Measurement of vitamin D₃ metabolites in smelter workers exposed to lead and cadmium

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Abstract

Objectives—To investigate the effects of lead and cadmium on the metabolic pathway of vitamin D₃.

Methods—Blood and urinary cadmium and urinary total proteins were measured in 59 smelter workers occupationally exposed to lead and cadmium. In 19 of these workers, the plasma vitamin D₃ metabolites, (25-hydroxycholecalciferol (25(OH)D₃), 24R, 25-dihydroxycholecalciferol (24R,25(OH)D₃) and 1a, 25-dihydroxycholecalciferol (1a,25(OH)D₃)) were measured together with blood lead. Vitamin D₃ metabolites were measured by radioimmunoassay, (RIA), lead and cadmium by atomic absorption spectrophotometry, and total proteins with a test kit.

Results—Ranges for plasma 25(OH)D₃, 24R,25(OH)D₃, and 1a,25(OH)D₃ were 1.0–51.9 ng/ml, 0.6–5.8 ng/ml, and 0.1–75.7 pg/ml, respectively. Ranges for blood lead were 1–3.7 µmol/l, (21–76 µg/dl), blood cadmium 6–145 nmol/l, and urinary cadmium 3–161 nmol/l. Total proteins in random urine samples were 2.1–32.6 mg/dl.

Concentrations of lead and cadmium in blood showed no correlation (correlation coefficient -0.265) but there was a highly significant correlation between blood and urinary cadmium. Concentrations for 24R,25(OH)D₃ were depressed below the normal range as blood and urinary cadmium increased, irrespective of lead concentrations. High cadmium concentrations were associated with decreased plasma 1a,25(OH)D₃ when lead concentrations were <1.9 µmol/l and with above normal plasma 1a,25(OH)D₃ when lead concentrations were >1.9 µmol/l, Kruskal-Wallis analysis of variance (K-W ANOVA) \( \chi^2 = 10.3, p=0.006 \). Plasma 25(OH)D₃ was negatively correlated with both urinary total proteins and urinary cadmium, but showed no correlation with plasma 24R,25(OH)D₃, 1a,25(OH)D₃, blood lead, or blood cadmium.

Conclusion—Continuous long term exposure to cadmium may result in a state of equilibrium between blood and urinary cadmium. Cadmium concentrations in blood could be predicted from the cadmium concentration of the urine, (regression coefficient 0.35 SE 0.077). Exposure to cadmium alone decreased the concentrations of 1a,25(OH)D₃, and 24R,25(OH)D₃, whereas exposure to both cadmium and lead increased the concentrations of 1a,25(OH)D₃.

Keywords: vitamin D₃ metabolites; lead; cadmium; total proteins; blood; urine; smelter workers

This study sought to investigate the effect of exposure to the heavy metals lead and cadmium on the plasma concentrations of vitamin D₃ metabolites and to determine the effects of dual exposure. The metabolic pathway of vitamin D₃, cholecalciferol, involves the gut, liver, kidneys, and bones, all of which are affected by these metals.1,2 The liver and kidneys are target organs for lead and cadmium, so that a potential biochemical effect might be found in the vitamin D₃ pathway to explain the changes in bone metabolism found after exposure to lead or cadmium.1,3 Damage caused by cadmium to the renal tubules5 was considered especially relevant to the formation of the more active dihydroxy metabolites, 24R, 25-dihydroxycholecalciferol (24R,25(OH)D₃) and 1a, 25-dihydroxycholecalciferol, (1a,25 (OH)₃D₃).

In the human, vitamin D₃ is obtained either from the diet10 or is metabolised from 7-dehydrocholesterol by the epidermis in the presence of sunlight or ultraviolet light.11,12 The enzymatic conversion of vitamin D₃ to 25-hydroxycholecalciferol, 25(OH)D₃, takes place in the liver,13 whereas the second hydroxylation of 25(OH)D₃ to form the dihydroxy metabolites, 24R,25-(OH)₂D₃ and 1a,25(OH)₂D₃, occurs mainly in the kidney.14 It has been suggested that this second hydroxylation is catalysed by a single enzyme; however, earlier studies identified two membrane bound multiprotein enzyme complexes in the mitochondria of cells in the proximal convoluted and straight tubular sections of the nephron.15,16 Other sites postulated for this hydroxylation include the placenta17 and the epidermis, especially in the keratinocytes,18 which are extremely active in psoriatic patients.19

Absorption of calcium from the gut is increased by 1a,25(OH)D₃,20 whereas it has been shown that 24R,25(OH)D₃ does not affect this regulation of calcium uptake from the small intestine.21 Both metabolites are implicated in the turnover of calcium in bone, with 1a,25(OH)D₃ considered more
important in the control of osteoclasts,25 26 and 24R,25(OH)2D3 more effective on the osteoclasts.27 Dietary lead intake affects the absorption of calcium from the gut28 and interferes with bone formation.29 30 Calcium and lead compete for binding sites on mucosal proteins in the intestine,11 although more recent work by these authors suggested that the primary effect was on the cholecalciferol endocrine system, rather than the interaction of calcium and lead at the intestinal level.32 In children with increased lead absorption, a reduction in 1α,25 (OH)2D3 concentrations has been reported,33 which returned to normal once bone lead decreased to <30 µg/dl (1.4 µmol/l). It has been suggested that chronic cadmium exposure is linked with the subsequent appearance of osteomalacia and osteoporosis.19

In smelter workers with a known exposure to both lead and cadmium there was a possibility that if the vitamin D3 metabolic pathway was affected, this should be apparent in the plasma concentrations of the active dihydroxy metabolites. The effects of exposure to a combination of lead and cadmium were also investigated. In this study, the plasma concentrations of three metabolites of vitamin D3, 25(OH)D3, 24R,25(OH)2D3, and 1α,25 (OH)2D3 blood concentrations of lead and cadmium, and urinary concentrations of cadmium and total proteins were measured.

Materials and methods

BLOOD AND URINARY SAMPLES

During their routine health check, male workers in a large non-ferrous metal smelter took part in a survey of the effects of long term low level cadmium exposure.3 9 In the five year follow up study3 9 of these workers were available for retesting (follow up group). Blood samples from 19 of these participants were collected consecutively, (study group), were stored in conditions suitable for analysing vitamin D3 metabolites. These blood samples were collected into tubes containing lithium heparin anticoagulant, mixed, and stored at 4°C until assayed for vitamin D3 metabolites. The urine samples were collected into tubes containing lithium heparin anticoagulant, mixed, and stored at 4°C in light proof containers. Samples were divided into two; one batch was stored at −20°C until analysed for lead and cadmium, whereas the other was centrifuged and the plasma separated and stored in glass vials at −20°C, protected from light, until assayed for vitamin D3 metabolites. The urine samples were stored in acid washed plastic vials at −20°C. These were spot urine samples, not 24 hour collections, thus all urinary calculations are based on concentration rather than excretion rates. Apart from the difficulty of obtaining collections of 24 hour urine from these volunteers, the probability of cadmium contamination was a deciding factor in collecting spot samples.

CHEMICALS

Tritiated (H) vitamin D3 metabolites were obtained from Amersham International, Little Chalfont, Bucks, UK, Sephadex LH20 was from Pharmacia Biotech, St Albans, Herts, UK, 25(OH)D3, binding globulin was prepared in our laboratory from normal rat serum, 1α,25(OH)2D3 binding protein was kindly donated by Dr S Edelstein (Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel), and the control samples for metals in blood and urine were from Behring Diagnostics, Hounslow, Middlesex, UK. All other chemicals and reagents were from Merck BDH, Lutterworth, Leics, UK and were of AnalR grade unless stated otherwise.

VITAMIN D3 METABOLITES

Figure 1 shows the analytical procedures, which were based on the methods developed by Edelstein (personal communication). Extraction methods were based on the procedure described by Lidor et al. Trace amounts of all three tritiated metabolites were added to all samples to monitor the recovery of the metabolites throughout the procedure. An adjustable rack supplied by Ilacon, Tonbridge, Kent, UK, that held the vials in a water bath at 35°C while a stream of nitrogen was directed at each individual vial, was used to evaporate all samples to dryness when changing solvents or reducing sample volumes. For high pressure liquid chromatography (HPLC), a Waters M45 HPLC solvent delivery system was used with a µPorasil 27477 column, Waters, Watford, Herts, UK, calibrated with 25(OD)3, 24R,25(OH)2D3, H-24R,25(OH)2D3 and H-1α,25(OH)2D3, run separately to establish the positions of the three peaks. The eluting solvent was iso-propanol:hexane (1:9), degassed under vacuum.

The radioimmunoassay (RIA) was a modified protein binding method, with activated dextran/charcoal separation and 3H -25(OH)D3, or H 1α,25(OH)2D3. For 25(OH)D3, and 24R,25 (OH)2D3, the binding protein was vitamin D3 binding globulin from rat serum and for 1α,25(OH)2D3 cytosol from the duodenum of rachitic chicks was used. Samples, standards, controls, and blanks were dissolved in absolute ethanol, each sample was assayed in duplicate, and each standard in quadruplet. Barbitone buffer, pH 8.6, was used in the assay for 25(OH)D3, or 24R,25(OH)2D3, and TRIS-molybdate buffer, pH 7.4, for the 1α,25 (OH)2D3. Assays were left overnight at 4°C, dextran/charcoal suspension was added and after 30 minutes on ice, the assay samples were filtered into counting vials with an Ilacon filter unit. Radioactivity was measured with a Packard Tricarb β-counter, Packard Instruments, Caversham, Berks, UK. Standard curves were calculated with the data transformation, the sample concentrations were calculated, and the values were adjusted for percentage recovery. Matched volumes of a plasma pool used as a laboratory quality control were included in each run. The RIA for vitamin D3 metabolites had minimum detection limits of 0.5 ng/tube for 25(OH)D3, and 24R,25(OH)2D3, and 0.5 pg/tube for 1α,25 (OH)2D3. The working concentration ranges were 30–200 ng/ml for 25(OH)D3, and 24R, 25(OH)2D3, and 20–300 pg/ml for 1α,25 (OH)2D3, calculated from the relevant precision profile. Control ranges for vitamin D3 metabolites measured in this laboratory in adults with no known exposure to lead or cadmium were as follows, 25(OH)D3, 12.2–26.2 ng/ml, 24R,
BLOOD LEAD, AND BLOOD AND URINARY CADMIUM

Samples were analysed for lead and cadmium with an IL Video 11 spectrophotometer and IL 655 furnace atomiser with Fastac II autosampler, Thermo Electron, Warrington, Cheshire, UK. A single matrix matched calibration curve was used to determine concentrations and all samples were analysed in duplicate with two replicates per sample; reproducibility was <10% CV. Control blood samples for metals were used at two concentrations, lead 15 and 40 µg/dl, and cadmium 4.5 and 57.9 nmol/l, control urine samples for cadmium were used at three concentrations 30, 180, and 410 nmol/l. The laboratory participated in external quality assurance schemes for blood lead and for blood and urinary cadmium. The results obtained were as follows, Blood Lead Proficiency Testing, Centers for Disease Control, Atlanta, Georgia, State Laboratory of Hygiene, all concentrations were within target ranges; UK National External Quality Assessment Scheme (NEQAS) for lead and cadmium in blood, Queen Elizabeth Hospital, Birmingham, the mean running variance index score, (MRVIS) for lead was MRVIS=24, (MRVIS=44 for all participants), for cadmium MRVIS=23 (MRVIS=57 for all participants); trace element quality assessment scheme (TEQAS), Analytical Unit, Robens Institute, University of Surrey, UK, for blood lead and blood and urinary cadmium, all the cumulative scores (CUSUM) were above the acceptable concentration of CUSUM=20, blood lead CUSUM=85.8, blood cadmium CUSUM=71, urinary cadmium CUSUM=36.4.
URINARY TOTAL PROTEINS

These were measured with a BioRad total protein test kit, BioRad Laboratories, Watford, Herts, UK, based on Macart's modification of the Bradford method in which protein in the urine is bound to an acidified dye reagent (Coomassie brilliant blue (G250) dye). The observed change in absorbance was proportional to the protein concentration and the test was linear up to 150 mg/dl with a sensitivity of 1.1 mg/dl. Assays were carried out at room temperature in glass tubes and all measurements were in duplicate. Saline solution, 0.9%, was used as the reagent blank and extra control standards were prepared with a mixture of human albumin and globulin in a 1:1 ratio. Dye/protein colour was stable for 60 minutes and all samples were read within that time with a Varian Techtron spectrophotometer, series 634, Varian, Walton on Thames, Surrey, KT12 2QF, UK.

STATISTICAL ANALYSIS

The statistical package SPSS for Windows was used for all calculations. Frequency distributions were measured for all variables and log transformations used for skew variables. Correlation coefficients and regression analyses were calculated. The non-parametric Kruskal-Wallis one way analysis of variance (K-W one way ANOVA) was used to measure the relation between 1α,25(OH)2 D3 and cadmium with lead.

Results

Table 1 shows the concentrations obtained for plasma 25(OH)D3, 24R,25(OH)2D3 and 1α,25(OH)2D3, blood lead, blood and urinary cadmium, and urinary total proteins. For the follow up group, results for blood and urinary cadmium and urinary total proteins were obtained in 55 of the 59 cases. In the study group, a complete set of concentrations was obtained in all 19 cases. Table 2 shows a correlation matrix with significance levels for all variables.

Figure 2 shows the regression line for log blood cadmium and log urinary cadmium, the regression coefficients being +0.35 (SE 0.077) and the residual SD (in logs to the base 10) was 0.20, corresponding to a 95% prediction width of 2.5-fold. Of the 19 concentrations, 16 (84%) plasma 25(OH)D3 results were in the normal range, but only six (21%) of the 24R,25(OH)2D3 and six (31%) of the 1α,25(OH)2D3 results.

The K-W one way ANOVA showed significant differences in the plasma 1α,25(OH)2D3 concentrations, χ2=10.3, df 2, p=0.006, (fig 3), when these were grouped according to the following combination of blood lead and blood or urinary cadmium concentrations: group 1, low Pb/high Cd, blood lead <1.9 µmol/l with urinary cadmium >8 mmol/l or blood cadmium >27 mmol/l; group 2, raised Pb/low Cd, blood lead >1.4 µmol/l with urinary cadmium < 8 mmol or blood cadmium <27 mmol/l; group 3, high Pb/high Cd, blood lead >1.9 µmol/l with...
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Figure 3 The effect of lead and cadmium on plasma 1α,25(OH)₂D₃ concentrations. Error bars show mean and 95% confidence interval (95% CI). Group 1 = blood lead <1.9 µmol/l with urinary cadmium >8 nmol/l or blood cadmium >27 nmol/l; group 2 = blood lead >1.4 µmol/l with urinary cadmium <8 nmol/l or blood cadmium <27 nmol/l; group 3 = blood lead >1.9 µmol/l with urinary cadmium >8 nmol/l or blood cadmium >27 nmol/l.

Figure 4 The effect of cadmium on plasma 24R, 25(OH)₂D₃ concentrations. Error bars show mean and 1R,25(OH)₂D₃ concentrations as logscales for both axes.

Discussion

These results suggest that increased blood and urinary cadmium and blood lead catalyze perturbation of the conversion of 25(OH)D₃ to 24R,25(OH)₂D₃ and 1α,25(OH)₂D₃. In the presence of cadmium, lead seems to be associated with increased conversion of 25(OH)D₃ to 1α,25(OH)₂D₃. Unlike the effect in children, 32 in whom the conversion of 25(OH)D₃ to 1α,25(OH)₂D₃ may be reduced when the blood lead concentration is >1.4 µmol/l (>30 µg/dl), in these adults the conversion seemed to be enhanced as blood lead concentrations increased. The negative correlation found in children between blood lead and 1α,25(OH)₂D₃ from 25(OH)D₃ was not found in these adults.

The membrane bound enzyme complexes that catalyse the formation of 24R,25(OH)₂D₃ and 1α,25(OH)₂D₃ from 25(OH)D₃ are termed 25OH-D₃-24R-hydroxylase (24R-OHase) and OHD₃-1α-hydroxylase (1α-OHase) respectively. 44 They are found in the mitochondria of the cells lining the proximal convoluted tubules and the straight tubular segments of the nephron. Phosphorylation of an inactive phosphatase is necessary for the activation of the 1α-OHase, which catalyses the formation of 1α,25(OH)₂D₃ and a calcium dependant protein kinase converts this enzyme complex to the fully active 24R-OHase. It has been suggested that lead interferes with phosphate linked reactions and that perturbations of calcium mediated processes are an early effect of lead toxicity at the cellular level. 33 Cadmium induces osteomalacia by a direct action on bone through abnormal calcium homeostasis 46 and has an effect on bone repair by reduction of alkaline phosphatase activity in osteoblastic cells. 47

Lead is initially accumulated in renal tissue and may be taken up by the renal tubular epithelial cells from the basolateral side by active transport as the free ion. 48 Both the structure and function of mitochondria are very sensitive to lead, which is rapidly taken up by these organelles by an active transport process that may be comparable with the transport process associated with calcium. Granules found in mitochondria exposed to lead are thought to be precipitates of both calcium and lead phosphates. 49 50 Lead may therefore interfere with both calcium and phosphate metabolism and thus affect enzymatic function in the vitamin D pathway. The kidney matures gradually from infancy and thus these membrane bound enzyme complexes that catalyse the formation of 24R,25(OH)₂D₃ and 1α,25(OH)₂D₃ from 25(OH)D₃ may be more susceptible to the effects of lead in children as opposed to adults.

The whole enzyme complex may be affected, inhibiting the 1α-OHase and the 24R-OHase so that both 1α,25(OH)₂D₃ and 24R,25(OH)₂D₃ are low in children with raised lead concentrations. Cadmium damage to the renal tubules may affect the folding, cleavage, and reorientation of the active sites of these enzymes. 44 Therefore cadmium and lead could have a combined effect on this enzyme complex inhibiting the inactivation of the 1α-OHase and its conversion to partially active 24R-OHase. This would subsequently prevent the formation of fully active 24R-OHase, resulting in raised concentrations of 1α,25(OH)₂D₃ and depleted concentrations of 24R,25(OH)₂D₃.

Concentrations of lead and cadmium in blood are indicators of present or recent exposure to these metals, whereas the concentrations of urine indicate previous or long term exposure. 42 51 However, a significant correlation has been shown between cadmium concentrations in blood and urine in this study, which suggests that continuous long term exposure to cadmium produces a state of equilibrium. The interrelation between blood and urinary cadmium concentrations and the decrease in 24R,25(OH)₂D₃ may have appeared after exposure to cadmium of as yet unknown duration. As these workers were still exposed to
cadmium and were taking part in a five year follow up study, it is possible to state that they had been continuously exposed to cadmium for at least five years. The preferential incorporation of lead rather than cadmium into bone and the osteomalacia and osteoporosis found after cadmium ingestion may be linked with decreased 24R,25(OH)2D3 concentrations, but this remains unproved. Epidemiological and clinical studies in Japan since 1962 have shown that bone is affected only after very high exposure to cadmium. However, where there is exposure to both lead and cadmium, as in this report, the effects on bone, mediated through the vitamin D pathway, may possibly be found at lower exposures.

The negative correlation of 25(OH)D3, with total urinary proteins agrees with that found by Saha, who also found that patients with heavy proteinuria had abnormal regulation of 1α,25(OH)2D, and a dependency on 25(OH)D pathway, may possibly be found at lower exposures.

Conclusions
The highly significant correlation between the cadmium concentrations in blood and urine suggested that continuous long term exposure to cadmium can result in an equilibrium between blood and urinary concentrations. In this situation, it is possible to estimate cadmium blood concentrations from the analysis of spot urine samples. Cadmium and lead have an effect on vitamin D, metabolism at blood and urinary concentrations below those at which there is clinical evidence of toxicity. Cadmium decreased the concentrations of 1α,25(OH)D3, whereas cadmium and lead increased the concentrations of 1α,25(OH)D2. Biochemical changes may occur that could have long term health implications. Although many sources of lead and cadmium have been removed, exposure in industrial situations is still possible. The simultaneous exposure to more than one contaminant may lower threshold levels for the appearance of health effects and this should be taken into account in routine monitoring.

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