prevalence of low-activity NQO1 polymorphisms in the Asian population. Previous studies have variously demonstrated that Chinese people have up to 29% slow metabolizer profile (Gaedigk et al., 1998), whereas a 24% prevalence of the slow metabolizer phenotype has been reported in a mixed Asian population (Kelsey et al., 1997). Similarly, the prevalence of the slow metabolizer phenotype has been reported at only 5% in Caucasians, thereby supporting the significant activity differences observed with ethnicity (Kelsey et al., 1997; Gaedigk et al., 1998).

That we have not genotyped the livers used here for NQO1 polymorphisms is an acknowledged shortcoming of the study. However, as detailed earlier, we have been able to use mathematical population-based analysis and published genotype/phenotype frequencies to determine the relative contribution of the slow metabolizer phenotype to our findings. Additionally we are able to tease out an apparent “extensive metabolizer” subset of individuals who we know cannot be polymorphic, which probably represents environmental plasticity in NQO1 in this population. Although the effects of NQO1 slow metabolizer polymorphisms/phenotypes have been extensively studied in the context of susceptibility to cancer and effects on drug metabolism, demonstrating environmental mechanisms for enzyme plasticity in human tissues is far more rare (David et al., 2003; Guha et al., 2008; Goodrich et al., 2009; Han et al., 2009; Martin et al., 2009; Vijayakrishnan and Houlston, 2010; Kolesar et al., 2011; Tsvetkov et al., 2011; Li and Zhou, 2014; Lin et al., 2014).

Based on the published data cited here, the maximum number of slow metabolizers expected in this cohort is 12, comprising four Caucasians (out of 82), four Asians (out of 12), two Hispanics (out of eight), and one African American (out of 9). Hence, poor metabolizers are unlikely to account for all of the skewness in the data presented. Visually, Fig. 4 shows a significant number of extensive metabolizers far to the right of the frequency curve peak, causing skewness. The number of individuals demonstrating extensive NQO1 activity is almost twice the number of expected (via the literature) and observed (via population fit). Because the extensive metabolizer phenotype did not localize to a single age, ethnicity, sex, or weight group, we do not believe that demographic variables and/or underlying genetics are driving the extensive metabolizer phenotype. Rather, we postulate that the data presented are reflective of up-regulation of NQO1 in response to environmental stimuli, as has been previously demonstrated with Ah-receptor ligands, the antioxidant response element, and hypoxia-inducible-1α in several tissue types (Waleh et al., 1998; Collier et al., 2006; Jetten et al., 2014). These data therefore provide interesting insights into gene-environment interactions of hepatic NQO1.

Allometric and PBPK modeling were used to investigate maturity in hepatic clearance via the NQO1 pathway by using DCPIP as a probe substrate. The maximum predicted clearance was not markedly different between well-stirred and parallel tube equations for either allometry or PBPK, but allometric models predict a slightly higher clearance rate of 4.36 l·h⁻¹ with both equations as compared with approximately 4.06 l·h⁻¹ for well-stirred and parallel tube, respectively, returned by PBPK modeling. The predicted age of maximum clearance was 23 years for allometry (both equations). In the PBPK simulation, the age of clearance maturation was 26.0 and 24.9 years in the well-stirred and parallel tube equations, respectively.

Both models predict clearance maturation at the unrealistically late age of >23 years, which is not supported by the early detection of NQO1 protein and activity demonstrated herein. This result would suggest that physiologically based liver development is a significant contributing factor to hepatic clearance, more than biochemical maturation of NQO1. In the PBPK model the adults were also modeled to take into account body surface area and thus should alleviate differences due to obesity. Hence, PBPK is likely a more appropriate and possibly more accurate prediction tool than simple allometry (R² of 0.3 compared with 0.1, respectively), as others have suggested previously (Jones et al., 2006). Also of note is that the allometric and PBPK models diverged greatest in individuals with high enzymatic activity. This is as expected as the parallel tube model takes into account metabolism of the drug as it passed through a tube, or tissue, and continuously reducing the concentration whereas the well-stirred is an instantaneous metabolism model. Thus, the parallel tube predicted slightly higher clearance rate (4.18 to 4.06 l·h⁻¹), which would be expected with higher metabolic turnover.

Although DCPIP is not used clinically, these data have relevance as a proxy measure for the development of the NQO1 metabolic pathway, which may affect the efficacy and toxicity of a number of anticancer drugs as well as the function(s) of the innate antioxidant defense network. The specific compound DCPIP has been investigated as an anticancer agent with effects in a number of human cancer cell lines, including colon cancer (Cabello et al., 2011), breast cancer (Mondalek et al., 2010), and melanoma (Cabello et al., 2009). Additionally, the growth of breast cancer and melanoma xenografts in severe combined immunodeficiency-mice could be arrested by DCPIP treatment (Cabello et al., 2009; Cabello et al., 2011). In these studies, NQO1 was activating the compound and causing cytotoxicity to the tumor. Additionally, the
DCPIP analog EO9, which is also an analog of mitomycin C, has come to market for use in bladder cancer with the trade name Apaziquone (Phillips et al., 2013). This demonstrates that the NQO1 pathway is still of interest, although primarily for activating produgs in chemotherapy.

In conclusion, we propose that hepatic NQO1 be classified as a class II developmental enzyme—stable from neonatal to adult life biochemically. However, in terms of hepatic clearance, which combines physiologic and biochemical development, the NQO1 pathway may take longer to approximate adult levels than would be predicted by biochemistry alone. Our other major finding is that the effects of obesity on NQO1 expression and activity in children should be further studied, particularly with respect to fatty liver disease and oxidative stress. It would appear that obesity increases NQO1 protein levels and activities in children but not adults and that the mechanisms of these effects on liver function, as well as on drug and chemical metabolism, are deserving of greater study.

**Authorship Contributions**

**Participated in research design:** Rougée, Collier.

**Conducted experiments:** Rougée, Berman, Colliver.

**Performed data analysis:** Rougée, Riches, Collier.

**Wrote or contributed to the writing of the manuscript:** Rougée, Riches, Berman, Colliver.

**References**


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