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Urinary Porphyrin Excretion in Children with Mercury Amalgam Treatment: Findings from the Casa Pia Children’s Dental Amalgam Trial

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Increases in the urinary concentrations of pentacarboxyl- and coproporphyrins and the appearance of the atypical precoproporphyrin have been defined in relation to mercury (Hg) body burden in animal studies, and this change in the porphyrin excretion pattern has been described as a biomarker of occupational Hg exposure and toxicity in adult human subjects. In the present studies, urinary porphyrins were determined in relation to Hg exposure in children and adolescents, 8–18 yr of age, over the 7-yr course of a clinical trial designed to evaluate the neurobehavioral and renal effects of dental amalgam in children. Subjects were randomized to either dental amalgam or composite resin treatments. Urinary porphyrins and creatinine concentrations were measured at baseline and annually in all subjects. Results were evaluated using linear regression analysis. No significant differences between treatment groups (amalgam versus composite) were found when comparing all subjects for any of the porphyrins of interest. However, incipient amalgam treatment-specific increases were observed in the mean concentrations of penta-, precopropo- and coproporphyrins especially when the analyses were restricted to younger subjects (8 to 9 yr old at baseline), and these increases were most apparent during 2 through 3 of follow-up, the period of highest mercury exposure from amalgam treatment. Based on the mean number of amalgam fillings received by children in this group (17.8), the renal Hg concentration associated with incipient increases in urinary porphyrins was estimated to be approximately 2.7 µg/g renal cortex. This value corresponds to an observed mean urinary Hg concentration of 3.2 µg/g creatinine, which is approximately fivefold less than that at which renal damage from Hg exposure is estimated to occur in children. These findings are consistent with growing evidence supporting the sensitivity of urinary porphyrins as a biological indicator of subclinical Hg exposure in children.

Porphyrins are formed as intermediates in the biosynthesis of heme, a process that proceeds in essentially all eukaryotic tissues. In humans and other mammals, porphyrins with eight, seven, six, five, and four carboxyl groups are commonly formed in excess of that required for heme biosynthesis and are excreted in the urine in a well-established pattern (Bowers et al., 1992; Woods et al., 1993). In previous studies specific changes in urinary porphyrin excretion patterns (porphyrin profiles) associated with prolonged exposure of both animals and humans to mercury (Hg) and other metals were described (Woods & Fowler, 1977, 1978; Fowler et al., 1987). The etiology of these changes was shown to involve both metal-induced inhibition of specific heme pathway enzymes in target tissues and metal-facilitated oxidation of reduced porphyrins that accumulate in tissue cells because of impaired porphyrin metabolism (Woods et al., 1990; Miller & Woods, 1993; Woods, 1995). Changes in porphyrin excretion patterns are largely metal specific, correlate with metal concentrations in tissue cells, and occur prior to the onset of target tissue injury. These findings support the potential utility of porphyrin profile measurements as biomarkers of metal exposure and toxicity in human subjects (Marks, 1985; Woods, 1995).

Of particular interest to the investigation of metal-mediated changes in porphyrin metabolism are findings from studies in methylmercury (MeHg)-exposed rats demonstrating highly specific changes in the urinary porphyrin excretion pattern due primarily to Hg-induced alterations of heme biosynthesis in the
kidney (Pingree et al., 2001). These changes are characterized by dose- and time-related increases in urinary concentrations of pentacarboxyl (5-carboxyl) and copro (4-carboxyl) porphyrins and also by the appearance of “precoproporphyrin,” an atypical porphyrin (molecular weight [mw] = 668) that elutes on high-performance liquid chromatography (HPLC) prior to coproporphyrin (mw = 655) (Woods et al., 1991). Precoproporphyrin appears to be specific to Hg exposure, and its mechanistic etiology in this regard was previously described (Woods et al., 2005; Heyer et al., 2006). Studies in human subjects with occupational exposure to elemental mercury (Hg0) vapor demonstrated responses precisely comparable to those observed in MeHg-treated animals, attesting to the utility of porphyrin profiles as a specific measure of Hg exposure in either organic or inorganic (elemental) form. The sensitivity of porphyrin changes as a measure of Hg exposure and body burden was also described (Pingree et al., 2001). Notably, statistically significant increases in the concentrations of penta-, precoopro-, and coproporphyrins were demonstrated in animals with renal cortical Hg concentrations as low as 14 µg/g (Woods et al., 1991), nearly fourfold less than the renal Hg concentration at which significant changes in more conventional bioindicators of mercury exposure in humans were reported (Buchet et al., 1980; Rosenman et al., 1986).

Studies of urinary porphyrins in children with well-defined, prolonged Hg exposure have not been reported. Here, urinary porphyrin concentrations in children 8 to 18 yr of age, with and without dental amalgam fillings, were determined over the course of a recently completed clinical trial that was designed to evaluate the potential health consequences of prolonged exposure to Hg from dental amalgam fillings (DeRouen et al., 2006).

Materials and Methods

The Study Population

The children’s cohort was derived from the recently completed Casa Pia clinical trial of the health effects of dental amalgam fillings in children (DeRouen et al., 2006). Subjects (n = 507) were boys and girls (54:46%), 70% white, aged 8–12 yr at inception, who were residents of the Casa Pia school system in Lisbon, Portugal. Eligibility requirements precluded children with preexisting neurological or developmental disabilities. Subjects were initially randomized to Hg amalgam (treatment) or composite resin (control) dental treatment groups. Children were evaluated at baseline and at seven subsequent annual intervals after initial dental treatment with an extensive battery of neurobehavioral, neurological, renal function, and urinary porphyrin assessments. Follow-up data were obtained on a similar number of subjects in each treatment group. The study protocol was approved by the institutional review boards at the University of Washington and the University of Lisbon.

All parents or guardians gave written consent, and all children provided signed assent. Principal design and analytical issues involved in this trial (DeRouen et al., 2002) as well as principal outcome measures (DeRouen et al., 2006) have been reported.

Procedures for Urine Collection and Measurement of Urinary Porphyrins and Creatinine

A urine sample (~50 ml) was collected from each child at baseline and at each subsequently scheduled annual visit to the University of Lisbon School of Dental Medicine for dental, neurological, and neurobehavioral evaluations. Immediately following urine collection, a 10-ml aliquot was removed and acidified with 1 N HCl for use in Hg analysis by continuous-flow, cold-vapor spectrophotometry (Woods et al., 2007). Porphyrins were quantitated in the remaining unacidified portion of the urine sample by high-performance liquid chromatography, as previously described (Woods et al., 1991; Bowers et al., 1992). Urinary creatinine concentrations were also measured in unacidified urine using a standard colorimetric procedure (Sigma number 555-A).

Statistical Analyses

Urine samples with uro- or coproporphyrin concentrations less than 0.2 µg/L were deemed a consequence of analytical error and excluded from the analysis. Concentrations of other porphyrins that were below the limit of detection in those samples that had acceptable uro- and coproporphyrin values were set at 0.05 µg/L so that a positive value for these porphyrins could be included in the log transformations for computation of geometric means. Such samples comprised less than 5% of the total, and this approach did not introduce a significant bias in the analysis. Analyses were performed on log-transformed porphyrin concentrations, as is common practice when analyzing concentration data, to account for positive skewness and het-
eroskedasticity (variances increased with increasing mean value). Mixed models were used to adjust log-transformed porphyrin/creatinine ratios for the baseline log-transformed porphyrin/creatinine ratio and to account for inter-individual variation in porphyrins. The resulting geometric means of adjusted porphyrin values were plotted on graphs as depicted in the figures presented herein. Separate linear regression models were used for each porphyrin to test whether treatment group assignment (amalgam or composite) had an effect on average log transformed urinary porphyrin (µg/L) during follow up (1–7 yr). Each regression model adjusted for age (quadratic), gender (male/female), race (white/non-white), follow-up year (quadratic), log-transformed urinary creatinine (g/L), and baseline log-transformed porphyrin/creatinine ratio (µg/g). Robust standard errors were used to account for possible within-child correlation not accounted for by adjustment variables.
RESULTS

The demographic characteristics of subjects were similar in the two treatment groups, as previously reported (DeRouen et al., 2006; Woods et al., 2007). Baseline mean urinary Hg levels were 1.8 µg/g creatinine in the amalgam group and 1.9 µg/g creatinine in the composite group. Changes in urinary Hg and creatinine concentrations over the course of the study were previously described (Woods et al., 2007; Martin et al., 2008). In summary, mean urinary Hg concentrations in the amalgam group increased to a peak of approximately 3.2 µg/g at yr 2 and then slowly declined to near baseline levels by yr 7 of follow-up. In contrast, no changes in the mean urinary Hg levels were observed in the composite group during the 7-yr follow-up period. Mean urinary creatinine concentrations increased as a function of age among all study participants irrespective of treatment, from approximately 0.8 to approximately 1.5 g/L over the course of follow-up.

Table 1 presents demographics at baseline (BL) and at each year of follow-up for all study subjects for whom porphyrin analyses were available (n = 479). Notably, subjects at baseline ranged in age from 8 to 12 yr, with mean ages of 10.2 and 10 yr among those randomized to amalgam and composite groups, respectively. Table 2 presents demographics at baseline and at each year of follow-up when subjects were restricted to 8- and 9-yr-olds at baseline (n = 195). The mean number of cavities among all subjects in the amalgam group at baseline was 15.6 and among 8- to 9-yr-olds in the amalgam group at baseline was 17.8.

Creatinine-adjusted geometric mean urinary porphyrin concentrations for all participants at baseline and for each year of follow-up are shown in Figure 1. When comparing all subjects for the porphyrins of interest, no effects of amalgam treatment on the mean concentrations of uro- (8-carboxyl), hepta- (7-carboxyl), or hexa- (6-carboxyl) porphyrins were found. However, the mean concentrations of penta-, precopro-, and coproporphyrins were slightly elevated among amalgam-treated subjects, particularly at follow-up yr 3, the year corresponding to the highest urinary Hg concentrations (Woods et al., 2007). These effects became more pronounced when analyses were restricted to younger subjects (8-9 yr old at baseline). As shown in Figure 2, children who were 8 to 9 yr old at baseline showed treatment-specific increases in penta-, precopro-, and coproporphyrin that were most apparent during yr 2 through 3 of follow-up, compared with those in the composite group. None of these changes was statistically significant, however, owing to the small number of subjects in each comparison group.

Possible gender- and race-related differences in porphyrin excretion in children and adolescents with and without amalgam treatment were also evaluated. Findings showed comparable effects at all years of follow-up among boys and girls who were 8–9 yr at baseline, with amalgam treatment-specific increases in penta-, precopro- and coproporphyrin, the three porphyrins that are selectively sensitive to Hg exposure (not shown). No statistically significant interaction was observed between gender and follow-up year over time. Similarly, no significant effects of race (black versus white) on porphyrin excretion in children and adolescents with and without amalgam treatment were found.

DISCUSSION

Previous studies characterized changes in the urinary porphyrin excretion pattern in animals with prolonged exposure to MeHg and defined the quantitative association of urinary porphyrin levels with kidney Hg content and Hg body burden (Woods et al., 1991; Pingree et al., 2001). Additionally, the utility of urinary porphyrins as a biological indicator of prolonged Hg exposure and Hg body burden in adult human subjects with occupational exposure to mercury as Hg⁰ was previously described (Woods et al., 1993, 2005; Echeverria et al., 1995; Gonzalez-Ramirez et al., 1995). The present findings

<table>
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are the first to describe urinary porphyrin excretion among children with Hg exposure predominantly from dental amalgam fillings. Notably, dietary MeHg was found not to be a significant source of Hg exposure in this population (Evens et al., 2001). Whereas no significant changes in urinary porphyrin concentrations were apparent when comparing amalgam- and composite-treated subjects within the entire study cohort, it is of interest that subtle but distinct increases in the three porphyrins that are indicative of Hg exposure were evident, and became more apparent, when comparing treatment groups among the younger members of the study population (8 to 9 yr old at baseline), suggesting potentially greater Hg accumulation in the kidneys of younger children than occurs in older subjects as a basis of this incipient porphyrinogenic response. In this regard, Woods et al. (1991) previously reported a renal cortical total Hg (Hg^{2+} + CH_{3}Hg^{+}) concentration of approximately 14 µg/g, or a Hg^{2+} concentration of 4 to 6 µg/g, as the threshold at which significant increases in the urinary concentrations of the 3 porphyrins associated with Hg exposure are first observed in rats during prolonged exposure to methylmercury (5 or 10 ppm) in the drinking water. To estimate the contribution of amalgam to kidney Hg burden in these children as a basis for interpreting the observed incipient changes in urinary porphyrin levels, the World Health Organization (WHO) (1976) general equation described by Clarkson et al. (1988) was employed to estimate renal Hg content from amalgam fillings as a function of [daily mercury intake × f × t_{1/2}/ln2], where daily intake = 0.5 µg Hg/day/amalgam filling, f is the the fraction of daily dose deposited in the kidney (50% for children), and t_{1/2} is the half-life of Hg in the kidney (64 d) (Hursh et al., 1976). For a child with kidney mass ~150 g and 17.8 amalgam fillings (the mean number of carious surfaces filled in amalgam group children who were 8-9 yr old at baseline), the calculated renal total Hg concentration is 2.7 µg/g. This kidney Hg burden corresponds to the highest mean urinary Hg concentration of 3.2 µg/g creatinine observed in the present trial (Woods et al., 2007), which is approximately fivefold less than that at which renal damage from Hg exposure is estimated to be first observed in children.

FIG. 1. Creatinine-adjusted geometric mean urinary porphyrin concentrations for all participants at baseline and for each year of follow-up, corrected for baseline porphyrin/creatinine ratio and individual variation.
FIG. 2. Creatinine-adjusted geometric mean urinary porphyrin concentrations for subjects 8 and 9 yr of age at baseline and for each year of follow-up, corrected for baseline porphyrin/creatinine ratio and individual variation.

(de Burbure et al., 2003). Based on these computations as well as findings from animal studies described earlier, this incipient porphyrinogenic effect is interpreted as a highly sensitive biological response to Hg exposure at renal cortical concentrations considerably below those associated with tissue injury in children.

Elemental Hg is a well-established neurotoxicant (Echeverria et al., 1998; Coulter & Buchanan, 2004). In considering urinary porphyrins as a bioindicator of neurobehavioral effects of Hg, previous studies (Echeverria et al., 1995) reported that the urinary concentrations of the 3 porphyrins that are affected by Hg were highly correlated with cognitive and other neurological effects associated with chronic occupational Hg exposure among dental professionals in a study where urinary Hg levels differed by as much as 30 μg/g between Hg-exposed and unexposed groups. In the present study, urinary Hg levels differed by less than 2 μg/g between treatment groups at the peak of exposure (follow-up yr 2–3), and no significant differences were observed in any of the neurological or neurobehavioral parameters over the 7 yr of follow-up (DeRouen et al., 2006). The incipient porphyrinogenic response observed here is therefore interpreted as indicative of Hg exposure at levels below the threshold of that eliciting detectable neurological effects within the cohort of subjects evaluated here. Of note, also, is the possible contribution of genetic predisposition to atypically increased porphyrin excretion in response to low-level Hg exposure, as previously reported (Woods et al., 2005; Echeverria et al., 2006). The potential role of genetic susceptibility in the incipient porphyrinogenic response to Hg observed here is currently under investigation.

In conclusion, the present findings describe incipient increases in the urinary concentrations of porphyrins previously defined in association with Hg body burden, in children and adolescents with dental amalgam Hg exposure. These findings
attest to the sensitivity of porphin changes in relation to Hg exposure and may be useful within the context of risk assessment for low-level Hg exposure in children.

REFERENCES


