

The initial pathogenesis of cadmium induced renal toxicity

Julian L. Griffin^a, Lee A. Walker^b, Jeff Troke^a, Dan Osborn^b, Richard F. Shore^b,
Jeremy K. Nicholson^{a,*}

^aBiological Chemistry, Biomedical Sciences Division, The Sir Alexander Fleming Building, Exhibition Road, Imperial College of Science, Medicine and Technology, University of London, London SW7 2AZ, UK

^bNERC Centre for Ecology and Hydrology, Monks Wood, Huntingdon, Cambridgeshire PE28 2LS, UK

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Abstract The novel application of magic angle spinning ¹H NMR spectroscopy, coupled with pattern recognition techniques, has identified biochemical changes in lipid and glutamate metabolism that precede classical nephrotoxicity. These changes occurred in the bank vole (*Clethrionomys glareolus*) after chronic dosing, at a low level of exposure and at a renal Cd²⁺ concentration (8.4 µg/g dry wt) that was nearly two orders of magnitude below the WHO critical organ concentration (200 µg/g wet wt). These early stage effects of Cd²⁺ on the biochemistry of renal tissue may reflect adaptation mechanisms to the toxic insult or the preliminary stages of the toxicological cascade. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cadmium toxicity; ¹H NMR spectroscopy; Nephrotoxicity; Renal tubular acidosis; Glutamate; *Clethrionomys glareolus*

1. Introduction

Cadmium has known multi-system impacts on human and animal health following acute and sub-acute exposure [1–6]. However, the effects of low-level chronic exposure and the relationship between tissue residues and demonstrable toxicological effects remain controversial. This is reflected in the wide range of tissue levels that are thought to be acceptable [1–3,5–8]. Cd²⁺ is a global contaminant that is readily transferred through food chains [9,10]. It is a kidney, liver and testicular toxin in both humans and wild mammals [11–14]. Although nephrotoxic lesions occur at levels substantially below those previously thought to cause renal toxicity [5–7], the underpinning early functional biochemical changes have not been reported. Major obstacles to understanding the health implications of Cd²⁺ have been: (a) the absence of low-level exposure studies that have identified sub-clinical measures or biomarkers of early nephrotoxicity, (b) the need to establish the mechanistic link between such biomarkers and later histopathological developments and clinical signs of illness, and (c) lack of demonstrable relationships between chronic and short-term exposure regimes.

Conventional liquid state ¹H NMR spectroscopy of biofluids has proven to be a powerful tool for investigating the biochemical effects of drugs and toxins in man and animals [14,15], particularly when coupled with pattern recognition

(PR) techniques [15–17]. With the advent of high resolution (HR) magic angle spinning (MAS) ¹H NMR spectroscopy techniques, it has recently become possible to study small (8–12 mg), intact tissue samples directly with superior sensitivity to that obtained in conventional solution NMR spectroscopies [18–22]. MAS ¹H NMR spectroscopy allows the simultaneous detection of both aqueous and lipid soluble metabolites along with constituents of biomembranes. We have used the novel combination of MAS ¹H NMR and PR methods to investigate the biochemical effects of environmentally realistic levels of Cd²⁺ on the bank vole (*Clethrionomys glareolus*), a widely distributed European rodent with pollution indicator potential. This study indicates that a coupled MAS–NMR–PR approach to biochemical abnormalities in animal tissue may provide an invaluable assessment of the impact of environmental toxins as well as elucidating the underlying metabolic pathways caused by a toxic insult and the adaptive responses of the tissue.

2. Materials and methods

Laboratory-bred male bank voles were kept throughout the study in standard plastic cages in a constant environment (15.5 L:8.5 D, 20°C). Before the experiment, they were fed ad libitum on standard laboratory feed (RM1; Special Diet Services, Witham, UK). They were then sub-divided into two groups (*n*=5), one of which was maintained on the normal (control) diet, the other was given the same diet to which 40 ppm cadmium chloride had been added. Male voles were chronically exposed to 40 ppm Cd²⁺ for 14 days, receiving the equivalent of a daily intake of ~6 µg/g body weight (bw). This resulted in the accumulation of a mean (±S.E.M.) renal Cd²⁺ concentration of 8.4±2.6 µg Cd²⁺/g dry weight (dw), as measured by atomic absorption spectroscopy. The daily intake of Cd²⁺ is close to the range of exposure thought likely to occur on heavily contaminated terrestrial sites [4]. Each animal was killed by cervical dislocation and its left kidney was immediately excised. A small section of inner cortex was removed and frozen in liquid nitrogen for HRMAS ¹H NMR spectroscopy and the remainder of the kidney was analysed for Cd²⁺ by atomic absorption spectrophotometry. Time from animal death to freezing of tissue samples was typically within 20 s.

Intact renal tissue samples were analysed directly by HRMAS ¹H NMR at 600 MHz (Bruker DRX spectrometer) using a modified solvent suppressed Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence [90-(τ-180-τ)_{*n*} acquisition] as a T₂ filter (*n*=20; 2 s relaxation delay) [18]. A high resolution inverse ¹H/¹³C magic angle spinning probe was used. Samples were spun at 5000 Hz at the magic angle (54.7°) in the presence of 50 µl of D₂O to provide a deuterium lock. D₂O was spiked with TSP to provide a chemical shift reference (δ=0.0). The MAS ¹H NMR spectra generated metabolic profiles for Cd²⁺ exposed bank vole kidney cortex and were compared with those of control animals fed the same, but uncontaminated diet.

For the purposes of PR analysis, ¹H NMR spectra were data reduced (AMIX v.2 program, Bruker, Karlsruhe, Germany), by being sub-divided into 0.04 ppm designated regions from δ 0.4 to 10.0

*Corresponding author. Fax: (44)-20-7594 3226.
E-mail: j.nicholson@ic.ac.uk

[15–17]. The residual water peak area was excluded from analysis and then the area of the resonances within each remaining region was integrated. Each spectral region was normalised to the summation of spectral regions for the entire spectrum, which accounted for bulk mass differences from sample size. Correlation principal components analysis (PCA) was performed using Pirouette v.2.6 (InfoMetrix, Woodinville, WA, USA). PCA is an unsupervised method of classification, requiring no training set of data, that mathematically correlates the variation detected in a data set to a spatial representation of the data. The first PC generated represents the maximum variation that can be correlated together for the data. Subsequent PCs represent less variation and are chosen to be orthogonal to the first PC. Both mean centred and autoscaling routines were applied to the spectral data prior to independent PCA. Mean centre analysis involved subtraction of the mean of the integral for a designated spectral region prior to analysis and measures variance about this mean. Thus, PCs in mean centred data are influenced by regions with the maximum variance about the mean. Autoscale analysis sets each integral region to unit variance, ensuring the sum of a single PC across all observables will be zero. This weights spectra so that features with lower spectral intensity contribute equally to the high concentration metabolites to the PC analysis. Spectral data were reduced from 240 spectral buckets to a number of PCs (less than five) as determined by the maximisation of cumulative total variation in the data that is predicted by those components. Loading plots from the PC analysis were used to identify resonances to predict metabolite changes within renal tissue exposed to Cd^{2+} . These predictions were then cross correlated with spectra visually and manual integration of the regions.

3. Results

During the study, there were no overt clinical signs of ill-health in the bank voles fed Cd^{2+} and no gross internal abnormalities detected at post-mortem. To determine which metabolites were present in the renal tissue, MAS ^1H NMR spectroscopic resonances were assigned in one- and two-dimensional spectra (Fig. 1). Renal tissue MAS ^1H NMR spec-

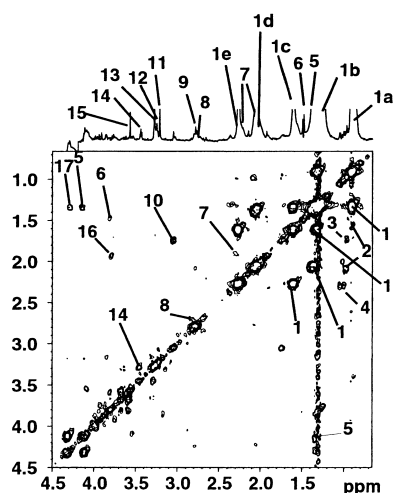


Fig. 1. A HRMAS gradient ^1H - ^1H correlation spectroscopy (COSY) spectrum with a one-dimensional spectrum projection from renal cortical tissue exposed to Cd^{2+} showing resonance assignments. Spectrum acquired at 600.13 MHz with 48 scans across time domains of 4096 and 256 points with a sweep width of 14.2 ppm. Key: 1. Lipid triglyceride signals (letters signify resonance: a CH_3 , b CH_2 , c COCH_2CH_2 , d $\text{HC}=\text{CHCH}_2$, e COCH_2); 2. isoleucine; 3. leucine; 4. valine; 5. lactate; 6. alanine; 7. glutamate; 8. aspartate; 9. dimethylamine; 10. lysine; 11. choline; 12. phosphocholine; 13. phosphatidylcholine; 14. taurine; 15. glycine; 16. arginine; 17. threonine.

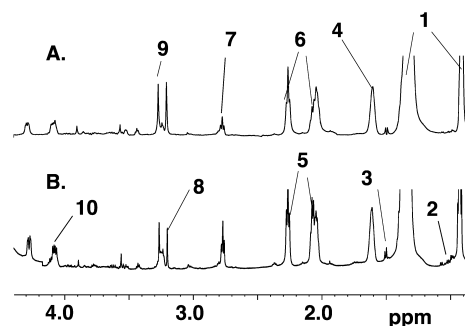


Fig. 2. Comparison of HRMAS ^1H CPMG spectra of renal tissue from voles exposed to cadmium (B) or control diet (A). Resonances from $-\text{HC}=\text{CHCH}_2$ lipid triglycerides (5) and $-\text{COCH}_2$ lipid triglycerides (6) were increased in renal tissue from voles exposed to cadmium. Key: 1. Lipid triglycerides CH_3 and CH_2 ; 2. leucine, isoleucine and valine; 3. alanine; 4. lipid triglyceride COCH_2CH_2 ; 5. lipid triglycerides ($\text{HC}=\text{CHCH}_2$) (and small glutamate contribution); 6. lipid triglycerides (COCH_2) (and small glutamine/glutamate contribution); 7. dimethylamine (singlet) and lipid triglyceride ($\text{CH}_2\text{C}=\text{C}$); 8. choline; 9. phosphatidylcholine; 10. carbohydrates and lactate.

tra indicated that the group of resonances around δ 2.05, due largely to $\text{HC}=\text{CHCH}_2$ lipid triglycerides, and δ 2.74 from $-\text{CH}_2\text{C}=\text{C}$ groups were increased in intensity while the resonances at δ 2.34 from glutamate and δ 1.34 from $-\text{CH}_3$ lipid moieties were decreased in intensity (Fig. 2) in Cd^{2+} exposed

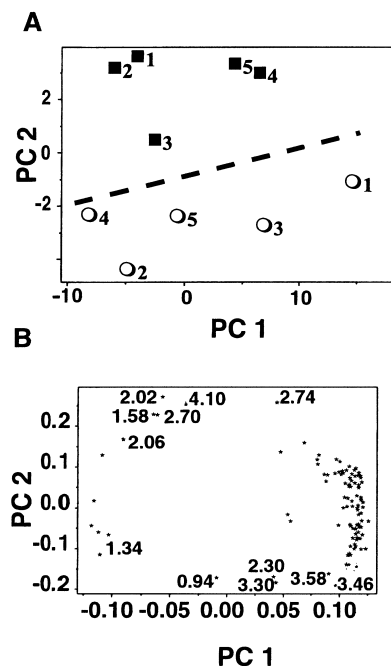


Fig. 3. A: Autoscale PC analysis of control and cadmium exposed renal tissue. Each point represents the mapping of one CPMG ^1H spectrum. The broken line visually shows the separation of control spectra from spectra obtained from cadmium exposed renal tissue along PC2 (factor 2). B: Plot of metabolic integral regions that contribute to PCs 1 and 2 in the above autoscale PCA analysis. Metabolites that were most perturbed in concentration are labelled. Key: $\circ$ control; $\blacksquare$ cadmium exposed; numbers indicate experiment number; * metabolite integral region. Numbers indicate integral region. 0.94 leucine/isoleucine; 1.34 CH_3 ; 1.58 COCH_2CH_2 ; 2.02–2.06 $\text{HC}=\text{CHCH}_2$; 2.30 glutamate; 2.74 $\text{CH}_2\text{C}=\text{C}$; 3.39 phosphatidylcholine; 3.46 taurine; 3.58 glycine; 4.10 lactate.

animals. Some of the resonances of low molecular weight metabolites were obscured by lipid triglyceride resonances and could not be quantified reliably as a result (notably the CH_3 lactate and $\beta\text{-CH}_2$ glutamate resonances).

PR using autoscaled PC analysis readily separated in PC2 ^1H NMR spectra from renal tissue of Cd^{2+} exposed animals from those of controls (Fig. 3A) (mean of PC2: $\text{Cd}^{2+} = 2.7 \pm 0.5$; control = -2.7 ± 0.5 ; $P < 0.001$, Student's unpaired t -test). On inspection of the PCs and their respective loadings (Fig. 3B), PC2 was found to be most influenced by the low molecular weight metabolites lactate (δ 4.10; increased for exposure to Cd^{2+}), glutamate (δ 2.30; decreased), isoleucine and leucine (δ 0.94; decreased), taurine (δ 3.46; decreased) and glycine (δ 3.58; decreased). PC2 was also affected by several lipid and membrane constituents, being most affected by the functional units $-\text{CH}_3$ (δ 1.34, decreased), phosphatidylcholine (δ 3.30, decreased), $\text{HC}=\text{CHCH}_2$ (δ 2.02–2.06; increased) and $\text{CH}_2\text{C}=\text{C}$ (δ 2.74–2.78; increased). In total PC2 accounted for 9.9% of the total variance (compared with 0.42% that each integral region initially represented). Separation of spectra across PC1 did not separate dosed and undosed animals and was probably caused by biovariation between animals. In mean centred PC analysis, separation of control and Cd^{2+} exposed animals occurred across PC4. Loadings (metabolites contributing to the description) for this PC were caused largely by lipid moieties including $\text{HC}=\text{CHCH}_2$ (δ 2.02–2.06; increased), $-\text{COCH}_2-$ (δ 2.26; increased) and $-\text{CH}_3$ lipid triglyceride units (δ 1.30; decreased), as well as leucine/isoleucine (δ 0.90; decreased) and glycerophosphocholine (δ 3.26; decreased) (mean of PC4: $\text{Cd}^{2+} = 11.4 \pm 1.9$; control = -11.3 ± 2.2 ; $P < 0.001$, Student's unpaired t -test).

Using the metabolites identified by autoscaled PC analysis it was possible to determine resonance integrals for each metabolite indicated by that PC for the individual spectra and compare dosed and untreated spectra using the AMIX data used to generate the PC analysis maps (Table 1). Each integral represents a ratio of the integral region across 0.04 ppm to that of the entire region from δ 0.4 to 10.0 excluding water. While resonance integrals are dependent on relaxation effects,

particularly for the CPMG pulse sequence used, between tissue samples and for a given metabolite the integral value is directly proportional to concentration. Lactate and lipid resonances associated with $\text{HC}=\text{CHCH}_2$ and COCH_2CH_2 groups were increased in resonance intensity while glutamate was decreased in Cd^{2+} exposed tissue.

4. Discussion

Using PR coupled with HRMAS ^1H NMR spectroscopy, we were not only able to detect that vole renal tissue had been exposed to Cd^{2+} , despite the low exposure dose used, but also to determine the metabolic consequences of the toxic insult. Cd^{2+} exposure caused two distinct metabolic changes in renal tissue. The first involved low molecular weight metabolites, with a concentration increase in lactate, and decreases in glutamate, taurine, leucine, isoleucine and glycine being detected. The second involved an alteration in the ratio of lipid triglycerides and cell membrane constituents. Increased $-\text{CH}=\text{CHCH}_2-$, $\text{CH}_2\text{C}=\text{C}$ and $-\text{COCH}_2\text{CH}_2-$ lipid moieties were contrasted by decreased $-\text{CH}_3$ lipid moieties and phosphatidylcholine in renal tissue from voles exposed to Cd^{2+} .

The relatively low dose and short exposure period used in the present study resulted in a sum exposure for the bank voles that was much lower than that normally associated with adverse effects. Previously, laboratory rats given chronic intra-peritoneal Cd^{2+} doses of 9–24 $\mu\text{mol/kg}$ bw [14] had demonstrated metabolic abnormalities including reduced urinary citrate, 2-oxoglutarate and 2-oxaloacetate concentrations. However, intra-peritoneal injection circumvents gut absorption of Cd^{2+} , which reduces tissue enrichment to 5–8% of the initial oral dose [1,9], and the final enrichment of Cd^{2+} into the renal tissue was never determined. According to the World Health Organisation, the generally accepted critical cadmium concentration in the renal cortex is 350–1000 $\mu\text{g/g}$ dw in mammals [1–3,8,9], over 10 times greater than the concentration in voles in the present study. Biochemical changes have been detected by electron microscopy in the kidneys of laboratory mice and wild seabirds at renal Cd^{2+} concentrations of 110–260 and 60–480 $\mu\text{g/g}$ dw respectively [5,7] and necrotic changes were observed in laboratory rats at renal concentrations of 105 $\mu\text{g/g}$ dw [23], although these are still at least an order of magnitude greater than those found in bank voles in this study.

While there are no previously published reports that Cd^{2+} affects lipid metabolism directly, Cd^{2+} exposure is known to increase mitochondrial density [5,7] and may alter the proportion of lipid biomembranes within a tissue. Targeted cellular necrosis may also alter the relative proportions of lipid moieties by reducing the contribution made by certain cell membranes to the total lipid pool for the kidney. The concentration changes of two of the low molecular weight metabolites have been documented following higher exposure levels of Cd^{2+} . Glutamate decreases in concentration during cellular acidosis as accompanies Cd^{2+} toxicity [24]. During cellular acidosis in the kidney induced by Cd^{2+} inhibition of carbonic anhydrase, uptake and deamination of glutamine is stimulated. Glutamine is the main source of NH_4^+ ions appearing within the glomerular filtrate, with NH_4^+ production in turn being the main mechanism to reduce cellular acidosis in the kidney [25]. Oxoglutarate dehydrogenase is stimulated by a decrease in pH, decreasing cellular 2-oxoglutarate, and reduc-

Table 1
Normalised integral region values from control and Cd^{2+} exposed bank vole renal tissue for integral regions contributing to PC2 of the autoscaled PCA

Metabolite	Control renal tissue	Cd^{2+} exposed renal tissue
Lactate (δ 4.10)	8.74 ± 0.44	$11.06 \pm 1.02^*$
Glycine (δ 3.58)	5.85 ± 1.25	3.91 ± 0.95
Taurine (δ 3.46)	3.45 ± 0.69	1.94 ± 0.47
Phosphocholine (δ 3.30)	4.92 ± 1.09	3.59 ± 0.90
$\text{CH}_2\text{C}=\text{C}$ (δ 2.74)	3.50 ± 0.77	4.95 ± 0.58
$\text{CH}_2\text{C}=\text{C}$ (δ 2.70)	3.28 ± 0.98	3.03 ± 0.50
Glutamate (δ 2.30)	6.72 ± 0.47	$5.52 \pm 0.31^*$
$\text{HC}=\text{CHCH}_2$ (δ 2.06)	20.5 ± 2.75	$29.0 \pm 2.6^*$
$\text{HC}=\text{CHCH}_2$ (δ 2.02)	18.4 ± 1.45	$26.4 \pm 2.2^*$
COCH_2CH_2 (δ 1.58)	16.0 ± 1.7	$22.6 \pm 2.6^*$
CH_2 (δ 1.34)	136.8 ± 30	125.7 ± 13.3
Leucine/isoleucine (δ 0.94)	17.4 ± 2.7	13.3 ± 2.9

Each integral is a ratio of the total integral of metabolites (δ 0.4–10.0) multiplied by 1000. Metabolites below all contributed to the autoscale PC separation of dosed and undosed animals along PC2 as determined by the loading plot. The chemical shift for each integral region is shown alongside the metabolite found in highest concentration for that region. $^*P < 0.05$ by Student's unpaired t -test.

ing glutamate. As glutamate is an allosteric inhibitor of glutaminase, this stimulates NH_4^+ production from glutamine [24]. The net effect is a reduction in glutamate concentration, explaining the resonance intensity change detected. Furthermore, acidosis and Cd^{2+} exposure both increase lactate production by stimulating pyruvate reduction and limiting blood supply into renal tissue, respectively [14,24]. Alterations in renal tissue concentrations of taurine, glycine and leucine/isoleucine have not previously been reported following cadmium exposure, although it has been suggested that taurine may be an important osmoregulatory compound within the cell [26].

The effects that we have observed may be an early stage of a mechanistically linked cascade of effects that lead from biochemical to physiological damage. Following renal acidosis, detected by the reduction in glutamate and increase in lactate concentrations in this study, tricarboxylic acid (TCA) cycle efficiency in mitochondria will be reduced, resulting in the reduction in TCA cycle intermediates seen in urinary studies at higher exposure levels [14]. Reduced energy production within renal mitochondria produces aminoaciduria, by energetically limiting re-uptake of these compounds in the glomerulus and proximal tubule, possibly induces increased expression of mitochondria, but ultimately culminates in cellular necrosis or apoptosis, as seen in high Cd^{2+} exposure studies [5,7]. It is unknown whether the alteration in lipid profile detected in the present study arises from cellular necrosis or mitochondrial proliferation. An alternative hypothesis is that the biochemical changes detected were in fact compensatory mechanisms following Cd^{2+} exposure rather than the onset of toxicity per se. Indeed no pathological changes were detected during the basic autopsy at time of death. However, if metabolic changes, such as the decrease detected in glutamate, are indicative of carbonic anhydrase inhibition by Cd^{2+} , this suggests that low concentrations of Cd^{2+} can indeed perturb the biochemistry of renal tissue.

The low concentrations of Cd^{2+} found to have accumulated in renal tissue in animals in this study suggest that levels of exposure to Cd^{2+} found in the environment may be detrimental to the health of bank voles and possibly other wild vertebrates. Bank voles, along with other microtine rodents, may be particularly prone to cadmium toxicity [27], but the relatively low level and short duration of exposure required to produce renal impairment is disturbing and it is possible that other mammals may be adversely affected by exposure to much lower levels of Cd^{2+} in the environment than previously thought. Furthermore, the dietary concentration that we gave to bank voles was much lower than that found in some human foodstuffs, particularly shellfish (100–1000 $\mu\text{g/g}$) [10], and Cd^{2+} concentrations in cortical tissue of cigarette smokers and occupationally cadmium exposed workers (28 $\mu\text{g/g}$ and 47–317 $\mu\text{g/g}$, respectively [28,29]) are much larger than those found in the vole renal tissue. Our results may, therefore, also have implications for human health. However, it remains to be seen whether the changes detected in bank voles may in part result from protective mechanisms within renal tissue and further studies are necessary, particularly in other species, to assess how widely applicable these results are to mammalian, including human, physiology. The use of PR techniques in combination with MAS ^1H NMR spectroscopy of intact renal tissue provides a powerful analytical approach in such studies

that is also capable of predicting the underlying biochemical changes.

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